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Protein Phosphatase Magnesium-Dependent 1A–Mediated Inhibition of BMP Signaling Is Independent of Smad Dephosphorylation

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ABSTRACT

Phosphorylation of Smad1/5/8 at carboxyl-terminal serine residues by type I receptors activates downstream bone morphogenetic protein (BMP) signaling. Protein phosphatase magnesium-dependent 1A (PPM1A) has been shown to suppress BMP activity by dephosphorylating phospho-Smads. We report here that PPM1A suppresses BMP signaling via a novel mechanism. PPM1A inhibited a constitutively activated Smad1 mutant lacking BMP receptor phosphorylation sites. PPM1A reduced the protein levels not only of Smad1 but also of Smad5 and Smad8. A proteasome inhibitor blocked the inhibitory effects of PPM1A on Smad1, but the Smurf-binding motif in the Smad1 linker region was not involved in this inhibition. The phosphatase activity of PPM1A is essential for inhibition. Taken together, these findings suggest that through the dephosphorylation of unidentified substrate(s), PPM1A inhibits BMP signaling by decreasing Smad protein levels via the proteasome pathway. Moreover, knockdown of endogenous PPM1A stimulated osteoblastic differentiation, suggesting that PPM1A may physiologically suppress BMP signaling via Smads. © 2010 American Society for Bone and Mineral Research.

KEY WORDS: BMP; SMAD; PROTEASOME; PHOSPHATASE; PHOSPHORYLATION

Introduction

Bone morphogenetic proteins (BMPs) induce ectopic bone formation in muscle tissues and osteoblastic differentiation of myoblasts in vitro.[1] BMP signaling is transduced by two different types of transmembrane serine/threonine kinase receptors, termed *type I* and *type II receptors*.[2,3] The BMP-bound type II receptor phosphorylates the type I receptor kinase, and the activated BMP type I receptor, in turn, phosphorylates downstream receptor-regulated Smads (R-Smads), including Smad1, Smad5, and Smad8. Phosphorylated R-Smads form heteromeric complexes with Smad4 and translocate into the nucleus to regulate the transcription of target genes such as *Id1*.[4–6]

Activation of the BMP-regulated Smad pathway may play an important role in patients with fibrodysplasia ossificans progressiva (FOP), an autosomal dominant disorder characterized by heterotopic bone formation in muscle tissues.[7] Recently, several mutations in a BMP type I receptor, activin receptor-like kinase 2 (ALK2), have been identified in FOP patients.[7,8] We found that these mutant ALK2 molecules phosphorylate Smad1/5/8 in the absence of BMPs and activate downstream intracellular signaling pathways that lead to the induction of *Id1* expression.[9] To examine the roles of BMP-regulated Smads, we generated a constitutively active form of Smad1, termed *Smad1(DVD)*, in which the carboxy-terminal serine residues have been substituted by aspartic acids. Evaluation of this mutant revealed that its overexpression induced osteoblastic differentiation of C2C12 myoblasts (Nojima et al., submitted for publication). These findings suggest that phosphorylation of the carboxy termini of Smad1/5/8 by BMP type I receptors plays an important role in BMP-induced bone formation. Moreover, these data suggest that Smad inhibitors may be useful for the treatment of FOP because they may prevent heterotopic bone formation.

BMP signal transduction is negatively regulated at several critical steps. BMP antagonists such as Noggin, Chordin, and DAN family members prevent the binding of BMPs to their receptors in the extracellular space.[10] A pseudoreceptor, BAMBI, acts as a dominant-negative receptor for members of the transforming growth factor β (TGF-β) superfamily on the plasma membrane.[11] Smad6 and Smad7 inhibit the kinase activity of type I receptors by direct interaction in the cytoplasm.[12,13] Smad ubiquitination regulatory factor 1 (Smurf1), a member of the HECT family of E3 ubiquitin ligases, has been found to interact with Smad1 and Smad5 via the PPAY motif in their linker regions, thereby triggering their ubiquitination and degradation.[14]

Recent studies have identified Smad phosphatases and have shed light on the roles of phosphorylation and dephosphorylation at the carboxy-terminal SXS motif in Smads.[15] Protein phosphatase magnesium-dependent 1A (PPM1A) was identified as a serine/threonine phosphatase in the PPM family.[16] PPM1A dephosphorylates the carboxy-terminal SXS motifs in Smad2/3 and Smad1 and suppresses the biologic activities of TGF-β and BMPs.[17,18] In this study, we examined the molecular mechanisms of the inhibitory activity of PPM1A on BMP signaling using Smad1(DVD), a constitutively active form of Smad1. We found that PPM1A inhibited Smad1(DVD) activity even though Smad1(DVD) lacks phosphoserine residues at the carboxy terminus. PPM1A reduced the protein levels of Smad1 via a proteasome-dependent mechanism, and its phosphatase activity was independent of a Smurf consensus sequence in the linker region. Moreover, knockdown of endogenous PPM1A stimulated BMP activity in C2C12 cells, suggesting that the inhibition by PPM1A may play an important role in physiologic BMP signaling in myoblasts.

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Materials and Methods

Plasmids

Plasmids encoding wild-type mouse Smad1, Smad1(DVD), Smad4, Smad7, and IdWT4F-luc have been described previously.[6] Smad6 and a Smad6 promoter-luciferase reporter were kindly provided by Dr. Kohei Miyazono of University of Tokyo and Dr. Mitsuyasu Kato of Tsukuba University, respectively.[13,19] Human Smurf1 and PPM1A (Accession Numbers NM_020429 and NM_021003, respectively) were obtained using a standard RT-PCR technique employing Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA, USA) and cloned into a pcDEF3 expression vector.[20] The Smad1 mutants, Smad1(AAAY-WT) and Smad1(AAAY-DVD), were generated from wild-type Smad1 and Smad1(DVD), respectively, by replacing proline 224 and proline 225 with alanine using a set of mutated PCR primers. A phosphatase activity–deficient PPM1A, PPM1A(R174G/D239N), was generated from wild-type PPM1A by replacing arginine 174 and aspartic acid 239 with glycine and asparagine, respectively, using sets of mutated PCR primers. All the final constructs were confirmed by sequencing.

Cell culture and transfection

C2C12 mouse myoblasts were maintained, treated with 100 ng/mL BMP-4 (R&D Systems, Minneapolis, MN, USA), and transfected with plasmids using Lipofectamine 2000 (Invitrogen) as described previously.[6,21] Cells were treated with 10 μM Lactacystin (Calbiochem, San Diego, CA, USA). Primary osteoblasts were prepared by sequential collagenase digestion of newborn mouse calvaria as described previously.[22]

Luciferase assays

Luciferase assays were performed using IdWT4F-luc, Smad6 promoter-luc, and phRL-SV40 (Promega, Madison, WI, USA) with the Dual-Glo Luciferase Assay System (Promega) as described previously.[6]

Immunohistochemistry and Western blot analysis

The following antibodies were used for immunohistochemistry and Western blot analysis: α-FLAG antibody (clone M2, Sigma Aldrich Chemicals, St. Louis, MO, USA), α-Myc polyclonal antibody (Medical & Biological Laboratories Co., Nagoya, Japan), and α-PPM1A mouse monoclonal antibody (Abcam, Cambridge, MA, USA). For immunohistochemical analysis, target proteins were visualized using an Alexa488- or Alexa594-conjugated secondary antibody (Invitrogen). For Western blot analysis, the target proteins were detected using a horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG antibody (GE Healthcare UK, Ltd., Buckinghamshire, England).

Reverse-transcriptase PCR and real-time PCR analysis

Total RNA was isolated from C2C12 cells using Trizol (Invitrogen) and then reverse transcribed into cDNA. cDNA was amplified by PCR using primers that were specific for murine *Id1*, *Osterix (Osx)*, *Runx2*, and *GAPDH*, which was used as a control (QIAGEN, Hilden, Germany). SYBR Green-based real-time PCR was performed in a 96-well plate format using SYBR Premix Ex Taq (TaKaRa, Ohtsu, Japan) on an iCycler Thermal Cycler (Bio-Rad, Richmond, CA, USA).

RNAi design and transfection

RNAi Stealth oligonucleotides were designed against murine PPM1A (No. MSS207887, Invitrogen), and a scrambled RNAi was used as a negative control (Invitrogen). Cells were transfected with the RNAi Stealth oligonucleotides using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

Statistical analysis

Comparisons were made using unpaired Student's *t* test. Results are presented as means ± SD. Statistical significance is displayed as $^{*}p < .05$ or $^{**}p < .01$.

Results

PPM1A inhibits BMP signaling induced by a constitutively active Smad1

First, we compared the effect of PPM1A with that of Smad7 and Smurf1 on osteoblastic differentiation of C2C12 myoblasts as induced by treatments with BMP-4, overexpression of a constitutively active ALK2(Q207D) receptor, or overexpression of a constitutively active Smad1(DVD). ALP activity was measured as a typical marker of osteoblastic differentiation. ALP activity induced by BMP-4 or ALK2(Q207D) was suppressed by Smad7 and Smurf1. As reported previously, although Smad6 also suppressed the ALP activity induced by ALK2(Q207D), it was

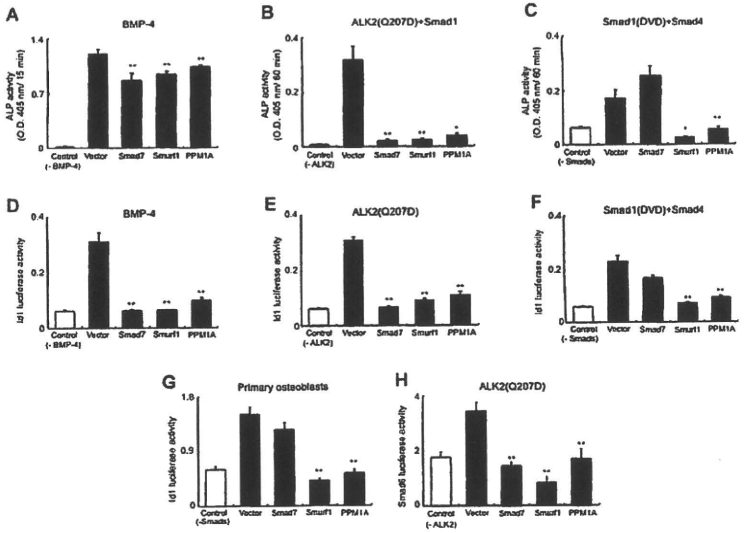

Fig. 1. PPM1A inhibits BMP signaling induced by constitutively active Smad1. (*A–C*) Inhibition of ALP activity by Smad7, Smurf1, and PPM1A in C2C12 cells. ALP activity was induced by BMP-4 treatment (*A*), overexpression of ALK2(Q207D) with wild-type Smad1 (*B*), and overexpression of Smad1(DVD) with Smad4 (C). (*D–F*) Inhibition of IdWT4F-luc activity by Smad7, Smurf1, and PPM1A in C2C12 cells. IdWT4F-luc activity was induced by BMP-4 treatment (*D*), overexpression of ALK2(Q207D) (*E*), and overexpression of Smad1(DVD) with Smad4 (*F*). (*G*) Inhibition of IdWT4F-luc activity by Smad7, Smurf1, and PPM1A in primary osteoblasts. IdWT4F-luc activity was induced by overexpression of Smad1(DVD) with Smad4. (*H*) Inhibition of Smad6 promoter-luc activity by Smad7, Smurf1, and PPM1A in C2C12 cells. ALP activity was induced by overexpression of ALK2(Q207D) with wild-type Smad1. Data are presented as means ± SD ($n = 3$). $^{*}p < .05$; $^{**}p < .01$ compared with empty vector transfection in each group.

weaker than Smad7 (data not shown).[9,23] Unexpectedly, ALP suppression likewise was detected in cultures transfected with Smad1(DVD), which lacked serine residues at the carboxy terminus (Fig. 1*A–C*). Next, we examined the inhibitory effect of PPM1A on Smad1 using IdWT4F-luc, which contained the Smad-binding element from the *ID1* gene. PPM1A suppressed the luciferase activity that was induced not only by the ligand and receptor but also by Smad1(DVD) (see Fig. 1*D–F*). A similar suppression by PPM1A was observed in primary osteoblasts (see Fig. 1*G*). PPM1A suppressed not only Id1 promoter activity but also Smad6 promoter-luc activity in C2C12 cells (see Fig. 1*H*).

