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Bone morphogenetic protein-2 down-regulates miR-206 expression by blocking its maturation process

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that are emerging as important post-transcriptional gene regulators. miR-206 is unique in that it is expressed only in skeletal muscle, including the myoblastic C2C12 cell line. In C2C12 cells, miR-206 expression was reduced dramatically after bone morphogenetic protein (BMP)-2 treatment. The down-regulation of miR-206 expression was also observed after co-transfection with constitutively-active Smad1 and Smad4, which are the intracellular signaling molecules of the BMP pathway. BMP-2 also reduced miR-206 expression in the presence of α-amanitin in a similar manner to that in the absence of α-amanitin. Moreover, the expression of pri-miR-206 was increased upon BMP-2 treatment for 6 h compared to that in the absence of BMP-2. These results suggested that BMP-2 down-regulates miR-206 expression at the post-transcriptional level, by inhibiting the processing of pri-miR-206 into mature miR-206, and that BMP-2 could regulate miRNA biogenesis by a novel mechanism.

Introduction

MicroRNAs (miRNAs) are a recently-discovered class of small non-coding RNAs that are approximately 22 nucleotides (nt) in length. In mammalian cells, miRNAs are transcribed as long primary transcripts (pri-miRNAs) in which the miRNA sequence is located within the stem of a local hairpin structure. These transcripts are cleaved into precursor hairpins of ~70 nt, which are referred to as pre-miRNAs, by the nuclear ribonuclease Drosha. Each pre-miRNA is exported out of the nucleus by exportin-5, and then cleaved by the cytoplasmic ribonuclease Dicer into a 22 nt miRNA duplex. One strand of this short-lived duplex is degraded by an unknown nuclease, while the other strand is selected and incorporated into the effector complex known as the RNA-induced silencing complex (RISC). RISC interacts with the mRNA target in a sequence-specific manner, and regulates translational inhibition or mRNA degradation [1,2].

More than 500 miRNAs have been discovered in mammals, and some of them are expressed in a tissue-specific manner, which suggests that they have specific roles in the specification of tissues during differentiation and development [3]. A small number of striated-muscle-specific miRNAs, such as miR-1, miR-133a, and miR-206, have been identified [4,5]. Among them, miR-1 and miR-133a are highly expressed in heart and skeletal muscle in both human and mouse [6]. miR-206 is unique in that it is only expressed in skeletal muscle, including the multipotent mouse myoblastic C2C12 cell line. Upon initiation of differentiation in these cells, there is a steady induction of miR-206, which indicates that miR-206 might play an important role in cell differentiation and cell identity [7,8].

Bone morphogenetic proteins (BMPs), which are members of the transforming growth factor-β (TGF-β) superfamily, regulate the proliferation, differentiation and apoptosis of various types of cells and organs [9]. Signaling by BMPs is initiated by their binding to two types of serine/threonine kinase receptor (type I and type II), which form a complex. Eight Smad proteins have been shown to play critical roles in the intracellular BMP signaling pathway in mammals [10,11]. The receptor Smads (R-Smads), which comprise Smad1–3, Smad5, and Smad8, are phosphorylated directly by type I receptors and then form complexes with the common-mediator Smad (Co-Smad), Smad4, and move into the nucleus. Here they bind to the regulatory regions of the target genes and regulate their expression. At least 15 types of BMP have been identified in humans. Among them, BMP-2 is sufficient to induce ectopic bone formation when it is implanted into the tissues of rodents. BMP-2 is reported to trigger osteoblast differentiation and to up-regulate the expression of most of the genes that encode osteoblastic phenotype-related proteins *in vitro* [9]. BMP-2 not only converts the differentiation pathway into that of osteoblasts but also

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inhibits myogenic differentiation [12]. C2C12 is a multipotent cell line and it is a well-characterized model system that has been reported to differentiate not only into myotubes but also into osteoblasts. As shown previously, BMP-2 induces osteoblastic differentiation and inhibits myotube formation in C2C12 cells, and the cells that remain as unfused mononuclear polygonal cells do not express myosin heavy chain, which is a marker for mature muscle cells [13]. Until now, little was known of the regulation of miR-206 expression by BMP-2.

miRNA expression is controlled mainly at the level of transcription [14]. Rosenberg et al. [15] demonstrated that the transcription factor MyoD1 directly regulates transcription of the primary miR-206 transcript AK132542 and, in the absence of MyoD1, AK132542 is not expressed [15]. However, like other RNAs, miRNA expression could potentially be controlled at the post-transcriptional level. Post-transcriptional regulation of miRNA expression has been reported to occur in a tissue-specific [16] and developmentally-regulated fashion [17]. In addition, certain pri-miRNAs are highly expressed in human and mouse embryonic stem cells, and tumors; however, the corresponding mature miRNAs are not detectable, which suggests that there may be a block in miRNA biogenesis [18]. The developmentally-regulated RNA-binding protein Lin28 blocks processing of miRNAs of the let-7 family in embryonic cells [19]. Recently, it has been reported that miR-21 mediates the induction by BMP-4 of a contractile phenotype in vascular smooth muscle cells. BMP-4 acts via a novel mechanism at the post-transcriptional level to increase miR-21 expression by promoting the processing of pri-miR-21 into pre-miR-21 by the Drosha complex. Upon BMP signaling, the BMP-specific Smad signaling molecules interact with the DEAD-box RNA helicase p68, which is a component of the Drosha Microprocessor complex, and the Smad-p68 complex is recruited to pri-miR-21 [20]. These results open new avenues for the study of BMP signaling and miRNA biogenesis. Here, we report that miR-206 expression is down-regulated by BMP-2 at a post-transcriptional level in C2C12 cells.

