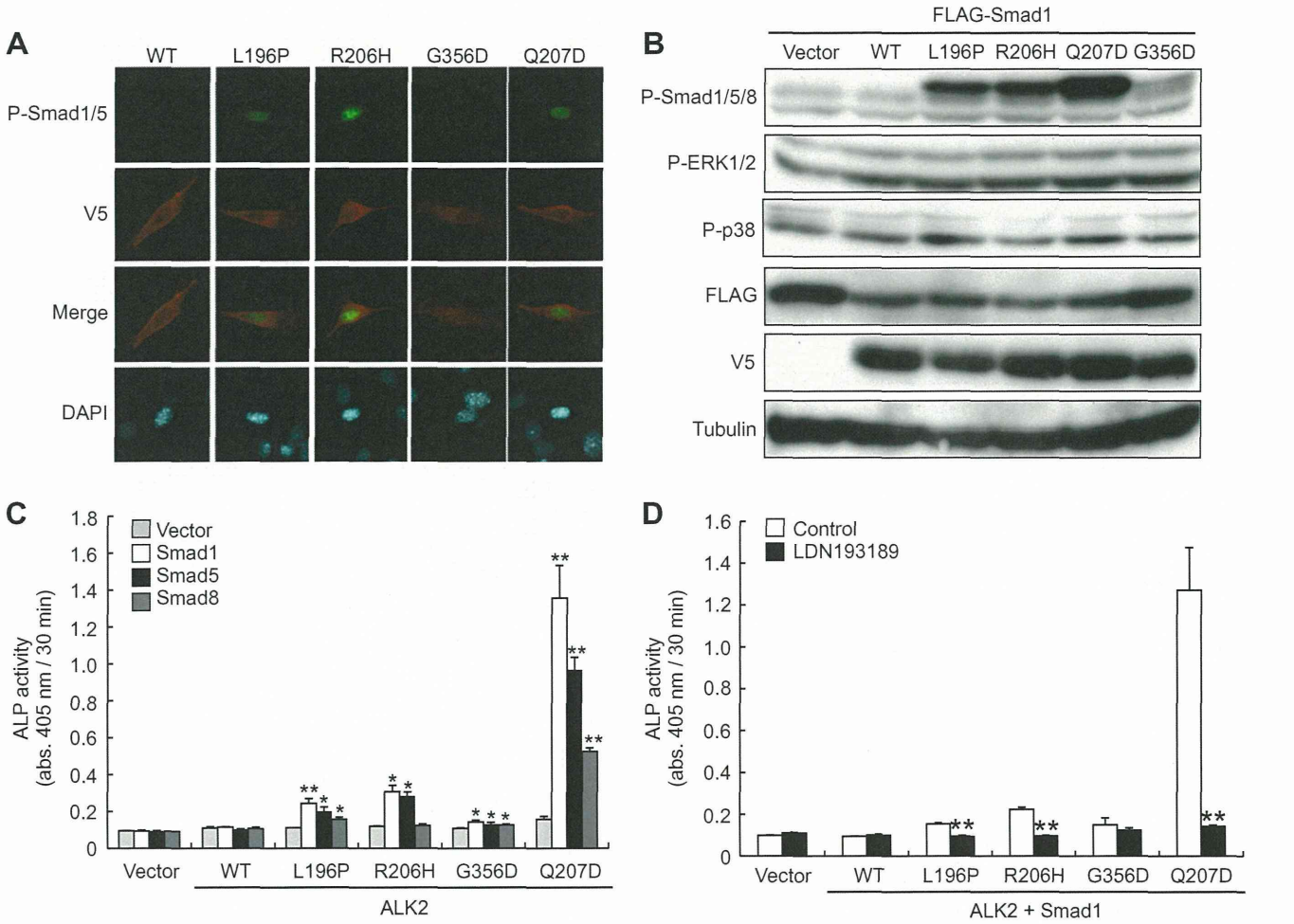

Fig. 2. ALK2(L196P) induces the Smad1/5-dependent pathway but not the non-Smad- or the Smad2/3-dependent pathways. (A) Immunohistochemical analysis of endogenous phospho-Smad1/5. C2C12 cells transfected with one of the V5-tagged ALK2 plasmids indicated were double-stained with anti-phospho-Smad1/5 (green) and anti-V5 (red) antibodies. (B) Western blot analysis of intracellular signaling pathways activated by ALK2 receptors. C2C12 cells were co-transfected with FLAG-tagged Smad1 and one of the V5-tagged ALK2 constructs. Cell lysates were immunoblotted with antibodies against phospho-Smad1/5/8, phospho-Erk1/2, phospho-p38, FLAG-tag, V5-tag and a-tubulin. (C) ALP activity induced by the cooperation of ALK2 and Smad1/5/8. C2C12 cells were co-transfected with one of the ALK2 plasmids as indicated and FLAG-tagged Smad1, Smad5 or Smad8 and cultured for three days in the absence of BMPs. The results are presented as the means ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison with cells transfected with an empty vector in each group. (D) Activation of the BMP-regulated Smads is involved in osteoblastic differentiation induced by ALK2. C2C12 cells were co-transfected with one of the ALK2 plasmids as indicated and FLAG-tagged Smad1 and cultured for three days with or without 100 nM LDN-193189. Results are presented as the means ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with controls.

3.3. *Effects of protein and chemical inhibitors on mutant ALK2 activities*

To examine the molecular mechanisms of the mild phenotypes of the FOP patient with activated ALK2(L196P), we examined the sensitivity of ALK2(L196P) to protein inhibitors and compared it to that of ALK2(R206H). Co-expression of Smad6, Smad7 or FKBP-12 dose-dependently suppressed the IdWT4F-luc activity induced by ALK2(L196P) or ALK2(R206H). The IC_{50} values of inhibitors against ALK2(L196P) were higher than those of ALK2(R206H) (Fig. 3A–C). The IdWT4F-luc induced by ALK2(L196P) or ALK2(R206H) was dose-dependently suppressed by the chemical inhibitor LDN-193189 (Fig. 3D). However, ALK2(L196P) was slightly more resistant than ALK2(R206H) to LDN-193189 (Fig. 3D).

4. Discussion

In the present study, we examined the molecular mechanisms of a novel ALK2 mutation, ALK2(L196P), found in the most benign case of FOP reported in the literature thus far [14]. We speculated that ALK2(L196P) was a more weakly activating mutation than other ALK2 mutations found in typical and atypical FOP patients because the patient with this mutation did not have toe malformations at birth and had delayed induction of heterotopic ossification in skeletal muscle [14]. Unexpectedly, however, ALK2(L196P) showed higher activity than ALK2(G356D) and equivalent activity to ALK2(R206H), a typical FOP variant mutation, suggesting that ALK2(L196P) activity may be suppressed by a novel mechanism in this patient. To examine this possibility, we compared the sensitivity of ALK2(L196P) receptors to different protein inhibitors, including FKBP12, that have been shown to be involved in the activation of ALK2 in FOP [20]. However, ALK2(L196P) was more resistant rather than more sensitive to Smad6 and FKBP12 than was ALK2(R206H). Thus, a novel inhibitor may be involved in the regulation of ALK2(L196P) *in vivo*. It is possible that such an inhibitory mechanism of ALK2 activity will aid in the establishment of novel therapeutic strategies for FOP patients with other ALK2 mutations. Further studies will be required to examine this possibility.

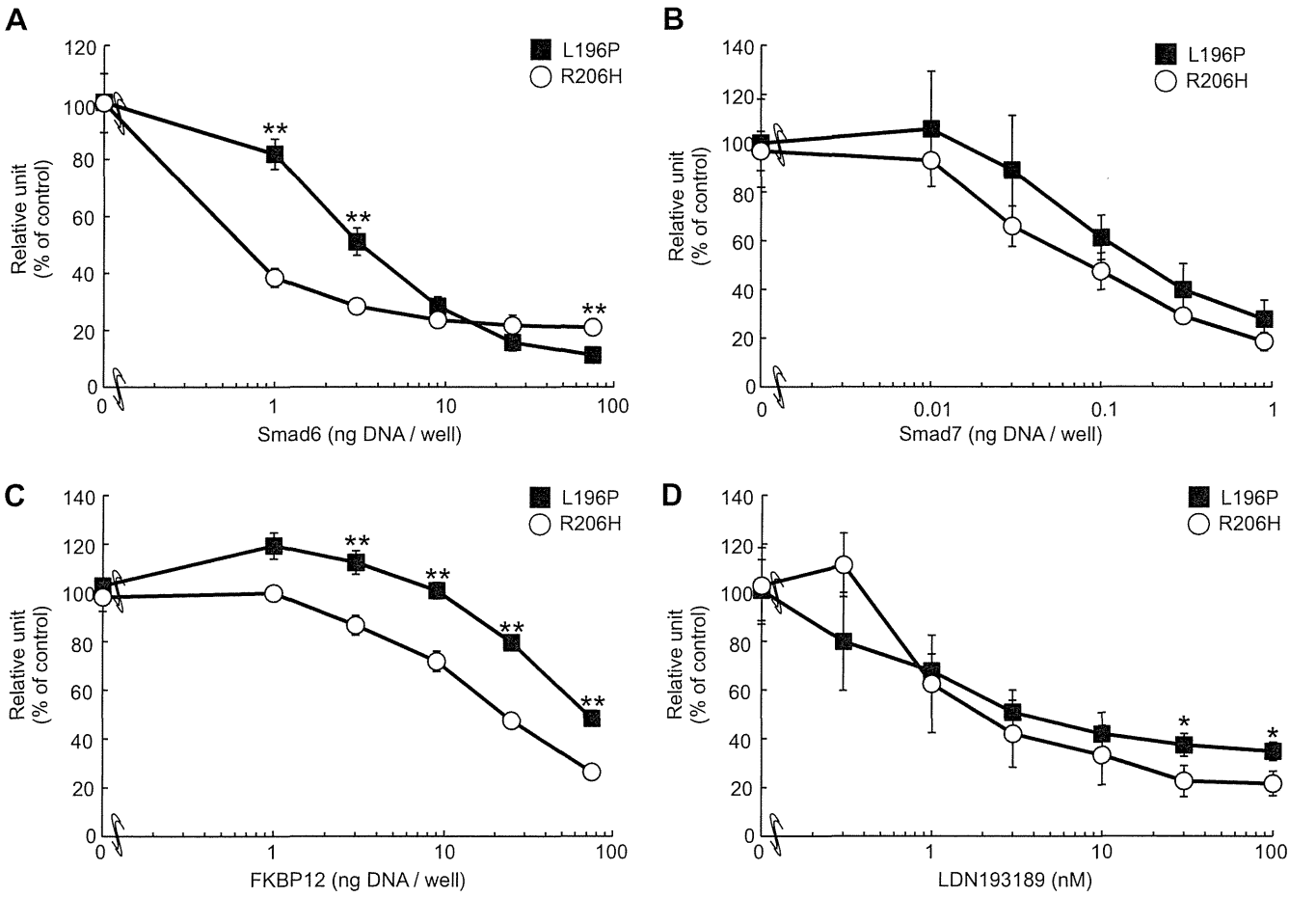

Fig. 3. Effects of protein and chemical inhibitors on ALK2(L196P) and ALK2(R206H). The BMP-specific IdWT4F-luc was activated by the transfection of C2C12 cells with ALK2(L196P) (closed squares) or ALK2(R206H) (open circles). The effects of inhibitors (Smad6 (A), Smad7 (B), FKBP12 (C) and LDN-193189 (D)) on ALK2(L196P) and ALK2(R206H) were determined using IdWT4F-luc in C2C12 cells. The cells were co-transfected with IdWT4F-luc, one of the ALK2 plasmids as indicated and varying amounts of Smad6 (A), Smad7 (B), or FKBP12 (C). The cells were cultured in the presence of varying concentrations of LDN-193189 (D). Total amounts of transfected DNA were adjusted equally with an empty vector. Luciferase activity was determined on day 1. Results are presented as the means (% of control) ± SD ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ in comparison with transfection with L196P and R206H.

The induction of the osteoblastic differentiation of myoblasts by ALK2 seems to be dependent on its kinase activity, which phosphorylates Smad1/5 at its carboxyl termini [13]. The degree to which ALP activity was induced by the co-transfection of ALK2 and Smad1 was the same as the degree of the phosphorylation levels of Smads by ALK2 mutants. Moreover, ALK2(L196P) and other mutant ALK2 variants did not activate the non-Smad MAPK or the TGF-b-specific Smad pathways. Recently, we showed that a constitutively activated Smad1, in which the carboxyl phosphorylation sites of BMP receptors were substituted with different acidic amino acids, induced osteoblastic differentiation of C2C12 myoblasts in the absence of BMPs [16]. Taken together, these findings suggest that the phosphorylation of Smad1/5 by mutant ALK2 receptors is one of the most critical targets to prevent heterotopic ossification in FOP. Indeed, the osteoblastic differentiation of C2C12 cells induced by ALK2 mutants was blocked by the chemical inhibitors dorsomorphin and LDN-193189 [10,13]. LDN-193189 has been shown to suppress heterotopic ossification induced by ALK2(Q207D) *in vivo* [21].