PPM1A decreases Smad protein levels via the proteasome pathway

Next, we examined the effect of PPM1A on Smad1(DVD) protein levels in C2C12 cells. The number of Smad1(DVD)-positive cells and the protein level of Smad1(DVD), as determined by Western blot analysis, were reduced by coexpression of PPM1A (Fig. 2*A, B*). Protein levels of wild-type Smad1, Smad5, and Smad8 also were reduced by PPM1A expression (see Fig. 2*C*).

Because the proteasome pathway plays a key role in Smad degradation,[24,25] we examined whether this pathway is also involved in the action of PPM1A. Treatment of C2C12 cells with lactacystin, which is a specific inhibitor of the proteasome, increased the number of cells that were positive for both Smad1 and PPM1A and prevented a decrease in Smad1 protein levels (Fig. 3*A, B*). Lactacystin also reduced the inhibitory activity of PPM1A on IdWT4F-luc activity that was induced by Smad1(DVD) (see Fig. 3*C*).

PPM1A decreases Smad1 protein levels independent of a Smurf1 consensus sequence

Smurf1 is a Smad ubiquitin ligase that stimulates Smad degradation via a proteasome pathway by interacting with a conserved PPAY motif in the linker regions of Smads.[14,26,27] Because substitution of the PPAY motif with AAAY destroys the

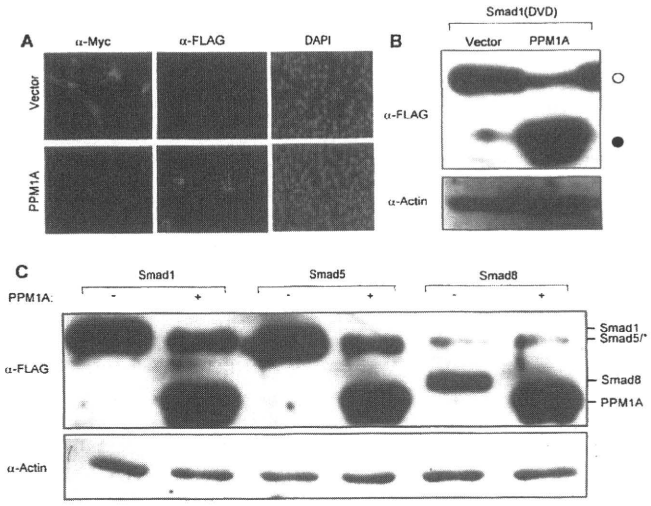

Fig. 2. PPM1A decreases protein levels of Smad1, Smad5, and Smad8 in C2C12 cells. (*A*) C2C12 cells were cotransfected with Myc-Smad1(DVD) and FLAG-PPM1A or with an empty vector, followed by immunohistochemical analysis on day 3 after transfection. Original magnification ×40. (*B*) C2C12 cells were cotransfected with FLAG-tagged Smad1(DVD) (*open circle*) and FLAG-PPM1A (*closed circle*) or an empty vector and analyzed by Western blotting on day 3 after transfection. (*C*) Effects of PPM1A on Smad1, Smad5, and Smad8. C2C12 cells were cotransfected with Smad1, Smad5, or Smad8 along with FLAG-PPM1A or with an empty vector. The cells then were analyzed by Western blotting on day 3 after transfection. Lanes for Smad8 contained nonspecific faint bands (*) with the same mobility as Smad5.

above-mentioned interaction, we introduced this mutation into wild-type Smad1 and Smad1(DVD), which we labeled Smad1(AAAY-WT) and Smad1(AAAY-DVD), respectively. Coexpression of PPM1A with Smad1(AAAY-WT) or Smad1(AAAY-DVD) reduced the protein levels of both types of Smad1 (Fig. 4*A, B*). This reduction was confirmed by immunohistochemical analysis of C2C12 cells (unpublished data). Moreover, PPM1A expression almost completely suppressed Smad1(AAAY-DVD)-induced IdWT4F-luc activity (see Fig. 4*C*). Taken together, these findings suggest that the interaction between Smurf1 and Smad1 via the PPAY motif is not involved in the inhibitory action of PPM1A.

Phosphatase activity is necessary for the inhibition of BMP signaling by PPM1A

To determine whether phosphatase activity is required for the action of PPM1A on Smads, we generated a phosphatase activity–deficient version of PPM1A (D239N/R174G).[28] In contrast to wild-type PPM1A, the mutant PPM1A did not suppress the IdWT4F-luc activity induced by BMP-4 or Smad1(DVD) (Fig. 5*A, B*). Moreover, mutant PPM1A did not decrease the number of Smad1-positive cells or the Smad1 protein level in C2C12 cells (see Fig. 5*C, D*). These findings indicate that the phosphatase activity of PPM1A is necessary for the inhibition of BMP signaling. They also suggest that a molecule(s) other than the Smads may be dephosphorylated by PPM1A.

Knockdown of endogenous PPM1A enhances BMP signaling

Finally, we examined the role of endogenous PPM1A on BMP signaling in C2C12 cells using RNAi (Fig. 6*A*). Transfection of C2C12 cells with siRNA specific for PPM1A efficiently reduced the levels of endogenous PPM1A protein and increased the IdWT4F-luc activity induced by Smad1(DVD) (see Fig. 6*A, B*). Moreover, the induction of Id1, Osx, and Runx2 mRNA levels by Smad1(DVD) with Smad4 or BMP-4 was increased by PPM1A-specific siRNA transfection, suggesting that endogenous PPM1A physiologically suppresses osteoblastic differentiation via Smads in C2C12 cells (see Fig. 6*C–H*).

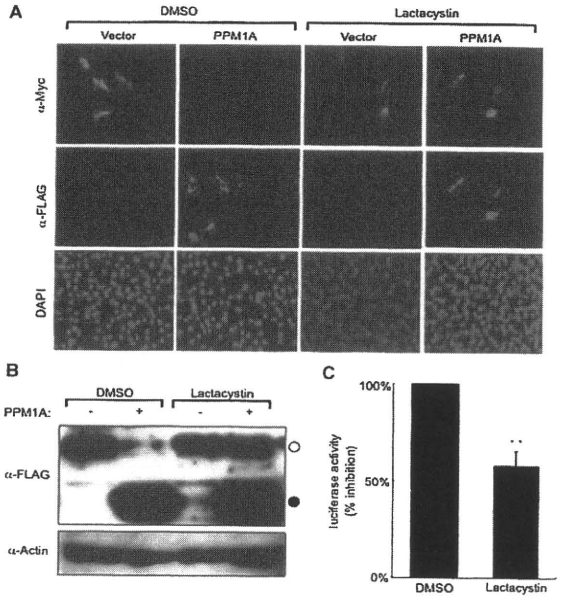

Fig. 3. The proteasome inhibitor lactacystin prevents the inhibitory activity of PPM1A on Smad1. (*A*) Immunohistochemical analysis of Myc-Smad1 and FLAG-PPM1A in C2C12 cells incubated with and without 10 μM lactacystin. Original magnification ×40. (*B*) Western blot analysis of FLAG-Smad1 (*open circle*) and FLAG-PPM1A (*closed circle*) in C2C12 cells incubated with and without 10 μM lactacystin. (*C*) Effect of lactacystin on Id1-luc activity, which is suppressed by PPM1A. C2C12 cells were transfected with IdWT4F-luc and PPM1A and treated with 10 μM lactacystin or vehicle (DMSO) for 8 hours. Data are expressed as percent inhibition relative to the vehicle control. Data are presented as means ± SD ($n = 3$). $^{**}p < .01$.

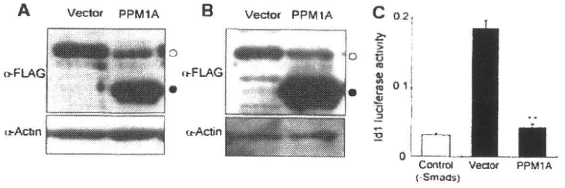

Fig. 4. PPM1A suppresses the activity of Smad1 carrying a mutation in the PPAY motif in the linker region. (*A, B*) C2C12 cells were cotransfected with FLAG-Smad1(AAAY-WT) (*open circle*) (*A*) or FLAG-Smad1(AAAY-DVD) (*open circle*) (*B*) along with FLAG-PPM1A (*closed circles*) or with an empty vector alone. Cells were analyzed by Western blotting on day 3 after transfection. (*C*) C2C12 cells were cotransfected with Smad1(AAAY-DVD), IdWT4F-luc, and PPM1A expression vector or an empty vector. Luciferase activity was analyzed on day 3 after transfection. Data are presented as means ± SD ($n = 3$). $^{**}p < .01$.

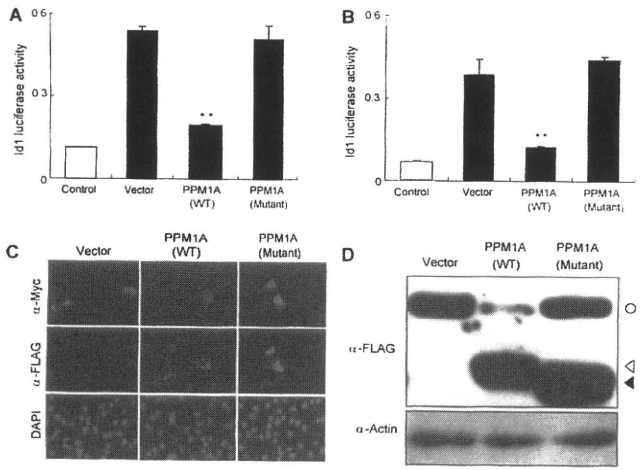

Fig. 5. The phosphatase activity of PPM1A is essential for Smad1 inhibition. (*A, B*) Suppression of IdWT4F-luc activity induced by treatment with BMP-4 (*A*) or by overexpression of Smad1(DVD) (*B*) via wild-type or mutant PPM1A. Data are presented as means ± SD ($n = 3$). $^{**}p < .01$. (*C*) C2C12 cells were cotransfected with Myc-Smad1 and wild-type FLAG-PPM1A, FLAG-PPM1A(R174G/D239N), or empty vector. The cells were submitted to immunohistochemical analysis on day 3 after transfection. Original magnification ×40. (*D*) C2C12 cells were cotransfected with FLAG-Smad1 (*open circle*) and wild-type FLAG-PPM1A (*open triangle*), FLAG-PPM1A(R174G/D239N) (*closed triangle*), or empty vector, followed by Western blot analysis on day 3 after transfection.

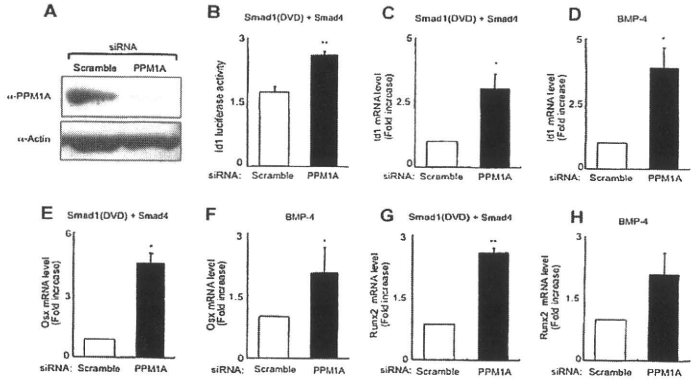

Fig. 6. Knockdown of endogenous PPM1A enhances BMP signaling in C2C12 cells. (*A*) C2C12 cells were transfected with 20 nM PPM1A RNAi or scrambled RNAi. The levels of PPM1A were examined by Western blotting at 24 hours after transfection. (*B*) Transfection of 20 nM PPM1A RNAi stimulated the IdWT4F1-luc activity induced by Smad1(DVD). (*C–H*) Real-time PCR analysis of Id1 (*C, D*), Osx (*E, F*), and Runx2 (*G, H*) mRNA levels in C2C12 cells. Twenty nanomoles of PPM1A siRNA or scrambled oligonucleotide was transfected, and BMP signaling was induced by Smad1(DVD) with Smad4 (*C, E, G*) or BMP-4 (*D, F, H*). Results are presented as means ± SD ($n = 3$). $^{*}p < .05$, $^{**}p < .01$ compared with scrambled RNAi transfection in each group.