Materials and methods

Cell cultures. Cells of the mouse myoblast cell line C2C12 and the mouse osteoblastic cell lines MC3T3-E1 and ST-2 were cultured as

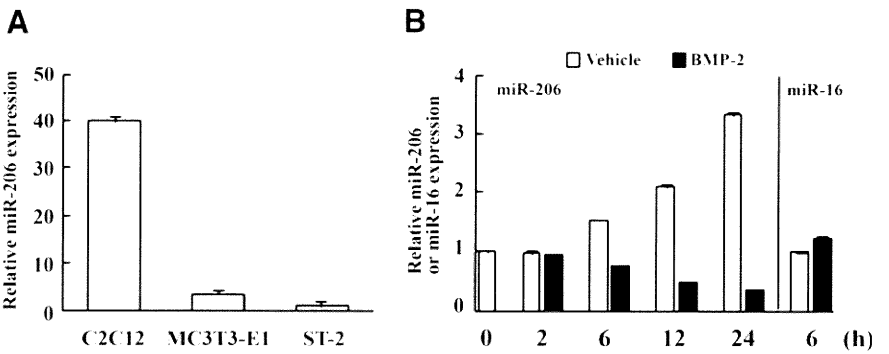

Fig. 1. Expression of miR-206 and the down-regulation of expression by bone morphogenetic protein (BMP)-2. (A) C2C12 cells, MC3T3-E1 cells and ST-2 cells were harvested at confluence, and total RNA was extracted from the cells. The endogenous expression level of miR-206 was analyzed. The expression levels were normalized against those of sno234 RNA. (B) C2C12 cells were plated and cultured with 10% FBS. After 24 h, the culture medium was replaced with fresh medium containing 2.5% FBS, and BMP-2 (300 ng/ml) (+) or vehicle (−) was added, after which the cells were cultured for the additional times indicated. The expression of miR-206 and miR-16 was analyzed.

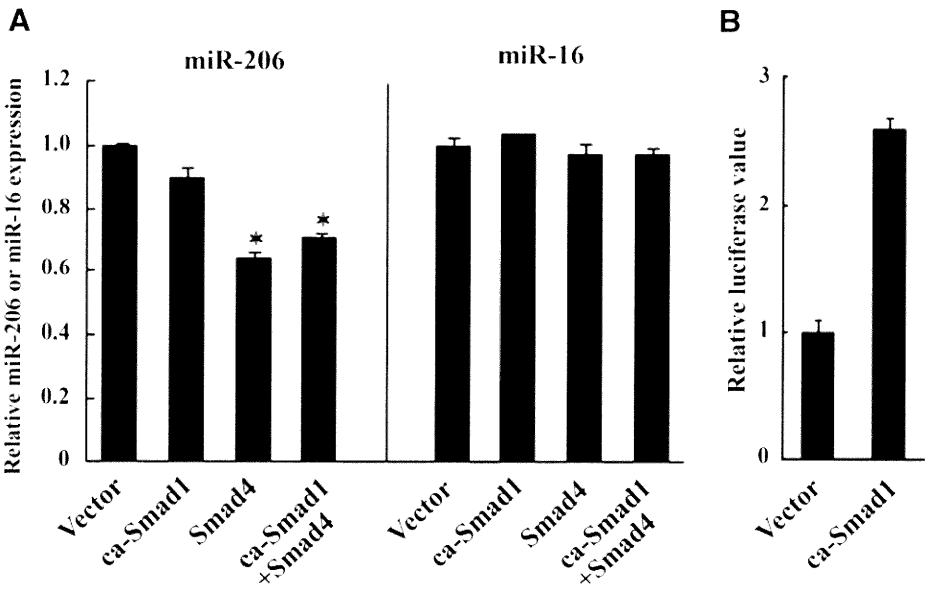

Fig. 2. Regulation of miR-206 expression by constitutively-active Smad1 (ca-Smad1) and Smad4 in C2C12 cells. Cells were plated and cultured with 10% FBS. After an overnight incubation, the cells were transiently transfected with 100 ng/ml of the expression construct for ca-Smad1 or Smad4 or an empty expression plasmid. After an overnight incubation, the culture medium was replaced with fresh medium containing 2.5% FBS, and the cells were then cultured for 6 h. Total RNA was extracted and miR-206 expression was analyzed (A). Luciferase activities were determined (B). Asterisks indicate significant differences ($p < 0.05$, t test for paired data).

described previously [13]. For the specific inhibition of RNA polymerase II, subconfluent C2C12 cells were treated with 10 μg/ml α-amanitin (Calbiochem, San Diego, CA) for 6 h.

