ORIGINAL

Role of matrix metalloproteinase-10 in the BMP-2 inducing osteoblastic differentiation

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Abstract. Fibrodysplasia ossificans progressiva (FOP) is a skeletal disorder with progressive heterotopic ossification in skeletal muscle. A mutation causing constitutive activation in a bone morphogenetic protein (BMP) type 1 receptor [ALK2(R206H)] is found in most patients with FOP. However, the details in the heterotopic ossification of muscle in FOP and the role of matrix metalloproteinase-10 (MMP-10) in bone remain to be fully elucidated. In the present study, we investigated the role of MMP-10 in the differentiation of mouse myoblastic C2C12 cells into osteoblasts. MMP-10 was extracted as a factor, whose expression was most extensively enhanced by ALK2 (R206H) transfection in C2C12 cells. MMP-10 significantly augmented the levels of Osterix, type 1 collagen, alkaline phosphatase (ALP) and osteocalcin mRNA as well as ALP activity enhanced by BMP-2 in C2C12 cells. Moreover, a reduction in endogenous MMP-10 levels by siRNA significantly decreased the levels of Runx2, Osterix, type 1 collagen, ALP and osteocalcin mRNA enhanced by BMP-2 in these cells. In addition, MMP-10 increased the phosphorylation of Smad1/5/8 as well as enhanced the levels of Smad6 and Smad7 mRNA induced by BMP-2. In conclusion, the present study first demonstrated that MMP-10 promotes the differentiation of myoblasts into osteoblasts by interacting with the BMP signaling pathway. MMP-10 may play some important role in the heterotopic ossification of muscle in FOP.

Key words: Matrix metalloproteinase-10, Bone morphogenetic protein, Osteoblast, Fibrodysplasia ossificans progressiva, Muscle ossification

FIBRODYSPLASIA OSSIFICANS PROGRESSIVA (FOP) is a rare autosomal dominant disorder of skeletal malformations, which characterized by postnatal progressive heterotopic ossification in soft tissues, especially skeletal muscle [1]. A mutation causing constitutive activation (617G>A; R206H) in a bone morphogenetic protein (BMP) type 1 receptor, the activin receptor type 1 (ACVR1) /activin-like kinase 2 (ALK2), is found in patients with the classic form of FOP [2]. Constitutive activation of BMP 1 receptor followed by activation of BMP signaling pathway induces ectopic bone formation in FOP [3]. Song *et al.* reported that the mild activation of the BMP signaling pathways by the ALK2 (R206H) mutant in the absence of exogenous BMP is due to its impaired binding to FKBP1/FKBP-12, a molecule that is known to safeguard against the leakage of BMP signaling [4].

Moreover, we revealed that Tmem119 might be a crucial molecule in the heterotopic ossification of muscle in FOP due to the induction of BMP-2 levels and interaction with the BMP signaling pathway [5]. However, the details of the heterotopic ossification of muscle in FOP remain to be fully elucidated.

BMP-2 plays important roles in bone formation and bone cell differentiation [6-8]. BMPs act through a complex of type I and type II transmembrane serine/threonine kinase receptors. The ligand bound type II receptor phosphorylates the type I receptor, which activates Smad1/5/8. Activated Smad1/5/8 molecules form a complex with the common Smad, Smad4. The complex translocates to the nucleus and regulates transcription of genes important for controlling the induction of bone formation.

Matrix metalloproteinases (MMPs) are members of a family of zinc-dependent proteinases, which are able to cleave many non-extracellular matrix and extracellular matrix (ECM) components, such as collagens and proteoglycans, and they have a role in normal development and tissue damage in various pathophysiological

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ical conditions involving wound healing and tissue remodeling [9]. MMPs are classified into subgroups, including collagenases, stromelysins, gelatinases and membrane type MMPs [10]. MMP-10, also called as stromelysin-2, initially identified in a human adenocarcinoma cDNA library, is demonstrated to be expressed in osteoblasts at sites of bone formation as well as in chondrocytes of the growth plate in neonatal ribs in human [11]. Inflammatory cytokine, such as interleukin- β , activates MMP-3 and MMP-10, and these MMPs are considered to expand the degradative potential of cancer cells by targeting no-collagen matrix proteins such as fibronectin and laminin as well as activating latent MMP-1 [12, 13]. Moreover, MMP-10 is enhanced in patients with atherosclerosis and in pathological conditions with increased thrombin generation [14, 15]. Orbe *et al.* suggested that MMP-10 can enhance tissue plasminogen activator-induced fibrinolysis *via* a thrombin-activatable fibrinolysis inhibitor inactivation-mediated mechanism [16], and we recently reported that plasminogen has a crucial role in bone repair process in mice [17]. In addition, plasminogen activator inhibitor-1 is involved in diabetic bone loss in female mice [18]. However, the role of MMP-10 in osteoblast biology and differentiation has not been reported yet.

We previously performed a comparative DNA microarray analysis between empty vector-transfected and ALK2 (R206H)-transfected mouse myoblastic C2C12 cells [5]. In that study, MMP-10 was extracted as a factor, whose expression was most extensively enhanced by ALK2 (R206H) transfection. In the present study, we therefore investigated the role of MMP-10 in the differentiation of myoblastic cells into osteoblasts.