Discussion

Type I BMP receptors phosphorylate the carboxy-terminal serine residues of Smad1/5/8 and thereby activate the transcriptional activity of Smads. Importantly, substitution of a phosphorylatable amino acid with an acidic residue (such as aspartic acid or glutamic acid) mimics the phosphorylated state of some proteins, including type I receptors of the TGF-β superfamily. Recently, we found that substitution of the two carboxy-terminal serine residues in Smad1 with aspartic acid residues induced osteoblastic differentiation via constitutive activation of Smad1 (Nojima et al., submitted for publication). Similar mutations at the carboxy-terminal phosphorylation sites of Smad3 have been shown to induce active trimer complex formation.[29] These constitutively activated Smads are useful for examining the role of PPM1A because they activate intracellular signaling just as the receptor-phosphorylated wild-type Smads do, but they are not sensitive to phosphatases targeting their carboxy termini.

In this study we have shown that the phosphatase PPM1A suppresses the induction of BMP signaling by Smad1 even when Smad1's carboxy terminus cannot be dephosphorylated. This finding suggests that PPM1A suppresses BMP signaling via a novel mechanism. Our data suggest that suppression by PPM1A depends on its phosphatase activity through a proteasome pathway. Moreover, we found that knockdown of endogenous PPM1A enhanced BMP activity. Although PPM1A suppressed the BMP signaling of an AAAY mutant, we cannot rule out the possibility that Smurf1 is still involved in the inhibition through interactions with I-Smads or other Smad-interacting molecules. The identification of novel endogenous substrates of PPM1A may help to elucidate the mechanism(s) by which PPM1A inhibits BMP signaling.

In addition to PPM1A, small C-terminal-domain phosphatases (SCPs) have been identified as Smad phosphatases.[30] In our preliminary experiments, SCP1 showed a much weaker inhibitory activity toward Smad1(DVD) than PPM1A, suggesting that dephosphorylation at the carboxy terminus of Smad1 is critical for its suppression by SCP1. Phosphorylation of the linker region by mitogen-activated kinases (MAPKs) inhibited Smad1 activity.[31,32] SCP1 has been shown to dephosphorylate Smad1 not only at its carboxy terminus but also at its linker region.[13] To investigate further, we generated additional Smad1 mutants lacking the ERK and GSK3β phosphorylation sites in the linker region. Similarly to wild type and the AAAY mutant, the ALP activity induced by these mutants was suppressed by PPM1A and SCP1 (data not shown). Further studies are needed to elucidate the molecular mechanisms of PPM1A- and SCP1-dependent inhibition of BMP signaling.

At present, no effective treatment is available to prevent heterotopic bone formation in FOP. A possible cause of this condition is constitutive activation of Smad pathways by the BMP receptor ALK2.[9,33] We have shown that a specific chemical inhibitor of the Smad pathway blocks the biologic activities of mutant ALK2, which is normally found in FOP patients.[9,33] These finding suggest that activation of Smad1/5 by phosphorylation may play an important role in heterotopic bone formation and that Smads should be evaluated as potential targets for novel FOP therapies. Because PPM1A inhibits Smad activity, a stimulator of PPM1A expression in muscle tissues may prevent heterotopic bone formation in FOP.

In conclusion, we have found that PPM1A suppresses BMP signaling by reducing the protein levels of Smad via a proteasome pathway. Thus PPM1A may indirectly regulate BMP signaling by dephosphorylating molecule(s) other than Smads.

Disclosures

The authors state that they have no conflicts of interest.

Acknowledgments

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Differentiation 80 (2010) 46–52

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Canonical Wnts and BMPs cooperatively induce osteoblastic differentiation through a GSK3β-dependent and β-catenin-independent mechanism

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ABSTRACT

Both BMPs and Wnts play important roles in the regulation of bone formation. We examined the molecular mechanism regulating cross-talk between BMPs and Wnts in the osteoblastic differentiation of C2C12 cells. Canonical Wnts (Wnt1 and Wnt3a) but not non-canonical Wnts (Wnt5a and Wnt11) synergistically stimulated ALP activity in the presence of BMP-4. Wnt3a and BMP-4 synergistically stimulated the expression of type I collagen and osteonectin. However, Wnt3a did not stimulate ALP activity that was induced by a constitutively active BMP receptor or Smad1. Noggin and Dkk-1 suppressed the synergistic effect of BMP-4 and Wnt3a, but Smad7 did not. Overexpression of β-catenin did not affect BMP-4-induced ALP activity. By contrast, inhibition or stimulation of GSK3β activity resulted in either stimulation or suppression of ALP activity, respectively, in the presence of BMP-4. Taken together, these findings suggest that BMPs and canonical Wnts may regulate osteoblastic differentiation, especially at the early stages, through a GSK3β-dependent but β-catenin-independent mechanism.

1. Introduction

Bone formation is regulated by various hormones and cytokines. Among them, bone morphogenetic proteins (BMPs) and Wnts are suggested to play important roles in bone metabolism in humans (Katagiri et al., 2008; Milat and Ng, 2009). BMPs are members of the transforming growth factor-β (TGF-β) superfamily, and they were originally identified as factors responsible for the induction of ectopic bone formation (Katagiri, 2010; Katagiri et al., 2008; Miyazono et al., 2005). BMP signaling is transduced by two different types of serine/threonine kinase receptors, termed type I and type II receptors. The ligand-bound type II receptor activates the type I receptor kinase via phosphorylation. Activated BMP type I receptor kinases, in turn, phosphorylate receptor-regulated Smads (R-Smads) including Smad1, Smad5 and Smad8 in the cytoplasm. Phosphorylated R-Smads form heteromeric complexes with Smad4 and translocate to the nucleus to regulate the transcription of various target genes including Id1 (Katagiri et al., 2002; Korchynskyi and ten Dijke, 2002; Lopez-Rovira et al., 2002). Dysfunction of BMP signaling has been implicated in several diseases such as primary pulmonary hypertension and brachydactyly (Morrell et al., 2001; Lane et al., 2000; Nickel et al., 2005). Recently, a recurrent heterozygous mutation at c.617G > A in the ACVR1 gene encoding the BMP type I receptor ALK2 was identified in both familial and sporadic patients with fibrodysplasia ossificans progressiva (Shore et al., 2006). We found that this mutation results in a constitutively activated BMP receptor and cooperatively induces osteoblastic differentiation with Smad1 and Smad5 (Fukuda et al., 2008, 2009).

Wnt proteins are small, cysteine-rich glycoproteins that regulate embryonic development, cell differentiation and cell fate determination (Huelsken and Birchmeier, 2001; Moon et al., 2002; Westendorf et al., 2004). Wnts transduce two types of intracellular signaling through canonical and non-canonical pathways. During the activation of canonical intracellular signaling, Wnts bind to a complex containing frizzled and low-density lipoprotein receptor-related protein (LRP)-5 or 6. This complex regulates the kinase activity of glycogen synthase kinase 3β (GSK3β). In the absence of Wnt, activity of β-catenin, a downstream effecter of the canonical pathway, is blocked by a phosphorylation-dependent degradation mechanism induced by GSK3β. Canonical Wnt signaling stabilizes β-catenin by inhibiting GSK3β activity. Stabilized β-catenin translocates to the nucleus and interacts with T cell factor/lymphoid enhancer factor 1 (Tcf/LEF1) to activate gene expression (Miller et al., 1999). Conversely, non-canonical Wnt ligands activate a β-catenin-independent signaling pathway and inhibit canonical Wnt signaling (Mikels and Nusse, 2006; Topol et al., 2003). In osteoblasts, canonical Wnt signaling has been shown to play an important role in the regulation of bone formation and the maintenance of bone mass. In both humans and mice, loss-of-function of LRP5 leads to decreased bone formation (Gong et al., 2001; Kato et al., 2002), and a gain-of-function mutation in LRP5 results in a high bone mass phenotype (Boyden et al., 2002; Little et al., 2002).

Several canonical Wnt proteins have been shown to induce osteoblastic differentiation in mesenchymal cells (Bennett et al., 2005; Friedman et al., 2009). BMP-2 was also shown to increase the levels of β-catenin in the nuclei of pre-osteoblastic cells, and to induce the expression of Wnt3a (Bain et al., 2003; Mbalaviele et al., 2005; Rawadi et al., 2003). In osteoblasts, the loss of the BMP type I receptor BMPR-IA causes the up-regulation of canonical Wnt signaling, which results in increased bone mass (Kamiya et al., 2008a, 2008b, 2009). These findings suggest that an interaction between BMP and Wnt signaling exists that affects osteoblastic differentiation; however, the mechanisms are poorly understood. In this report, we examined a molecular mechanism that regulates cross-talk between BMPs and canonical Wnts during osteoblastic differentiation.

2. Methods

2.1. Materials and reagents

Recombinant BMP-4, BMP-6, Noggin, Wnt3a and Wnt5a were purchased from R&D Systems (Minneapolis, MN). A GSK3β-specific inhibitor, 6-bromoindirubin-3′-oxime (BIO), was purchased from Calbiochem (Dermstadt, Germany). cDNAs of murine BMP-4, Wnt1, Wnt3a, Wnt5a, Wnt11, Dkk-1, GSK3β and β-catenin were obtained by a standard RT-PCR technique and each cDNA was subcloned into the pcDEF3 expression plasmid (Goldman et al., 1996). A constitutively active BMP type I receptor, ALK2(Q207D), was described previously (Fukuda et al., 2008). A constitutively active β-catenin construct in which four phosphorylation sites are mutated to alanine (S33A, S37A, T41A and S45A) was generated by PCR according to the method of Nelson et al. (1999). All expression plasmids were confirmed by nucleotide sequencing.

2.2. Cell cultures

C2C12 mouse myoblasts were maintained in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum (Katagiri et al., 1994). Primary myoblasts were prepared from mouse extensor digitorum longus muscles using a single fiber isolation method (Ono et al., 2010).

2.3. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was measured on day 3 as an early marker of osteoblastic differentiation (Katagiri et al., 1994; Kodaira et al., 2006). In brief, cells were incubated with a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$ and 1 mg/ml of p-nitrophenylphosphate) (Kodaira et al., 2006). After 20 min incubation at room temperature, the reactions were terminated by adding 3 M NaOH and the absorbance was measured at 405 nm. Results are expressed as the mean ± SD ($n=3$). To examine ALP histochemically, cells were incubated at room temperature for 20 min with a mixture of 0.1 mg/ml naphthol AS-MX phosphate (Sigma, St. Louis, MO), 0.5% N, N-dimethylformamide, 2 mM $MgCl_2$ and 0.6 mg/ml fast blue BB salt (Sigma) in 0.1 M Tris–HCl, pH 8.5 (Katagiri et al., 1994).