Plasmids and transfection. Constitutively-active Smad1 (ca-Smad1), Smad1(DVD), in which the C-terminal serines in the SXS phosphorylation motifs were substituted with aspartic acid [21], was constructed by site-directed mutagenesis (Nojima et al., unpublished). Smad4 expression plasmids and IdWT4F-luc were generated as described previously [22]. The plasmid DNA was transfected into cells using the cationic lipid reagent Lipofectamine 2000 (Invitrogen). In brief, cells were plated 24 h before transfection at a density of 0.5×10^5 cells per ml on 24-well plates and cultured in α-MEM supplemented with 10% FBS. The cells were transfected with 100 ng/well of expression plasmid. After an overnight incubation, the culture medium was replaced with fresh medium containing 2.5% FBS and then cultured for 6 h. The luciferase reporter assay was performed as described previously [13]. All experiments were performed on samples in triplicate and were repeated independently three times.

Detection of miRNA expression. Total RNA was extracted from the cells at the indicated time points using Isogen (Nippon Gene, Toyama, Japan). cDNA was synthesized with specific miRNA primers from the TaqMan MicroRNA Assays (Applied Biosystems) and reagents from the TaqMan MicroRNA Reverse Transcription kit. The resulting cDNA was amplified by PCR using the TaqMan MicroRNA Assay system and analyzed with the StepOne® Real-Time PCR System. The relative levels of miRNA expression were quantified using the comparative C_t method with sno234 RNA as the endogenous control.

Detection of the primary miRNA transcript. Total RNA was extracted from the cells at the indicated time points using Isogen. Complementary DNA was synthesized with Omniscript Reverse Transcriptase (RT) (Qiagen) using an oligo $(dT)_{15}$ primer. Two microliters of the RT reaction were used for PCR analysis. To analyze the levels of the pri-miRNA-206 transcript, specific primers were designed to target sequences 170 bp upstream and downstream of the miRNA stem-loop (pre-miRNA) as described previously [23,24]. The primer sequences for pri-miRNA-206 were 5′-CCCAACAAGCTCTGCCTG-3′ (forward) and 5′-GGGAGCATAGTTG ACCTGAAAC-3′ (reverse), and gave an expected product size of 401 bp. Pri-miRNA expression was normalized to ribosomal protein L26 (*Rpl26*) expression. The *Rpl26* PCR primers were as follows: 5′-CGAGTCCAGCGAGAGAAGG-3′ (forward) and 5′-GCAGTC TTTAATGAAAGCCGTG-3′ (reverse). The amplification products were electrophoresed on 2% agarose gels. For quantitation, the gels were scanned, and the pixel intensity for each band was determined using the ImageJ program (NIH Image, Bethesda, MD) and normalized to the amount of Rpl26 product.

Quantitation of gene expression by reverse transcription-polymerase chain reaction (RT-PCR). PCR was used to analyze the transcript levels of Drosha, exportin-5, and Dicer1. Total RNA was extracted from the cells at the indicated time points using Isogen and treated with RNase-free DNase (Qiagen) to remove any contaminating genomic DNA, according to the manufacturer's protocol. RT-PCR was performed as described previously [13]. The primers were designed to have a T_m of ~60 °C and to amplify a fragment of 200 bp from the 3′ end of the coding sequence. The primer sequences for each gene were as follows: Drosha, 5′-GGATAGGCTGTGGGAAAG GA-3′ (forward), 5′-CTTCTTGATGTCTTCAGCCTCC-3′ (reverse); exportin-5, 5′-CCACTTCAAACGTCTAATCGCT-3′ (forward), 5′-GCC GGAGAAGGATGCC-3′ (reverse); Dicer1, 5′-TGCTCGAGATGGAAC CAGA-3′ (forward), 5′-TCAGCTGTTAGGAACCTGA GGC-3′ (reverse). To account for any difference in the amount of starting RNA, *Rpl26* was chosen as our endogenous control and amplified using the primers described above. The amplification products were electrophoresed on 2% agarose gels.

Reagents. Recombinant human BMP-2 was kindly supplied by Astellas Pharma Inc. (Tokyo, Japan).

Results and discussion

BMP-2 and Smad1/4 down-regulate miR-206 expression in C2C12 cells

We investigated the cell type-specific expression of miR-206 in a myoblastic cell line and two osteoblastic cell lines. The expres-

