Fibrodysplasia Ossificans Progressiva-related Activin-like Kinase Signaling Enhances Osteoclast Formation during Heterotopic Ossification in Muscle Tissues*

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Fibrodysplasia ossificans progressiva is characterized by extensive ossification within muscle tissues, and its molecular pathogenesis is responsible for the constitutively activating mutation (R206H) of the bone morphogenetic protein type 1 receptor, activin-like kinase 2 (ALK2). In this study, we investigated the effects of implanting ALK2 (R206H)-transfected myoblastic C2C12 cells into nude mice on osteoclast formation during heterotopic ossification in muscle and subcutaneous tissues. The implantation of ALK2 (R206H)-transfected C2C12 cells with BMP-2 in nude mice induced robust heterotopic ossification with an increase in the formation of osteoclasts in muscle tissues but not in subcutaneous tissues. The implantation of ALK2 (R206H)-transfected C2C12 cells in muscle induced heterotopic ossification more effectively than that of empty vector-transfected cells. A co-culture of ALK2 (R206H)-transfected C2C12 cells as well as the conditioned medium from ALK2 (R206H)-transfected C2C12 cells enhanced osteoclast formation in Raw264.7 cells more effectively than those with empty vector-transfected cells. The transfection of ALK2 (R206H)-transfected C2C12 cells into myoblasts elevated the expression of transforming growth factor (TGF)-β, whereas the inhibition of TGF-β signaling suppressed the enhanced formation of osteoclasts in the co-culture with ALK2 (R206H)-transfected C2C12 cells and their conditioned medium. In conclusion, this study demonstrated that the causal mutation transduction of fibrodysplasia ossificans progressiva in myoblasts enhanced the formation of osteoclasts from its precursor through TGF-β in muscle tissues.

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The abbreviations used are: FOP, fibrodysplasia ossificans progressiva; BMP, bone morphogenetic protein; ALK2, activin-like kinase 2; RANKL, receptor activator of nuclear factor κ B ligand; OPG, osteoprotegerin; qCT, quantitative computed tomography; TRAP, tartrate-resistant acid phosphatase; MNC, multinucleated cell; CM, conditioned medium; ALP, alkaline phosphatase; Ctsk, cathepsin K.

**The significance of this study:**
Fibrodysplasia ossificans progressiva (FOP) is a rare autosomal dominant disorder of skeletal malformations and progressive extraskeletal ossification (1). A heterozygous constitutively activating mutation (c.617G>A; p.R206H) in the bone morphogenetic protein (BMP) type I receptor, activin receptor type I (ACVR1/activin-like kinase 2 (ALK2)), was detected in patients with the classic form of FOP (2). The constitutive activation of ALK2 signaling has been shown to induce heterotopic ossification in skeletal muscle and other connective tissues such as aponeuroses, fascia, ligaments, and tendons in FOP, whereas subcutaneous tissues are spared from FOP (1, 3, 4). A previous study demonstrated that acute heterotopic ossification was induced by muscle injury, such as accidental trauma and surgery (4). However, there is currently no effective medical treatment to prevent the formation of heterotopic bone in FOP (4). Therefore, elucidating the mechanism responsible for the progression of FOP is needed to meet the clinical demand for FOP treatments.

Recent progress has been made in clarifying the relationships between bone metabolism and several organs. Previous studies indicated that an increase in muscle mass was closely related to an increase in bone mass and a decrease in fracture risk in postmenopausal women (5, 6). Richards et al. (7) showed that muscle flaps applied to autogenous bone grafts improved bone healing, whereas coverage with skin did not. We previously demonstrated that Tmem119 played a crucial role in the commitment of myoprogenitor cells to the osteoblast lineage related to the pathogenesis of FOP (8, 9). Moreover, we and others reported that some humoral factors produced from muscle tissues possessed bone anabolic activity (10, 11). These findings indicated that muscle tissues play some important...
physiological and pathophysiological roles through certain interactions between muscle tissues and bone metabolism.

Osteoclastogenesis is regulated by osteoblast lineage cells through several factors such as receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) (12, 13). Osteoclastogenesis was also previously shown to be enhanced by cytokines and growth factors, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and transforming growth factor (TGF)-β (13, 14). Moreover, several studies reported that BMP signaling enhanced osteoclast formation (15), which suggested that osteoclasts may play some role in the pathogenesis of FOP. However, to the best of our knowledge, the role of osteoclasts in FOP has not yet been investigated, even though the molecular pathogenesis of this disease is related to the constitutive activation of BMP signaling. Previous studies showed that osteoclasts induced the formation of bone by releasing anabolic growth factors from the bone matrix and secreting several factors such as sphingosine 1-phosphate and cardiotrophin-1 in vivo and in vitro (16–18). These findings suggested that osteoclasts may play an important role in the formation of bone as well as heterotopic ossification.

The mechanisms underlying the heterotopic ossification of skeletal muscle in FOP remain to be fully elucidated. Several case studies have suggested that bisphosphonates may effectively treat FOP (19, 20). These findings indicate that osteoclasts may regulate pathological ossification in FOP. Moreover, recent studies suggested an interaction between muscle tissues and bone metabolism (21). However, whether muscle tissues play some role in osteoclast formation has yet to be confirmed.