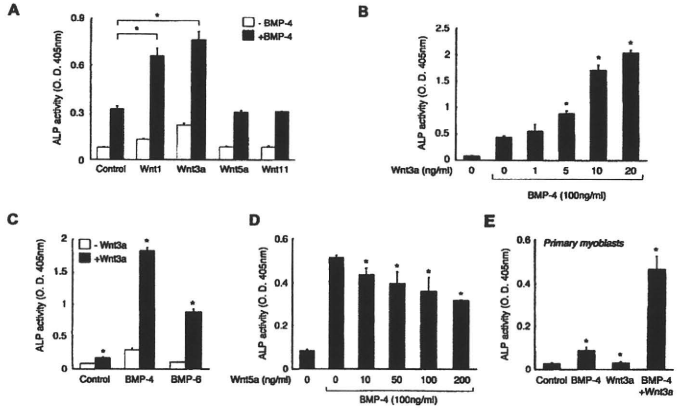

Fig. 1. Canonical Wnts cooperatively enhance osteoblastic differentiation in cooperation with BMPs. (A) Wnt1 and Wnt3a increase ALP activity in the presence of BMP-4. C2C12 cells were transiently co-transfected with BMP-4 and each Wnt expression vector, and ALP activity was determined. *$p < 0.05$ compared with the BMP-4 treatment in the control group. (B) Wnt3a dose dependently induces ALP activity. C2C12 cells were treated with 100 ng/ml of BMP-4 and graded concentrations of Wnt3a. *$p < 0.05$ compared with BMP-4 treatment alone. (C) C2C12 cells were treated with 20 ng/ml of Wnt3a and 100 ng/ml of BMP-4 or BMP-6, and ALP activity was determined. *$p < 0.05$ compared with the no-Wnt3a treatment in each group. (D) Wnt5a failed to increase ALP activity in the presence of BMP-4. C2C12 cells were treated with 100 ng/ml of BMP-4 and graded concentrations of Wnt5a. *$p < 0.05$ compared with BMP-4 treatment alone. (E) A synergistic effect was observed in primary myoblasts. Cells were treated with 20 ng/ml of Wnt3a and 100 ng/ml of BMP-4. ALP activity was determined on Day 3. *$p < 0.05$ compared with the control.

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2.4. Quantitative RT-PCR

Quantitative RT-PCR was performed as described (Kokabu et al., 2010). In brief, total RNA was isolated from C2C12 cells using TRizol (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase I (Takara, Ohtsu, Japan) to remove contaminating genomic DNA. cDNAs were synthesized using Superscript III reverse transcriptase (Invitrogen). The cDNAs were amplified by PCR using specific primers for murine ALP, osteonectin, type I collagen and osteocalcin (Takara). Atp5f1 was used as internal control. SYBR Green-based real-time PCR was performed in a 96-well plate format using SYBR Premix Ex Taq (Takara) with a Thermal Cycler Dice Real Time system

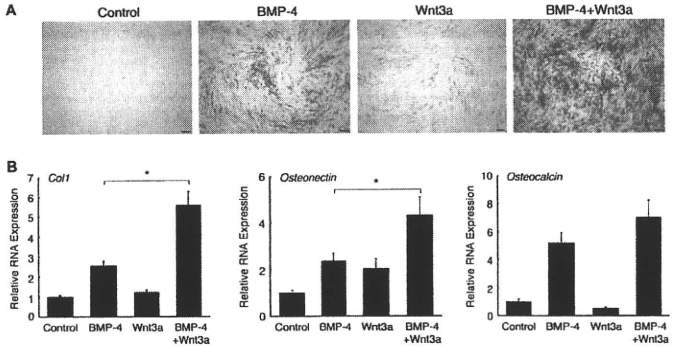

Fig. 2. BMP and Wnt3a cooperatively induce expression of collagen α2 (I) and osteonectin but not osteocalcin. (A) ALP staining. C2C12 cells were treated with 20 ng/ml of Wnt3a and 100 ng/ml of BMP-4 for 3 days and then stained for ALP activity. Scale bar, 200 μm. (B) Quantitative RT-PCR analysis for the osteoblastic differentiation marker genes type I collagen (Col1), osteonectin and osteocalcin. Expression levels of Col1 and osteonectin were significantly higher after treatment with BMP-4 and Wnt3a for 3 days. *$p < 0.05$ compared with treatment with BMP-4 alone.

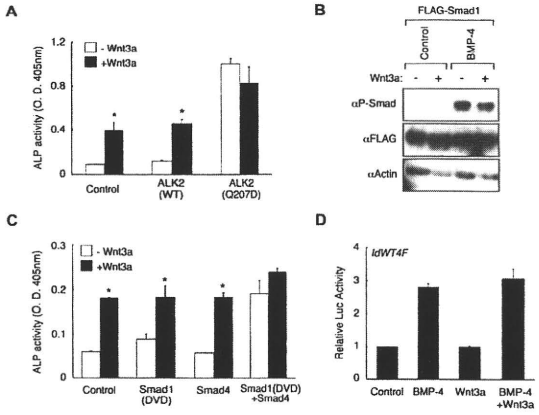

Fig. 3. Wnt3a does not cross-talk with BMP-specific intracellular signaling molecules. (A) Wnt3a failed to increase the ALP activity induced by a constitutively active BMP type I receptor. C2C12 cells were transiently transfected with ALK2(WT), ALK2(Q207D) or an empty plasmid and then cultured in the presence or absence of 20 ng/ml of Wnt3a. ALP activity was determined on day 3. (B) Wnt3a does not affect the phosphorylation levels of Smads. C2C12 cells transfected with FLAG-tagged Smad1 were stimulated for 1 h with BMP-4 (100 ng/ml) in the presence or absence of Wnt3a (20 ng/ml). Cell lysates were immunoblotted with anti-phospho-Smad1/5/8, anti-FLAG and anti-actin antibodies. (C) C2C12 cells were transiently transfected with the indicated plasmids and were treated with 20 ng/ml of Wnt3a. Next, ALP activity was determined on day 3. (D) Luciferase reporter analysis of the effect of Wnt3a on BMP signaling. C2C12 cells transfected with an IdWT4F-luc reporter plasmid were treated with BMP-4 (100 ng/ml), Wnt3a (20 ng/ml), or both. After 24-h treatment, luciferase activity was determined. *$p < 0.05$ compared with the no-Wnt3a treatment in each group.

TP800 (Takara). Fold differences in gene expression were calculated using the ΔΔCt method.

2.5. Immunoblotting

Immunoblotting was performed as described (Kanomata et al., 2009; Nojima et al., 2010). Cells were lysed in TNE buffer [10 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA and 1% Nonidet P-40] containing protease and phosphatase inhibitors. Lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Western blots were probed using the following antibodies: anti-FLAG (clone M2, Sigma), anti-phosphorylated Smad1/5/8 (Cell Signaling, Beverly, MA), anti-V5 (Invitrogen) and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were detected using an enhanced chemiluminescence (ECL) system (GE Healthcare Japan, Tokyo, Japan).

2.6. Dual-luciferase reporter assays

Transcriptional activation induced by BMP and Wnt were determined using IdWT4F-luc (Katagiri et al., 2002) and Super8xTOPflash (kindly provided by Dr. Randall Moon), respectively. C2C12 cells were plated in 96-well plates at a concentration of 1×10^4 cells/well. Cells were transfected with the reporter constructs and with a phRL-SV40 Renilla luciferase construct using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. The transfected cells were treated with BMP-4 and/or Wnt3a as indicated in the figure legends. After a 24-h treatment, the luciferase activity was measured using the Dual-Glo Luciferase assay system (Promega, Madison, WI).

2.7. Statistical analysis

Statistical analyses were conducted using Student's *t*-test; the results are shown as means ± S.D. Statistical significance is indicated as *$p < 0.05$.

3. Results

3.1. Canonical Wnts and BMPs synergistically induce osteoblastic differentiation in C2C12 cells

To investigate the function of Wnt3a on osteoblastic differentiation induced by BMPs, we used C2C12 myoblasts. C2C12 cells from a myoblastic cell line are a well-characterized model system for the study of the osteoblastic differentiation activity of BMPs. C2C12 cells can differentiate into not only into myotubes, but also into osteoblasts when incubated in the presence of BMPs for 48–72 h (Katagiri et al., 1994). These culture conditions were previously reported to give rise to cells that express many osteoblastic markers including ALP, the bone matrix proteins osteopontin and osteocalcin (Katagiri et al., 1994). First, we examined the effects of canonical (Wnt1 and Wnt3a) and non-canonical Wnts (Wnt5a and Wnt11) on osteoblastic differentiation induced by BMPs in C2C12 myoblasts. To determine the expression of an osteoblastic phenotype, we measured the activity of ALP, a well-defined marker of early osteoblastic differentiation. Co-transfection of Wnt1 and Wnt3a, but not Wnt5a or Wnt11, increased ALP activity in the presence of BMP-4; however, transfection of any individual Wnt induced only minimal ALP activity (Fig. 1A). Treatment of C2C12 cells with recombinant Wnt3a also stimulated ALP activity in the presence of BMP-4 in a concentration-dependent manner (Fig. 1B). Similar cooperative stimulation was observed in the presence of BMP-6 (Fig. 1C). In contrast to the canonical Wnt3a, treatment with the non-canonical Wnt5a reduced ALP activity induced by BMP-4 in a concentration-dependent manner (Fig. 1D). Furthermore, we examined the cooperative effects of treatment with Wnt3a plus BMP-4 on osteoblastic differentiation in primary myoblasts, mouse embryonic fibroblasts and Sol8, another myoblastic cell line derived from mouse soleus muscle. Like C2C12 cells, Wnt3a and BMP-4 synergistically increased ALP activity (Fig. 1E and data not shown).

3.2. Wnt3a and BMP-4 synergistically induce preosteoblast marker expression in C2C12 cells

During osteogenesis, pluripotent mesenchymal stem cells differentiate into preosteoblasts, which then differentiate into mature osteoblasts that deposit the necessary components to form bone matrix and to promote subsequent mineralization. Upon differentiation into osteoblasts, the cells express proteins related to their stage of differentiation at high levels, such as ALP, type I collagen (Col1), osteonection, parathyroid hormone receptor and bone sialoproteins and osteocalcin (Aubin, 2001).

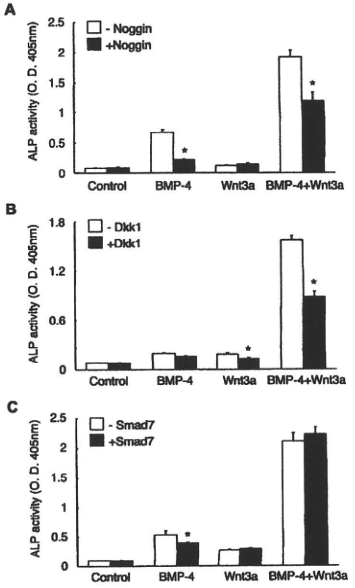

Fig. 4. Effects of specific inhibitors of Wnts or BMPs on ALP activity induced by Wnt3a and BMP-4. (A) ALP activity was reduced by treatment with Noggin. C2C12 cells were treated with BMP-4 (100 ng/ml), Wnt3a (20 ng/ml) or both in the presence or absence of Noggin (100 ng/ml). (B and C) Dkk-1 but not Smad7 significantly reduced ALP activity. C2C12 cells transfected with Dkk-1 (B) or Smad7 (C) were treated for 3 days with BMP-4 (100 ng/ml), Wnt3a (20 ng/ml) or both. *$p < 0.05$ compared with the no-Noggin treatment or to cells not transfected with Dkk-1 or Smad7.

Quantitative RT-PCR analysis revealed that expression of the early osteoblastic differentiation markers Col1, osteonectin and ALP was up-regulated by BMP-4 plus Wnt3a treatment, whereas a marker of mature osteoblasts, osteocalcin, was not affected (Fig. 2B and data not shown). Up-regulation of ALP was reconfirmed by ALP staining (Fig. 2A). Additionally, BMP-4 and Wnt3a did not alter total protein levels (control: 194.0 ± 6.29 μg/well, BMP-4: 219.0 ± 8.47 μg/well, Wnt3a: 175.2 ± 13.52 μg/well and BMP-4+Wnt3a: 204.3 ± 16.28 μg/well). These data indicate that treatment with BMP-4 and/or Wnt3a had no effect on cell growth or cell mortality.