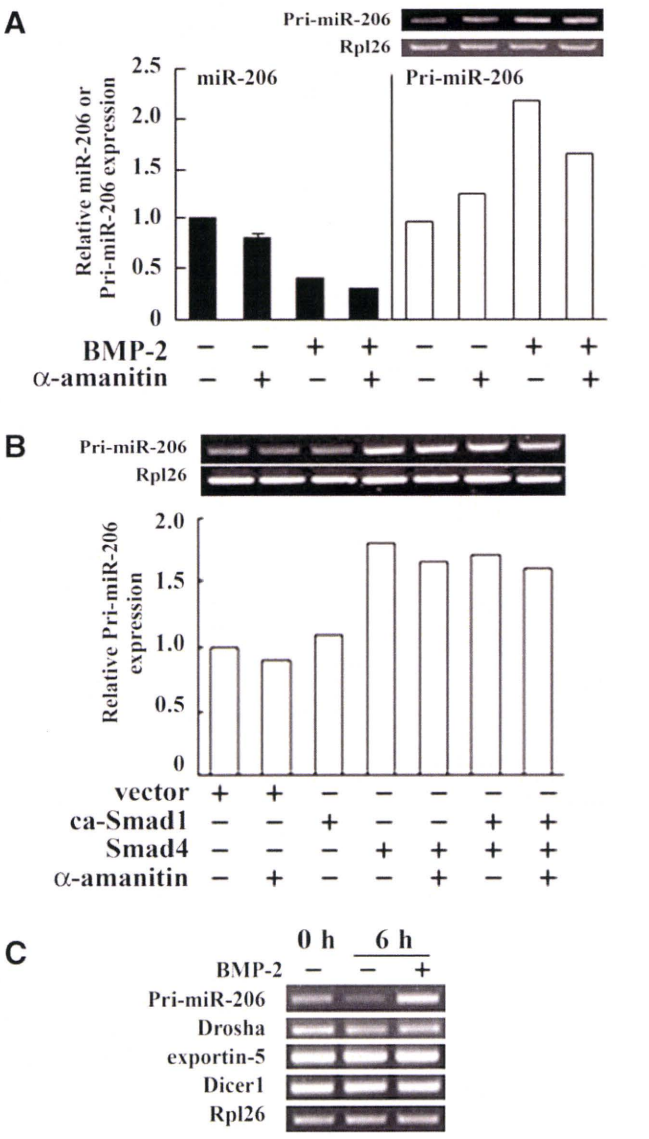

Fig. 3. Regulation of the expression of pri-miR-206 and Drosha, exportin-5 and Dicer1 mRNA by BMP-2. (A and C) C2C12 cells were plated and cultured with 10% FBS. After 24 h, the medium was replaced with fresh medium containing 2.5% FBS and α-amanitin (10 μg/ml) (+), BMP-2 (300 ng/ml) (+) or vehicle (−) was added. Then the cells were cultured for 6 h. The expression of miR-206 was analyzed (A, left panel). RT-PCR was performed to estimate the expression level of pri-miR-206. PCR products were resolved by 2% agarose gel electrophoresis (A, right upper). The expression level was quantitated and normalized to the amount of Rpl26 product (A, right lower). RT-PCR was performed to estimate the level of expression of pri-miR-206 and Drosha, exportin-5 and Dicer1 mRNA. RT-PCR for Rpl26 was performed as the endogenous control (C). (B) C2C12 cells were plated and cultured with 10% FBS. After an overnight incubation, the cells were transiently transfected with 100 ng/well of the expression construct for ca-Smad1 or Smad4 or an empty expression plasmid. After an overnight incubation, the culture medium was replaced with fresh medium containing 2.5% FBS and α-amanitin (10 μg/ml) (+) or vehicle (−) and then cultured for 6 h. RT-PCR was performed to estimate the expression level of pri-miR-206 (upper). The pri-miR-206 level was quantitated (lower).

sion level of miR-206 was determined by a quantitative real time RT-PCR assay. In myoblastic C2C12 cells, miR-206 was highly expressed, whereas its expression level in osteoblastic MC3T3-E1 and ST-2 cells was very low (Fig. 1A). To elucidate the role of BMP-2 in the regulation of miR-206 expression in C2C12 cells, we analyzed the effect of culturing cells in BMP-2 on the level of miR-206 over time. In the control cells, the level of miR-206 began to rise after 6 h and continued to increase for the remainder of the experimental period (24 h) (Fig. 1B). The addition of BMP-2 attenuated this increase in miR-206 expression (Fig. 1B). In contrast, miR-16 expression was not affected by BMP-2. Three muscle-specific microRNAs, miR-206, −1, and −133a, are induced during the differentiation of C2C12 myoblasts *in vitro*. Transfection of miR-206 promotes muscle differentiation of C2C12 cells despite the presence of serum, and inhibitors of myogenic transcription factors, Id1-3 and MyoR, are decreased upon the introduction of miR-206 [7]. Similar to our findings, it has been reported that BMP-2 decreases miR-206 expression in C2C12 cells for 2–6 days after BMP-2 treatment [25]. It was also shown previously that BMP-2 completely suppresses the induction of myogenic differentiation markers such as myogenin and muscle creatine kinase in C2C12 cells [12,13]. In our present study, we found that the induction of miR-206 was also suppressed by BMP-2, which opens up the possibility that miR-206 may be a potential stimulator of myoblastic differentiation.