Materials and Methods

Materials

Human recombinant BMP-2 was purchased from by Sigma-Aldrich Corp (St. Louis, MO, USA). Anti-phosphorylated Smad1/5/8 and anti- β -actin antibodies were from Cell Signaling Technology (Danvers, MA, USA). Anti-Smad1, anti-Smad5 and anti-MMP-10 antibodies, MMP-10 siRNA(m) and control siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human recombinant MMP-10 protein was from Abnova (Taipei, Taiwan). All other chemicals used were of analytical grade.

Cell culture

Mouse myoblastic C2C12 (ATCC) cells were cultured in Dulbecco's modified Eagle's medium (Wako Pure Chem. Osaka, Japan) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Wako Pure Chem.). The medium was changed twice a week, as previously described [19].

Protein extraction and Western blot analysis

Cells were lysed with Laemmli Sample Buffer (Bio-Rad Laboratories, Inc.) and heated at 100°C for 5 minutes. The same amount of protein aliquots were separated on 10% polyacrylamide-SDS gels. The proteins were transferred to a PVDF membrane in 25 mM Tris, 192 mM glycine, and 20% methanol to polyvinylidene difluoride. Blots were blocked with 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% Tween 20, and 3% dried milk powder. The membranes were immunoblotted with each primary antibody. The immune complexes were visualized using the appropriate secondary antibody and the ECL advance detection system (GE Healthcare, Tokyo, Japan), according to the manufacturer's instructions. The results depicted in each figure are representative of at least three independent cell preparations.

RNA extraction and real-time PCR

Total RNA was prepared from cells using TRIzol reagent. Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA, USA). Quantitative real-time PCR was assessed by the incorporation of SYBR Green into double-stranded DNA and performed on an ABI StepOne Real-Time PCR System (Applied Biosystems). The mRNA value for each gene was normalized relative to the mouse GAPDH mRNA levels in RNA samples. Primer sequence (forward and reverse) were as follows: GAPDH, 5'-GTGTAC ATGGTTCCAGTATGAGTCC-3' and 5'-AGTGAGT TGTCATATTTCTCGTGGT-3'; osteocalcin (OCN), 5'-CCTGAGTCTGACAAAGCCTTCA-3' and 5'-GCCGAGTCTGTTCACCTACCTT-3'; Runx2, 5'-AAAT GCCTCCGCTGTTATGAA-3' and 5'-GCTCCGGC CCACAAATCT-3'; alkaline phosphatase (ALP), 5'-ATCTTTGGTCTGGCTCCCATG-3' and 5'-TTTCCC GTTCACCGTCCAC-3'; Osterix, 5'-AGCGACCACT TGAGCAAACAT-3' and 5'-GCGGCTGATTGGCTTC TTCT-3'; Type I collagen (Col1), 5'-ATGCCTGG TGAACGTGGT-3' and 5'-AGGAGAGCCATCAGCA CCT-3'; MMP-10, 5'-GAGCCACTAGCCATCCTGG-

3' and 5'-CTGAGCAAGATCCATGCTTGG-3'; Smad6, 5'-CCACTGGATCTGTCCGATTCT-3' and 5'-GGTCGTACACCGCATAGAGG-3'; Smad7, 5'-GCATTCCTCGGAAGTCAAGAG-3' and 5'-CCAGGGGCCAGATAATTCGT-3'.

Small interfering (si) RNA

Mouse MMP-10 siRNA and control siRNA were transfected as recommended by the supplier (Sant Cruz) into cells seeded at 5×10^5 cells per well using lipofectamineTM RNAi MAX (Invitrogen), as previously described [19].

ALP activity assay

Cells were seeded in a 6-well plate (2.0×10^4 cells/well) and cultured in DMEM with or without MMP-10 for 48h, then collected with distilled water. ALP activity was measured with LabAssay ALP (Wako Pure Chem.) followed the manufacturer's instructions, as previously described [18].

Mineralization

Mineralization of C2C12 cells was determined in 6-well plates using Alizarin Red staining. After confluent cells were grown in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, and 10 mM of β -glycerophosphate for 3 weeks, cells were fixed with ice-cold 70% ethanol and stained with Alizarin Red (Sigma) to detect calcification. For quantitation, cells stained with Alizarin Red were destained with ethylpyridinium chloride (Wako Pure Chem.), the extracted stain was transferred to a 96-well plate, and absorbance at 562 nm was measured using a microplate reader as previously described [19].

Preparation of primary osteoblasts

Calvarial osteoblastic cells were obtained from C57BL6 mice according to the method described previously [17, 18]. Briefly, calvaria was removed from 3-day-old mice, cleaned of soft tissue, and digested 4 times with 1 mg/mL type IV collagenase and 0.25% trypsin for 20 min at 37°C with gentle agitation. Cells from the second, third, and fourth digestions were plated and grown in α -MEM with 10% fetal bovine serum (FBS). The cells were seeded in a 6-well plate (1.0×10^5 cells/well) and grown until confluent.

Statistics

All experiments were repeated at least three times.

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using ANOVA. P value < 0.05 was taken to indicate a significant difference.

Results

Induction of MMP-10 by ALK2(R206H) transfection

In our previous study, C2C12 cell clones stably expressing either empty vector or the ALK2 (R206H) construct, the most frequent ALK2 mutation in FOP patients, were prepared, and the comprehensive DNA microarray analysis between empty vector- and ALK2 (R206H)-expressed C2C12 cells was performed. From approximately 45,000 genes on the Affymetrix gene chip, we identified genes whose expression was increased at least 2 times in the experimental versus the control group (Supplemental Table 1). Of all these 40 genes, MMP-10 was a gene, whose expression was mostly marked (8.6 times) in the stable ALK2 (R206H)-transfected C2C12 cells, compared to empty vector-transfected cells [5]. The expressions of Osterix, ALP, Runx2, Col1a1 and Col1a2 were increased by 1.23, 2.00, 1.52, 0.13 and 0.54 times in the stable ALK2 (R206H)-transfected C2C12 cells, compared to empty vector-transfected cells. As shown in Fig. 1, we confirmed marked higher levels in MMP-10 mRNA of stable ALK2 (R206H)-transfected C2C12 cells, compared to empty vector-transfected cells.