In conclusion, a mutant ALK2, ALK2(L196P), found in the most benign case of FOP reported thus far is an activated BMP receptor in the absence of BMPs, and it has equivalent *in vitro* activity to a typical mutation, ALK2(R206H). The *in vivo* biological activity of ALK2(L196P) may be masked by a novel, as yet undiscovered molecular mechanism in this patient.

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References

[1] F.S. Kaplan, Q. Shen, V. Lounev, P. Seemann, J. Groppe, T. Katagiri, R.J. Pignolo, E.M. Shore, Skeletal metamorphosis in fibrodysplasia ossificans progressiva (FOP), J. Bone Miner. Metab. 26 (2008) 521–530.

[2] F.S. Kaplan, M. Le Merrer, D.L. Glaser, R.J. Pignolo, R.E. Goldsby, J.A. Kitterman, J. Groppe, E.M. Shore, Fibrodysplasia ossificans progressiva, Best Pract. Res. Clin. Rheumatol. 22 (2008) 191–205.

[3] T. Katagiri, Heterotopic bone formation induced by bone morphogenetic protein signaling: fibrodysplasia ossificans progressiva, J. Oral Biosci. 52 (2010) 33–41.
[4] K. Mishima, H. Kitoh, T. Katagiri, H. Kaneko and N. Ishiguro, Early clinical and radiological characteristics in fibrodysplasia ossificans progressiva. A report of two cases, J. Bone Joint Surg. (2011), in press.
[5] E.M. Shore, M. Xu, G.J. Feldman, D.A. Fenstermacher, T.J. Cho, I.H. Choi, J.M. Connor, P. Delai, D.L. Glaser, M. LeMerrer, R. Morhart, J.G. Rogers, R. Smith, J.T. Triffitt, J.A. Urtizberea, M. Zasloff, M.A. Brown, F.S. Kaplan, A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva, Nat. Genet. 38 (2006) 525–527.
[6] K. Miyazono, S. Maeda, T. Imamura, BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk, Cytokine Growth Factor Rev. 16 (2005) 251–263.
[7] T. Katagiri Suda, T.K. Miyazono, The bone morphogenetic proteins, In The TGF-b Family, in: R. Derynck, K. Miyazono (Eds.), Cold Spring Harbor Press, New York, 2008, pp. 121–149.
[8] M.R. Urist, Bone: formation by autoinduction, Science 150 (1965) 893–899.
[9] T. Katagiri, A. Yamaguchi, M. Komaki, E. Abe, N. Takahashi, T. Ikeda, V. Rosen, J.M. Wozney, A. Fujisawa-Sehara, T. Suda, Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage, J. Cell Biol. 127 (1994) 1755–1766.
[10] T. Fukuda, M. Kohda, K. Kanomata, J. Nojima, A. Nakamura, J. Kamizono, Y. Noguchi, K. Iwakiri, T. Kondo, J. Kurose, K.I. Endo, T. Awakura, J. Fukushi, Y. Nakashima, T. Chiyonobu, A. Kawara, Y. Nishida, I. Wada, M. Akita, T. Komori, K. Nakayama, A. Nanba, Y. Maruki, T. Yoda, H. Tomoda, P.B. Yu, E.M. Shore, F.S. Kaplan, K. Miyazono, M. Matsuoka, K. Ikebuchi, O. Akira, O. Hiromi, J. Eijiro, O. Ichiro, O. Yasushi, K. Takenobu, Constitutively activated ALK2 and increased smad1/5 cooperatively induce BMP signaling in fibrodysplasia ossificans progressiva, J. Biol. Chem. 284 (2009) 7149–7156.
[11] H. Furuya, K. Ikezoe, L. Wang, Y. Ohyagi, K. Motomura, N. Fujii, J. Kira, Y. Fukumaki, A unique case of fibrodysplasia ossificans progressiva with an ACVR1 mutation, G356D, other than the common mutation (R206H), Am. J. Med. Genet. A 146A (2008) 459–463.
[12] F.S. Kaplan, M. Xu, P. Seemann, J.M. Connor, D.L. Glaser, L. Carroll, P. Delai, E. Fastnacht-Urban, S.J. Forman, G. Gillessen-Kaesbach, J. Hoover-Fong, B. Koster, R.M. Pauli, W. Reardon, S.A. Zaidi, M. Zasloff, R. Morhart, S. Mundlos, J. Groppe, E.M. Shore, Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1, Hum. Mutat. 30 (2009) 379–390.
[13] T. Fukuda, K. Kanomata, J. Nojima, S. Kokabu, M. Akita, K. Ikebuchi, E. Jimi, T. Komori, Y. Maruki, M. Matsuoka, K. Miyazono, K. Nakayama, A. Nanba, H. Tomoda, Y. Okazaki, A. Ohtake, H. Oda, I. Owan, T. Yoda, N. Haga, H. Furuya, T. Katagiri, A unique mutation of ALK2, G356D, found in a patient with fibrodysplasia ossificans progressiva is a moderately activated BMP type I receptor, Biochem. Biophys. Res. Commun. 377 (2008) 905–909.
[14] C.L. Gregson, P. Hollingworth, M. Williams, K.A. Petrie, A.N. Bullock, M.A. Brown, J.H. Tobias, J.T. Triffitt, A novel ACVR1 mutation in the glycine/serine-rich domain found in the most benign case of a fibrodysplasia ossificans progressiva variant reported to date, Bone 48 (2011) 654–658.
[15] T. Katagiri, M. Imada, T. Yanai, T. Suda, N. Takahashi, R. Kamijo, Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis, Genes Cells 7 (2002) 949–960.
[16] J. Nojima, K. Kanomata, Y. Takada, T. Fukuda, S. Kokabu, S. Ohte, T. Takada, T. Tsukui, T.S. Yamamoto, H. Sasanuma, K. Yoneyama, N. Ueno, Y. Okazaki, R. Kamijo, T. Yoda, T. Katagiri, Dual roles of smad proteins in the conversion from myoblasts to osteoblastic cells by bone morphogenetic proteins, J. Biol. Chem. 285 (2010) 15577–15586.
[17] T. Katagiri, S. Akiyama, M. Namiki, M. Komaki, A. Yamaguchi, V. Rosen, J.M. Wozney, A. Fujisawa-Sehara, T. Suda, Bone morphogenetic protein-2 inhibits terminal differentiation of myogenic cells by suppressing the transcriptional activity of MyoD and myogenin, Exp. Cell Res. 230 (1997) 342–351.
[18] H. Suzuki, K. Yagi, M. Kondo, M. Kato, K. Miyazono, K. Miyazawa, c-Ski inhibits the TGF-beta signaling pathway through stabilization of inactive Smad complexes on Smad-binding elements, Oncogene 23 (2004) 5068–5076.
[19] K. Kodaira, M. Imada, M. Goto, A. Tomoyasu, T. Fukuda, R. Kamijo, T. Suda, K. Higashio, T. Katagiri, Purification and identification of a BMP-like factor from bovine serum, Biochem. Biophys. Res. Commun. 345 (2006) 1224–1231.
[20] Q. Shen, S.C. Little, M. Xu, J. Haupt, C. Ast, T. Katagiri, S. Mundlos, P. Seemann, F.S. Kaplan, M.C. Mullins, E.M. Shore, The fibrodysplasia ossificans progressiva R206H ACVR1 mutation activates BMP-independent chondrogenesis and zebrafish embryo ventralization, J. Clin. Invest. 119 (2009) 3462–3472.
[21] P.B. Yu, D.Y. Deng, C.S. Lai, C.C. Hong, G.D. Cuny, M.L. Bouxsein, D.W. Hong, P.M. McManus, T. Katagiri, C. Sachidanandan, N. Kamiya, T. Fukuda, Y. Mishina, R.T. Peterson, K.D. Bloch, BMP type I receptor inhibition reduces heterotopic ossification, Nat. Med. 14 (2008) 1363–1369.

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Lactacystin, a proteasome inhibitor, enhances BMP-induced osteoblastic differentiation by increasing active Smads

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ABSTRACT

Proteasome inhibitors enhance bone formation and osteoblastic differentiation *in vivo* and *in vitro*. In the present study, we examined whether the molecular mechanisms of lactacystin, one of many proteasome inhibitors, stimulated the osteoblastic differentiation of C2C12 cells that is induced by bone morphogenetic proteins (BMPs). Pretreatment with lactacystin enhanced the alkaline phosphatase (ALP) activity induced by BMP2, BMP4 or BMP7, but lactacystin did not induce ALP in the absence of BMPs. In addition, lactacystin-stimulated BMP2 induced mRNA expression of *ALP*, *type I collagen*, *osteonectin*, *osteocalcin*, *Id1*, *Osterix*, and *Runx2*. Lactacystin maintained BMP2-induced phosphorylation of Smad1/5/8 and increased the length of time that these Smads were bound to target DNA. Moreover, lactacystin prevented BMP receptor-induced Smad degradation. This enhancement of BMP2-induced ALP activity and Smad phosphorylation by lactacystin was also observed in primary osteoblasts. These findings suggest that pretreatment with lactacystin accelerates BMP-induced osteoblastic differentiation by increasing the levels of phosphorylated Smads, which are maintained because BMP receptor-induced degradation is inhibited. We propose that optimized stimulation by proteasome inhibitors in a clinical setting may facilitate autogenous or BMP-induced bone formation in areas of defective bone.

1. Introduction

Bone morphogenetic proteins (BMPs), which are members of the transforming growth factor (TGF)-β superfamily, are known to be important in bone formation during postnatal skeletal development, growth, and regeneration in vertebrates. Dysfunction of BMP signaling has been implicated in several diseases, such as brachypodism, which is characterized by skeletal abnormalities restricted to the limbs and limb joints, and in a severe reduction in bone formation [1,2]. Linkage analysis suggested that *BMP2* is associated with osteoporosis in humans [3]. BMP2 has been shown to be essential for fracture healing in mice [4].

The ubiquitin–proteasome pathway regulates degradation of various intracellular signaling molecules involved in cell proliferation, differentiation and death [5,6]. Inhibition of the proteasome results in the accumulation of these proteins, thereby changing cell fate. Consequently, the ubiquitin–proteasome pathway has become an attractive target for pathway-directed bone formation. Proteasome inhibitors, such as lactacystin, proteasome inhibitor-1 (PS-1) and epoxomicin, stimulate bone formation *in vivo* and *in vitro* and are suppressed by noggin, a specific antagonist of BMPs, suggesting that BMPs are involved in the stimulation of bone formation by targeting proteasome inhibitors [7]. Indeed, the expression of *Bmp2* was increased by treatment with proteasome inhibitors.

The intracellular signaling induced by BMPs is mediated by morphogen interactions with two types of serine/threonine kinase receptors, type I and type II, which form a complex [1,2]. The BMP-bound type II receptor phosphorylates the type I receptor kinase, which results in the activation of the BMP type I receptor. Consequently, downstream receptor-regulated Smads (R-Smads),

Abbreviations: BMP, bone morphogenetic protein; ALP, alkaline phosphatase; PBS, phosphate buffered saline; TGF, transforming growth factor.

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including Smad1, Smad5 and Smad8, are phosphorylated. Phosphorylated R-Smads form heteromeric complexes with Smad4 and translocate to the nucleus where they regulate the transcription of target genes such as *Id1* and *Smad6* [1,2]. The overexpression of a constitutively active form of Smad1 induced osteoblastic differentiation of C2C12 myoblasts in the absence of BMPs [8]. Furthermore, specific inhibitors of Smad1/5/8 phosphorylation by BMP type I receptors prevented ectopic bone formation *in vivo* [9]. These results indicate that phosphorylation of the carboxyl terminus of Smad1/5/8 by BMP type I receptors plays an important role in BMP-induced bone formation.

Smad functions can be regulated not only by carboxyl terminus phosphorylation but also by protein stability through a ubiquitin–proteasome proteolytic pathway. Upon activation of the BMP type I receptor, proteasomal degradation of Smad1 is induced via interaction with an E3 ubiquitin ligase [1,2,10]. Smurf1, a member of the Hect domain family of E3 ubiquitin ligases, inhibits osteoblastic differentiation and bone formation [11]. In the present study, we examined the effects of a proteasome inhibitor, lactacystin, on osteoblastic differentiation in C2C12 myoblasts and murine primary osteoblasts. We found that lactacystin stimulated BMP-induced osteoblast differentiation by increasing phosphorylated Smad levels through the prevention of Smad degradation.

2. Materials and methods

2.1. Reagents

Recombinant human BMP2, BMP4, and BMP7 were purchased from R&D Systems (Minneapolis, MN). Lactacystin was purchased from Calbiochem (San Diego, CA).