3.3. Wnt3a does not cross-talk directly with a BMP-specific receptor or Smad1

To elucidate the molecular mechanisms underlying the cooperative activity between Wnts and BMPs, we examined the effects of Wnt3a on BMP intracellular signaling. Wnt3a treatment failed to stimulate ALP activity induced by a constitutively active BMP type I receptor—ALK2(Q207D) or ALK2(R206H)—or the phosphorylation of Smad1/5/8, which is induced by BMP-4 (Figs. 3A, 2B and data not shown). Moreover, Wnt3a treatment failed to increase ALP activity induced by constitutively active Smad1 [Smad1(DVD)] (Nojima et al., 2010), even in the presence of Smad4 (Fig. 3C). Wnt3a treatment also failed to alter the transcriptional activity of BMP-specific Smads, which was evaluated using the IdWT4F-luc reporter (Fig. 3D).

The synergistic effects of Wnt3a and BMP-4 on the osteoblastic differentiation of C2C12 cells were suppressed by treatment with Noggin or by transfection with Dkk-1, which are specific extracellular inhibitors of BMPs and canonical Wnts, respectively (Figs. 4A and B). However, the overexpression of Smad7, an inhibitor of BMP type I receptor kinases, failed to reduce ALP activity that was induced by BMP-4 and Wnt3a (Fig. 4C).

3.4. GSK3β but not β-catenin plays an important role in the synergism between Wnt3a and BMP-4 during osteoblastic differentiation

To examine the effect of BMP on Wnt signaling, we performed luciferase assays. BMP-4 did not increase the activity of SuperTOPflash, which is a specific β-catenin reporter that is induced by Wnt3a (Fig. 5A). We next investigated the effect of β-catenin on osteoblastic differentiation using wild-type and constitutively active β-catenin (β-catenin C. A.). The activity of β-catenin C. A. was confirmed by luciferase assay (Fig. 5B). Overexpression of neither wild-type β-catenin nor β-catenin C. A. affected ALP activity in the presence of BMP-4; however, overexpression of GSK3β suppressed the ALP activity induced by Wnt3a together with BMP-4 (Fig. 5C). Moreover, treatment with BIO, a specific inhibitor of GSK3β (Yan et al., 2009) synergistically increased ALP activity in the presence of BMP-4 in C2C12 cells (Fig. 5D).

4. Discussion

In the present study, we investigated the synergism of Wnts and BMPs, which are important regulators of bone formation. We found that canonical Wnts (Wnt1 and Wnt3a) but not non-canonical Wnts (Wnt5a and Wnt11) stimulated osteoblastic differentiation induced by BMPs. These results were further supported by the finding that GSK3β appears to be involved in this synergism. Interestingly, however, activation of the β-catenin pathway did not show synergism with BMP signaling, suggesting that another substrate of GSK3β may be involved in this effect. Kugimiya et al. (2007) showed that heterozygous GSK3β-deficient mice displayed an increase in bone formation due to enhanced transcriptional activity of Runx2, a master transcription factor of osteoblastic differentiation. GSK3β inhibits DNA binding and transcriptional activities through phosphorylation of Runx2 at S369, S373 and S377 residues. It has been reported that Runx2 stimulated osteoblastic differentiation in cooperation with BMPs

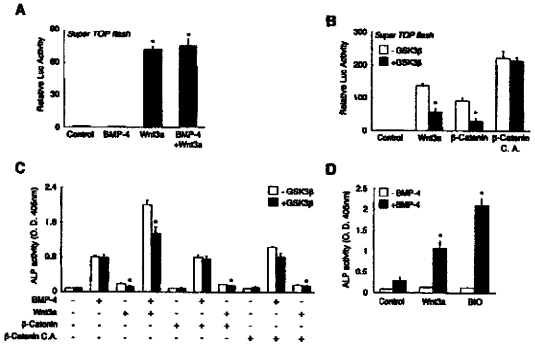

Fig. 5. GSK3β but not β-catenin is required for the synergism of Wnt3a and BMP-4. (A) Luciferase analysis of the effect of BMP-4 on Wnt signaling. C2C12 cells transfected with a SuperTOPflash reporter plasmid were treated with BMP-4 (100 ng/ml), Wnt3a (20 ng/ml) or both. After 24-h treatment, reporter activity was determined. (B) The activity of β-Catenin C. A. was confirmed by a luciferase reporter assay. C2C12 cells were transfected with a SuperTOPflash reporter and the indicated plasmids and then cultured for 24 h. (C) C2C12 cells transfected with GSK3β or empty vector and with wild-type or a constitutively active β-catenin plasmid were treated with BMP-4 (100 ng/ml), Wnt3a (20 ng/ml) or both and ALP activity was subsequently determined. (D) Effect of BIO on ALP activity. C2C12 cells were treated for 3 days with BMP-4 (100 ng/ml) plus Wnt3a (20 ng/ml) or BIO (5 μM). *$p < 0.05$ compared with the no-GSK3β transfection or no-BMP-4 treatment in each group.

(Lee et al., 2000; Phimphilai et al., 2006). Further studies will be required to examine the role of Runx2 and other novel substrates of GSK3β in the synergism of Wnts and BMPs.

In the present study, BMP-4 and Wnt3a were demonstrated to induce the expression of ALP, Col1 and osteonectin mRNAs, which are typical markers of early osteoblastic differentiation. In contrast, the expression of osteocalcin, a marker of late osteoblastic differentiation, was not affected. These data suggest that BMP-4 and Wnt3a synergistically induce early osteoblastic differentiation. BMPs induce osteoblastic differentiation in various types of mesenchymal cells, including C2C12 myoblasts. We and others have reported that the overexpression of a constitutively activated BMP type I receptor reproduces the induction of osteoblastic differentiation, and R-Smads may play critical roles in this event. However, in this study, Wnt3a did not cross-talk with either constitutively active BMP type I receptor or constitutively active Smad1. Moreover, Noggin but not Smad7 suppressed the synergism of Wnt3a and BMP-4. Taken together, these findings suggest that BMP ligands may activate a novel non-Smad intracellular signaling pathway during this synergism with canonical Wnts. Recent studies have suggested that BMP signaling may upregulate Wnt signaling to synergistically regulate osteoblast genesis (Chen et al., 2007; Mbalaviele et al., 2005), possibly through an autocrine or paracrine loop (Rawadi et al., 2003). These results are in contrast with our previous studies using a reporter assay showing that BMP failed to induce Wnt signaling, which indicated that BMP did not regulate β-catenin nuclearization or interaction with Tcf/Lef in C2C12 cells. This discrepancy may be due to the use of different cell lines or coercive culture conditions. BMP activates not only Smad-dependent signaling but also MAPK and PI3K to induce their respective intracellular signaling pathways (Iwasaki et al., 1999). Therefore, we cannot rule out the possibility that MAPK and/or PI3K signaling plays a role in this process. Further studies are essential for elucidating the molecular mechanisms of synergistic induction of osteoblastic differentiation in vitro and bone formation in vivo by BMPs and canonical Wnts.

In conclusion, we found that BMP and canonical Wnts cooperatively induce early osteoblastic differentiation through a GSK3β-dependent mechanism. This study could also help to elucidate the molecular mechanisms of cross-talk between BMP and the Wnt signaling pathway.

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進行性骨化性線維異形成症の発症メカニズム

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進行性骨化性線維異形成症の発症メカニズム

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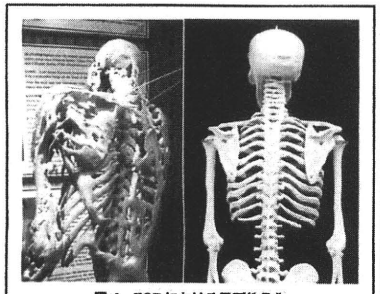

図1 FOPにおける異所性骨化
米国の博物館に保存されているFOPの骨格標本(左)と，人体骨格模型(右)．

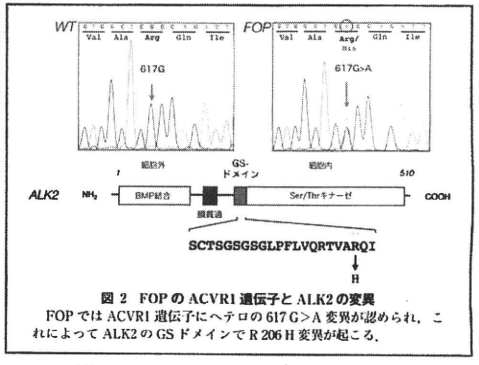

図2 FOPのACVR1遺伝子とALK2の変異
FOPではACVR1遺伝子にヘテロの617G>A変異が認められ，これによってALK2のGSドメインでR206H変異が起こる．

進行性骨化性線維異形成症(FOP)の特徴

進行性骨化性線維異形成症(Fibrodysplasia ossificans progressiva，FOP)は，骨格筋や靱帯，靭帯などの異所性骨化を特徴とする遺伝性疾患である(図1)[1~3]．筋組織における異所性骨化の進行は関節や複数の骨組織の癒合を招くため，咀嚼を含む全身の可動域が極度に制限される．FOPの異所性骨化は出生時にはほとんど認められず，成長に伴い徐々に進行する．現時点でFOPの異所性骨化を抑制する治療法は確立されていない．異所性に形成された骨組織も，生化学的に正常な骨組織と同等なため，異所性骨のみを既存の薬物で除去することは難しい．

FOPでは筋組織を損傷すると異所性骨化が急激に進行する．そのため，異所性骨を外科的に除去してもさらに異所性骨化を招く．新たな異所性骨化を誘発するため，FOPでは手術や生検などの筋組織に対する侵襲的医療行為は禁忌とされる．智歯(親知らず)の抜歯後，顎関節の骨化による開口障害を招いた症例がある．

FOPの発症頻度は世界的に約200万人に1人で，国内患者数は約60人と推定されている．遺伝形式は常染色体優性遺伝で，多くは孤発例であるが，少数の家族性FOPや一卵性双生児の症例が知られている．FOPの特徴として，出生時から認められる足の親指の外反母趾様変形があり，FOPの早期診断に極めて重要な表現形質である[1~3]．これは，FOPの異所性骨化を起こす原因が胎生期の指の発生にも関与することを示唆する．

FOPは2007年にわが国の難治性疾患克服研究事業の対象疾患(いわゆる難病)の一つに認定され，研究班が組織されている．難病認定以前から「埼玉医科大学FOP診療・研究プロジェクト」も組織されており，FOP専門研究組織として遺伝子診断などFOP全般の研究を行っている．

FOPにおけるALK2の変異

2006年，FOPの責任遺伝子としてACVR1遺伝子が同定され，617番目のグアニン(G)からアデニン(A)への変異が家族性FOPと孤発性FOPに共通することが判明した(図2)[4]．

ACVR1遺伝子によってコードされるALK2は膜貫通型セリンスレオニンキナーゼ受容体で，細胞外領域で骨形成を促すbone morphogenetic protein(BMP)を結合する(図2)[5]．BMPを結合したALK2は，細胞内領域のキナーゼが転写因子Smad 1/Smad 5/Smad 8などをリン酸化する．BMPの初期応答遺伝子のId 1の場合，遺伝子上流1 kbにあるGCに富む配列に，核内に移行したSmadが結合することで転写が誘導される．

FOPで見出されたACVR1遺伝子の617G>A変異は，ALK2の206番アルギニンをヒスチジンに置換する〔ALK2(R206H)〕(図2)[4]．この変異はBMP受容体のキナーゼ活性制御領域と考えられる「GSドメイン」に位置し，近傍の立体構造を変化させる[4]．これまでにALK2内で異なる部位のアミノ酸変異を伴う10種類の変異が同定された(図3)[6]．これは，FOPがALK2の機能変化に基づく疾患であり，ACVR1単一遺伝子の変異に起因することを示唆する．

FOPにおけるALK2の活性化

FOPで同定されたALK2(R206H)は，BMP非存在下でも，培養細胞で転写因子Smadのリン酸化や，BMP特異的初期応答遺伝子の発現などを誘導する(図3)[7,8]．筋芽細胞C2C12にALK2(R206H)を発現させると，BMP処理と同様に，筋分化の抑制と共に骨芽細胞様細胞への分化誘導が起こる[7]．また，ニワトリ肢芽由来の細胞では，ALK2(R206H)の過剰発現により軟骨細胞の分化が