Role of MMP-10 in BMP-2 induced-differentiation of myoblasts into osteoblasts

We examined the effects of MMP-10 on the differentiation of myoblasts into osteoblasts induced by

Fig. 1 Effects of MMP-10 levels by ALK2(R206H)

Total RNA was extracted from C2C12 cells stably transfected with empty vector (V) or ALK2(R206H), and then real-time RT-PCR was performed. Data are expressed relative to the GAPDH mRNA value. * $p < 0.01$ relative to V-transfected cells.

BMP-2. Human recombinant MMP-10 did not affect the levels of osteogenic marker gene mRNA, such as Runx2, Osterix, Col1, ALP and OCN of C2C12 cells in the absence of exogenous BMP-2 (Fig. 2A). Moreover, it did not affect ALP activity in these cells in the absence of exogenous BMP-2 (Fig. 2B). On the other hand, MMP-10 significantly augmented the expressions of Osterix, Col1, ALP and OCN mRNA enhanced by BMP-2 in C2C12 cells (Fig. 2A). Moreover, it significantly augmented ALP activity enhanced by BMP-2

in these cells (Fig. 2B). Meanwhile, MMP-10 did not affect the levels of Runx2, an early osteoblast differentiation marker, enhanced by BMP-2 (Fig. 2B). As shown in Fig. 2C and 2D, MMP-10 enhanced mineralization induced by BMP-2, although it did not affect it in the absence of exogenous BMP-2 addition. As shown in Fig. 3A and 3B, the effects of MMP-10 was observed at the concentration of 1 ng/mL and more, and the time course study revealed that MMP-10 effects were observed at 48 hr, but not within 24 hr.

Fig. 2 Effects of MMP-10 on the differentiation of myoblasts into osteoblasts

(A) C2C12 cells were treated with or without 1 ng/mL recombinant human MMP-10 protein in the absence or presence of 200 ng/mL BMP-2 for 48 h. Total RNA was extracted, and real-time RT-PCR for Runx2, Osterix, Col1, ALP, OCN or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. *, $p < 0.01$ relative to C2C12 cells treated without MMP-10 with BMP-2. (B) C2C12 cells were treated with or without 1 ng/mL MMP-10 in the absence or presence of 200 ng/mL BMP-2 for 48 h. Then, ALP activity was measured as described under Materials and Methods. Each value is the mean $\pm$ SEM of 4 determinations. *, $p < 0.01$ relative to C2C12 cells treated without MMP-10 in the presence of BMP-2. (C) C2C12 cells were cultured for 3 weeks with or without 1 ng/mL recombinant human MMP-10 protein in the absence or presence of 100 ng/mL BMP-2 in the presence of 50 μ g/mL ascorbic acid and 10 mM β -glycerophosphate, and Alizarin Red staining was performed. (D) Mineralization was quantified by Alizarin Red staining as described under Materials and Methods.

We assume that the level of MMP-10 exogenously added was pharmacological, but not physiological. Because serum levels of MMP-10 in human seemed to be less than 0.5 ng/mL, as previously reported [20].

Role of endogenous MMP-10 in BMP-2-induced differentiation of myoblasts into osteoblasts

Next, we explored the effects of a reduction in endogenous MMP-10 levels by siRNA on the differentiation of myoblasts into osteoblasts. A reduction in endoge-

nous MMP-10 levels by siRNA significantly decreased the levels of Runx2, Osterix, Col1, ALP and OCN mRNA enhanced by BMP-2 in C2C12 cells (Fig. 4).

Effects of MMP-10 on the phosphorylation of Smads and BMP-induced genes

We examined the phosphorylation of Smad1/5/8 and the levels of Smad6 and Smad7 in C2C12 cells. As shown in Fig. 5A, MMP-10 increased the phosphorylation of Smad1/5/8 in C2C12 cells. Moreover,

Fig. 3 Augmentation of MMP-10 on BMP-2-induced differentiation of myoblasts into osteoblasts

(A) Total RNA was extracted from C2C12 cells cultured in the absence or presence of 200 ng/mL BMP-2 with or without the indicated concentrations of MMP-10 for 48 h, and then real-time RT-PCR was performed. (B) Total RNA was extracted from C2C12 cells treated with 1 ng/mL BMP-2 for the indicated times in the absence or presence of 200 ng/mL BMP-2 for 48 h, and then real-time RT-PCR was performed. Data are expressed relative to the GAPDH mRNA value. *, $p < 0.01$ relative to C2C12 cells treated with BMP-2 and without MMP-10.

Fig. 4 Effects of a reduction in endogenous MMP-10 on BMP-2 induced differentiation of myoblasts into osteoblasts (A) Total RNA was extracted from C2C12 cells transfected with control siRNA or MMP-10 siRNA in the absence or presence of 200 ng/mL BMP-2 for 48 h. Then real-time RT-PCR for Runx2, Osterix, Col1, ALP, OCN or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. *, $p < 0.01$ relative to C2C12 cells transfected with control siRNA in presence of BMP-2. (B) Total protein from C2C12 cells transfected with control siRNA or MMP-10 siRNA was extracted, and Western blot analysis for anti-MMP-10 and anti-β-actin antibodies were performed.