Several reports have demonstrated that BMP-2 signaling results in the direct transcriptional activation of BMP-responsive promoters by transcription factors that include Smad1 and Smad4 [22]. Therefore, to explore the effect of Smad1/4 on miR-206 expression, we transfected expression plasmids for these proteins into C2C12 cells, instead of adding BMP-2. Although pseudo-phosphorylated Smad1 (constitutively-active Smad1: ca-Smad1) induced the expression of luciferase from IdWT4F-luc (a luciferase reporter plasmid that contains four copies of a 29-bp BMP responsive fragment) (Fig. 2B), miR-206 expression was suppressed after the transfection of Smad4 but not ca-Smad1 (Fig. 2A). In addition, the miR-206 expression level was decreased significantly by the co-transfection of ca-Smad1 and Smad4. In contrast, miR-16 expression was not affected by ca-Smad1 or Smad4 (Fig. 2A). Our results suggested that the regulation of miR-206 expression by BMP-2 depends on the Smad signaling pathway.

The BMP-2 signaling pathway controls the processing of miR-206

miRNA expression can be controlled at either the transcriptional or post-transcriptional level [2]. The transcription factor MyoD1 directly regulates transcription of the primary miR-206 transcript [15,25]. Although BMP-2 completely suppresses myogenin expression in C2C12 cells, we observed previously that BMP-2 does not affect the expression of MyoD1 [13]. Therefore, regulation of miR-206 expression by BMP-2 could potentially be controlled at the post-transcriptional level. We therefore examined miR-206 expression in the presence or absence of α-amanitin, a specific inhibitor of pol II-dependent transcription [26]. Incubation of cells with α-amanitin alone slightly reduced miR-206 expression at 6 h (Fig. 3A). However, BMP-2 reduced miR-206 expression either in the presence or absence of α-amanitin, which suggested that the effect of BMP-2 on miR-206 expression might be independent of transcription (Fig. 3A, left).

Next, we examined the level of primary miR-206 gene transcripts (pri-miR-206) in C2C12 cells using RT-PCR. The pri-miR-206 level was increased after BMP-2 treatment for 6 h compared to that in the absence of BMP-2 (Fig. 3A, right). In addition, the pri-miR-206 level was up-regulated by Smad4 but not ca-Smad1 (Fig. 3B), which indicated that the effects of BMP-2 on miR-206 biogenesis could be mediated by Smad4. In the presence of α-amanitin, the level of pri-miR-206 transcripts was also increased by BMP-2 (Fig. 3A, right) or Smad4 (Fig. 3B). These results suggested that BMP-2 could act at the post-transcriptional level to decrease the amount of miR-206, perhaps by inhibiting the processing of

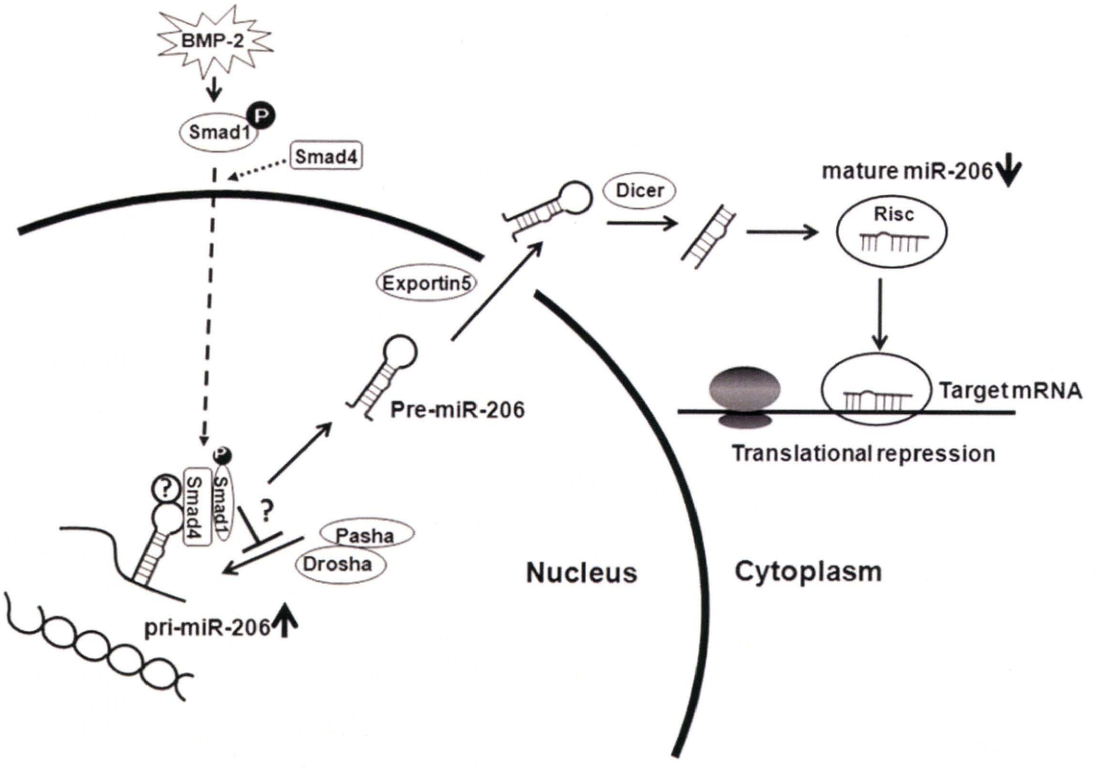

Fig. 4. Schematic model of the hypothetical involvement of BMP-2 and Smad1/4 in miR-206 maturation processes. BMP-2 or Smad1/4 (the intracellular signaling molecules for BMP-2) might inhibit the processing of pri-miR-206 into miR-206. Pri-miR-206 would then accumulate in these cells.

pri-miR-206 to miR-206. This would result in the accumulation of pri-miR-206. Turnover of mature miR-206 in C2C12 cells could be rapid. In embryonic tissue, the processing of several pri-miRNAs is blocked, with the activation of processing only occurring as development proceeds [18]. Our results indicated that not only developmental processes but also extracellular signaling molecules regulate the processing of miRNAs. Others have reported that miR-21 processing can also be regulated by BMP signaling [20].