2.2. Cell cultures

C2C12 mouse myoblast cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 mg/ml of streptomycin (Sigma–Aldrich, St. Louis, MO) in a humidified atmosphere of 5% CO_2 at 37 °C [12]. Primary osteoblasts (POBs) were prepared from the calvaria of 1-day-old *ddY* mice by digestion with 0.1% collagenase (Wako) and 0.2% dispase (Godo Shusei, Tokyo, Japan). POBs were maintained in α-minimum essential medium (αMEM) containing 10% FBS and antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C [13].

2.3. Alkaline phosphatase activity

The day before treatment, C2C12 cells or POBs were cultured in 96-well plates with DMEM containing 5% FBS. The cells were pretreated with vehicle (DMSO) or 10 μM lactacystin for 30 min and washed twice with PBS. Subsequently, the cells were treated with various concentrations of BMPs for 72 h. Next, the cells were fixed with an acetone/ethanol mixture (50:50, v/v) and incubated in a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$, and 10 mg/ml *p*-nitrophenyl phosphate). The reaction was terminated by adding 5 M NaOH, and absorbance was measured at 405 nm using a microplate reader (iMark; Bio-Rad Laboratories, Tokyo, Japan).

2.4. Reverse-transcriptase PCR and real-time PCR analysis

Total RNA was isolated from C2C12 cells using TRIzol (Invitrogen, Carlsbad, CA) and then reverse transcribed into cDNA. cDNA was amplified by PCR using primers that were specific for ALP and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time PCR was performed using SYBR Green PCR master mix and the 7300 Real-time PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Samples were matched to a standard curve generated by amplifying serially diluted products using the same PCR parameters. GAPDH expression served as an internal control. The primer sequences have been described previously [13].

2.5. Luciferase assay

C2C12 cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. BMP2-induced Id1 luciferase assays were performed using the IdWT4F-luc reporter plasmid [10] and the phRL-SV40 vector (Promega, Madison, WI) with the Dual-Glo Luciferase Assay System (Promega) as previously described [13,14].

2.6. Chromatin immunoprecipitation (ChIP) assays

ChIP was performed with a ChIP Assay Kit (Upstate Biotechnology, Waltham, MA) according to the manufacturer's instructions, using antibodies against phosphorylated Smad1/5/8 or normal IgG. The purified DNA was analyzed by PCR using primers that amplify sequences containing the Id1 promoter, which harbors a BMP2-responsive element (BRE) to which Smad proteins bind [14]. The primer pairs for the Id1 promoters were 5′-TAAGTTGAC CCTTGGTCAGC-3′ (forward) and 5′-GACGTCACCCATTCATAAAAC-3′ (reverse) [15].

2.7. Transfections and immunoblotting

Plasmids encoding wild-type murine Smad1 and Smad4 and a constitutively active BMP type I receptor, ALK2 (Q207D), have been described previously [5,6]. C2C12 cells were maintained and transfected with plasmids using Lipofectamine 2000 transfection reagent. Twelve hours after transfection, cells were treated with vehicle or with 10 μM lactacystin for 4 h. 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) and subjected to immunoblotting as described previously [13,15]. The following antibodies were used: anti-FLAG (clone M5), anti-β-actin (clone AC-15) (Sigma–Aldrich), anti-phosphorylated Smad1/5/8 (#9511), anti-Smad1 (#9743) (Cell Signaling, Beverly, MA), and anti-V5 (Invitrogen).

2.8. Statistical analysis

Comparisons were made using Student's *t*-test. The results were expressed as the means ± SD. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Lactacystin stimulates BMP-induced osteoblastic differentiation in C2C12 cells

We examined the effects of lactacystin on BMP-induced osteoblastic differentiation to elucidate the molecular mechanisms involved in proteasome inhibitor-mediated enhancement of bone formation *in vivo* [7]. C2C12 cells were pretreated with lactacystin for 30 min and cultured for 72 h in the presence of graded concentrations of BMP2, BMP4 or BMP7. Although pretreatment with lactacystin did not induce ALP activity in the absence of BMPs, ALP activity was induced in the presence of BMPs in a dose-dependent manner (Fig. 1A–C). In the presence of BMP-2,

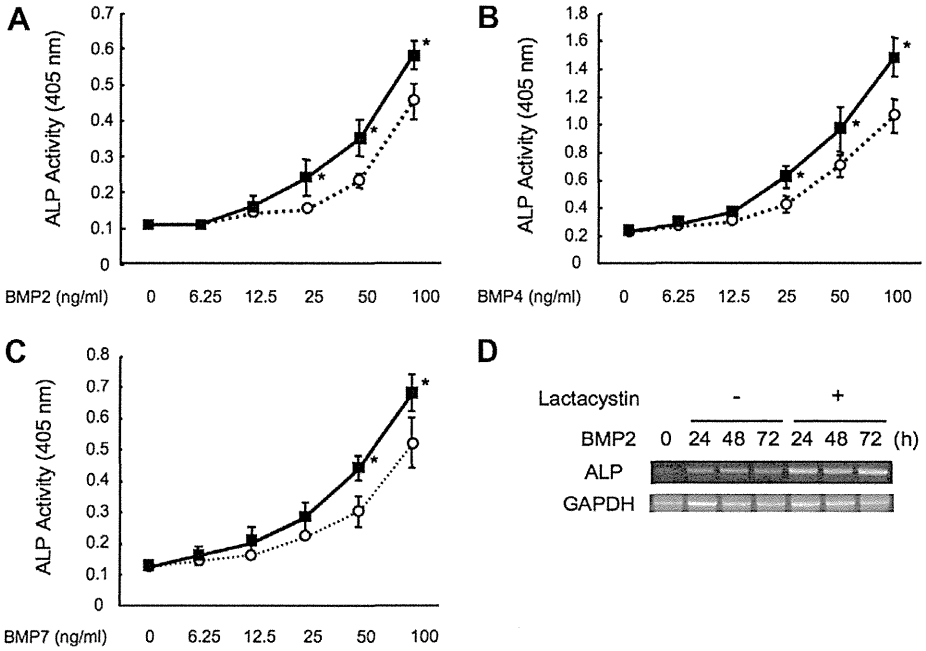

Fig. 1. Effects of lactacystin on BMP-induced osteoblast differentiation in C2C12 cells. C2C12 cells were pretreated with vehicle (open squares) or with 10 μM lactacystin (closed circles) for 30 min, washed twice with PBS, and cultured with or without various concentrations of (A) BMP2, (B) BMP4 or (C) BMP7. On day 3, ALP activity, a marker of osteoblastic differentiation, was determined. The data are represented as the means ± SD ($n = 3$). Similar results were obtained in three independent experiments. *$p < 0.01$ versus BMP-treated cultures. (D) C2C12 cells were pretreated with vehicle or with 10 μM lactacystin for 30 min, washed twice with PBS, and cultured with or without 100 ng/ml BMP2 for the indicated times. Total RNA was prepared, and ALP mRNA levels were analyzed using real-time PCR.

lactacystin increased ALP mRNA levels within 24 h of treatment, suggesting that lactacystin stimulated ALP at the transcriptional level (Fig. 1D).

We next measured the mRNA expression levels of other genes that associated with osteoblastic differentiation [16]. BMP2-induced expression of *type I collagen* (*Coll*), *osteonectin*, and *osteocalcin* was increased by lactacystin pretreatment (Fig. 2A). In addition, the expression levels of transcription factors involved in osteoblastic differentiation, such as *Id1*, *Osterix* and *Runx2*, were also increased by lactacystin in the presence of BMP2 (Fig. 2B). We found that lactacystin stimulated the luciferase activity of the BMP-specific reporter driven by a BMP-responsive element (BRE) in the *Id1* gene [14] (Fig. 2C).

3.2. Lactacystin enhances BMP2-induced phosphorylation and DNA-binding of Smads by preventing their degradation

As Smads bind to the BRE in the *Id1* gene, leading to regulation of its expression [14], we examined the effects of lactacystin on Smads. The BMP2-induced phosphorylation of Smad1/5/8 reached a maximum at 30 min and decreased thereafter without lactacystin. However, phosphorylated Smads were detected up to 120 min when treated with lactacystin (Fig. 3A). Moreover, in the ChIP assays, 10 min of lactacystin treatment increased the recruitment of phosphorylated Smad1/5/8 to the BRE in the *Id1* gene, and the increased recruitment persisted throughout the 60 min of treatment (Fig. 3B).

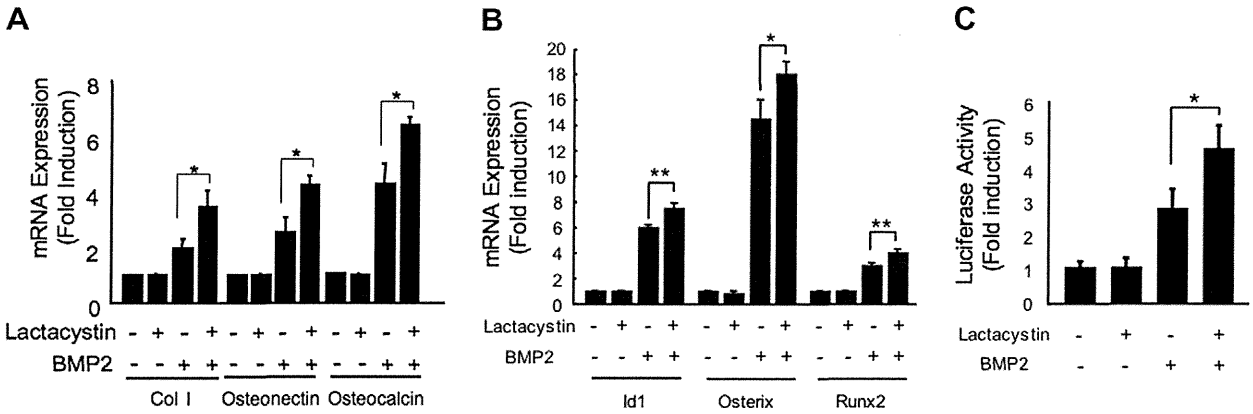

Fig. 2. Lactacystin enhances expression of BMP-induced osteoblastic differentiation markers and transcription factors, and BMP2-induced Id1-luciferase activity. C2C12 cells were pretreated with vehicle or with 10 μM lactacystin for 30 min, washed twice with PBS, and cultured with or without 100 ng/ml BMP2 for 72 h. Total RNA was prepared, and (A) *type I collagen*, *osteonectin*, and osteocalcin, and (B) *Id1*, *Osterix*, *Runx2* and *GAPDH* mRNA levels were analyzed using real-time PCR. The data are represented as the means ± SD of the expression levels of osteogenic genes, *Id1*, *Osterix* or *Runx2* relative to *GAPDH* ($n = 3$). Similar results were obtained in three independent experiments. (A) *$p < 0.01$ versus BMP2-treated cultures and (B) *$p < 0.05$ and **$p < 0.01$ versus BMP2-treated cultures. (C) C2C12 cells were transfected with the IdWT4F-luc reporter plasmid using the Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. Cells were pretreated with vehicle or with 10 μM lactacystin for 30 min, washed twice with PBS, and further cultured with or without 100 ng/ml BMP2. BMP2-induced Id1 luciferase activity was measured as previously described. The data are represented as the means ± SD ($n = 3$). Similar results were obtained in three independent experiments. *$p < 0.01$ versus BMP2-treated cultures.