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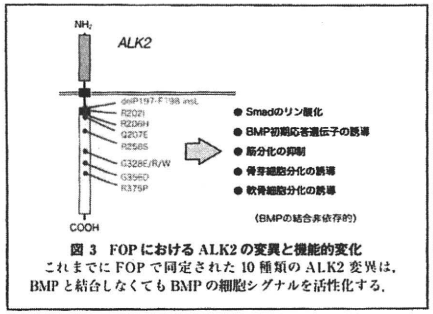

図 3　FOP における ALK2 の変異と機能的変化
これまでに FOP で同定された 10 種類の ALK2 変異は，BMP と結合しなくても BMP の細胞シグナルを活性化する．

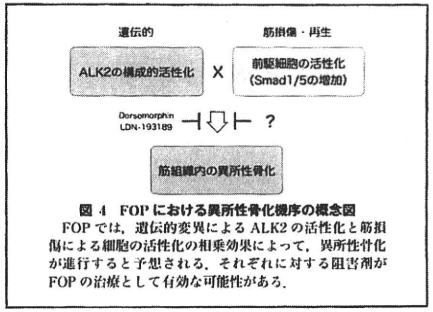

図 4　FOP における異所性骨化機序の概念図
FOP では，遺伝的変異による ALK2 の活性化と筋損傷による細胞の活性化の相乗効果によって，異所性骨化が進行すると予想される．それぞれに対する阻害剤が FOP の治療として有効な可能性がある．

亢進する[8]．これらの結果は，ALK2(R 206 H)がリガンド非依存的に活性化された機能獲得型変異体であり，この細胞内シグナルによって FOP の異所性骨形成が誘導されることを示唆する(図 3)．

筋再生による BMP シグナルの亢進

前述のように，FOP の異所性骨化は筋組織の損傷によって急激に誘発される．ALK2(R 206 H)は構成的活性型受容体であるものの，in vitro のキナーゼ活性が低いことを考え合わせると，筋損傷・再生の一連の過程が ALK2(R 206 H)による BMP シグナルを相乗的に促進する可能性が考えられる．

マウスの筋再生モデルを解析したところ，ALK2 を含む BMP 受容体の発現量に大きな変化はないが，下流の Smad 1 と Smad 5 の発現量が増加した[7]．ALK2 と Smad 1 または Smad 5 を同時に筋芽細胞で発現させると，野生型 ALK2 では変化はないが，ALK2 (R 206 H)は Smad 1/5 のリン酸化を誘導し，骨芽細胞様細胞への分化を相乗的に誘導した[7]．Smad 1 や Smad 5 の発現が亢進した細胞は単核細胞で，最近，異所性骨化における軟骨細胞や骨芽細胞の前駆細胞が，血管周囲の細胞である可能性が報告されている[7,9]．

これらの結果より，遺伝的な変異による ALK2 の活性化と，筋損傷・再生による前駆細胞の活性化(転写因子 Smad 1/5 の増加)，という 2 つの要因の相乗効果によって BMP の細胞内シグナルが亢進し，FOP の異所性骨化が進行すると予想される(図 4)．日常生活での筋組織の代謝も，個々の細胞レベルで損傷・再生を誘発している可能性がある．

発症機序の解析に基づく FOP 治療法の確立

FOP では，遺伝的変異により ALK2 が活性化されていることが発症機序の中心をなすと考えられる．そこで，ALK2 の活性を阻害する化合物が FOP 治療薬として期待されている(図 4)．Dorsomorphin と命名された低分子化合物は，ALK2 を含む BMP 受容体を阻害する活性物質として発見された．最近，さらに活性の高い Dorsomorphin 誘導体(LDN-193189)が開発され，ALK2 変異体による筋組織における異所性骨形成を抑制することが確認された[10]．

FOP の発症機序に 2 つの要因が関与することを考えると，今後，筋損傷を標的とした全く新しい治療法が確立されることも期待される(図 4)．

むすび

FOP が単一遺伝子の変異による疾患で，そこに筋損傷が起こると発症することが明らかとなってきた．これらの発見により遺伝子診断の有効性が確立され，論理的な治療法確立に向けた研究が可能となりつつある．今後の研究により，早期に FOP が克服されることが期待される．

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進行性骨化性線維異形成症（FOP）の病態と新規治療法の開発

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進行性骨化性線維異形成症（FOP）の病態と新規治療法の開発

片桐　岳信*

進行性骨化性線維異形成症（fibrodysplasia ossificans progressiva：FOP）は，骨格筋などで異所性骨化が起こる遺伝性疾患である。FOP患者から，bone morphogenetic proteinの受容体ALK2に変異が同定された。この発見により，FOPの病態モデルマウスの樹立や，新しい治療法の確立に向けた研究が進行している。

The genetic basis for skeletal disease.

Establishment of novel treatments for fibrodysplasia ossificans progressiva (FOP).

Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University.

Takenobu Katagiri

Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder characterized by progressive heterotopic bone formation in skeletal muscle tissue. In patients with FOP, heterozygous mutations have been identified in the BMP type I receptor, ALK2. This finding stimulates establishment of some animal models of FOP and development of novel treatments for FOP.

はじめに

骨形成は既存の骨組織が成長する「骨伝導」と，新たに骨が形成される「骨誘導」に分類することができる。多くの骨疾患は，既存の骨組織において量や形が変化するものであるが，まれに本来骨組織のない組織に異所性の骨形成が認められる疾患がある。進行性骨化性線維異形成症（fibrodysplasia ossificans progressiva：FOP）は後者に属す遺伝性疾患の一種で，成長に伴って全身の骨格筋を中心に靭帯や腱で異所性骨化が進行する。

筋組織での異所性骨形成は，Bone morphogenetic protein（BMP）の移植によって誘導できる

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ことが半世紀以上前から知られていた。このため，FOPはBMPのシグナル異常に起因すると予想されてきたが，症例数が極めて少ないために責任遺伝子の同定はなかなか進まなかった。しかし，2006年，FOPの責任遺伝子としてBMP受容体の1種が同定されたことにより，FOPに関する研究は大きな転機を迎えた。現在は，FOPの発症機序に関する分子レベルの解明が進みつつあると同時に，新しいFOP病態モデルの樹立や論理的な新しい治療法の確立が進んでいる。

FOPの遺伝

FOP（OMIM # 135100）は，骨格筋を主として腱，靭帯などで異所性骨化が起こる遺伝性疾患である[1)~3)]。一般には「筋組織が骨に変わる」とも表現されるが，正確には筋組織内における異所性骨組織の形成である。

FOPの発症率は極めて低く，世界的に約200万人に1人と推定されている[1)~3)]。わが国では，約60名のFOP患者がいると考えられるが，正確な患者数は把握されていない。FOPの遺伝様式は常染色体優性遺伝で，これまでに報告されたFOP症例の多くは孤発例である[1) 2)]。世界的には，少数の家族性FOPや一卵性双生児の症例が見出されている。後述するように，家族性FOPの解析から，FOPの責任遺伝子としてACVR1/ALK2が同定された。

BMPシグナルとFOP

筋組織内で異所性骨形成を誘導する因子として，BMPが知られている[2)]。この異所性骨誘導活性はBMPに特徴的なため，FOPにおける異所性骨化には，BMPシグナルの遺伝的変異が関与すると予想された。

BMPの骨誘導作用は，標的細胞の細胞膜上に存在する2種類のセリン・トレオニンキナーゼ活性をもつBMP受容体により伝達される（図1）[2)]。BMPを結合したⅡ型受容体はⅠ型受容体を活性化し，Ⅰ型受容体は転写因子Smad1/5/8をリン酸化によって活性化する。活性化されたSmad1/5/8は，Smad4と複合体を形成して核内へ移行し，さまざまな標的遺伝子の転写を制御する。

これまでに，FOPの責任遺伝子としていくつかのBMPシグナル関連因子が報告された（表1）。1996年，FOP由来のリンパ球で，初めてBMP-4の過剰発現が報告された[4)]。しかし，BMP-4の発現はBMPシグナルで亢進することから，二次的な影響である可能性もある。また，2000年には，FOPの連鎖解析からSmad1の遺伝子座を含む領域（4q27-q31）との関連が示唆されたが[5)]，後に同グループによって否定された。

Nogginは，細胞外でBMPと受容体の結合を阻害するアンタゴニストの1種である。1999年以降，フランスの研究グループが，複数のFOP症例でNoggin遺伝子の欠失や点変異を報告している[6)]。しかし，Nogginの欠失が報告された同一患者をアメリカの研究グループが解析した結果，Nogginの変異は確認されず，代わりに次に述べるACVR1遺伝子の変異が同定された[7)]。

2006年，家族性FOPの解析から，BMP受容体の一種であるALK2をコードするACVR1遺伝子のヘテロ接合変異（617G>A）が同定された[7)]。このACVR1/ALK2の変異は，わが国の30症例以上を含む世界的な孤発性FOP症例でも確認された[8) 9)]。その後の多くの研究から，今日ではACVR1/ALK2がFOPの責任遺伝子と考えられている。

FOPにおけるALK2の変異

FOPで同定されたACVR1遺伝子の617G>A変異は，ALK2タンパク質の細胞内GSドメインに位置する206番目のアルギニン残基（R）をヒスチジン（H）に変異させる（図2）[7)]。この領域は，Ⅰ型受容体のキナーゼ活性をリガンド依存的に制御するスイッチの役割を果たすことで知られている。

このスイッチ機能には，免疫抑制剤FK506の結合タンパク質として知られるFKBP12が重要な役割を果たすと考えられている（図2）[10)]。FKBP12は，リガンドを結合していないⅠ型受容体のGSドメインに結合し，キナーゼ活性を不活性な状態に保つ。受容体がリガンドを結合すると，FKBP12がGSドメインから解離してキナーゼが活性化される。

コンピュータ・シミュレーションによると，FOPで同定されたALK2（R206H）変異体では，アミノ酸置換によってGSドメインの立体構造が変化する[7)]。生化学的な実験からも，ALK2（R206H）はFKBP12との親和性が低下し，BMPを結合した

BMP：Bone morphogenetic protein，FOP：fibrodysplasia ossificans progressiva（進行性骨化性線維異形成症）

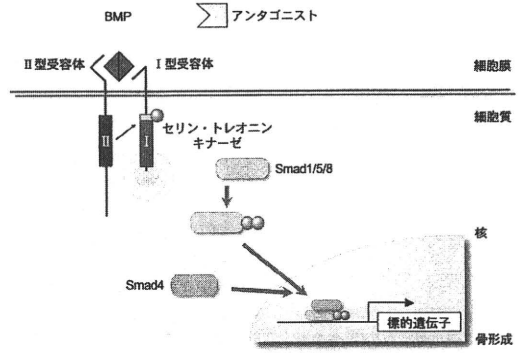

図1　BMPの細胞内シグナル伝達機構

BMPは標的細胞の2種類の受容体複合体に結合し，Smadを中心とした細胞内情報伝達系を活性化することで，最終的に骨形成を誘導する。BMPと受容体の結合は，アンタゴニストによって阻害される。

（筆者作成）

表1　FOPの原因候補として報告されたBMPシグナル関連因子

これまでに，複数のBMPシグナル関連因子がFOPの原因として報告されたが，現在はALK2が最も有力である。

分類	因子	遺伝子座	文献
リガンド	BMP-4	14q22-q24	Shafritz et al. (1996)
アンタゴニスト	Noggin	17q22	Luccotte et al. (1999)
Smad	Smad1	4q28	Feldman et al. (2000)
Ⅰ型受容体	ALK2	2q23-q24	Shore et al. (2006)