Fig. 5 Effects of MMP-10 on BMP signaling pathway. (A) Total protein from C2C12 cells treated with or without 1 ng/mL MMP-10 for 24 h was extracted, and Western blot analysis for anti-Smad1, Smad5, phosphorylated Smad1/5/8 and β-actin antibodies were performed. (B) C2C12 cells were treated with or without 1 ng/mL MMP-10 in the absence or presence of 200 ng/mL BMP-2 for 48 h. Total RNA was extracted, and real-time RT-PCR for Smad6, Smad7 or GAPDH was performed. Data are expressed relative to the GAPDH mRNA value. *, $p < 0.01$ relative to C2C12 cells treated with BMP-2 without MMP-10.

Fig. 6 Effects of MMP-10 on myogenic differentiation. Total RNA was extracted from wild-type C2C12 cells cultured with or without 2% horse serum for 6 days in the absence or presence of MMP-10, and real-time RT-PCR for MyoD, myogenin, or GAPDH was performed. *, $p < 0.01$ relative to C2C12 cells without horse serum. **, $p < 0.01$, relative to C2C12 cells with horse serum without MMP-10.

MMP-10 enhanced the levels of Smad6 and Smad7 mRNA induced by BMP-2 (Fig. 5B).

Effects of MMP-10 on myotube differentiation

Finally, we examined the effect of MMP-10 on the differentiation of myoblasts into myotubes. The levels of MyoD, an early myogenic differentiation gene, and myogenin mRNA were similar between with or without 1 ng/mL MMP-10 before the addition of horse serum to induce myotube differentiation (data not shown). As shown in Fig. 6, MMP-10 significantly decreased the levels of myogenin mRNA induced by horse serum, although its reduction on MyoD mRNA induced by horse serum was not significant, indicating that MMP-10 suppresses the differentiation of myoblasts into myotubes.

Discussion

The previous studies showed that ALK2 (R206H) constitutively activates BMP signaling through Smad1/5/8, thereby contributing to the ectopic bone formation in FOP, a disease characterized by marked heterotopic ossification in muscle tissues [1-4]. We therefore performed a comparative DNA microarray analysis between empty vector- and ALK2 (R206H)-stably transfected mouse myoblastic C2C12 cells to

explore some additional factors that contribute to the ossification of muscle, as previously described [5]. We then identified several factors whose expression is increased by the constitutively active ALK2 (R206H) expression, including MMP-10, NMB (Gpnmb), Frizzled-3 and Tmem119. Transmembrane glycoprotein NMB (Gpnmb), also known as osteoactivin, is a type I transmembrane glycoprotein that is found at the cell surface and lysosomal membranes. It is highly expressed in bone and is related to the regulation of osteoblast maturation [21]. Fzd3 is related to Wnt/Frizzled signaling through several pathways, including the canonical Wnt/ β -catenin and Wnt/Ca²⁺ pathways [22]. Tmem119 promotes the differentiation of myoblasts into osteoblasts by inducing BMP-2 levels and interacting with the BMP signaling pathway [5, 23]. Among them, MMP-10 was the gene, whose expression was most extensively enhanced by ALK2 (R206H) expression in C2C12 cells, as confirmed in the present study. We have therefore tried to characterize the role of MMP-10 in the differentiation of myoblastic cells into osteoblastic cells.

The previous observations in humans with spontaneous mutations and in mice with targeted mutations in MMP genes provide evidence that MMPs have several roles in skeletal development and remodeling. NAO syndromes (nodulosis, arthropathy, osteolysis) characterized by multicentric osteolysis are due to mutations in MMP-2, which result in the absence of detectable serum MMP-2 activity in affected subjects [24]. Later, the phenotype of short snouts, hypertelorism and dome-shaped skulls similar to patients with NAO syndromes recurred in Mmp2-null mice [25]. DNA sequence analysis of the SEMD_{MO}, one subform of spondyloepimetaphyseal dysplasias (SEMDs) with moderate to severe metaphyseal changes, mild epiphyseal involvement, pearshaped vertebrae in childhood, rhizomelic shortening especially of the lower limbs and genu varum deformities, revealed a missense mutation in the pro-region domain of MMP-13 [26]. Moreover, growth plate abnormalities had just been identified as a major phenotype of Mmp13-null mice [27]. The hypertrophic zones of long bones were increased in Mmp9^{-/-} mice [28]. Mt1-mmp null mice developed a severe skeletal phenotype that included cranial abnormalities such as short snout, orbital protrusions, hypertelorism and dome-shaped skull. Ossification of calvarial bones was delayed and suture closure was never completed [29]. As for MMP-10 and bone, the previous studies

indicated that MMP-10 is expressed in osteoblasts as well as chondrocytes, but not in osteocytes, although the controversy exists about its expression in osteoclasts [11, 30]. MMP-10 has broad substrate specificity including proteoglycans, laminin and fibronectin [31]. The present study revealed that MMP-10 augmented the differentiation of myoblastic cells into osteoblastic cells induced by BMP-2, although it did not possess the activity of osteoinduction in the absence of exogenous BMPs. Moreover, a reduction of endogenous MMP-10 levels by siRNA significantly suppressed the levels of osteogenic marker mRNA enhanced by BMP-2 in myoblastic cells. On the other hand, our preliminary study suggested that MMP-10 had little effects on osteogenic marker expression in mouse primary osteoblast cultures in the absence of exogenous BMP-2 (data not shown). These findings suggest that MMP-10 might play some role in the pathogenesis of muscle ossification in FOP, although it does not seem to be critical for osteoblastic bone formation physiologically.