In this study, we investigated osteoclast formation during heterotopic ossification in the muscle and cutaneous tissues of nude mice, in which ALK2 (R206H)-transfected myoblastic C2C12 cells were implanted to address the role of osteoclasts in the pathogenesis of FOP.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant BMP-2 was provided by Pfizer Inc. (Groton, CT). Anti-TGF-β, anti-Smad1, and anti-Smad5 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-alkaline phosphatase (ALP) antibody was from Abnova (Taipei, Taiwan). Anti-phosphorylated p38 mitogen-activated protein kinase (MAPK), anti-p38 MAPK, anti-phosphorylated Smad1/5/8, anti-phosphorylated Smad2/3, anti-Smad2/3, and anti-β-actin antibodies were from Cell Signaling Technology (Danvers, MA). An anti-TGF-β antibody and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-alkaline phosphatase (ALP) antibody was from Abnova (Taipei, Taiwan). Anti-phosphorylated p38 mitogen-activated protein kinase (MAPK), anti-p38 MAPK, anti-phosphorylated Smad1/5/8, anti-phosphorylated Smad2/3, anti-Smad2/3, and anti-β-actin antibodies were from Cell Signaling Technology (Danvers, MA). A neutralizing anti-TGF-β antibody and normal rabbit IgG were purchased from R&D Systems (Minneapolis, MN). SB431542 was from Tocris Cookson Ltd. (Bristol, UK). Human recombinant TGF-β1, PD98059, SB203580, and curcumin were purchased from Sigma. The wild-type pcDEF3-ALK2 and pcDEF3-ALK2 (R206H) V5-tagged construct was described previously (3).

Cell Culture—Mouse myoblastic C2C12 cells (ATCC, Manassas, VA), mouse monocytic RAW264.7 cells (ATCC), and mouse fibroblastic NIH3T3 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chem., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The medium was changed twice a week.

Stable Transfection—ALK2 (R206H) was transfected into C2C12 or NIH3T3 cells with Lipofectamine (Invitrogen) as described previously (9, 10). Six hours later, the cells were supplied with fresh DMEM containing 10% FBS. The cells were passaged to generate stably transfected C2C12 cells after incubation in DMEM containing 10% FBS for 48 h, and clones were selected in DMEM supplemented with G418 (700 μg/ml; Invitrogen) and 10% FBS. Twenty four clones were selected after 3 weeks of culture in G418. Several clones were selected after Western blotting with the anti-V5 antibody and semi-quantitative RT-PCR, as described previously (9). At least three independent clones for each stable transfection were characterized to rule out the possibility of clonal variation. Empty vector-transfected cell clones were used as the control. Stably transfected C2C12 or NIH3T3 cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 700 μg/ml G418.

Animals—Seven-week-old male nude mice (BALB/c nu/nu) were purchased from Japan Clea Laboratory (Tokyo, Japan). They were acclimated to our animal facilities for 1 week and then used in experiments. All experiments were performed according to the guidelines of the National Institutes of Health and the institutional rules for the use and care of laboratory animals at Kinki University.

Heterotopic Ossification Model—Heterotopic ossification was induced as described previously (22) with modifications. Briefly, a 2.5-μl aliquot containing 5 μg of recombinant human BMP-2 was adsorbed onto a 5 × 5-mm-thick atelocollagen sponge (Koken Co., Ltd., Tokyo, Japan). Under anesthesia induced with pentobarbital sodium (50 mg/kg, intraperitoneally), the sponge was implanted into a pouch created in the quadriceps muscle or subcutaneous tissue with stable empty vector- or ALK2 (R206H)-transfected C2C12 cells (1 × 10⁶ cells) in nude mice. Body temperature was maintained at 37 °C during surgery by a heating pad.

To treat mice, SB431542 and SB203580 were dissolved in 50% sterile dimethyl sulfoxide in saline at 5 and 25 mg/ml, respectively. SB431542 at 5 mg/kg and SB203580 at 25 mg/kg were injected intraperitoneally in mice daily for 14 days. An injection of the vehicle alone was used as the control.

In Vivo Quantitative Computed Tomography (qCT) Analysis—The in vivo qCT analysis was performed according to the American Society for Bone and Mineral Research guidelines (23) and our previous study (22). Mice were anesthetized using 2% isoflurane, and the BMP-2-induced heterotopic bone was scanned using an x-ray CT system (Latheta LCT-200; Hitachi Aloka Medical, Tokyo, Japan). Parameters used for the CT scans were as follows: tube voltage, 50 kV peak; tube current, 500 μA; integration time, 3.6 ms; axial field of view, 48 mm, with an isotropic voxel size of 48 μm. Images were generated by the integration of three signal averages for the heterotopic bone. Total scan time was ~9 min for the heterotopic bone. Volume-rendered three-dimensional CT pictures were reconstructed using VGStudio MAX2.2 software (Nikon Visual Science, Tokyo, Japan). The mineral content of the heterotopic bone was calculated using Latheta software (Hitachi Aloka
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Medical. A threshold density of 160 mg/cm³ was selected to distinguish mineralized from unmineralized tissue. The density range was calibrated daily with a manufacturer-supplied phantom.