（筆者作成）

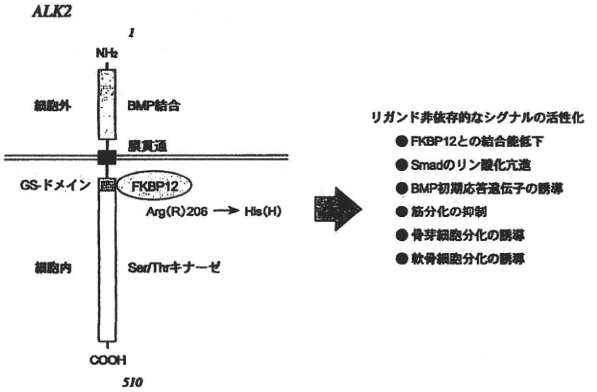

図2　ALK2の構造とFOPにおける変異

FOPで最初に見出されたALK2の変異は，細胞内のGSドメインにおけるR206H変異であった。BMP-4を結合していない受容体のGSドメインには，FKBP12が結合してキナーゼ活性を抑制する。R206H変異によりFKBP12との親和性が低下し，ALK2はリガンド非依存的に種々の細胞内シグナルを構成的に活性化する。

（筆者作成）

野生型ALK2と同様な細胞内局在を示すことが報告されている[10][12]。ALK2（R206H）を培養細胞などに過剰発現させると，BMP非依存的に種々のBMPシグナルが活性化される（図2）[9][11][12]。従って，FOPにおける異所性骨化は，FKBP12との解離によって構成的活性型BMP受容体となったALK2変異体が，常にBMPシグナルを伝達し続けることにより誘導されると考えられる。

最近，臨床症状が少し異なるFOP症例から，R206Hとは異なるALK2の変異が相次いで同定されている[13][14]。これまでに10種類のALK2変異体が見出されており，これらはGSドメインに限らず，キナーゼ領域内にも分布する[13][14]。FOPがすべてALK2の変異に基づくという発見は，FOPが単一遺伝子の疾患であることを示し，同時にALK2の遺伝子診断が有効であることを意味する。

FOPと筋再生

FOPでは，筋損傷・再生が異所性骨形成を誘発する。筋組織の再生は，線維間に存在する単核の前駆細胞が増殖・分化・融合によって起こることが知られている（図3）[15]。この筋組織に存在する幹細胞として，筋衛星細胞が重要な役割を果たす[15]。BMP刺激によって骨芽細胞様細胞に分化するC2C12細胞は，筋衛星細胞由来の株細胞と考えられている。これは，筋損傷によって活性化された幹細胞が，BMPシグナルによって骨芽細胞や軟骨細胞に分化誘導される可能性を示す（図3）。最近，Tie2を発現する細胞が，BMP誘導性の異

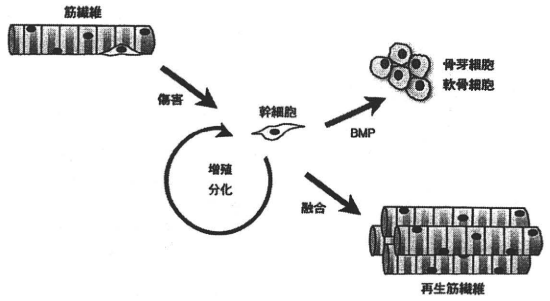

図3　筋組織の幹細胞による筋再生と異所性骨形成

筋の再生をつかさどる幹細胞は，BMPシグナルによって骨芽細胞や軟骨細胞に分化する可能性がある。

（筆者作成）

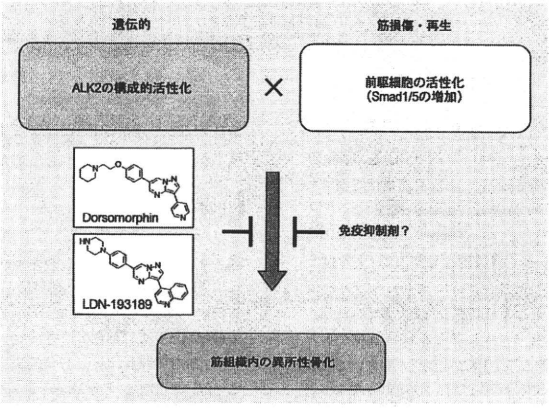

図4　FOPにおける異所性骨化機序の概念図

FOPでは，遺伝的変異によるALK2の活性化と，筋損傷による細胞の活性化の協調作用によって異所性骨化が進行すると予想される。それぞれの過程に対する阻害因子が，FOPの治療に有効な可能性がある。

（筆者作成）

所性骨化の前駆細胞であることが報告された[16]。

マウスの筋再生モデルを調べると，再生過程で一過性にSmad1とSmad5の発現が亢進した[9]。さらに，筋芽細胞にSmad1やSmad5とALK2（R206H）を共発現させると骨芽細胞分化が亢進した[9]。これらの結果から，FOPでは遺伝的に活性化されたALK2（R206H）と，筋損傷・再生に伴って増加したSmad1/5が協調的に作用することで，局所的な骨形成が進行する可能性が考えられる（図4）。

FOPの病態モデル

現在，FOPの病態モデルとして，いくつかの遺伝子改変マウスが用いられている（表2）。

そのひとつは，ニューロン特異的エノラーゼプロモーターの下流でBMP-4を発現させるトランスジェニック（Tg）マウスである[17]。興味深いことに，このマウスで筋損傷・再生を促すと，高頻度に異所性骨形成が起こるという。

FOPでは見出されていない変異であるが，構成的活性型となるALK2のQ207D変異をもつ誘導型floxedマウスが樹立されている。本マウスは，未刺激の状態ではALK2変異体を発現しないものの，Cre酵素を発現するアデノウイルスの局所投与でALK2変異体の誘導が起こり，約30日で異所性骨形成が誘導される[18]。本マウスでも，アデノウイルスが惹起する筋組織での炎症反応が異所性骨誘導に重要な可能性が示されている。

このほかに，FOPのR206Hを組み込んだALK2のノックインマウスが作製されつつある[19]。本マウスでは，FOP症例と同様に母指の形成不全が認められており，指の発生過程でALK2が重要なことが示された。しかし，何らかの理由でこのマウスの表現形は子孫に遺伝せず，現時点で安定的なALK2（R206H）マウスは樹立されていない。

発症機序の解析に基づくFOP治療法の確立

FOPの責任遺伝子の同定に基づく発症機序の解析から，活性化されたALK2の阻害因子がFOPに有効な治療薬として期待されている（図4）。これまでに，Dorsomorphin，およびさらに比活性の高い誘導体LDN-193189が開発されている。LDN-193189は，前記のALK2（Q207D）マウスの異所性骨形成モデルにおいて，筋組織における異所性骨形成を抑制することが確認された[18]。

FOPの1症例で，骨髄移植に伴い免疫抑制剤を投与された症例において，14年間もの間，異所性骨化が抑制されたことが報告された[20]。この機序は不明であるが，免疫抑制剤が筋損傷・再生の過程等に影響した可能性も考えられる。FOPの異所性骨化には，遺伝的ALK2の活性化と筋再生の過程が関わると予想されることから，今後，筋再生の過程を標的とした全く新しい治療法が確立されることも期待される（図4）。

表2　FOPの病態モデルマウス

現在，FOPの病態モデルとして，いくつかの遺伝子改変マウスが用いられている。

モデル	特徴	文献
BMP-4 Tg	・ニューロン特異的エノラーゼ（ENOS2）プロモーターによるBMP-4 Tgマウス ・筋再生による異所性骨化の誘導	Kan et al. (2004)
ALK2 (Q207D) Tg	・誘導型ALK2 (Q207D) floxed Tgマウス ・Cre-アデノウイルスの局所投与による異所性骨化の誘導	Yu et al. (2008)
ALK2 (R206H) KI	・ALK2 (R206H) のノックインマウス ・母指の形成不全	Chakkalkal et al. (2008)

（筆者作成）

おわりに

FOPは，長い間BMPシグナルの異常が予想されながら，症例数が少ないために責任遺伝子の同定が困難であった。しかし，ALK2の遺伝的変異が明らかとなったことで，発症機序が少しずつ解明されつつある。さらに，発症機序の解明に基づいて，論理的な治療法や診断法の確立も進んでいる。今後のさらなる研究により，早期にFOPの克服が期待される。

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BMP シグナルと進行性骨化性線維異形成症

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特集

BMP シグナルと進行性骨化性線維異形成症

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進行性骨化性線維異形成症 (fibrodysplasia ossificans progressiva : FOP) は，骨格筋などの異所性骨化を伴う遺伝性疾患である。2007 年，FOP がわが国の難治性疾患克服研究事業対象疾患のひとつとして認定され，研究班による FOP の発症機序の解明や治療法の確立に向けた研究が開始された。これにより，FOP の克服に向けた取り組みが進みつつある。

Cytokines in bone diseases.

BMP signaling and fibrodysplasia ossificans progressiva.

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Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder characterized by progressive heterotopic bone formation in skeletal muscle tissue. In 2007, FOP was authorized as one of the Intractable Disorders by the Ministry of Health, Labour and Welfare of Japan. The Research Committee on FOP is working on the molecular mechanisms underlying heterotopic bone formation and the development of new treatments for FOP.

はじめに

我々の身体は，筋と骨格を使いさまざまな運動を可能にしている。日常の運動にも，しなやかな筋組織となめらかな関節の協調的な連携が重要な役割を果たす。

進行性骨化性線維異形成症（fibrodysplasia ossificans progressiva, FOP;OMIM＃135100）は，骨格筋を主として腱，靭帯などにおいて，成長に伴い異所性骨化が起こる疾患である。形成される異所性骨組織により身体が変形したり，顎関節を含めた関節の可動域が極度に制限されるため，さまざまな運動が困難となる。FOPの発症頻度は200万人に1人と低い上に，骨化を誘発するため病変部の侵襲的な解析が禁忌であるなど，FOPの研究は多くの困難を伴った。

しかし，ここ数年の間にFOPが厚生労働省の難治性疾患克服研究事業対象疾患のひとつとして認定され，責任遺伝子が同定されたことにより，FOP研究は急速に進展しつつある。

FOPにおける異所性骨化

FOPは小児期頃から筋組織において異所性骨化が起こる疾患で[1~3]，関節が癒合したり，異所性骨が既存の骨組織と癒合することにより，脊柱弯曲症など骨格の変形を引き起こす（**図1**）。肩甲骨近傍はFOPで早期から異所性骨化が起こる部位のひとつであり，骨化が上肢帯の成長を妨げて肩幅が狭くなったり，上腕の運動を制限して「万歳」の所作が困難となる。異所性骨化が顎骨周囲に生じると，開口障害を示す。FOPの異所性骨化は，一般に体幹部から末梢へ，上部から下部へ，背部から腹部へと進行する傾向がある。異所性に形成された骨組織は，正常な骨組織と同様に骨髄を伴って骨リモデリングを受ける。従って，形成された異所性骨のみを薬剤等で除去することは難しい。

FOPでは，筋組織の損傷が異所性骨化を誘発することが知られており，手術や生検，筋肉内注射など侵襲的医療行為は禁忌とされる。筋再生に関与する組織特異的な幹細胞が，FOPの異所性骨化に関与する可能性がある。しかし，病変部の組織学的知見は極めて乏しく，特に発症初期に関与する細胞群などに関してはほとんど明らかにされていない。しばしば，FOPは「筋肉が骨に変わる」とも表現されるが，筋繊維が骨組織に変化するのではなく，主に内軟骨性骨化により筋組織で新たな骨組織の形成が進行する。

歯科治療時の無理な開口や，特殊な局所麻酔も異所性骨化を誘発するとされ，十分な注意が必要である。通常の歯肉・歯根膜への局所麻酔は比較的安全とされるが，開口障害の著しい場合，多数歯の抜歯が必要な場合は，全身麻酔下での抜歯がむしろ安全なことがある[4]。