Osteoblast, chondrocytes, adipocytes and myotubes are derived from common precursors, multipotential mesenchymal stem cells derived from mesoderm [32]. Several osteotropic factors differentially regulate the commitment of the multipotential stem cells into various cell lineages [33, 34]. BMPs are necessary for different steps of chondrogenesis, chondroprogenitor determination and/or condensation and subsequent differentiation into chondrocytes [35]. Moreover, BMPs inhibit myoblast differentiation by inducing the basic helix-loop-helix protein, hair enhancer-of-split-related 1 (HesR1), which transcriptionally represses myogenesis by forming an inactive complex with MyoD [36]. In the present study, MMP-10 alone did not affect the expression of osteoblast differentiation markers and ALP activity in myoblasts, although it augmented the differentiation of myoblasts into osteoblasts enhanced by BMP-2. These findings suggest that MMP-10 is not the central molecule for the pathogenesis of muscle ossification in FOP in contrast to BMPs, although it might play some role in BMP pathways. In present study, MMP-10 enhanced the phosphorylation of Smad1/5/8 in mouse myoblastic cells. Moreover, MMP-10 enhanced the levels of Smad6 and Smad7 mRNA, BMP-signal-responsive genes, induced by BMP-2 in these cells. These findings suggest that MMP-10 regulates BMP signaling pathway. In the present study, we could not clarify the precise mechanism

of MMP-10 effects on the differentiation of myoblastic cells into osteoblasts. MMP-10 effects might not be due to its direct effects on BMP-Smad pathway, because its effects on the differentiation of myoblastic cells took more than 24 hr and many matrix proteins are included in the substrates for MMP-10. For example, fibronectin, one of substrates of MMP-10, modulates osteoblast function [37]. MMP-10 might affect BMP-induced differentiation of myoblasts into osteoblasts through the release of bone matrix proteins induced by MMP-10. Further studies will be necessary to clarify the precise mechanism underlying these phenomena.

The mechanisms by which ALK2(R206H) transfection induces MMP-10 levels in C2C12 cells are still unknown in the present study. BMP family proteins, including BMP-2, 4, 6 and 7, affect cells through ALK2, and the ALK2 (R206H) mutation mildly activates BMP signaling, as previously reported [4]. Although the most crucial ligand of ALK2 for the pathogenesis of FOP have not been determined yet, our preliminary study revealed that exogenous BMP-2 addition did not induce the levels of MMP-10 mRNA in C2C12 cells (data not shown). Therefore, the ligands other than BMP-2 may be responsible for the induction of MMP-10 by an activation of ALK2 signaling.

In conclusion, MMP-10 was identified as a molecule whose expression was increased in myoblastic cells overexpressing the constitutively activating ALK2 mutation responsible for FOP. The present study first demonstrated that MMP-10 promotes the differentiation of myoblasts into osteoblasts by interacting with the BMP signaling pathway. MMP-10 may play some important role in the heterotopic ossification of muscle in FOP, and the further studies of the mechanisms whereby it acts may lead to understanding for the pathogenesis of muscle ossification as well as the development of novel treatments for FOP and other-related mineralization disorders.