Histological Analysis—Mice were anesthetized using pentobarbital sodium (50 mg/kg, intraperitoneally) on day 14 after the implantation of transfected cells. The heterotopic bone was removed, fixed in 4% paraformaldehyde, demineralized in 22.5% formic acid and 340 mm sodium citrate solution for 24 h, and embedded in paraffin. Thereafter, 4-μm-thick sections were obtained.

Immunostaining for ALP was performed as described previously (22). Briefly, the sections were incubated with the anti-ALK2 antibody at a dilution of 1:100, followed by incubation with the appropriate secondary antibody conjugated with horseradish peroxidase. Positive signals were visualized using a tyramide signal amplification system (PerkinElmer Life Sciences). These sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and photographed under a fluorescence microscope (E800; Canon, Tokyo, Japan) with a CCD camera. The number of ALP-positive cells in the microscopic fields was quantified in a blinded evaluation.

Sections were stained with tartrate-resistant acid phosphatase (TRAP) by a TRAP staining kit (Wako), according to the manufacturer’s instructions. These sections were counterstained with hematoxylin. The number of TRAP-positive multinucleated cells (MNCs) on the bone surface per 1 mm was measured in the heterotopic bone in a blinded evaluation.

Preparation of Primary Cutaneous Fibroblasts—Primary fibroblasts were isolated from the skin of newborn mice, as described previously (24), with some modifications. Briefly, the skin was removed, cut into 1-mm² fragments, and digested with 1 mg/ml collagenase (Sigma) and 0.25% trypsin (Invitrogen) in DMEM for 30 min at 37 °C. The cell suspension was passed through a 40-μm cell strainer (Falcon). The cells were collected by centrifugation and plated in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were grown until confluent and then used in experiments.

Osteoclast Formation—Raw264.7 cells were seeded at a density of 1 × 10⁵ cells/cm² in minimum essential medium-α modification supplemented with 10% FBS, 1% penicillin/streptomycin, and 50 ng/ml RANKL (Wako Pure Chemical) for 5 days, as described previously (25). In the co-culture system, wild-type, stable empty vector-, or ALK2 (R206H)-transfected C2C12 cells were seeded at a density of 1 × 10⁴ cells/cm² and co-cultured with Raw264.7 cells in minimum essential medium-α modification supplementation with 10% FBS, 1% penicillin/streptomycin, and 50 ng/ml RANKL for 5 days. The medium was changed every 2 days. The cells were fixed and stained for TRAP by a TRAP staining kit (Wako Pure Chemical). Osteoclasts were identified as TRAP-positive cells containing three or more nuclei.

Preparation of Conditioned Medium (CM)—To prepare CM, C2C12 cells and primary fibroblasts were cultured in 10-cm dishes. When the cells had grown to confluence, the medium was replaced with flash minimum essential medium-α modification supplemented with 10% FBS and 1% penicillin/streptomycin. The conditioned medium was collected and replaced by the fresh medium after 24 or 48 h.

RNA Extraction and Real Time PCR—Total RNA was prepared from cells using TRIzol reagent (Invitrogen). cDNA was synthesized using a High Capacity cDNA reverse transcription kit for RT-PCR (Applied Biosystems, Foster City, CA). Specific mRNA was quantified by StepOnePlus real time PCR systems (Applied Biosystems) with a Fast SYBR Green master mix (Applied Biosystems), according to the manufacturer’s standard protocol. The specific mRNA amplification of the target was determined as the Ct value followed by normalization with the Gapdh level. Primer sequences (forward and reverse) were as follows: cathepsin K (Ctsk), 5'-GGTACTCCAGTCAA-GAACCAGG-3' and 5'-TCTGCTGCAGTTTAGGAAGG-3'; Trap, 5'-GCAACATCCCTGATTGTG-3' and 5'-GCA-GAGCTATGAAGGCTG-3'; ALK2, 5'-AGGGCTATCCA-CCACCAAATG-3' and 5'-TTCCGCAACACTCCACAG-3'; Tgf-β1, 5'-GCAAATCTTCCCTGGTTACC-3' and 5'-CGCTGAATCAGAAGCCTGTA-3'; Id1, 5'-GGTGGAAT-CCTCTGACAGT-3' and 5'-CAAGCTGCAGTCCCTG-ATG-3'; and Gapdh, 5'-AGGTCCGTGTGTAACGGAT-TTG-3' and 5'-GGGGTCTTGTGATTGCACA-3'.

Microarray Analysis—Microarray analysis was performed, as described previously (9). Total RNA was extracted from C2C12 cells stably transfected with the empty vector or ALK2 (R206H) using TRIzol reagent. Total RNA was purified using the RNasy MiniElute clean up kit (Qiagen, Tokyo, Japan) to yield an A260/280 > 1.90. Double-stranded cDNA was synthesized using a T7-oligo(dT) primer with the 3'IVT express kit (Affymetrix Inc., Santa Clara, CA). Hybridization samples were prepared and processed according to the GeneChip Expression Analysis Technical Manual. The Affymetrix Murine Genome 430 2.0 set was used to compare gene expression. Data were analyzed using the Affymetrix Operating Software Version 1.4 (Affymetrix 690036) according to the manufacturer’s standard protocol. The specific mRNA amplification of the target was determined as the Ct value followed by normalization with the Gapdh level. Primer sequences (forward and reverse) were as follows: cathepsin K (Ctsk), 5'-GGTACTCCAGTCAA-GAACCAGG-3' and 5'-TCTGCTGCAGTTTAGGAAGG-3'; Trap, 5'-GCAACATCCCTGATTGTG-3' and 5'-GCA-GAGCTATGAAGGCTG-3'; ALK2, 5'-AGGGCTATCCA-CCACCAAATG-3' and 5'-TTCCGCAACACTCCACAG-3'; Tgf-β1, 5'-GCAAATCTTCCCTGGTTACC-3' and 5'-CGCTGAATCAGAAGCCTGTA-3'; Id1, 5'-GGTGGAAT-CCTCTGACAGT-3' and 5'-CAAGCTGCAGTCCCTG-ATG-3'; and Gapdh, 5'-AGGTCCGTGTGTAACGGAT-TTG-3' and 5'-GGGGTCTTGTGATTGCACA-3'.