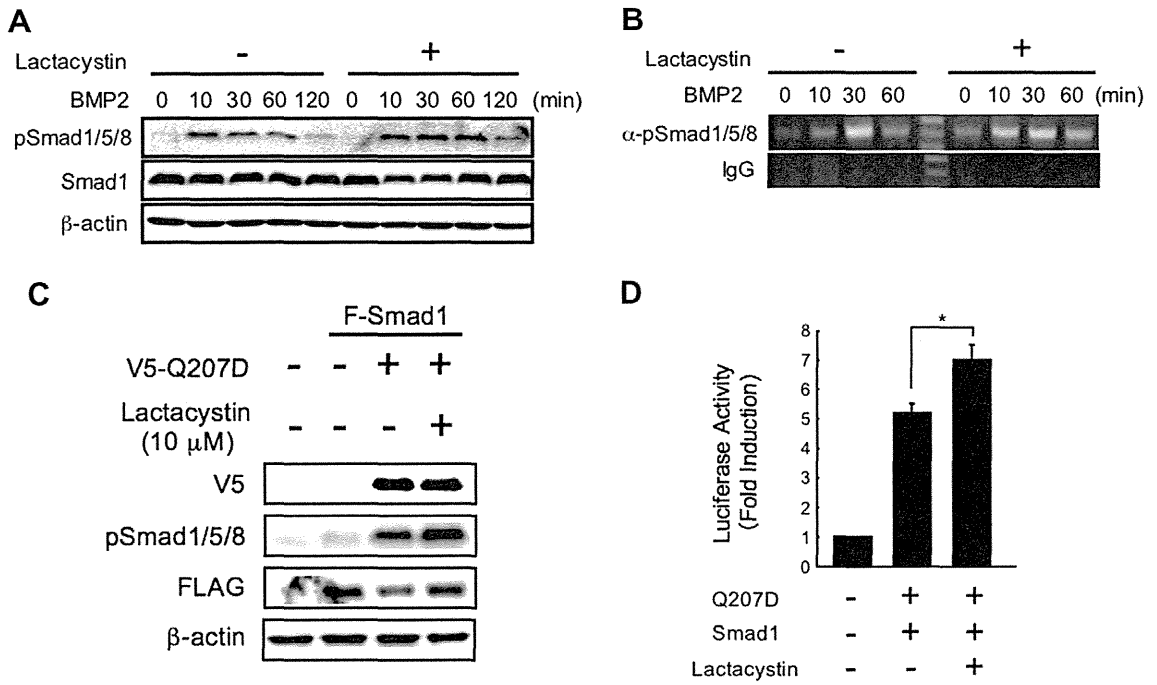

Fig. 3. Lactacystin enhances BMP2-induced Smad1/5/8 phosphorylation and DNA-binding activity. C2C12 cells were pretreated with vehicle or 10 μM lactacystin for 30 min, washed twice with PBS, and cultured with or without 100 ng/ml BMP2 for the indicated times. (A) Total cell lysates were prepared, and equal amounts of protein were subjected to SDS–PAGE. Anti-phosphorylated Smad1/5/8 and anti-Smad1 antibodies were used. β-Actin was used as a loading control. (B) Chromatin immunoprecipitation was performed with a ChIP Assay Kit according to the manufacturer's instructions, using antibodies against phosphorylated Smad1/5/8 or normal IgG. The purified DNA was analyzed by PCR using primers that amplify sequences containing the Id1 promoter. (C) Lactacystin prevents ligand-induced Smad1 degradation and stimulates phosphorylation of Smad1/5/8. C2C12 cells were transfected with 0.5 μg of FLAG-Smad1 with or without 0.5 μg of the I receptor R1Q207D. Cells were treated for 4 h with vehicle or with10 μM lactacystin before the cell lysates were collected and subjected to SDS–PAGE and western blot analyses using anti-V5 (top panel), anti-phospho-Smad 1/5/8 (2nd panel), anti-Flag (3rd panel), and anti-β-actin antibodies (bottom panel). (D) C2C12 cells were transfected with FLAG-Smad1 and the V5-activated BMP type I receptor R1Q207D. Id1 luciferase activity was measured after the cells were treated with vehicle or with 10 μM lactacystin for 4 h and cultured for 24 h. The data are represented as the means ± SD ($n = 3$). Similar results were obtained in three independent experiments. $^{*}p < 0.01$ versus BMP2-treated cultures.

The FLAG-Smad1 protein levels decreased when co-expressed with a constitutively active BMP type I receptor, ALK2 (Q207D) (Fig. 3C). Lactacystin treatment blocked the reduction in total FLAG-Smad1 levels induced by BMP signaling and increased phosphorylated Smad1/5/8 levels (Fig. 3D). Moreover, lactacystin enhanced the transcriptional activity of Smad1 induced by ALK2 (Q207D) in a BMP-specific luciferase reporter assay (Fig. 3D).

3.3. Lactacystin stimulates BMP-induced osteoblastic differentiation in primary osteoblasts

A previous study showed that proteasome inhibitors stimulated bone formation by increasing *Bmp2* mRNA expression [7]. Therefore, we examined the direct effects of lactacystin on the osteoblastic differentiation of POBs *in vitro*. Pretreatment of POBs with lactacystin slightly, but not significantly, increased the expression of *Bmp2* mRNA (Fig. 4A). Consistent with our findings in C2C12 cells, the pretreatment of POBs with lactacystin increased ALP activity in the presence of BMP2, although lactacystin did not show such activity in the absence of exogenous BMP2 (Fig. 4B). Again, the BMP2-induced phosphorylation of Smad1/5/8 was enhanced by the pretreatment with lactacystin compared to the vehicle treatment in POBs (Fig. 4C).

4. Discussion

In the present study, we investigated the effect of lactacystin, a natural product of *Streptomyces* sp. OM-6519, on BMP2-induced osteoblast differentiation of C2C12 cells. Pretreatment with lactacystin enhanced BMP2-induced osteoblastic differentiation of C2C12 cells and POBs. Activation of the BMP type I receptor decreased the protein levels of Smad1. However, treatment with lactacystin stimulated the transcriptional activity of the Smads, increased the levels of phosphorylated Smads and increased the time for which the Smads were bound to the target DNA. Lactacystin blocked the decrease of Smad protein levels upon activation of the BMP receptor. Taking these findings together, we suggest that the long-lasting Smad phosphorylation may contribute to the stimulation of bone formation by proteasome inhibitors.

A previous study suggested that increased *Bmp2* expression is involved in the stimulation of bone formation by proteasome inhibitors in organ culture systems [7]. We confirmed that lactacystin increased *Bmp2* expression in POBs. However, lactacystin alone failed to induce ALP activity, even in cultures with increased *Bmp2* mRNA. These findings suggest that the endogenous levels of BMP2 increased by lactacystin were too low to stimulate osteoblastic differentiation by lactacystin. Indeed, compared with PS-1 or epoxomicin, lactacystin induced less *Bmp2* expression and new bone formation [7]. The proteasome inhibitors may stabilize phosphorylated Smad1/5/8 only in the cells activated by an excess amount of BMPs. It is also possible that proteasome inhibitors may target different types of cells, in addition to osteoblastic cells, to stimulate BMP expression during bone formation *in vivo*. Further studies will be required to examine these possibilities.

In mammals, the degradation of various signaling molecules involved in the cell cycle and inflammation, such as p53, $p27^{kip1}$, and IκBα, is regulated by the ubiquitin–proteasome pathway [17–19]. Inhibition of the proteasome results in the accumulation of these proteins, thereby promoting cell cycle arrest and inhibition of inflammation. Consequently, the ubiquitin–proteasome pathway has become an attractive target for pathway-directed bone

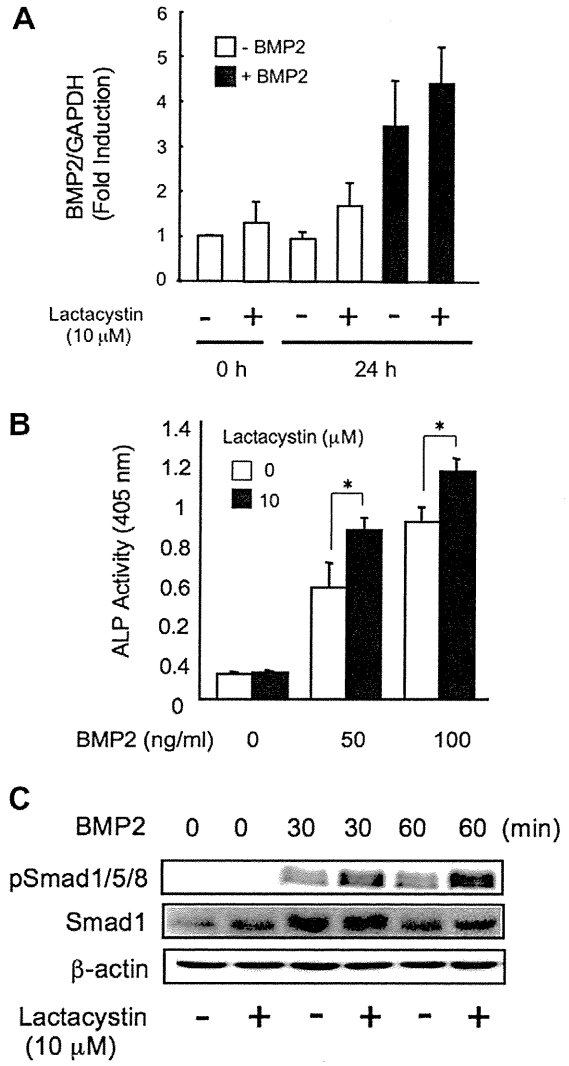

Fig. 4. Lactacystin stimulates BMP-induced osteoblastic differentiation in primary osteoblasts. (A) Primary osteoblasts were pretreated with vehicle or with 10 μM lactacystin for 30 min, washed twice with PBS, and cultured with or without 100 ng/ml BMP2 for 24 h. Total RNA was prepared, and the BMP2 and GAPDH mRNA levels were analyzed using real-time PCR. The data are represented as the means ± SD of BMP2 relative to GAPDH ($n = 3$). Similar results were obtained in three independent experiments. (B) Primary osteoblasts were pretreated with vehicle (open columns) or with 10 μM lactacystin (closed columns) for 30 min, washed twice with PBS, and cultured with or without various concentrations of BMP2. On day 3, ALP activity was determined. The data are represented as the means ± SD ($n = 3$). Similar results were obtained in three independent experiments. $^*p < 0.01$ versus BMP-treated cultures. (C) Primary osteoblasts were pretreated with vehicle or with 10 μM lactacystin for 30 min, washed twice with PBS, and cultured with or without 100 ng/ml BMP2 for the indicated times. Total cell lysates were prepared, and equal amounts of protein were subjected to SDS–PAGE. Anti-phosphorylated Smad1/5/8 and anti-Smad1 antibodies were used. β-Actin was used as a loading control.

formation. The activation of BMP signaling has been demonstrated to induce the expression of the cyclin-dependent kinase inhibitors $p21^{waf1/cip}$ and $p27^{Kip1}$, which results in osteoblast differentiation [20]. We have recently shown that activation of NF-κB inhibits BMP2-induced osteoblast differentiation by inhibiting Smad DNA binding [15]. Treatment with a selective inhibitor of NF-κB restored the suppression of BMP2-induced osteoblast differentiation by TNFα and stimulated osteoblastic differentiation [15,21]. These results suggest that lactacystin may enhance BMP2-induced osteoblast differentiation, in part, through the regulation of cell cycle proteins or NF-κB signaling. Additionally, lactacystin may target multiple steps in osteoblast differentiation induced by BMP signaling.

In conclusion, our results indicate that pretreatment with lactacystin accelerates BMP-induced osteoblastic differentiation in C2C12 cells and POBs through the activation of Smad signaling.

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References

[1] J. Massagué, J. Seoane, D. Wotton, Smad transcription factors, Genes Dev. 19 (2005) 2783–2810.
[2] T. Katagiri, T. Suda, K. Miyazono, The bone morphogenetic proteins, in: K. Miyazono, R. Derynck (Eds.), The TGF-β Family, Cold Spring Harbor Press, New York, 2008, pp. 121–149.
[3] U. Styrkarsdottir, J.B. Cazier, A. Kong, et al., Linkage of osteoporosis to chromosome 20p12 and association to BMP2, PLoS Biol. 1 (2003) E69.
[4] K. Tsuji, A. Bandyopadhyay, B.D. Harfe, et al., BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing, Nat Genet. 38 (2006) 1424–1429.
[5] A.M. Weissman, Themes and variations on ubiquitylation, Nat. Rev. Mol. Cell Biol. 2 (2001) 169–178.
[6] A. Hershko, The ubiquitin system for protein degradation and some of its roles in the control of the cell division cycle, Cell Death Differ. 12 (2005) 1191–1197.
[7] I.R. Garrett, D. Chen, G. Gutierrez, et al., Selective inhibitors of the osteoblast proteasome stimulate bone formation in vivo and in vitro, J. Clin. Invest. 111 (2003) 1771–1782.
[8] J. Nojima, K. Kanomata, Y. Takada, et al., Dual roles of smad proteins in the conversion from myoblasts to osteoblastic cells by bone morphogenetic proteins, J. Biol. Chem. 285 (2010) 15577–15586.
[9] P.B. Yu, D.Y. Deng, C.S. Lai, et al., BMP type I receptor inhibition reduces heterotopic ossification, Nat. Med. 14 (2008) 1363–1369.
[10] C. Gruendler, Y. Lin, J. Farley, et al., Proteasomal degradation of Smad1 induced by bone morphogenetic proteins, J. Biol. Chem. 276 (2001) 46533–46543.
[11] M. Zhao, M. Qiao, S.E. Harris, et al., Smurf1 inhibits osteoblast differentiation and bone formation in vitro and in vivo, J. Biol. Chem. 279 (2004) 12854–12859.
[12] T. Katagiri, A. Yamaguchi, M. Komaki, et al., Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage, J. Cell Biol. 127 (1994) 1755–1766.
[13] S. Hirata, C. Kitamura, H. Fukushima, et al., Low-level laser irradiation enhances BMP-induced osteoblast differentiation by stimulating the BMP/Smad signaling pathway, J. Cell. Biochem. 111 (2010) 1445–1452.
[14] T. Katagiri, M. Imada, T. Yanai, et al., Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis, Genes Cells 7 (2002) 949–960.
[15] M. Yamazaki, H. Fukushima, M. Shin, et al., TNFA represses BMP signaling by interfering with the DNA binding of Smads through the activation of NF-κB, J. Biol. Chem. 284 (2009) 35987–35995.
[16] J.E. Aubin, Regulation of osteoblast formation and function, Rev. Endocr. Metab. Disord. 2 (2001) 81–94.
[17] P.G. Richardson, C. Mitsiades, T. Hideshima, et al., Bortezomib: proteasome inhibition as an effective anticancer therapy, Annu. Rev. Med. 57 (2006) 33–47.
[18] C.G. Maki, J.M. Huibregtse, P.M. Howley, In vivo ubiquitination and proteasome-mediated degradation of p53(1), Cancer Res. 56 (1996) 2649–2654.
[19] M.S. Rodriguez, J. Wright, J. Thompson, et al., Identification of lysine residues required for signal-induced ubiquitination and degradation of IκBα in vivo, Oncogene 12 (1996) 2425–2435.
[20] S.F. Chang, T.K. Chang, H.H. Peng, et al., BMP-4 induction of arrest and differentiation of osteoblast-like cells via p21 CIP1 and p27 KIP1 regulation, Mol. Endocrinol. 23 (2009) 1827–1838.
[21] N. Alles, N.S. Soysa, J. Hayashi, et al., Suppression of NF-κB increases bone formation and ameliorates osteopenia in ovariectomized mice, Endocrinology 151 (2010) 4626–4634.