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Supplemental Table 1

Gene name	GenBank accession no.	Ratio	Gene name	GenBank accession no.	Ratio	Gene name	GenBank accession no.	Ratio
Mmp10	NM_019471	8.6	Acsf6	NM_001033597	2.6	B4galnt1	NM_008080	2.1
Ppt-2	NM_019441	8.0	Arhgdig	NM_008113	2.6	BC004044	NM_030565	2.1
Sgcg	NM_011892	7.7	Col5a1	NM_015734	2.6	BC022687	NM_145450	2.1
Ctla2a	NM_007796	7.5	Cpsf6	NM_001013391	2.6	Bmp2k	NM_080708	2.1
Gpnmb (Osteoactivin)	NM_053110	7.5	Cxcr4	NM_009911	2.6	C030048B08Rik	NM_172991	2.1
Krox20	NM_010118	7.0	D330028D13Rik	NM_172727	2.6	C1qtnf3	NM_030888	2.1
LOC433762	NM_001012326	7.0	Hgf	NM_010427	2.6	Cdk6	NM_009873	2.1
Ociad2	NM_026950	6.5	Mterf	NM_001013023	2.6	Chst11	NM_021439	2.1
Serpnb9g	NM_011455	6.5	Nrp2	NM_001077403	2.6	Dgat2	NM_026384	2.1
Fzd3	NM_021458	5.7	Pdlim3	NM_016798	2.6	Dus4l	NM_028002	2.1
Cox2	NM_011198	5.3	Rcn3	NM_026555	2.6	Dyrk2	NM_001014390	2.1
Cyp1a1	NM_009992	5.3	Thbs1	NM_011580	2.6	Gcnt2	NM_008105	2.1
Foxa2	NM_010446	5.3	Zfpm1	NM_009569	2.5	Gper	NM_029771	2.1
LOC100039031	XM_001472121	5.3	1110059G02Rik	AV235558	2.5	Huwe1	NM_021523	2.1
Ngfr	NM_033217	5.3	5430404G13Rik	AK017264	2.5	Inhba	NM_008380	2.1
Afp	NM_007423	4.9	Akr1c12	NM_013777	2.5	Jag1	NM_013822	2.1
Clec4d	NM_010819	4.9	Aldh1a3	NM_053080	2.5	LOC544988	NM_001024712	2.1
LOC632297	XM_906349	4.9	B4galnt5	NM_019835	2.5	Neo1	NM_001042752	2.1
Lrrc4c	NM_178725	4.9	Bik	NM_007546	2.5	Nfib	NM_001002671	2.1
Ttbk2	NM_001024856	4.9	Cacna2d1	NM_001110843	2.5	Odc1	NM_013614	2.1
Zip8	NM_026228	4.9	Col5a3	NM_016919	2.5	Pcdhb16	NM_053141	2.1
Olr1	NM_138648	4.6	Dlx2	NM_010054	2.5	Pex1	NM_027777	2.1
Tmem176b	NM_023056	4.6	Gpc6	NM_001079844	2.5	Prkg2	NM_008926	2.1
Oasis	NM_011957	4.0	Hs6st1	NM_015818	2.5	Sepw1	NM_009156	2.1
Pde4b	NM_019840	4.0	Irak2	NM_001113553	2.5	Sgce	NM_001130188	2.1
Pik3r6	NM_001004435	4.0	Islr	NM_012043	2.5	Slc22a15	XM_001002464	2.1
Thbs1	NM_011580	4.0	Isyna1	NM_023627	2.5	Smc6	NM_025695	2.1
A430107O13Rik	NM_001081351	3.7	Lgr4	NM_172671	2.5	Spsb1	NM_029035	2.1
Car3	NM_007606	3.7	LOC100044376	XR_030576	2.5	St6galnac4	NM_011373	2.1
Emilin2	NM_145158	3.7	LOC553091	AK086074	2.5	Tmem158	NM_001002267	2.1
Il1rn	NM_001039701	3.7	Lonrf3	NM_028894	2.5	Trim54	NM_021447	2.1
Rragd	NM_027491	3.7	Mt2	NM_008630	2.5	Uap1l1	NM_001033293	2.1
Zfp677	NM_172486	3.7	Ncam1	NM_001081445	2.5	Whrn	NM_001008791	2.1
1810063B07Rik	NM_026209	3.7	Nid1	NM_010917	2.5	9330156P08Rik	AK034105	2.0
Crim1	NM_015800	3.5	Pard3b	NM_001081050	2.5	A130001G05Rik	AK037258	2.0
Cyp51	NM_020010	3.5	Ptpfr	NM_011213	2.5	Adamts6	NM_001081020	2.0
Pde2a	NM_001008548	3.5	Rasl11b	NM_026878	2.5	Adh7	NM_009626	2.0
Tagln	NM_011526	3.5	Sema3e	NM_011348	2.5	Alpl	NM_007431	2.0
Tmem119	NM_146162	3.5	Serpine1	NM_008871	2.5	Arhgap44	NM_001099288	2.0