Protein Extraction and Western Blot Analysis—Cells were lysed with Laemmli Sample Buffer (Bio-Rad). Proteins were transferred in 25 mm Tris, 192 mm glycine, and 20% methanol to a polyvinylidene difluoride membrane. Blots were blocked with 20 mm Tris-HCl (pH 7.5), 137 mm NaCl, and 0.1% Tween 20 containing 3% dried milk powder. Membranes were immunoblotted with each antibody. Antibody-antigen complexes were visualized using appropriate secondary antibodies (Cell Signaling Technology) and the ECL Prime Western blotting detection reagent (GE Healthcare), as recommended by the manufacturer. The results depicted in each figure were representative of at least three independent cell preparations.

Statistical Analysis—Data are expressed as the means ± S.E. Significance was evaluated using the Student’s t test for two group comparisons and an analysis of variance followed by Tukey’s test for multiple comparisons. The significance level was set at p < 0.05.
**RESULTS**

*Effects of the Implantation of ALK2 (R206H)-transfected Myoblastic Cells on BMP-2-induced Heterotopic Ossification*—BMP-2 absorbed onto an atelocollagen sponge was employed to induce heterotopic ossification in the muscle or subcutaneous tissues of nude mice. Heterotopic bone was formed in the muscle tissues of nude mice, in which empty vector- or ALK2 (R206H)-transfected C2C12 cells were implanted in the presence of BMP-2, as assessed by qCT (Fig. 1A). Total bone mineral content in the heterotopic bone was significantly higher in muscle tissues implanted with ALK2 (R206H)-transfected C2C12 cells than in tissues implanted with empty vector-transfected C2C12 cells (Fig. 1B). The number of ALP-positive cells in the heterotopic bone on day 14 (Fig. 1C) and the number of TRAP-positive MNCs in the heterotopic bone on day 14 (Fig. 1D) were also significantly higher in muscle tissues implanted with ALK2 (R206H)-transfected C2C12 cells than in tissues implanted with empty vector-transfected C2C12 cells (Fig. 1E and F). The number of TRAP-positive MNCs in the heterotopic bone on day 14 was also significantly higher in muscle tissues implanted with ALK2 (R206H)-transfected C2C12 cells than in tissues implanted with empty vector-transfected C2C12 cells (Fig. 1G). The results represent experiments performed on four or five mice in each group (A–H). Scale bars, 50 μm (C, E, and G). Data represent the mean ± S.E. of four or five mice in each group (B, D, F, and H). *, p < 0.05; **, p < 0.01.

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We examined the roles of ALK2 (R206H)-transfected mouse fibroblasts in osteoclast formation. We investigated the levels of several known osteoclast formation-related factors, such as Ccn2/connexin 26, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor-2, IGF-1, IGF-2, IGFBP-3, Id1, Rankl, Sost, Tnf-α, Wnt1, -2, -2b, -3, -3a, -4, -5a, -5b, -6, -7b, -8a, -8b, -9d, -10a, -10b, -10c, -11, and -12 were undetectable in empty vector- and ALK2 (R206H)-transfected C2C12 cells in this microarray analysis (data not shown). We confirmed that Tgfb-1 mRNA levels were higher in ALK2 (R206H)-transfected C2C12 cells than in empty vector-transfected cells, as assessed by real-time PCR and Western blot analyses (Fig. 4, B and C). Tgf-β1 mRNA levels were higher in mouse primary fibroblasts than in C2C12 cells (Fig. 4A). No significant differences were observed in Tgf-β1 mRNA levels among empty vector-, wild-type ALK2-, and ALK2 (R206H)-transfected C2C12 cells in this microarray analysis (data not shown). We confirmed that Tgfb-1 levels were higher in ALK2 (R206H)-transfected C2C12 cells than in empty vector-transfected cells, as assessed by real-time PCR and Western blot analyses (Fig. 4, B and C). Tgf-β1 mRNA levels were higher in mouse primary fibroblasts than in C2C12 cells (Fig. 4A). No significant differences were observed in Tgf-β1 mRNA levels among empty vector-, wild-type ALK2-, and ALK2 (R206H)-transfected mouse fibroblastic NIH3T3 cells (Fig. 4B). The addition of BMP-2 did not affect Tgf-β1 mRNA levels in C2C12 cells (Fig. 4A). TGF-β1 levels were not higher in wild-type ALK2-transfected C2C12 cells than in empty vector-transfected cells (Fig. 4A). A and B, osteoclast formation was induced in RAW264.7 cells treated with 20% CM from the primary cultures of fibroblasts of newborn mice or C2C12 cells. A, TRAP-positive MNCs were counted under a microscope. B, total RNA was extracted from RAW264.7 cells treated with CM, and real-time PCR for Ctsk, Trap, or Gapdh was performed. Data represent the mean ± S.E. of six wells (A) and three samples (B). **, p < 0.01 versus each fibroblast CM-treated group.