The pathway involved in the production of a mature miRNA from a pri-miRNA transcript is controlled principally by the activities of three proteins: two Rnase III endonucleases, Drosha and Dicer1, and the transporter exportin-5 [2]. A reasonable explanation for the inhibition of pri-miR-206 maturation would be a decrease in the expression of one or more of these components of the biogenesis pathway. However, semi-quantitative RT-PCR analysis showed that the expression levels of Drosha, exportin-5 and Dicer1 were unchanged by BMP-2 (Fig. 3C). Unless the protein levels of these components are affected, these results indicate that an alternative mechanism is involved in the inhibition of pri-miR-206 maturation.

Because the double-stranded RNA binding domain of Drosha is insufficient for substrate binding, Drosha needs an additional partner protein that provides the RNA recognition function. Parsha (also known as DGCR8) interacts with Drosha to form a functional complex called the "Microprocessor" [27]. Drosha-mediated pri-miRNA processing requires the DEAD-box RNA helicases p68 and p72 [28]. It has been reported that p68 interacts with Smad proteins [29]. More recently, the interaction of p68 with Smad2 was shown to promote the processing of pri-miR-21 into pre-miR-21 [20]. This effect is miRNA-specific: BMP signaling has no effect on the expression of other miRNAs such as miR-125a, miR-221, miR-15b, and miR-100. The authors speculated that the Smad MH1 domain may recognize an RNA sequence or structural element, and thus provide specificity for the BMP-target miRNA [20]. Our results show that BMP signaling through Smad4 suppresses miR-206 expression and increases the level of pri-miR-206 (Fig. 4). There are several possible explanations for this regulation of miR-206 processing. Firstly, Smad4 could interact directly with pri-miR-206, and then inhibit the processing of pri-miR-206 into pre-miR-206. It is unclear whether Smads interact with miRNAs in a sequence-specific manner. A second possibility is that Smad4 associates with additional factors such as p68 and p72, and this reduces the processing efficiency of the Microprocessor complex. This would be the opposite effect to that which Smad2 exerts on miR-21 processing [20]. Depending on the cell type and stage of differentiation, Smad binds to co-activators such as CBP and p300, co-repressors such as c-ski and SnoN, and other transcription factors such as β-catenin and Runx2 [10,11,30–33]. Therefore, Smad4 may interact with these proteins or unknown factors, and then interface with the Microprocessor complex. The precise mechanism by which Smad4 blocks miRNA processing, as well as its substrate specificity, is unknown. The exact nature of the regulatory mechanism awaits further investigation.

Acknowledgments

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miR-210 promotes osteoblastic differentiation through inhibition of *AcvR1b*

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ABSTRACT

Although microRNAs (miRNAs) are involved in many biological processes, the mechanisms whereby miRNAs regulate osteoblastic differentiation are poorly understood. Here, we found that BMP-4-induced osteoblastic differentiation of bone marrow-derived ST2 stromal cells was promoted and repressed after transfection of sense and antisense miR-210, respectively. A reporter assay demonstrated that the activin A receptor type 1B (*AcvR1b*) gene was a target for miR-210. Furthermore, inhibition of transforming growth factor-β (TGF-β)/activin signaling in ST2 cells with SB431542 promoted osteoblastic differentiation. We conclude that miR-210 acts as a positive regulator of osteoblastic differentiation by inhibiting the TGF-β/activin signaling pathway through inhibition of *AcvR1b*.

1. Introduction

MicroRNAs (miRNAs) are classified as regulatory RNAs, and have been reported to play important roles in the development, proliferation and differentiation of various types of cells. Among the various miRNAs, miR-143 regulates adipocyte differentiation, miR-206 regulates muscle differentiation and miR-133 regulates skeletal differentiation [1–3]. miR-223 regulates granulopoiesis by a feedback mechanism and is modulated competitively by the transcription factors nuclear factor I/A (NFI-A) and CCAAT/enhancer binding protein-α (C/EBPα) [4]. miR-223 was also recently reported to regulate osteoclastogenesis in RAW 264.7 cells [5]. Some miRNAs are known to be present in cancer cells and act as oncogenes or tumor suppressor genes [6–8]. miR-21 has been reported to be an oncogene that promotes tumor growth by down-regulating the tropomyosin 1 tumor suppressor (*TPM1*) gene [9,10]. Expression of miR-17 is regulated by the myelocytomatosis (c-Myc) oncogene and acts as an oncogene by regulating cell proliferation through E2F transcription factor 1 (E2F1) [11,12]. On the other hand, the let-7 miRNA suppresses the high mobility group AT-hook 2 (*HMGA2*) oncogene and acts as a tumor suppressor gene [13]. The let-7 miRNA is also known to suppress cell proliferation [14] and induce regression of colon cancer [15].