Early Clinical and Radiographic Characteristics in Fibrodysplasia Ossificans Progressiva

A Report of Two Cases

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Fibrodysplasia ossificans progressiva is an extremely rare and disabling genetic condition characterized by progressive heterotopic ossification of soft tissues such as muscles, ligaments, tendons, fasciae, and aponeuroses. The prevalence of fibrodysplasia ossificans progressiva is estimated to be about one in 2 million individuals[1]. Heterotopic ossification in fibrodysplasia ossificans progressiva usually begins in the first decade of life with the episodic development of inflammatory fibroproliferative masses in the axial skeleton. Most patients with fibrodysplasia ossificans progressiva are misdiagnosed as having soft-tissue sarcoma or aggressive juvenile fibromatosis before the definitive appearance of heterotopic ossification and undergo invasive procedures that usually lead to the acceleration of ossification[2]. Early correct diagnosis of fibrodysplasia ossificans progressiva is necessary to prevent additional iatrogenic harm or trauma.

At the molecular level, dysregulated bone morphogenetic protein (BMP) signaling is associated with the formation of heterotopic ossification in fibrodysplasia ossificans progressiva. Patients with classical features of the disease have the same heterozygous missense mutation in the glycine-serine activation domain of activin A receptor type-I gene (ACVR1), a BMP type-I receptor[3]. The causative mutation of fibrodysplasia ossificans progressiva is a recurrent single-nucleotide substitution at position 617 (c.617G>A; R206H) in the ACVR1 gene, which is one of the most specific disease-causing mutations in the

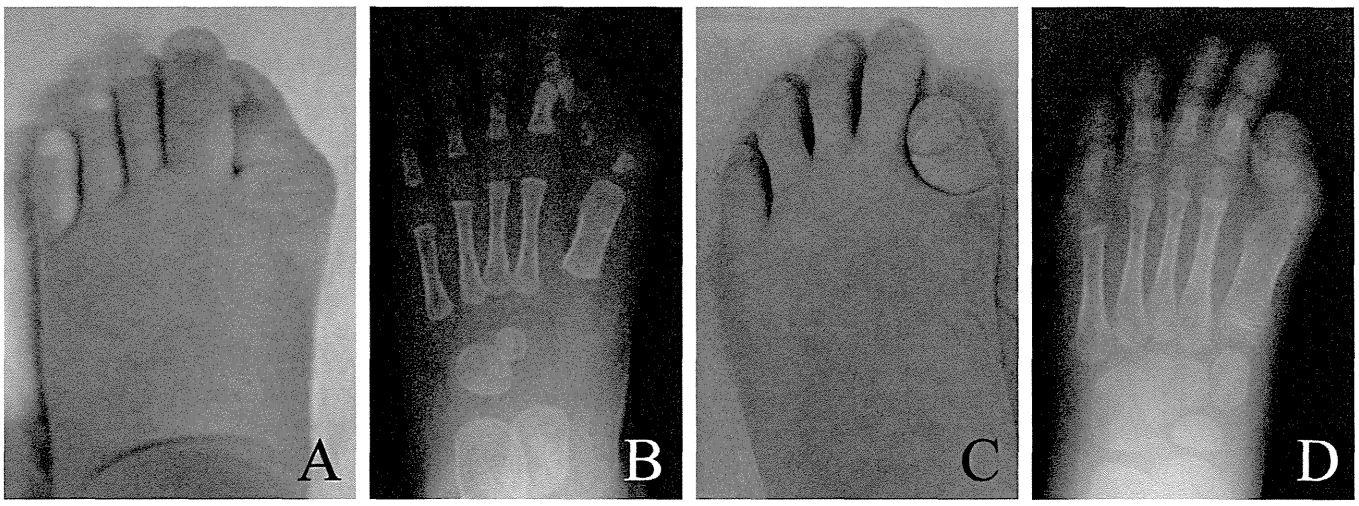

Fig. 1

Photographs and anteroposterior radiographs of the left foot of the first patient (Case 1) at the age of eleven months (*A* and *B*) and the second patient (Case 2) at the age of 7.1 years (*C* and *D*), demonstrating marked hallux valgus. Note the extra ossification center of the first metatarsal and hypoplasia of the proximal phalanx in the first patient (Case 1) (*B*). In contrast, monophalanx of the great toe and deformity of the distal end of the first metatarsal were observed in the second patient (Case 2) (*D*).

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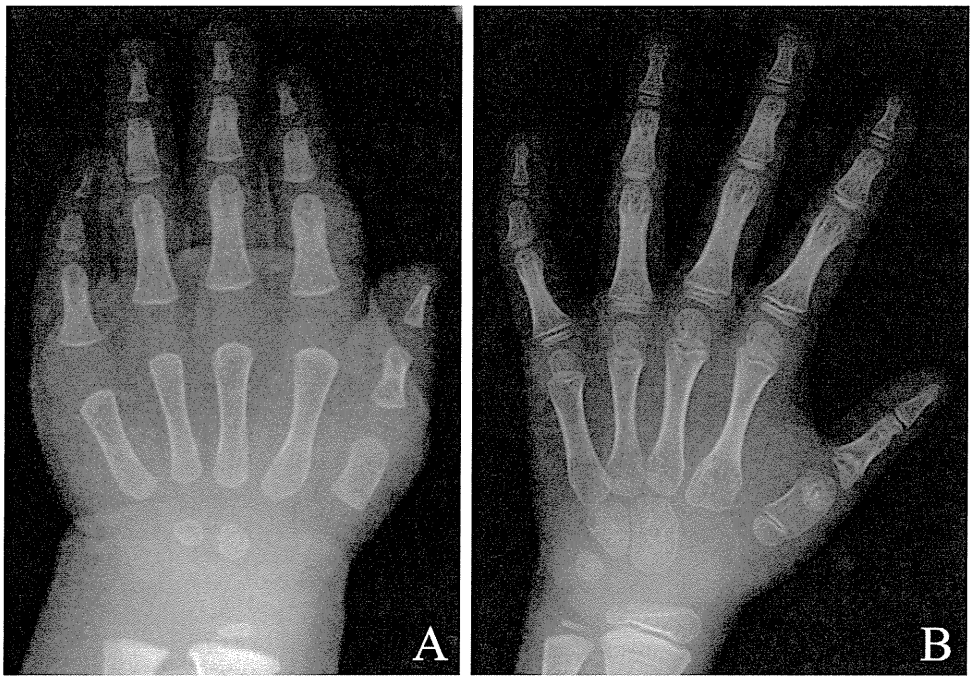

Fig. 2
Anteroposterior radiographs of the left hand of the first patient (Case 1) at the age of eleven months (*A*) and the second patient (Case 2) at the age of 8.4 years (*B*), showing shortening of the first metacarpal. The thumb of the second patient (Case 2) appears as a monophalanx associated with a cone-shaped epiphysis of the proximal phalanx and a fused epiphysis of the distal phalanx (*B*).

human genome. This mutant receptor constitutively activates BMP signaling without binding of ligands[4]. A unique and recurrent mutation has great relevance for diagnostic purposes in the case of fibrodysplasia ossificans progressiva. Definitive molecular diagnosis of fibrodysplasia ossificans progressiva is now available at early stages of disease development, when misdiagnosis is most likely to occur. Clinical awareness of the preosseous features of the disease is necessary for the early diagnosis of fibrodysplasia ossificans progressiva.

In the present study, we describe the cases of two patients in whom fibrodysplasia ossificans progressiva was genetically confirmed before the appearance of heterotopic ossification and demonstrate the clinical and radiographic features of systemic skeletal abnormalities. Father-to-son transmission

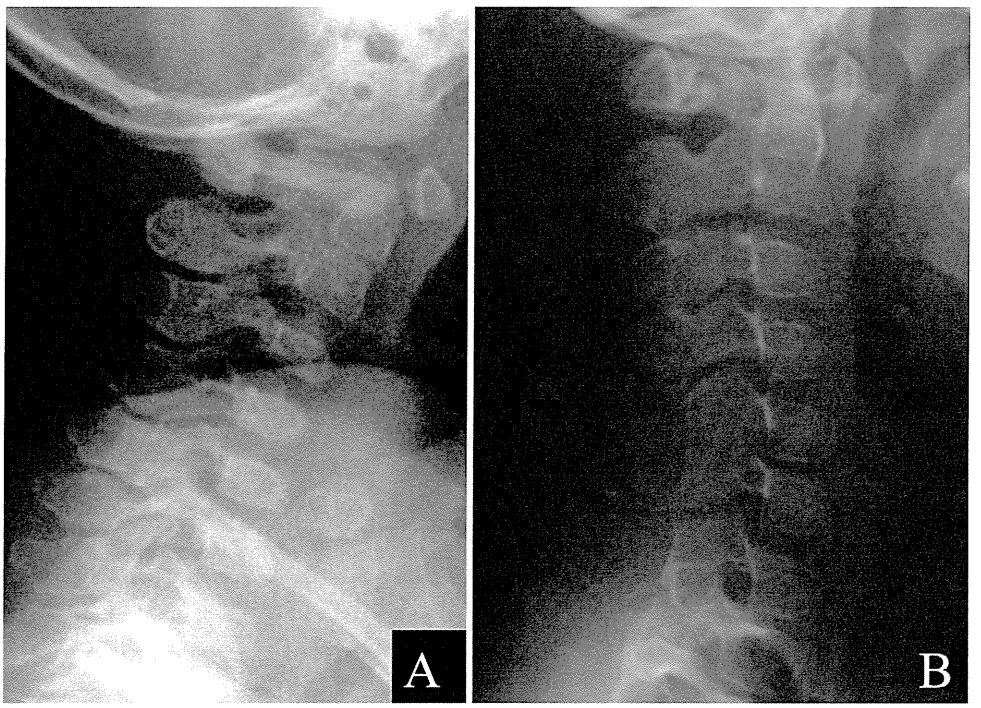

Fig. 3
Lateral radiographs of the cervical spine of the first patient (Case 1) at the age of eleven months (*A*) and the second patient (Case 2) at the age of 7.6 years (*B*), demonstrating hypertrophy of the laminae and spinous processes of the cervical vertebrae. Complete osseous fusions in facet joints and spinous processes were evident between C5/C6 and C7/T1 in the second patient (Case 2) (*B*).

was confirmed in the case of one of our patients; to our knowledge, this case represents the first reported instance of familial fibrodysplasia ossificans progressiva in Japan. Our study provides important information on the early clinical signs and symptoms of fibrodysplasia ossificans progressiva. The patients and their parents were informed that data concerning the cases would be submitted for publication, and they consented.

Case Reports

CASE 1. A ten-month-old boy was referred to our hospital with bilateral valgus deformity of the great toe. The patient's father also had had bilateral hallux valgus and had undergone corrective osteotomies at the age of three years. At the age of seven years, the father had had resection of a soft-tissue mass on the left posterolateral part of the neck. At the age of twelve years, the father had been diagnosed with fibrodysplasia ossificans progressiva on the basis of the clinical course, including progressive spinal deformity and joint contractures in the extremities due to heterotopic ossification. At the age of thirty-two years, the father was confirmed to have fibrodysplasia ossificans progressiva on the basis of genetic testing (617G>A; R206H in the ACVR1 gene).