Comparison of the Ability of Myoblasts and Fibroblasts to Support Osteoclast Formation—We examined the effects of CM from myoblastic C2C12 cells and primary subcutaneous fibroblasts from newborn mice on osteoclast formation in RAW264.7 cells. The number of TRAP-positive MNCs was significantly increased in RAW264.7 cells treated with CM from C2C12 cells than in those treated with CM from subcutaneous fibroblasts (Fig. 4A). The elevation induced in Ctsk and Trap mRNA levels in RAW264.7 cells was significantly higher by CM in C2C12 cells than by subcutaneous fibroblasts (Fig. 2B). These results suggested that osteoclast formation was markedly induced in the presence of myoblastic C2C12 cells rather than subcutaneous fibroblasts, and this was partly attributed to the release of some soluble factors from myoblasts.

Effects of ALK2 (R206H)-transfected Myoblasts on Osteoclast Formation—We examined the roles of ALK2 (R206H)-transfected C2C12 cells in osteoclast formation in vitro. The number of TRAP-positive MNCs was significantly higher in the co-culture of RAW264.7 cells with ALK2 (R206H)-transfected C2C12 cells than in the co-culture with empty vector-transfected C2C12 cells (Fig. 3A). Empty vector- and ALK2 (R206H)-transfected C2C12 cells did not affect the number of TRAP-positive MNCs in the absence of RANKL (data not shown). The levels of Ctsk and Trap mRNA were significantly higher in the co-culture of RAW264.7 cells with ALK2 (R206H)-transfected C2C12 cells than in the co-culture with empty vector-transfected C2C12 cells (Fig. 3, B and C). We then examined the effects of CM from empty vector- and ALK2 (R206H)-transfected C2C12 cells on osteoclast formation. The number of TRAP-positive MNCs was significantly higher in RAW264.7 cells treated with CM from ALK2 (R206H)-transfected C2C12 cells than in cells treated with CM from empty vector-transfected C2C12 cells (Fig. 3D). CM from ALK2 (R206H)-transfected C2C12 cells significantly elevated the levels of Ctsk and Trap mRNA in RAW264.7 cells (Fig. 3, E and F). CM from wild-type ALK2-transfected cells did not affect the number of TRAP-positive MNCs or levels of Ctsk and Trap mRNA in RAW264.7 cells (Fig. 3, D–F). These results indicated that the transfection of ALK2 (R206H) in myoblasts enhanced osteoclast formation, at least in part through the release of some soluble factors from myoblasts.

Increase in TGF-β Levels in ALK2 (R206H)-transfected C2C12 Cells—We hypothesized that the transfection of ALK2 (R206H) in myoblasts may induce osteoclast differentiation factors. We investigated the levels of several known osteoclast differentiation factor transcripts by a comparative DNA microarray analysis between stable empty vector- and ALK2 (R206H)-transfected C2C12 cells. The levels of several osteoclast formation-related factors, such as Ccn2/connexin 26, growth factor-β, growth differentiation factor-15 (Gdf-15), Tgf-β1, and Tgf-β2, were higher in stable ALK2 (R206H)-transfected C2C12 cells than in empty vector-transfected cells (Table 1). The levels of activin, Bmp-2, Bmp-4, Bmp-6, Bmp-7, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor-2, IGF-1, IGF-2, IGFBP-3, Ópg, Rankl, Sost, Tnf-α, Wnt1, -2, -2b, -3, -3a, -4, -5a, -5b, -6, -7b, -8a, -8b, -9d, -10a, -10b, -10c, and -11 were undetectable in empty vector- and ALK2 (R206H)-transfected C2C12 cells in this microarray analysis (data not shown). We confirmed that Tgf-β1 levels were higher in ALK2 (R206H)-transfected C2C12 cells than in empty vector-transfected cells, as assessed by real-time PCR and Western blot analyses (Fig. 4, B and C). Tgf-β1 mRNA levels were higher in mouse primary fibroblasts than in C2C12 cells (Fig. 4A). No significant differences were observed in Tgf-β1 mRNA levels among empty vector-, wild-type ALK2-, and ALK2 (R206H)-transfected mouse fibroblastic NIH3T3 cells (Fig. 4B). The addition of BMP-2 did not affect Tgf-β1 mRNA levels in C2C12 cells (Fig. 4A). TGF-β1 levels were not higher in wild-type ALK2-transfected C2C12 cells than in empty vector-transfected cells (Fig. 4A). A and B, osteoclast formation was induced in RAW264.7 cells treated with 20% CM from the primary cultures of fibroblasts of newborn mice or C2C12 cells. A, TRAP-positive MNCs were counted under a microscope. B, total RNA was extracted from RAW264.7 cells treated with CM, and real-time PCR for Ctsk, Trap, or Gapdh was performed. Data represent the mean ± S.E. of six wells (A) and three samples (B). **, p < 0.01 versus each fibroblast CM-treated group.