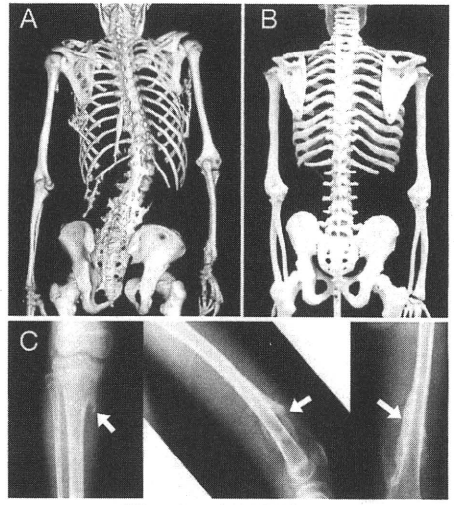

図1　FOPにおける異所性骨化

脊柱弯曲症を伴うFOP症例における左右肩甲骨および脊柱周囲に生じた異所性骨化（A）と人体骨格模型（B）。（C）FOP患者の下肢に生じた異所性骨化。骨端部の外骨腫形成は，FOP症例の多くに認められる。
（筆者ら提供）

異所性骨化とBMP

酸で脱灰した骨基質を筋組織内に移植すると，FOPと同様な異所性骨化を誘導できることが知られていた（**図2A**）。この活性本体として発見されたサイトカインが，Transforming Growth Factor-β（**TGF-β**）ファミリーのBone Morphogenetic Protein（**BMP**）で，相同性が高い15種類以上のBMPが存在する[2]。BMPは培養細胞でも骨芽細胞や軟骨細胞の分化誘導活性を示す。

BMP：Bone Morphogenetic Protein，TGF-β：Transforming Growth Factor-β

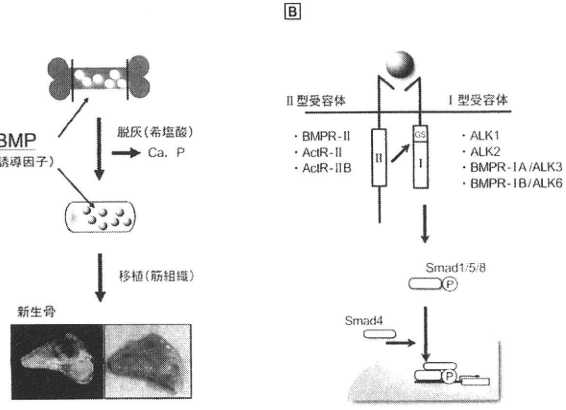

図2　BMPによる異所性骨誘導とBMPの細胞内シグナル伝達

(A)BMPは骨基質に含まれるサイトカインで，脱灰した骨基質を筋組織に移植すると異所性骨化を誘導する。

(B) BMPは標的細胞の2種類の受容体に結合し，Smadを中心とした細胞内情報伝達系を活性化して骨形成を誘導する。FOPの変異は，Ⅰ型受容体に分類されるALK2のGSドメインに同定された。

BMP：bone morphogenetic protein

(筆者ら作成)

今日，BMPは実験的な異所性骨化を誘導するのみならず，個体発生や組織再生の生理的な条件下で骨形成を促す重要なサイトカインと考えられている。

TGF-βファミリーの細胞内シグナルは，標的細胞の膜上でⅠ型およびⅡ型のセリン・スレオニンキナーゼ受容体との結合で伝達される（図2B）[2]。リガンドを結合したⅡ型受容体がⅠ型受容体の「GSドメイン」と呼ばれる領域をリン酸化すると，Ⅰ型受容体の立体構造が変化してキナーゼが活性化される。GSドメイン内を酸性アミノ酸と置換すると，リガンドと結合しなくとも細胞内シグナルを活性化できることから，このGSドメインがⅠ型受容体のスイッチとして機能すると考えられている[3]。

BMPのⅠ型受容体の下流では，転写因子Smad1/5/8がリン酸化を受ける（図2B）[2]。Smad1のリン酸化部位であるC末端を酸性アミノ酸に置換すると，リン酸化状態と類似した立体構造を取って構成的に標的遺伝子を活性化する[4]。最近，この構成的活性型Smad1が骨芽細胞様の分化を誘導することが明らかとなった[5]。これらの結果は，BMPのⅠ型受容体からSmad1/5/8へのシグナル伝達が骨形成に重要な経路であることを示す。一方，BMPによる筋分化の抑制作用には，Smad1と複合体を形成するSmad4がより重要らしい[6]。

FOPにおける責任遺伝子の同定

FOPは一卵性双生児の発症例や，まれな家族性症例の解析から，常染色体優性遺伝を示す遺伝性疾患であると考えられていた[1~3]。FOP責任遺伝子探索の結果，2006年，5例の家族性FOPの解析から，BMPのⅠ型受容体ALK2をコードする*ACVR1*遺伝子内に変異が同定された（図3）[7]。健常者では*ACVR1/ALK2*遺伝子の第4エクソンに位置する617番目の塩基がGであるのに対し，FOP症例ではGとAのヘテロ接合体である[7]。この変異は，ALK2タンパク質の206番ArgをHisに置換（R206H）する（図3）[7]。遺伝子レベルでも同一のR206H変異は，わが国の40症例以上を含めて，世界的な孤発性FOP症例でも確認された[7~9]。

最近，臨床症状が従来のFOPと異なるまれなFOP症例から，さらに9種類の異なる変異が*ACVR1/ALK2*遺伝子に同定されている（図3）[10~13]。このように，FOP症例における全ての遺伝子変異がひとつの遺伝子に収束することは，FOPが単一遺伝子疾患であることを示唆する。FOPの確定診断には遺伝子診断が有効で，異所性骨化発症前でも，*ACVR1/ALK2*遺伝子の617G＞A（R206H）の有無を簡便に検査できる。*ACVR1/ALK2*遺伝子はヒト2番染色体上に位置することから[7]，FOPは性別によらず50%の確率で子に遺伝する可能性がある。最近，わが国における家族性FOPが報告された[14]。

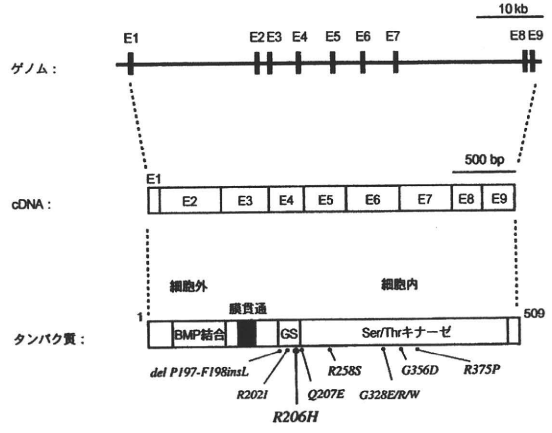

図3　*ACVR1/ALK2*遺伝子の構造とFOPにおける変異

*ACVR1/ALK2*遺伝子は，ゲノム上で9つのエクソンから構成される。FOPで同定されたcDNAの617G＞A変異は，GSドメインをコードする第4エクソンに含まれる。ALK2（R206H）は，構成的活性型受容体となる。最近，新しい変異がACVR1/ALK2に見出されている。

(筆者ら作成)

FOP における ALK2 の活性化

FOP で発見された ALK2 の R206H 変異は，受容体のスイッチとして機能する「GS ドメイン」内に位置する（**図3**）[7]。培養細胞に変異型 ALK2 (R206H) を過剰発現させると，BMP 非存在下でも Smad1/5/8 のリン酸化が亢進し，骨芽細胞分化や軟骨細胞分化の誘導が認められる[9,15,16]。ゼブラフィッシュで変異型受容体を過剰発現させた場合にも，BMP 刺激と同様のシグナルを伝達した[15]。従って，FOP の ALK2 (R206H) は，TGF-β ファミリーで初めて同定された I 型受容体の機能獲得型変異であり，受容体の構成的活性化が異所性骨形成の主たる原因と予想される。しかし，FOP でも出生時には異所性骨化が認められない点や，FOP の異所性骨化が筋損傷による誘導性である点などは，さらに複雑な機構が関与する可能性を示唆する。また，FOP で見出された R206H 以外の変異受容体に関しても，臨床症状の差が生じる機序等について，さらに検討する必要がある。

FOP の外反母趾様変形とフレアアップ

出生時の FOP では，筋組織における異所性骨化は認められないが，多くの症例で左右対称性に外反母趾様の変形が観察される（**図 4A**）[1~3]。最近のわが国の FOP 16 症例を対象とした研究によると，検討した 93.5% (31 足中 29 足) の母趾に何らかの形成不全が認められ，1 症例だけは左右の足趾に異常は認められなかったという[17]。

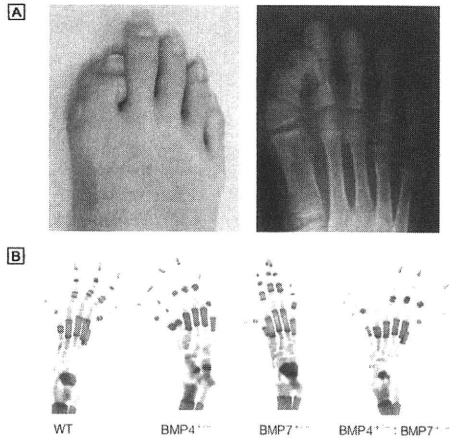

図4　FOP における外反母趾様変形と，*BMP* 遺伝子変異マウスにおける多指症

（A）FOP で出生時から認められる外反母趾様変形。
（B）野生型 (WT)，BMP4，BMP7，および BMP4 : BMP7 ヘテロ接合マウスの新生児骨格標本

（筆者ら提供）

BMP は個体発生時の体軸形成にも重要なサイトカインである。特に，発生初期には指の原基に BMP や受容体の発現が見られ，BMP や受容体遺伝子の機能喪失型変異が多指症や短指症など指の形成不全を招くことが知られている（**図 4B**）[18]。BMP 受容体の機能獲得型変異と考えられる FOP では，変異 ALK2 から BMP シグナルが過剰に伝達された結果，外反母趾様の変形が生じると予想される。

ほかに，FOP における異所性骨化前の特徴として，皮下軟部組織におけるフレアアップと呼ばれる腫瘤形成が知られている[1~3]。腫瘤は数日から数週間かけて形成と消退を繰り返す。初期の腫瘤内に軟骨組織や骨組織はなく，盛んに増殖する線維芽細胞が認められることが，FOP ががんと診断される一因となる。フレアアップと BMP シグナルの関連は明らかでない。

従来，診断法が確立されていなかったこともあり，FOP の確定診断が遅れた結果，医療行為や日常生活によって異所性骨化を招いた症例がある。現在は遺伝子診断が可能であることから，外反母趾様変形とフレアアップが乳児や小児に認められた場合，早期に FOP の可能性を検討することが望まれる。

FOP 治療法の開発に向けて

FOP が活性化された ALK2 によると予想されることから，FOP に有効な BMP 受容体阻害分子として，Dorsomorphin の誘導体 LDN-193189 が期待されている[9,19]。LDN-193189 は，変異 ALK2 マウスの異所性骨形成モデルで，筋組織における異所性骨化を抑制した[19]。

これとは別に，ヒト FOP 症例で免疫抑制剤が骨化を抑制した可能性が報告されている。それによると，再生不良性貧血による骨髄移植に伴い 14 年間免疫抑制剤を投与された FOP 症例で，この期間は異所性骨化が抑制されたという[20]。投与中止後に異所性骨化が再発したことから，骨髄移植ではなく，免疫抑制剤が異所性骨化を抑制した可能性が考えられる。この作用機序は不明で，骨髄移植を行っていないほかの FOP 症例でも骨化を抑制するか否か明らかでないが，FOP に関する新しい情報を提供する可能性がある。

おわりに

FOP は，責任遺伝子が同定されるまで，異所性骨化の発症機序が説明できない疾患であった。しかし，ALK2 の構成的活性型変異が見出されたことで，骨化の一部を説明できるようになり，この発見に基づく BMP 受容体阻害剤のような新しい治療法の開発も始まった。今後のさらなる組織的な研究により，一日も早く FOP が克服されることが期待される。

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