Although miRNAs are known to play a number of regulatory roles, little is known about their roles in osteoblastic differentiation. As a functional miRNA, we recently reported that miR-125b negatively regulates osteoblastic differentiation by mediating cell proliferation [16]. However, there are no reports of miRNAs that positively regulate osteoblastic differentiation.

In the present study, we demonstrate positive regulation of osteoblastic differentiation by miR-210. The expression profiles of miRNAs during osteoblastic differentiation of mouse ST2 mesenchymal stem cells were obtained by miRNA microarray analyses, and miR-210 was found to be highly expressed in these cells. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) confirmed that miR-210 expression was upregulated during osteoblastic differentiation. We also analyzed the function of miR-210 in osteoblastic differentiation by transfection

Abbreviations: miRNA, microRNA; TGF-β, transforming growth factor-β; AcvR1b, activin A receptor type 1B; Alk, activin-like kinase; ALP, alkaline phosphatase; OC, osteocalcin

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of exogenous miR-210 or its antisense strand into ST2 cells. The miRNA target gene databases MiRanda and TargetScan predicted that the activin A receptor type 1B (*AcvR1b*; also known as Alk4) gene could be a target for miR-210. The regulatory relationship between miR-210 and *AcvR1b* was confirmed by reporter assays. The role of transforming growth factor-β (TGF-β)/activin signaling for osteoblastic differentiation in ST2 cells was also validated.

2. Materials and methods

2.1. Cell culture

ST2 cells and NRG cells were obtained from the RIKEN BioResource Center (BRC, Tsukuba, Japan) and cultured according to the supplier's protocols using RPMI-1640 medium and DMEM, respectively, supplemented with 10% fetal bovine serum (FBS).

2.2. Osteoblastic differentiation

Osteoblastic differentiation was induced by replacing the medium with fresh medium containing 10% FBS and 100 ng/ml of bone morphogenetic protein-4 (BMP-4) (R&D Systems, Minneapolis, MN) every third day. SB431542 (1 μM; Sigma, St. Louis, MO) was added to fresh medium containing the above components for inhibition of TGF-β/activin signaling.

2.3. miRNA microarray analysis

Small RNA fractions of total RNA were purified using a flashPAGE Fractionator System (Ambion, Austin, TX) and labeled with a *mir*Vana miRNA Labeling Kit (Ambion), followed by loading on a custom-made miRNA array on which a *mir*Vana miRNA Probe Set (Ambion) was printed according to the manufacturer's instructions. After 16 h of hybridization, the signals were detected by a microarray scanner (G2565BA; Agilent Technologies, Santa Clara, CA) and quantified by the software Feature Extraction ver. 8.1 (Agilent Technologies).

2.4. Alkaline phosphatase (ALP) staining and its measurement

Cells were fixed with 10% formalin for 20 min, followed by fixation for 1 min in an ice-cold solution of equal volumes of ethanol and acetone, and then washed with phosphate-buffered saline. The ALP staining solution was prepared by dissolving 1 mg of Naphthol AS-MX (Sigma) in one droplet of *N,N*-dimethylformamide (Wako, Osaka, Japan) and suspending the droplet in 10 ml of 0.1 M Tris–HCl buffer (pH 8.5) containing 2 mM $MgCl_2$. Six micrograms of Fast Blue BB salt (Sigma) was added and the ALP staining solution was filtered. The fixed cells were incubated in the ALP staining solution at 37 °C for 20 min. A *p*-Nitrophenyl Phosphate Liquid Substrate System (Sigma) was added to determine the ALP activity and the absorbances at 405 nm were measured using an ARVO MX plate reader (Perkin–Elmer, Norwalk, CT). ALP staining and ALP activity measurements were performed in triplicate wells in two independent experiments.

2.5. Cell proliferation assay

After plating cells and changing to fresh media containing 100 ng/ml BMP-4 and 10% FBS, the total cell count was measured every day for 6 days using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. The absorbances at 490 nm were measured with an ARVO MX plate reader (Perkin–Elmer). Background absorbances from empty wells were subtracted from those of the sample wells.