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These results suggested that the ALK2 (R206H) mutation enhanced the expression of TGF-β in myoblasts.

Involvement of TGF-β in the ALK2 (R206H)-transfected C2C12 Cell-mediated Osteoclast Formation—We examined the involvement of TGF-β in ALK2 (R206H)-transfected C2C12 cell-mediated formation of osteoclasts. TGF-β significantly increased the number of TRAP-positive MNCs at concentrations of 0.1 and 1 ng/ml in Raw264.7 cells and the co-culture with C2C12 cells (Fig. 5A and B). SB431542 specifically inhibits ALK5 (TGF-β receptor type I kinase) and the subsequent phosphorylation of Smad2/3 and was therefore considered to be useful for assessing the effects of endogenous TGF-β (26, 27). SB431542 suppressed the increase in the number of TRAP-positive MNCs in Raw264.7 cells co-cultured with ALK2 (R206H)-transfected C2C12 cells (Fig. 5C). Moreover, SB431542 decreased the number of TRAP-positive MNCs and levels of Ctsk and Trap mRNA in Raw264.7 cells treated with CM from ALK2 (R206H)-transfected C2C12 cells (Fig. 5D and E).
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**TABLE 1** Gene transcripts in stable ALK2 (R206H)-transfected C2C12 cells versus vector alone-transfected cells

| Gene name | Ratio | Gene name | Ratio |
|-----------|-------|-----------|-------|
| Ccn2      | 1.5   | Il-34     | 0.7   |
| Egf       | 0.7   | Mgp-1     | 0.5   |
| Fgf-5     | 1.1   | Mgp-2     | 0.5   |
| Fgf-7     | 1.1   | Mgp-3     | 1.5   |
| Fgf-9     | 0.9   | MCSF      | 1.0   |
| Gdf-11    | 1.1   | Mip-1y    | 0.4   |
| Gdf-15    | 1.5   | Mip-2     | 0.4   |
| Igfbp-2   | 1.1   | Pdgfa     | 0.9   |
| Igfbp-5   | 0.8   | Pdgfb     | 1.4   |
| Igfbp-6   | 1.1   | Sema3b    | 0.6   |
| Igfbp-7   | 1.1   | Sema3c    | 0.7   |
| H-1B      | 1.1   | Sema3d    | 0.2   |
| H-6       | 1.0   | Tgf-β1    | 1.7   |
| H-7       | 0.2   | Tgf-β2    | 1.7   |
| H-11      | 0.7   | Tgf-β3    | 1.4   |
| H-12      | 0.7   | Vegfa     | 1.4   |
| H-16      | 0.9   | Vegfb     | 0.7   |
| H-18      | 0.4   | Wnt-7a    | 0.6   |
| H-33      | 0.1   | Wnt-9a    | 1.1   |

E) Because SB431542 inhibits ALK4, ALK7, and casein kinase Ib, we employed a neutralizing anti-TGF-β antibody. The enhanced formation of osteoclasts by CM from ALK2 (R206H)-transfected C2C12 cells was antagonized by the neutralizing anti-TGF-β antibody (Fig. 5F). These results suggested that ALK2 (R206H)-transfected myoblasts may enhance osteoclast formation via TGF-β.

**Roles of the TGF-β Signaling Pathways in Raw264.7 Cells during Osteoclast Formation Mediated by ALK2 (R206H)-transfected Myoblasts**—TGF-β signaling is known to be transduced by the phosphorylation of Smad2 and Smad3, which converts them into transcriptional regulators that complex with Smad4. TGF-β also activates MAPK signaling pathways such as the p38 MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2), and Jun N-terminal kinase (JNK). Therefore, we investigated the involvement of TGF-β signaling pathways in Raw264.7 cells during the formation of osteoclasts mediated by ALK2 (R206H)-transfected C2C12 cells. The inhibitor of p38 MAPK, SB203580, significantly suppressed the increase in the number of TRAP-positive MNCs induced by CM from ALK2 (R206H)-transfected C2C12 cells (Fig. 6A). Conversely, PD98059, an inhibitor of ERK1/2, increased the number of osteoclasts formed in Raw264.7 cells treated with CM from empty vector- and ALK2 (R206H)-transfected C2C12 cells (Fig. 6A), and these results were consistent with the findings of a previous study (25). Curcumin, an inhibitor of JNK, did not affect the number of TRAP-positive MNCs induced by CM from ALK2 (R206H)-transfected C2C12 cells (Fig. 6A). SB431542 and SB203580 effectively suppressed the phosphorylation of Smad2/3 and p38 MAPK induced by TGF-β in Raw264.7 cells, respectively (Fig. 6B). Moreover, SB431542 effectively suppressed the phosphorylation of Smad2/3 induced by TGF-β in C2C12 cells (Fig. 6C). These results suggested that Smad2/3 and p38 MAPK, but not ERK1/2 or JNK, contributed to the formation of osteoclasts mediated by ALK2 (R206H)-transfected C2C12 cells.

**Roles of TGF-β and p38 MAPK in Osteoclast Formation Enhanced by the ALK2 (R206H) Mutation in Vivo**—SB431542 and SB203580 significantly suppressed the formation of osteoclasts that had been enhanced by ALK2 (R206H)-transfected C2C12 cell implantation with BMP-2 in vivo (Fig. 7). These results indicated that TGF-β and p38 MAPK may be involved in the formation of osteoclasts enhanced by the ALK2 (R206H) mutation in vivo.