The patient, the first child for his parents, was delivered by cesarean section at thirty-eight weeks of gestational age because of breech presentation. He had an uneventful perinatal and postnatal clinical course with normal developmental milestones. Physical examination revealed no abnormal findings except for bilateral valgus deformity of the great toe (Fig. 1, *A*) and mild stiffness of the neck, especially in extension. A full radiographic skeletal survey demonstrated various malformations in the feet, hands, spine, and extremities. Hallux valgus was associated with the extra ossification center in the distal end of the first metatarsal bone and marked hypoplasia of the proximal phalanx (Fig. 1, *B*). Shortening of the first metacarpal and the proximal phalanx of the thumbs was noted (Fig. 2, *A*). In the cervical spine, enlarged posterior elements, including pedicles, laminae, and spinous processes, were seen (Fig. 3, *A*). Neither cartilaginous fusions of the posterior elements of the cervical vertebrae nor inflammatory processes in the posterior part of the neck were detected with magnetic resonance imaging (MRI) at the age of 1.5 years (data not shown). The femoral

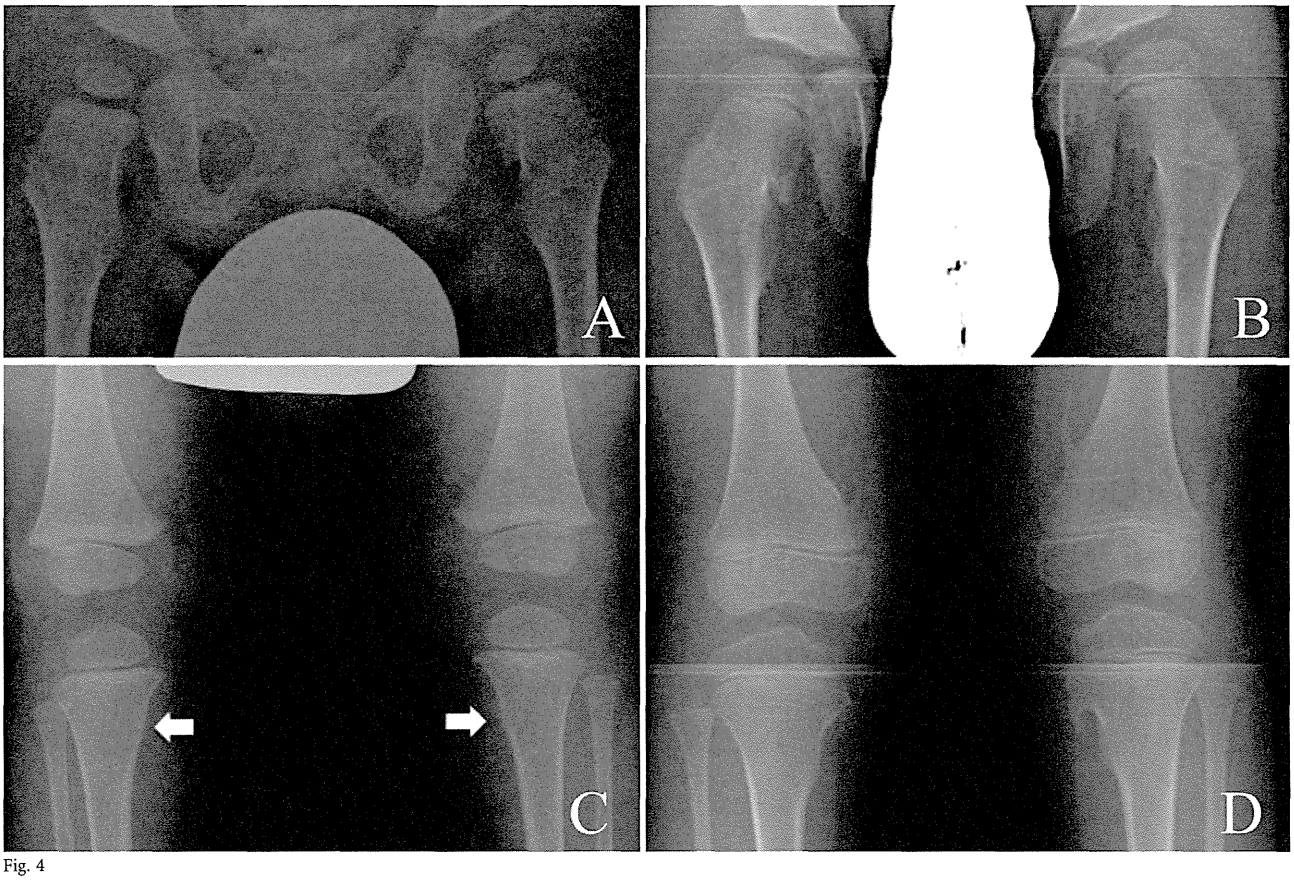

Fig. 4

Anteroposterior radiographs of the lower limb of the first patient (Case 1) at the age of eleven months (*A* and *C*) and the second patient (Case 2) at the age of 7.1 years (*B* and *D*). Note apparent coxa valga and broad femoral necks with the osteochondroma-like spurs in the metaphysis of the proximal part of the femur (*A* and *B*). Faint osseous protrusions are seen bilaterally on the metaphysis of the proximal part of the tibia (arrows) in the first patient (Case 1) (*C*), whereas multiple osteochondroma-like lesions are seen bilaterally at the medial side of the proximal part of the tibia in the second patient (Case 2) (*D*).

neck was broad, and an osteochondroma-like spur was seen in the proximal part of the left femur (Fig. 4, *A*). Faint osseous protrusions were found on the proximal-medial side of the tibia bilaterally (Fig. 4, *C*). Genomic DNA was obtained from the oral mucosa, and sequencing analysis demonstrated the presence of the same heterozygous single-nucleotide change (617G>A; R206H) in ACVR1 as had been demonstrated in his father.

CASE 2. A seven-year-old boy without any remarkable family history was referred to our hospital for genetic testing for fibrodysplasia ossificans progressiva. He was born as a low-birth-weight baby by means of cesarean section because of preterm rupture. Bilateral valgus deformity of the great toe was evident from birth. He had been repeatedly examined by several orthopaedists because of the hallux valgus but was never suspected as having fibrodysplasia ossificans progressiva. At the age of six years, the patient was referred to another physician, who made a provisional diagnosis of fibrodysplasia ossificans progressiva on the basis of the congenital malformations of the great toes and stiffness of the neck. The clinical course was unremarkable during follow-up periods.

Physical examination revealed remarkable bilateral shortening and valgus deformity of the great toe (Fig. 1, *C*). Although the range of motion of the upper and lower extremities was normal, neck motion was limited in all planes. The radiographic characteristics were similar to but more severe than those in the first patient (Case 1). He had monophalanx of the great toes, marked hallux valgus, and deformity of the distal end of the first metatarsal (Fig. 1, *D*), corresponding in position to the extra ossification center in the first patient (Case 1). The first metacarpals were extremely short, the epiphysis and metaphysis of the proximal phalanx of the thumb were deformed, and the thumb appeared to be a monophalanx as the ossification centers within the interphalangeal joint were very close together (Fig. 2, *B*). The posterior elements of the cervical spine were extremely hypertrophic, with fusions in facet joints and ligamentum interspinales between C5/C6 and C7/T1 (Fig. 3, *B*). The femoral necks were broad, with osteochondroma-like lesions hanging from the medial aspect of the proximal metaphysis, and bilateral distal femoral and proximal tibial osteochondromas were obvious (Fig. 4, *B* and *D*). Genomic DNA was extracted from peripheral blood, and sequencing analysis confirmed the same mutation (617G>A; R206H) in the ACVR1 gene.

Discussion

The present report describes the clinical and radiographic characteristics of two patients who had fibrodysplasia ossificans progressiva with no apparent heterotopic ossification. To our knowledge, there has been only one report that has documented genetically confirmed fibrodysplasia ossificans progressiva before the appearance of heterotopic ossification[5]. The current report is the first to present detailed characteristics of an infant with fibrodysplasia ossificans progressiva. In addition to remarkable deformity of the great toes, neck stiffness appears to be an important early clinical sign of fibrodysplasia ossificans progressiva. Characteristic radiographic features include a shortened and deformed first metacarpal and first metatarsal, hypertrophy or fusion of the posterior elements of the cervical spine, and osteochondroma-like lesions in the metaphysis of the long bones. These radiographic abnormalities appear to be caused by overactivity of the BMP signaling pathway during embryonic or postnatal skeletogenesis.

Dysregulated BMP signaling, which provides an important role in the regulation of condensation and differentiation of precursor cells into chondrocytes[6], has been postulated to be associated with malformed cartilaginous anlages of digits. However, the reasons why abnormalities of the hands and feet are restricted predominantly to the first digit have not been fully elucidated. Recently, various types of heterozygous ACVR1 mutations have been reported in patients with fibrodysplasia ossificans progressiva who showed atypical clinical features[7,8]. Such atypical fibrodysplasia ossificans progressiva is distinctly different from classic fibrodysplasia ossificans progressiva in terms of the severity of the malformations of the hands and feet, including severe truncation deformities of digits. Karamboulas et al. indicated that BMPs exert an anti-chondrogenic effect on early limb bud mesenchymal cells[9]. The differences in genotype of the ACVR1 may be related to the strength of the anti-chondrogenic effect on condensing mesenchymal cells via BMP signaling, leading to variety of phenotype in the hands and feet.

BMP activities may be modulated not only through gene expression and protein processing but also by interaction with antagonists such as Noggin and chordin[10]. Noggin-null mice exhibit fusions of the spinous processes and lateral masses of the cervical vertebrae[11], which are similar to radiographic manifestations of the cervical spine in the patients with fibrodysplasia ossificans progressiva described here. This null mutation should cause overactivity of BMP proteins because the BMP antagonist Noggin has been removed. Elevated BMP activity may cause misregulation of BMP-dependent genes, leading to hypertrophy of the axial skeleton.

With regard to osteochondroma-like lesions in fibrodysplasia ossificans progressiva, Deirmengian et al. described radiographic similarities between fibrodysplasia ossificans progressiva and multiple hereditary exostoses, including broad femoral necks and metaphyseal widening[12]. Multiple hereditary exostoses is an autosomal dominant condition characterized by numerous osteochondromas, caused by mutations in EXT1 or EXT2 genes, which encode tumor suppressors and glycosyltransferases involved in the biosynthesis of heparan sulfate proteoglycans (HSPGs)[13]. HSPGs bind to and modulate the activity of several growth factors and cell surface receptors such as Indian hedgehog (Ihh). Koziel et al. demonstrated that reduced HSPG synthesis in mice carrying a hypomorphic mutation in EXT1 resulted in an elevated range of Ihh signaling during embryonic chondrocyte differentiation[14]. The abnormal modulation of the tightly regulated Ihh/parathyroid hormone-related peptide (PTHrP)-negative feedback loop has been proposed as a molecular model of osteochondroma formation in multiple hereditary exostoses[15]. In contrast, Zhang et al.,

using a genetically engineered chicken limb bud model, showed that constitutively active ACVR1 mutation resulted in dramatic upregulation of Ihh at the perichondrium and a delay in chondrocyte differentiation[16]. Thus, osteochondroma formation in fibrodysplasia ossificans progressiva could be mediated by disruption of the BMP/Ihh/PTHrP-negative feedback loop at the perichondrium.

Diagnostic errors or delay have been common in fibrodysplasia ossificans progressiva. Kitterman et al. reported that obtaining an accurate diagnosis for fibrodysplasia ossificans progressiva required about four years or six physician evaluations from the onset of symptoms and noted that approximately 90% of affected individuals are given incorrect diagnoses, with nearly 70% receiving hazardous diagnostic procedures[2]. Although effective treatment for fibrodysplasia ossificans progressiva is not available to date, early clinical diagnosis, which can be confirmed with genetic testing, is extremely important to prevent iatrogenic harm or trauma. Several clinical and radiographic signs suggestive of fibrodysplasia ossificans progressiva were demonstrated in the present study, including apparent deformities of the great toes, neck stiffness, short and deformed first metacarpal and metatarsal bones, hypertrophy of the posterior elements of the cervical spine, and osteochondroma-like lesions in the metaphysis of the long bones. Mutational analysis for fibrodysplasia ossificans progressiva may not be so easy as it is available for research purposes only, but clinicians should become aware of these early detectable skeletal abnormalities for the consideration of genetic testing. ■

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References

1. Shore EM, Feldman GJ, Xu M, Kaplan FS. The genetics of fibrodysplasia ossificans progressiva. Clin Rev Bone Miner Metab. 2005;3:201-4.

2. Kitterman JA, Kantanie S, Rocke DM, Kaplan FS. Iatrogenic harm caused by diagnostic errors in fibrodysplasia ossificans progressiva. Pediatrics. 2005;116:e654-61.

3. Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, Choi IH, Connor JM, Delai P, Glaser DL, LeMerrer M, Morhart R, Rogers JG, Smith R, Triffitt JT, Urtizberea JA, Zasloff M, Brown MA, Kaplan FS. A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. Nat Genet. 2006;38:525-7.

4. Fukuda T, Kohda M, Kanomata K, Nojima J, Nakamura A, Kamizono J, Noguchi Y, Iwakiri K, Kondo T, Kurose J, Endo K, Awakura T, Fukushi J, Nakashima Y, Chiyonobu T, Kawara A, Nishida Y, Wada I, Akita M, Komori T, Nakayama K, Nanba A, Maruki Y, Yoda T, Tomoda H, Yu PB, Shore EM, Kaplan FS, Miyazono K, Matsuoka M, Ikebuchi K, Ohtake A, Oda H, Jimi E, Owan I, Okazaki Y, Katagiri T. Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva. J Biol Chem. 2009;284:7149-56.

5. Kaplan FS, Xu M, Glaser DL, Collins F, Connor M, Kitterman J, Sillence D, Zackai E, Ravitsky V, Zasloff M, Ganguly A, Shore EM. Early diagnosis of fibrodysplasia ossificans progressiva. Pediatrics. 2008;121:e1295-300.

6. Bandyopadhyay A, Tsuji K, Cox K, Harfe BD, Rosen V, Tabin CJ. Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. PLoS Genet. 2006;2:e216.

7. Kaplan FS, Xu M, Seemann P, Connor JM, Glaser DL, Carroll L, Delai P, Fastnacht-Urban E, Forman SJ, Gillessen-Kaesbach G, Hoover-Fong J, Köster B, Pauli RM, Reardon W, Zaidi SA, Zasloff M, Morhart R, Mundlos S, Groppe J, Shore EM. Classic and atypical fibrodysplasia ossificans progressiva (FOP) phenotypes are caused by mutations in the bone morphogenetic protein (BMP) type I receptor ACVR1. Hum Mutat. 2009;30:379-90.

8. Furuya H, Ikezoe K, Wang L, Ohyagi Y, Motomura K, Fujii N, Kira J, Fukumaki Y. A unique case of fibrodysplasia ossificans progressiva with an ACVR1 mutation, G356D, other than the common mutation (R206H). Am J Med Genet. 2008;146:459-63.

9. Karamboulas K, Dranse HJ, Underhill TM. Regulation of BMP-dependent chondrogenesis in early limb mesenchyme by TGFbeta signals. J Cell Sci. 2010;123:2068-76.

10. Brunet LJ, McMahon JA, McMahon AP, Harland RM. Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. Science. 1998;280:1455-7.

11. Wijgerde M, Karp S, McMahon J, McMahon AP. Noggin antagonism of BMP4 signaling controls development of the axial skeleton in the mouse. Dev Biol. 2005;286:149-57.

12. Deirmengian GK, Hebela NM, O'Connell M, Glaser DL, Shore EM, Kaplan FS. Proximal tibial osteochondromas in patients with fibrodysplasia ossificans progressiva. J Bone Joint Surg Am. 2008;90:366-74.

13. McCormick C, Leduc Y, Martindale D, Mattison K, Esford LE, Dyer AP, Tufaro F. The putative tumour suppressor EXT1 alters the expression of cell-surface heparan sulfate. Nat Genet. 1998;19:158-61.

14. Koziel L, Kunath M, Kelly OG, Vortkamp A. Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification. Dev Cell. 2004;6:801-13.

15. Stickens D, Brown D, Evans GA. EXT genes are differentially expressed in bone and cartilage during mouse embryogenesis. Dev Dyn. 2000;218:452-64.

16. Zhang D, Schwarz EM, Rosier RN, Zuscik MJ, Puzas JE, O'Keefe RJ. ALK2 functions as a BMP type I receptor and induces Indian hedgehog in chondrocytes during skeletal development. J Bone Miner Res. 2003;18:1593-604.

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SPATIAL AND TEMPORAL DISTRIBUTION OF GROWTH FACTORS RECEPTORS IN THE CALLUS: IMPLICATIONS FOR IMPROVEMENT OF DISTRACTION OSTEOGENESIS

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ABSTRACT

Management of bone deficits by distraction osteogenesis is an appreciated but lengthy procedure. To accelerate the consolidation of newly formed distraction callus, an administration of growth factors into the distraction gap has been suggested. Changes in expression of growth factors receptors in the distracted callus during consolidation were studied in order to improve our understanding of the underlying molecular mechanisms and to provide a scientific basis for clinical application of growth factors. In a model of rat bone lengthening the expression of receptors for: vascular endothelial growth factor, transforming growth factor β1, insulin like growth factor and platelet derived growth factor were evaluated semiquantitatively with immunohistochemistry and quantitatively with real time PCR in various callus zones at zero, one and two weeks of consolidation. Overall growth factors receptors' expression was highest at the beginning of consolidation. It was strongest in the trabecular bone and weakest in the fibrous zone. Transforming growth factor β receptor 1 was most abundant and vascular endothelial growth factor receptor 1, although scarce, showed the most consistent expression. In contrast to the osteogenic zones, the fibrous zone demonstrated a dramatic loss of the growth factors receptors over time. High growth factors receptors expression shortly after termination of the distraction may warrant the maximal callus' response to injected growth factors. Rapid decline of growth factors receptors in the fibrous zone may imply its decreasing sensitivity to growth factors and, as a consequence, a declining osteogenic potential.

Key Words: Distraction osteogenesis, Growth factors, Flt-1, TGFβR1, IGF-1R, PDGFRα

INTRODUCTION

Management of a leg length deficit or short stature poses a major challenge. Although distraction osteogenesis is the least invasive procedure, the formation and maturation of new bone during distraction is a time consuming process. Problems related to the treatment's duration (pin tract infection, failure of the distraction device), or to the biology itself (poor callus formation, delayed consolidation) are not uncommon.[1)] Therefore, it seems reasonable to seek improvements to this valuable method, thus possibly shortening its duration. Distraction osteogenesis consists of two

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phases – active distraction (new bone formation) and consolidation (maturation and remodeling). The optimal rate of distraction has been established both experimentally and clinically, and due to limited soft tissues regeneration must not be accelerated.[2] However, it is possible to reduce time of the second phase by enhancing consolidation and improving the quality of the callus. Acceleration of consolidation has been attempted with various methods and outcomes. The newest techniques favor autogenous materials. Local injections of bone marrow, stem cells or GFs,[3-6] or their combinations[7] have been reported.

Autologous platelet-rich plasma abundantly provides the key stimulators of bone development, e.g., VEGF (vascular endothelial growth factor), TGFβ1 (transforming growth factor β1), IGF1 (insulin like growth factor 1) and PDGF (platelet derived growth factor), etc. In addition PRP (platelet rich plasma) is widely accessible and easily applicable to the distraction gap and has provided good outcomes in preliminary clinical trials[5,6].

To most efficiently use growth factors, it is imperative to understand their cellular and molecular biology. However, bone formation is regulated not only by growth factors availability itself, but also by the functioning of their specific receptors. PDGFRα (receptor for PDGF) regulates the entire process of bone formation from hematoma briefly incurred after injury, until remodeling.[8-10] It is particularly active during the inflammation and promotes bone cell proliferation. Angiogenesis is an indispensible precursor of successful osteogenesis; bone apposition depends on the adequate function of Flt-1 (receptor for VEGF).[11,12] The pro-osteogenic effect assured by IGF is reduced in any case of IGF-R1 (receptor for IGF1) impairment.[3,14] Osteoblast proliferation depends on TGFβR1 (receptor for TGFβ1), and all isoforms of TGFβ1 receptor are necessary for ossification, the formation of mature bone and for bone turnover.[15-17]

In this study the performance of the above mentioned growth factors receptors was assessed in order to define their spatial distribution, sequence and quantity (most importantly the time of the highest receptors' expression) during the consolidation phase of distraction osteogenesis.

We discussed alterations in growth factors receptors' expression, especially in fibrous and bone regions of the callus, and the potential impact of these changes on bone formation and remodeling. We hypothesize that bone formation may benefit most from injected growth factors if they are applied at the times of the highest activity of their specific receptors. To the best of our knowledge, this is the first report evaluating receptors for VEGF, TGFβ1, IGF1 and PDGF in the rat model of long-bone distraction.

MATERIALS AND METHODS

Model of bone distraction

The research model was designed in accordance with general guidelines for the care and use of experimental animals and was approved by the animal experiment committee of the Nagoya University Graduate School of Medicine. Thirty-two 9- week-old Sprague-Dawley rats weighing 330–380 g underwent surgery under general anesthesia. Through a lateral incision, a mini external fixator-distractor (Nagoya Screw Manufacturing Co., Ltd, Nagoya, Japan) was secured to the rat's femoral bone using four threaded pins (Fig. 1A). The mid-third of the bone shaft was osteotomized (Fig. 1B), and the skin was closed. Full weight bearing was allowed immediately after recovering from anesthesia. Distraction was initiated after 7 days of latency at a rate of 0.375 mm twice a day, and lasted for 10 days (Fig. 1C), producing 7.5 mm of distraction callus. After termination of the distraction, callus' consolidation was allowed for 0 (Fig. 1C), 7 and 14 (Fig. 1D) days, after which tissue samples were harvested. The animals were randomly divided into five experimental groups. In two groups (after 0 and 14 days of consolidation) distraction

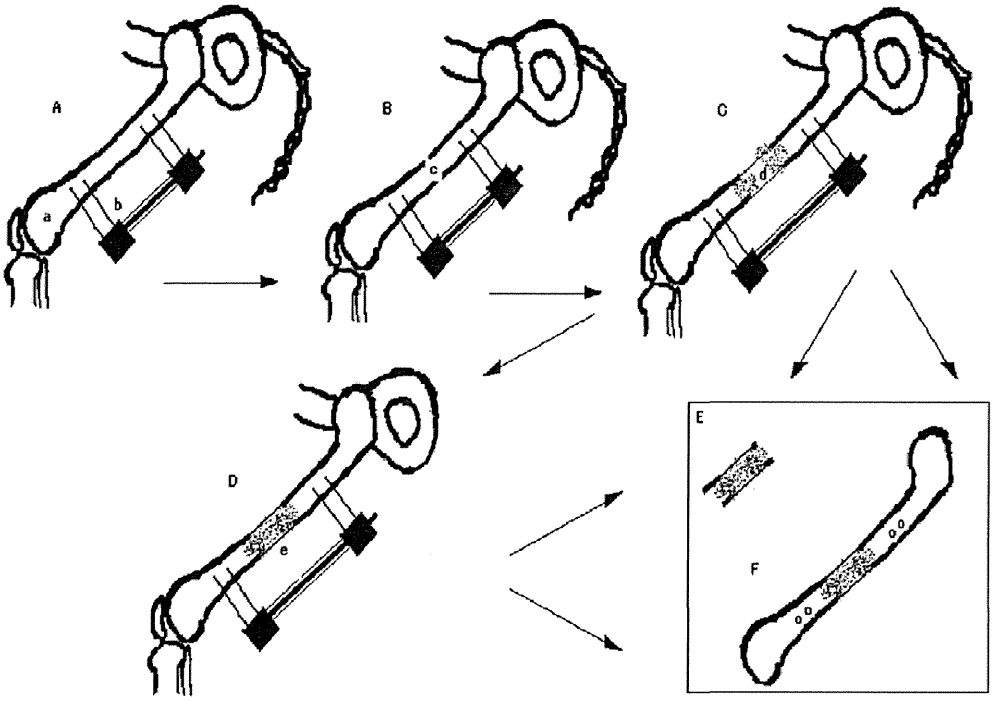

Fig. 1 Rat model of long bone lengthening. A – insertion of an external fixator-distractor device, B – osteotomy of the femoral bone, C – distraction of femoral bone and production of the distraction callus (0 days of consolidation), D – consolidation of distraction callus (7 or 14 days), E – harvesting of distraction callus for mRNA analysis (after 0 or 14 days of consolidation), F – harvesting of whole femoral bone for immunohistochemical analysis (after 0, 7 or 14 days of consolidation); a – femoral bone, b – external fixation-distraction device, c – site of bone osteotomy, d – distracted callus, e – consolidating callus.

callus (Fig. 1E) was harvested for mRNA analyses of growth factors receptors. In the remaining three groups (after 0, 7 and 14 days of consolidation) femoral bones (Fig. 1F) were harvested for histological and immunohistochemical analysis of growth factors receptors.

Histological and immunohistochemical analysis

Whole femoral bones were fixed in paraformaldehyde, embedded in paraffin, cut into 6 μm sections and mounted on slides.

Serial samples were stained with hematoxylin and eosin. The consistency of bone formation and changes in cellular characteristics during callus maturation was assessed under a light microscope with attention to four histological zones: woven and trabecular bone, fibrous tissue and chondral tissue.