Acta1	NM_009606	3.2	Sox11	NM_009234	2.5	Ass1	NM_007494	2.0
Adam19	NM_009616	3.2	Strn3	NM_052973	2.5	B230334L07Rik	AK046028	2.0
Al646023	NM_198860	3.2	Ugcg	NM_011673	2.5	Bzw2	NM_025840	2.0
Cd28	NM_007642	3.2	1500005K14Rik	NM_029658	2.3	Ccdc132	NM_024260	2.0
Epha4	NM_007936	3.2	2610528A11Rik	AK012157	2.3	Cdc42bpa	NM_001033285	2.0
Glt8d4	NM_198612	3.2	4833441D16Rik	AA409766	2.3	Ckb	NM_021273	2.0
Gpr19	NM_008157	3.2	9630019E01Rik	AK051384	2.3	Cndp2	NM_023149	2.0
Havcr2	NM_134250	3.2	Akap9	NM_194462	2.3	Creg1	NM_011804	2.0
Krit1	NM_030675	3.2	Ankib1	NM_001003909	2.3	Ctnna1	NM_009818	2.0
Plekhh2	NM_177606	3.2	Atp6v1h	NM_133826	2.3	D1Erd75e	C77922	2.0
Robo1	NM_019413	3.2	Atxn7l1	NM_001033436	2.3	Elavl1	NM_010485	2.0
Tnfrsf21	NM_178589	3.2	Cadm4	NM_153112	2.3	Ets1	NM_001038642	2.0
Tnnt2	NM_001130174	3.2	Cd68	NM_009853	2.3	Fam196a	NM_001143802	2.0
Whrn	NM_001008791	3.2	Cotl1	NM_028071	2.3	Fgfbbp1	NM_008009	2.0
0610010O12Rik	NM_001081365	3.0	Decr1	NM_026172	2.3	Gnpda2	NM_001038015	2.0
2810003C17Rik	NM_145144	3.0	Dub1	NM_007887	2.3	Gusb	NM_010368	2.0
6330416G13Rik	NM_144905	3.0	E030030I06Rik	NM_001254744	2.3	Itgb3	NM_016780	2.0
AA414768	XM_141626	3.0	Fgfr1	NM_001079908	2.3	Lamb1-1	NM_008482	2.0
Aldh3a1	NM_001112725	3.0	Gatad1	NM_026033	2.3	Lpp	NM_178665	2.0
Arnt2	NM_007488	3.0	Gbe1	NM_028803	2.3	Murc	NM_026509	2.0
Cmtm7	NM_133978	3.0	Glpr1	NM_028608	2.3	Ncapd3	NM_178113	2.0
Fam38b	NM_001039485	3.0	Gper	NM_029771	2.3	Ncdn	NM_011986	2.0
Mical2	NM_177282	3.0	Jam2	NM_023844	2.3	Ndr2	NM_139133	2.0
Mt1	NM_013602	3.0	Lrp11	NM_172784	2.3	Nqo1	NM_008706	2.0
Myor	NM_010827	3.0	Mylpf	NM_016754	2.3	Pdpx	NM_020271	2.0
Smox	NM_145533	3.0	Myo1d	NM_177390	2.3	Pip4k2a	NM_008845	2.0
Snai1	NM_011427	3.0	Nav3	NM_001081035	2.3	Polr3g	NM_001081176	2.0
2510017J16Rik	AV105737	2.8	Ngf	NM_001112698	2.3	Prkd3	NM_029239	2.0
9330118A15Rik	AK046320	2.8	Pdgfrb	NM_008809	2.3	Ptger4	NM_008965	2.0
Abcc4	NM_001033336	2.8	Plcl2	NM_013880	2.3	Ptpn3	NM_011207	2.0
Al256396	AK134520	2.8	Pmaip1	NM_021451	2.3	Pum2	NM_030723	2.0
Col4a1	NM_009931	2.8	Rab30	NM_029494	2.3	Rbm25	NM_025930	2.0
Dem1	NM_028457	2.8	Slc6a8	NM_133987	2.3	Rdh10	NM_133832	2.0
Dusp9	NM_029352	2.8	Stxbp2	NM_011503	2.3	Sdc3	NM_011520	2.0
Enpp3	NM_134005	2.8	Tcf7l2	NM_009333	2.3	Sh2b2	NM_018825	2.0
Gsta3	NM_001077353	2.8	Thbs2	NM_011581	2.3	Srxn1	NM_029688	2.0
Nmnat2	NM_175460	2.8	Txnrd1	NM_001042513	2.3	Steap1	NM_027399	2.0
Siglecg	NM_172900	2.8	Vegfc	NM_009506	2.3	Stxbp5	NM_001081344	2.0
Slc30a1	NM_009579	2.8	1500011B03Rik	AK018264	2.1	Tecpr1	NM_027410	2.0
Smad6	NM_008542	2.8	9130213B05Rik	NM_145562	2.1	Thsd7a	NM_001164805	2.0
100042016	XM_001478392	2.6	A130040M12Rik	NR_002860	2.1	Trim26	NM_001025599	2.0
9430011C21Rik	AK079978	2.6	Ahnak	NM_001039959	2.1	Tspan12	NM_173007	2.0