**DISCUSSION**

An increasing number of studies have suggested that muscle tissues play important physiological and pathophysiological roles through certain interactions with bone metabolism (21). Fractures that are covered with relatively intact muscle have been shown to improve more rapidly than fractures associated with more severe damage (28). Proinflammatory cytokines, in particular TNF-α, at the site of fracture were found to induce the differentiation of stromal cells present in muscle into osteoprogenitor cells, which subsequently promoted fracture healing (29). These findings suggested that muscle tissues may affect the ossification process. FOP is a rare skeletal disorder associated with progressive heterotopic ossification in soft tissues, especially skeletal muscle, but not subcutaneous tissues (1). A previous study showed that the constitutive activation of BMP signaling by the ALK2 mutation was responsible for the molecular pathogenesis of FOP (1). However, the mechanisms underlying the heterotopic ossification of skeletal muscle in FOP remain to be fully elucidated. We revealed that the implantation of ALK2 (R206H)-transfected myoblasts in the presence of exogenous BMP-2 induced more robust heterotopic ossification in muscle tissues than in subcutaneous tissues in nude mice, which indicated that muscle tissues rather than subcutaneous tissues more effectively support heterotopic ossification. Moreover, the results of this study suggest that the pathogenesis of FOP cannot only be explained by the activation of BMP signaling; however, muscle tissues may play some important roles in heterotopic ossification in FOP. We previously reported that Tnem119 may play a critical role in the commitment of myoprogenitor cells to the osteoblast lineage (8, 9). Leblanc et al. (30) reported that BMP-9, which is one of the ALK2 ligands, was involved in the pathophysiology of heterotopic ossification, and its activity depended on the skeletal muscle microenvironment in mice. These findings suggest that there may be some local regulators that specifically enhance ossification in muscle tissues in FOP.

Bone formation and resorption are regulated by multiple cellular interactions (31). Intercellular communication between osteoblasts and osteoclasts is crucial for bone homeostasis. The formation of osteoclasts is regulated by osteoblast lineage cells through several factors, including RANKL and OPG (13). Several studies demonstrated that BMP signaling enhanced the formation of osteoclasts (15, 32–34). Otsuka et al. (35) reported that BMP-2-treated myoblastic cells stimulated the formation of osteoclasts in vitro. These findings suggest that the activation of BMP signaling in myoblastic cells may enhance osteoclast formation. We showed that osteoclast formation in Raw264.7 cells was enhanced more by CM from myoblastic cells than from subcutaneous fibroblasts from newborn mice. Moreover, osteoclast formation in Raw264.7 cells was further increased in a co-culture with ALK2 (R206H)-transfected myoblasts in this study. These findings suggest that the constitutive activation of BMP signaling by ALK2 (R206H) in myoblasts may be
responsible for the enhancement in osteoclast formation following the implantation of ALK2 (R206H)-transfected myoblasts into nude mice in vivo.

Several case studies showed that bisphosphonates are effective for the treatment of FOP (19, 20). Moreover, we previously encountered in a patient with FOP higher levels of a bone resorption marker (36). These findings indicated that osteoclasts may be involved in the pathogenesis of FOP. Osteoclasts have been shown to release bone anabolic factors such as IGF-I and TGF-β from the bone matrix during the process of bone resorption (18). Recent studies reported that osteoclasts promoted bone formation through the release of several factors such as sphingosine 1-phosphate and cardiotrophin-1 in vivo and in vitro (16, 37). This evidence suggests that osteoclasts may be involved in the heterotopic ossification of muscle tissues in FOP. In this study, we showed that the implantation of ALK2 (R206H)-transfected myoblasts enhanced heterotopic ossification with osteoblastogenesis in muscle tissues in nude mice. At the bone sites in muscle tissues during heterotopic ossification, osteoclast formation was enhanced by myoblasts with the ALK2 (R206H) mutation. These results indicated that myoblasts with the constitutively active mutation of ALK2 enhanced osteoclastogenesis in muscle tissues. Taken together, ALK2 (R206H)-transfected myoblasts may enhance heterotopic ossification through osteoclastogenesis in FOP.
Muscle tissues produce local growth factors that affect bone tissues (21). Several studies demonstrated that TGF-β enhanced osteoclast formation by RANKL in bone marrow macrophages and Raw264.7 cells, which suggested that TGF-β may enhance the formation of osteoclasts both physiologically and pathologically (14, 38). We revealed that myoblasts expressed several osteoclast formation-related factors by DNA microarray analysis. Among them, TGF-β levels were higher in ALK2 (R206H)-transfected myoblasts than in empty vector-transfected myoblasts. Moreover, the ALK2 (R206H) transfection into myoblasts elevated TGF-β levels in cell extracts from cultured myoblasts. The enhancement in the formation of osteoclasts in Raw264.7 cells by the co-culture with ALK2 (R206H)-transfected myoblasts as well as by CM from ALK2 (R206H)-transfected myoblasts was suppressed by the inhibition of TGF-β signaling with an endogenous TGF-β signaling inhibitor. Furthermore, the enhancement in osteoclast formation in Raw264.7 cells by CM from ALK2 (R206H)-transfected myoblasts was antagonized by a neutralizing TGF-β antibody. The inhibition of the TGF-β signal also suppressed osteoclast formation facilitated by ALK2 (R206H)-transfected myoblast implantation with BMP-2 in nude mice. These findings indicate that TGF-β is responsible for the enhancement in osteoclast formation by ALK2 (R206H)-transfected myoblasts.
ALK2 Signaling Enhances Osteoclast Formation