For immunohistochemical analysis we used primary polyclonal anti-mouse Flt-1 (NeoMarkers, Fremont, CA, USA; catalog # RB-1527-R7) and primary rabbit polyclonal anti-human TGFβR1 (BioVision, Mountain View, CA, USA; catalog # 5636-100), IGF-1R (Spring Bioscience, Fremont, CA, USA; catalog # E12204) antibodies and PDGFRα Ab-1 (Thermo Scientific, Fremont, CA, USA; catalog # RB-1691). Bone sections were stained using the Simple Stain Rat MAX PRO Histofine (Nichirei Biosciences, Japan) and DAB (3, 3'-diaminobenzidine tetrahydrochloride) (Nichirei Biosciences, Japan). Negative controls were obtained by excluding primary antibodies

from the protocols. Blinded observers evaluated immunohistochemical stains of the callus under a light microscope. Two digital microphotographs were taken of each zone: fibrous, chondral, woven and trabecular. All images were evaluated at two points (a sixteen-point study of every specimen). All relevant cells in the analyzed zones (fibroblasts, chondroblasts/chondrocytes and osteoblasts/osteocytes respectively) were counted. The stained cells were expressed as a percentage of all counted cells; a semiquantitative approach was applied. In accordance with protocols applied in previous studies,[18-20] the areas were labeled by applying the following scale: – (no staining), + (≤ 25%), ++ (≤ 50%), +++ (≤ 75%), ++++ (≤ 100%) of staining. Such observations were repeated for each of the four receptors.

RNA Isolation, RT-PCR and quantitative real time analysis

For mRNA analysis each distraction callus was harvested and dry frozen. Total RNA was extracted by Trizol and purified with an RNeasy Mini Kit (QUIAGEN, Tokyo, Japan). A reverse Transcription System (Roche, New Jersey, USA) was used to obtain a total cDNA of every sample. In the Light Cycler instrument, using Light Cycler-Fast Start DNA Master SYBR Green I (Roche Molecular Biochemicals, Mannheim, Germany), one sample of total cDNA was amplified with oligonucleotide primer for GFR (designed by Nihon Gene Research Laboratories, Sendai, Japan) (Table 1). The PCR product (receptor-specific cDNA) was dyed with Loading Buffer 6x (Takara, Shiga, Japan), and underwent electrophoresis in the agarose gel to confirm the primer's specificity by visualization of a single band. With QIAquick Spin (QUIAGEN, Tokyo, Japan), cDNA was extracted from the agarose gel. Absorption of extracted cDNA was measured with a spectrophotometer (Biophotometer Plus, Eppendorf, Germany). cDNA concentration was calculated and serial dilutions were prepared as standards. The same procedure was repeated to generate standards for other GFRs' primers. Using the prepared standards, quantitative real-time PCR was carried out on the remaining samples in the Light Cycler instrument, with primers for GFRs and for GAPDH as a house-keeping gene. Values of Flt-1, TGFβR1, IGF-1R, and PDGFRα were calculated in respect to values for GAPDH, and further analyzed.

RESULTS

Histological examination:

A central part of the callus (fibrous zone, Fig. 2a) consisted of longitudinally aligned spindle-like fibrous cells (with regions of less organized fibroblasts). The length of the fibrous zone was

Table 1 Forward (F) and reverse (R) nucleotide sequence of four growth factors receptors' (GFRs') primers used for quantitative real-time RT-PCR and size of product.

GFR	Sequence (5'->3')	Product size
Flt1 F	GCCTACGTGTCCGCATTA	114
Flt1 R	GGTAGCAGGCTGGACAGTAA	
IGF R1 F	AGGAGGCTGAGTACCGTAAA	144
IGF R1 R	TGTCAGCTACCGTGGTGT	
TGFβR1 F	CCATTTGTTTGTGCACCATC	173
TGFβR1 R	ATAAGTGCAATGCAGACGAA	
PDGFRα F	GACAACTTGACCCTGATTGAG	116
PDGFRα R	CCGCTGTCTTCTTCCTTAG	

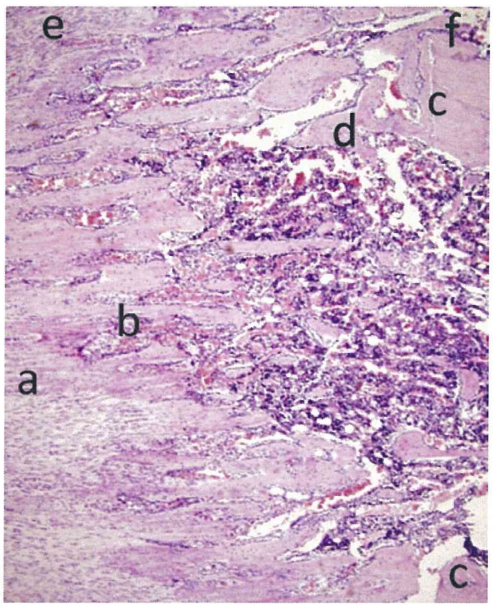

Fig. 2 Microphotograph of a distraction callus' regions: (a) central fibrous zone, (b) trabecular bone zone, (c) site of osteotomy with adjacent (d) woven bone zone, (e) nodules of chondral tissue, (f) zone of periosteal ossification (1:100).

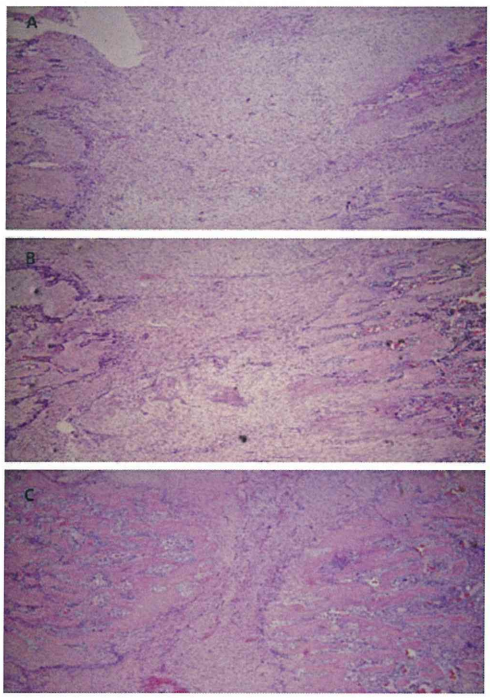

Fig. 3 Microphotograph of the central, fibrous region of distraction callus at 0 (A), 7 (B) and 14 (C) days of consolidation. The fibrous zone decreases with time as the front of osteogenesis invades towards the center of distraction callus (1:40).

decreasing with time (Fig. 3 A-C). A trabecular zone (Fig. 2b) was adjacent to the fibrous zone. The trabeculae were formed of columns of osteoblasts organized along the axis of distraction. This zone (active osteogenesis) was subject to most histological changes during consolidation. A large number of osteoblasts with big nuclei (day 0 of consolidation) was decreasing with time, yielding smaller osteocytes in a more abundant extracellular matrix (day 14 of consolidation). The most peripheral zone of the distraction callus adjacent to the osteotomy site (Fig. 2c) was occupied by disorganized woven bone (Fig. 2d) which was also invading a medullary canal in retrograde fashion. Although distraction callus forms through an intramembranous ossification, rare islands of chondrogenesis could be observed (Fig. 2e). Chondroblasts (briefly after distraction) or hypertrophied chondrocytes (later in the consolidation phase) were forming nodules within the fibrous tissue or paraperiosteally. The osteoid (similar to the woven zone) produced by periosteal reaction was surrounding the callus as a side layer (Fig. 2f).

Immunohistochemical examination (Table 2):

Temporal characteristics: Expression of all receptors was most abundant at the beginning of consolidation (day 0) but showed a general decrease with time (day 7 and day 14). These changes were, however, tissue- and receptor-specific. The reduction of staining was most significant in the chondral tissue (by ++ for most receptors). In the fibrous tissue, TGFβR1 and PDGFRα had already decreased in the 7th day of consolidation. In the bone zones, the decline was in the 25% ranged and was delayed to 14 days of consolidation for most receptors.

Tissue characteristics: The average expression of all receptors was highest in the trabecular zone which was the region of most active bone formation (over 75%) and remained over 50% after 2 weeks (with exception of Flt-1). However, since the absolute number of bone cells within the trabeculae was decreasing (due to maturation from osteoblasts to osteocytes), the overall number of stained cells was reduced significantly as well. Very intensive staining was evident in the cells lining the trabeculae (up to 100%). The woven bone had a relatively strong (+++/++++) stain as well, and the periosteal ossification zone showed similar receptor characteristics (data not shown).

The least staining (++) with rapid decrease was observed in the fibrous-like tissue (Fig. 4). Interestingly, highly organized and aligned spindle-like cells had fewer detectable receptors than in the areas of less ordered fibrous tissue. Compared to Flt-1 and IGF-1R, the expression of TGFβR1 and PDGFRα in this zone was at least 50% stronger.

In the immature chondroid, IGF-1R staining reached over 75%, but almost disappeared when the chondrocytes hypertrophied during consolidation. The remaining receptors also decreased in this zone, with the Flt-1 decline manifesting itself as early as already on day 7).

A number of vessels' lumen was also highlighted immunohistochemically.

Table 2 Immunohistochemical analysis of temporal changes of GFRs expression in four zones of distraction callus. An overall decrease in expression of all analyzed GFRs could be observed after 14 days of consolidation. [a] The virtually 100% Flt-1 staining in endothelium and trabeculae lining cells not included into average expression calculation.

	Chondral tissue				Fibrous tissue				Woven bone				Trabecular bone			
	IGF-1R	TGF-βR1	PDG-FRα	Flt-1	IGF-1R	TGF-βR1	PDG-FRα	Flt-1*	IGF-1R	TGF-βR1	PDG-FRα	Flt-1	IGF-1R	TGF-βR1	PDG-FRα	Flt-1[a]
0 days	++++	+++	+++	+++	+	+++	+++	+	++++	++++	++++	++	++++	++++	++++	+++
7 days	++	++	++	+	+	+	+	+	+++	++++	++++	++	+++	++++	++++	++
14 days	+	++	+	+	+	+	+	+	+++	+++	+++	++	+++	+++	+++	++

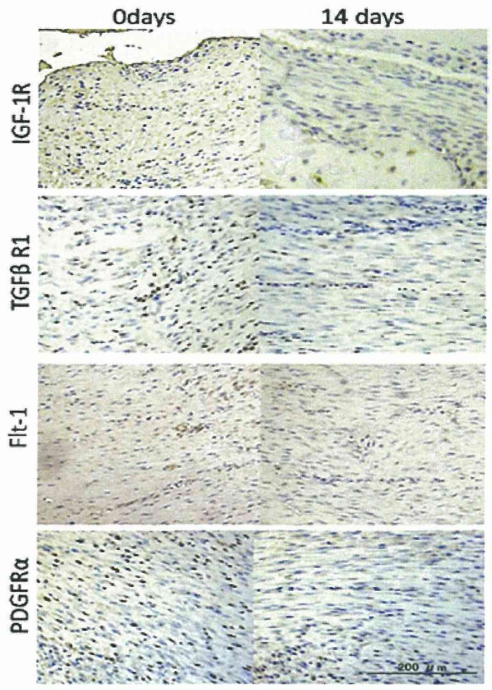

Fig. 4 Microphotographs of immunohistological staining of IGF-1R, TGFβR1, PDGFRα and Flt-1 at 0 and 14 days of consolidation in fibrous zone. Particularly large receptor reduction was observed for TGFβR1 and PDGFRα after 14 days of consolidation.

Receptor characteristics: TGFβR1 showed the most intense staining at all stages in all zones (only IGF-1R in immature chondroid exhibited stronger staining but lost its dominance after 1 week). PDGFRα represented the second most intensive staining after TGFβR1 (a weaker expression than TGFβR1 was observed only in hypertrophied chondrocytes). Both receptors decreased slowly in bony tissues but had lost their intensity after only 1 week in the fibrous zone. Except for chondral tissue, Flt-1 showed a low (+/++) but constant expression throughout consolidation. However, vessels and cells lining the surface of newly formed trabeculae were stained close to 100%, while no other receptor showed a similarly strong expression in analogous areas.

Quantitative real-time PCR (Fig. 5):

The overall decrease in mRNA receptors expression with time was consistent with immunohistochemical studies.

PDGFRα exhibited the strongest expression of all receptors at both 0 and 14 days of consolidation, reaching an almost two-fold reduction within those 2 weeks (5.13E-01 and 2.83E-01, respectively). IGF-1R was prevalent to Flt-1 and TGFβR1 while mRNA for Flt-1 displayed a relatively weak expression (5.20E-03 and 3.14E-03, respectively). The expression of TGFβR1 mRNA dropped from 1.06E-03 at 0 days to 3.71E-04 at 14 days.