References

- Shore EM, Kaplan FS (2008) Insights from a rare genetic disorder of extra-skeletal bone formation, fibrodysplasia ossificans progressiva (FOP). *Bone* 43: 427-433.
- Shore EM, Xu M, Feldman GJ, Fenstermacher DA, Cho TJ, et al. (2006) A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. *Nat Genet* 38: 525-527.
- Fukuda T, Kohda M, Kanomata K, Nojima J, Nakamura A, et al. (2009) Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva. *J Biol Chem* 284: 7149-7156.
- Song GA, Kim HJ, Woo KM, Baek JH, Kim GS, et al. (2010) Molecular consequences of the ACVR1(R206H) mutation of fibrodysplasia ossificans progressiva. *J Biol Chem* 285: 22542-22553.
- Tanaka K, Inoue Y, Hendy GN, Canaff L, Katagiri T, et al. (2012) Interaction of Tmem119 and the bone morphogenetic protein pathway in the commitment of myoblastic into osteoblastic cells. *Bone* 51: 158-167.
- Nishimura R, Kato Y, Chen D, Harris SE, Mundy GR, et al. (1998) Smad5 and DPC4 are key molecules in mediating BMP-2-induced osteoblastic differentiation of the pluripotent mesenchymal precursor cell line C2C12. *J Biol Chem* 273: 1872-1879.
- Yamaguchi A, Katagiri T, Ikeda T, Wozney JM, Rosen V, et al. (1991) Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation in vitro. *J Cell Biol* 113: 681-687.
- Bragdon B, Moseychuk O, Saldanha S, King D, Julian J, et al. (2011) Bone morphogenetic proteins: a critical review. *Cell Signal* 23: 609-620.
- Milner JM, Cawston TE (2005) Matrix metalloproteinase knockout studies and the potential use of matrix metalloproteinase inhibitors in the rheumatic diseases. *Curr Drug Targets Inflamm Allergy* 4: 363-375.
- Werner JA, Rathcke IO, Mandic R (2002) The role of matrix metalloproteinases in squamous cell carcinomas of the head and neck. *Clin Exp Metastasis* 19: 275-282.
- Bord S, Horner A, Hembry RM, Compston JE (1998) Stromelysin-1 (MMP-3) and stromelysin-2 (MMP-10) expression in developing human bone: Potential roles in skeletal development. *Bone* 23: 7-12.
- Armstrong D, Phelps LN, Vincenti MP (2009) CCAAT enhancer binding protein- β regulates matrix metalloproteinase-1 expression in interleukin-1 β -stimulated A549 lung carcinoma cells. *Mol Cancer Res* 7: 1517-1524.
- Nagase H, Visse R, Murphy G (2006) Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res* 69: 562-573.
- Orbe J, Montero I, Rodriguez JA, Belouqui O, Roncal C, et al. (2007) Independent association of matrix metalloproteinase-10, cardiovascular risk factors and subclinical atherosclerosis. *J Thromb Haemost* 5: 91-97.
- Orbe J, Rodriguez JA, Calvayrac O, Rodriguez-Calvo R, Rodriguez C, et al. (2009) Matrix metalloproteinase-10 is upregulated by thrombin in endothelial cells and increased in patients with enhanced thrombin generation. *Arterioscler Thromb Vasc Biol* 29: 2109-2116.
- Orbe J, Barrenetxe J, Rodriguez JA, Vivien D, Orset C, et al. (2011) Matrix metalloproteinase-10 effectively reduces infarct size in experimental stroke by enhancing fibrinolysis via a thrombin-activatable fibrinolysis inhibitor-mediated mechanism. *Circulation* 124: 2909-2919.
- Kawao N, Tamura Y, Okumoto K, Yano M, Okada K, et al. (2013) Plasminogen plays a crucial role in bone repair. *J Bone Miner Res* 28: 1561-1574.
- Tamura Y, Kawao N, Okada K, Yano M, Okumoto K, et al. (2013) Plasminogen activator inhibitor-1 is involved in streptozotocin-induced bone loss in female mice. *Diabetes* 62: 3170-3179.
- Tanaka K, Matsumoto E, Higashimaki Y, Katagiri T, Sugimoto T, et al. (2012) Role of osteoglycin in the linkage between muscle and bone. *J Biol Chem* 287: 11616-11628.
- Komosinska-Vassev K, Olczyk P, Winsz-Szczotka K, Kuznik-Trocha K, Klimek K, et al. (2011) Age- and gender-dependent changes in connective tissue remodeling: physiological differences in circulating MMP-3, MMP-10, TIMP-1 and TIMP-2 level. *Gerontology* 57: 44-52.
- Harada H, Tagashira S, Fujiwara M, Ogawa S, Katsumata T, et al. (1999) Cbfa1 isoforms exert functional differences in osteoblast differentiation. *J Biol Chem* 274: 6972-6978.
- Wodarz A, Nusse R (1998) Mechanisms of Wnt signaling in development. *Annu Rev Cell Dev Biol* 14: 59-88.
- Hisa I, Inoue Y, Hendy GN, Canaff L, Kitazawa R, et al. (2011) Parathyroid hormone-responsive Smad3-related factor, Tmem119, promotes osteoblast differentiation and interacts with the bone morphogenetic protein-Runx2 pathway. *J Biol Chem* 286: 9787-9796.
- Martignetti JA, Aqeel AA, Sewairi WA, Boumah CE, Kambouris M, et al. (2001) Mutation of the matrix metalloproteinase 2 gene (MMP2) causes a multicentric osteolysis and arthritis syndrome. *Nat Genet* 28: 261-265.
- Inoue K, Mikuni-Takagaki Y, Oikawa K, Itoh T, Inada M, et al. (2006) A crucial role for matrix metalloproteinase 2 in osteocytic canalicular formation and bone metabolism. *J Biol Chem* 281: 33814-33824.
- Kennedy AM, Inada M, Krane SM, Christie PT, Harding B, et al. (2005) MMP13 mutation causes spondyloepimetaphyseal dysplasia, Missouri type (SEMD(MO)). *J*

- Clin Invest* 115: 2832-2842.
27. Inada M, Wang Y, Byrne MH, Rahman MU, Miyaara C, et al. (2004) Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. *Proc Natl Acad Sci U S A* 101: 17192-17197.
 28. Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, et al. (1998) MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 93: 411-422.
 29. Holmbeck K, Bianco P, Chrysovergis K, Yamada S, Birkedal-Hansen H (2003) MT1-MMP-dependent, apoptotic remodeling of unmineralized cartilage: a critical process in skeletal growth. *J Cell Biol* 163: 661-671.
 30. Haeusler G, Walter I, Helmreich M, Egerbacher M (2005) Localization of matrix metalloproteinases, (MMPs) their tissue inhibitors, and vascular endothelial growth factor (VEGF) in growth plates of children and adolescents indicates a role for MMPs in human postnatal growth and skeletal maturation. *Calcif Tissue Int* 76: 326-335.
 31. Somerville RP, Oblander SA, Apte SS (2003) Matrix metalloproteinases: old dogs with new tricks. *Genome Biol* 4: 216.
 32. Fang J, Hall BK (1997) Chondrogenic cell differentiation from membrane bone periosteal. *Anat Embryol (Berl)* 196: 349-362.
 33. Komori T (2002) Runx2, a multifunctional transcription factor in skeletal development. *J Cell Biochem* 87: 1-8.
 34. Stein GS, Lian JB, van Wijnen AJ, Stein JL, Montecino M, et al. (2004) Runx2 control of organization, assembly and activity of the regulatory machinery for skeletal gene expression. *Oncogene* 23: 4315-4329.
 35. Pizette S, Niswander L (2000) BMPs are required at two steps of limb chondrogenesis: formation of prechondrogenic condensations and their differentiation into chondrocytes. *Dev Biol* 219: 237-249.
 36. Sun J, Kamei CN, Layne MD, Jain MK, Liao JK, et al. (2001) Regulation of myogenic terminal differentiation by the hairy-related transcription factor CHF2. *J Biol Chem* 276: 18591-18596.
 37. Bentmann A, Kawelke N, Moss D, Zentgraf H, Bala Y, et al. (2010) Circulating fibronectin affects bone matrix, whereas osteoblast fibronectin modulates osteoblast function. *J Bone Miner Res* 25: 706-715.