FIGURE 6. Activation of TGF-β signal pathway during myoblast-mediated osteoclast formation. A, osteoclast formation was induced in Raw264.7 cells treated with 20% CM from stable vector-transfected or ALK2 (R206H)-transfected C2C12 cells with 1 μM SB203580, 20 μM PD98059, 5 μM curcumin, and vehicle (Cont). Raw264.7 cells were pretreated with SB203580, PD98059, and curcumin for 1 h before the treatment of CM. The number of TRAP-positive MNCs was counted. Data represent the mean ± S.E. of six wells. **, p < 0.01. B, total protein was extracted from Raw264.7 cells treated in the absence or presence of 5 ng/ml TGF-β for 1 h (upper panel) or 15 min (lower panel) with or without 1 μM SB431542 or 1 μM SB203580 pretreatment, and Western blot analysis for phosphorylated p38 MAPK, p38 MAPK, phosphorylated Smad2/3, Smad2/3, and β-actin was performed. C, total protein was extracted from C2C12 cells treated in the absence or presence of 5 ng/ml TGF-β for 1 h with or without 3 μM SB431542 pretreatment, and Western blot analysis for phosphorylated Smad2/3, Smad2/3, and β-actin was performed.

In Fig. 4A, subcutaneous fibroblasts expressed significantly higher amounts of Tgf-β1, compared with C2C12 cells. Because exogenous TGF-β addition enhances osteoclast formation, fibroblasts might secrete some factors that suppress osteoclast formation induced by TGF-β. Further studies will be necessary to clarify this issue.

TGF-β signaling is initiated by ligand binding to two cell surface receptors, type I and II TGF-β receptors. The activation of TGF-β receptors subsequently phosphorylates Smad2 and Smad3 (39). TGF-β is also known to mediate its signaling through a Smad-independent pathway by activating the MAPK pathways of ERK1/2, JNK, and p38 MAPK (39). Previous studies reported that activation of the p38 MAPK pathway enhanced osteoclast formation in primary bone marrow cells and Raw264.7 cells, which suggested that the p38 MAPK signaling pathway plays an important role in osteoclast formation (40, 41). Moreover, several groups showed that TGF-β promoted osteoclastogenesis in monocytes by stimulating p38 MAPK (42, 43). In this study, we showed that the inhibition of p38 MAPK as well as ALK5 abolished the enhancement in osteoclast formation by the treatment with CM from ALK2 (R206H)-transfected myoblasts. Moreover, the inhibition of p38 MAPK as well as the TGF-β signal suppressed osteoclast formation facilitated by ALK2 (R206H)-transfected myoblasts. Taken together, our results indicate that TGF-β production from myoblasts, which was enhanced by the ALK2 (R206H) transfection, stimulated osteoclast formation through Smad2/3 and p38 MAPK pathways in osteoclast precursor Raw264.7 cells.

The implantation of ALK2 (R206H)-transfected C2C12 cells in nude mice induced a marked increase in osteoclast formation in the presence of exogenous BMP-2 addition, whereas the coculture with ALK2 (R206H)-transfected C2C12 cells as well as the conditioned medium from ALK2 (R206H)-transfected C2C12 cells enhanced osteoclast formation from Raw264.7 cells more effectively than those with empty vector-transfected cells in the absence of exogenous BMP-2. The reasons for the differences between in vivo and in vitro assays are currently unclear. The activation of BMP signaling through Smad1 or Smad5 by the ALK2 (R206H) mutant is considered to be mild in the absence of the exogenous addition of BMP. Because the ALK2 (R206H) mutation causes impaired binding to FKBP12/FKBP-12, a molecule that is known to safeguard against the leakage of BMP signaling, and an altered subcellular distribution, the ALK2(R206H) mutant is mainly located at the plasma membrane regardless of the stimulation with BMP-2 (44). Moreover, the presence of RANKL may be related to the differences between in vivo and in vitro assays because RANKL was exogenously added to the in vitro osteoclast formation assay in this study.
There are currently no effective medical treatment options to prevent the formation of heterotopic bone in FOP (4). Glucocorticoids have been used to manage FOP symptoms (4). Nonsteroidal anti-inflammatory drugs have been shown to manage chronic pain and progression of the disease (4). These findings suggest that inflammation contributes to disease progression in FOP. Inflammatory stimuli, including TGF-β, enhance osteoclastogenesis. Because our findings imply that TGF-β-mediated osteoclast formation is involved in the heterotopic ossification in FOP, a further in vivo study using osteoclast-targeting agents is warranted for the treatment of heterotopic ossification in FOP.

In conclusion, the results of the present study provide novel evidence that ALK2 (R206H)-transfected myoblasts enhance osteoclast formation through TGF-β in muscle tissues. Further studies are necessary to clarify the pathological role of osteoclasts induced by the FOP mutation.

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