2.6. qRT-PCR

Total RNA was isolated from cultured cells using a Nucleospin column (Machery Nagel, Düren, Germany), according to the manufacturer's instructions. A *mir*Vana miRNA Isolation Kit (Applied Biosystems, Forster City, CA) was used for purification of miRNAs. The yield and quality of the RNA samples were determined using a NanoDrop spectrometer (NanoDrop Technology, San Diego, CA). The expression levels were measured by qRT-PCR. Total RNA was reverse-transcribed by Transcriptor (Roche, Mannheim, Germany) with oligo-dT primers or specific primers for miR-210 (Applied Biosystems) according to the manufacturer's instructions. Using the resulting cDNAs as templates, the gene expression levels were measured using an Mx3000P system (Stratagene, La Jolla, CA) and Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The primer sequences for measurement of mRNA expression were: 5′-TGGAGAAACCTGCCAAGTATG-3′ (glyceraldehyde phosphate dehydrogenase (GAPDH)-forward); 5′-GGAGACAACCTGGTCCTCAG-3′ (GAPDH-reverse); 5′-CTCTGTCTCTCTGACCTCACAG-3′ (osteocalcin (OC)-forward); 5′-GGAGCTGCTGTGACATCCATAC-3′ (OC-reverse); 5′-CGTCCTCTCTG CTTGAGGAA-3′ (Osterix-forward); 5′-TTCCCCAGGGTTGTTGAGT-3′ (Osterix-reverse); 5′-AACGAGATCGAGCTCAGAGG-3′ (Col1a1-forward); 5′-GGGAGGTCTTGGTGGTTTTG-3′ (Col1a1-reverse); 5′-CTG GCTACAAGGTGGTGGAC-3′ (ALP-forward); 5′-GTCTTCTCCACC GTGGGTCT-3′ (ALP-reverse); 5′-GCTTGGCTTATGGACTGAGG-3′ (Osteopontin-forward); 5′-CGCTCTTCATGTGAGAGGTG-3′ (Osteopontin-reverse); 5′-GAGGGCACAAGTTCTATCTG-3′ (Runx2-forward); 5′-GCTCGGATCCCAAAAGAAG-3′ (Runx2-reverse). Specific primers for miR-210 and U6 (Applied Biosystems) were used for measurement of miRNA expression.

2.7. Transfection

The sense strand of miR-210 (miR-210) and the antisense strand of miR-210 (anti-miR-210) (Applied Biosystems) were used to promote and inhibit miR-210 activity, respectively. Negative controls were used for both reactions. For transfection, Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA) was mixed with 20 nM of the above-mentioned RNAs according to the manufacturer's instructions, and these solutions were directly mixed with ST2 cells in 24-well culture plates at a density of 3.2×10^4 cells/well. For osteoblastic differentiation, the medium was replaced with fresh medium containing 10% FBS and 100 ng/ml of BMP-4 at 4 h after transfection.

2.8. Luciferase reporter assay

The miR-210 target region of the *AcvR1b* sequence (5′-ATTCTCCAGACTCAAACGCACAT-3′, part of the NCBI RefSeq ID of NM_007395) or its mutated sequence (5′-ATTCTCCAGAACCAA CTATGTCT-3′) was inserted downstream of the pGL4.13 luciferase plasmid (Promega) and the resulting constructs were named pGL4.13-AcvR1b and pGL4.13-AcvR1-mut, respectively. Cotransfection of 200 ng of pGL4.13-AcvR1b or pGL4.13-AcvR1-mut with 20 nM miR-210 or anti-miR-210 was carried out in 24-well plates. A native pGL4.13 luciferase plasmid was cotransfected with the same concentration of miR-210 or anti-miR-210 in separate wells as a control. Twenty nanograms of a Renilla luciferase vector, pGL4.74 (Promega), was also transfected into all samples for

Table 1
miRNAs whose expression are changed during osteoblastic differentiation (day 14) comparing to Control (without BMP-4) (1 day).

miRNA	log 2 ratio (B14/C1)
Upregulated	
let-7d	2.97
let-7a-1	2.72
miR-210	2.20
let-7f-2	2.16
let-7f-1	1.79
miR-16-1	1.65
let-7b	1.55
Downregulated	
miR-329	−4.31
miR-122a	−2.01
miR-23a	−1.12
miR-147	−1.04
miR-198	−1.01

normalization. Transfections with all plasmid and RNA combinations were repeated three times. After 48 h, the cells were harvested with PLB reagent (Promega) and the firefly luciferase activity was measured in each well using the LARII substrate and a Dual Luciferase Assay Kit (Promega) with the ARVO MX plate reader. The relative firefly luciferase activity was calculated by normalization to the Renilla luciferase activity.

3. Results

3.1. Expression of miR-210 is upregulated during osteoblastic differentiation

To elucidate the differential expression of miRNAs during osteoblastic differentiation, a microarray analysis for miRNAs was performed. We found that the expressions of several miRNAs changed during osteoblastic differentiation (Table 1). Several let-7 family miRNAs, miR-210 and miR-16-1 were listed as upregulated. The let-7 family miRNAs are known to be broadly expressed in many different cells and tissues, and have several known functions. In contrast, the function of miR-210 has been poorly understood to date. Therefore, we focused on the possibility of a regulatory role for miR-210 in osteoblastic differentiation. qRT-PCR with the miRNA measurement protocol was performed to further analyze the expression pattern of miR-210 during osteoblastic differentiation of ST2 cells. As shown in Fig. 1A, miR-210 expression was greatly upregulated at day 12 when ST2 cells were cultured with BMP-4, suggesting its involvement in osteoblastic differentiation of ST2 cells. To test whether the increase in miR-210 was a dose-responsive effect to BMP-4, increasing amounts of BMP-4 were introduced for osteoblastic differentiation. The expression level of miR-210 increased until the BMP-4 concentration reached 50 ng/ml (Fig. 1B), indicating that the induction of

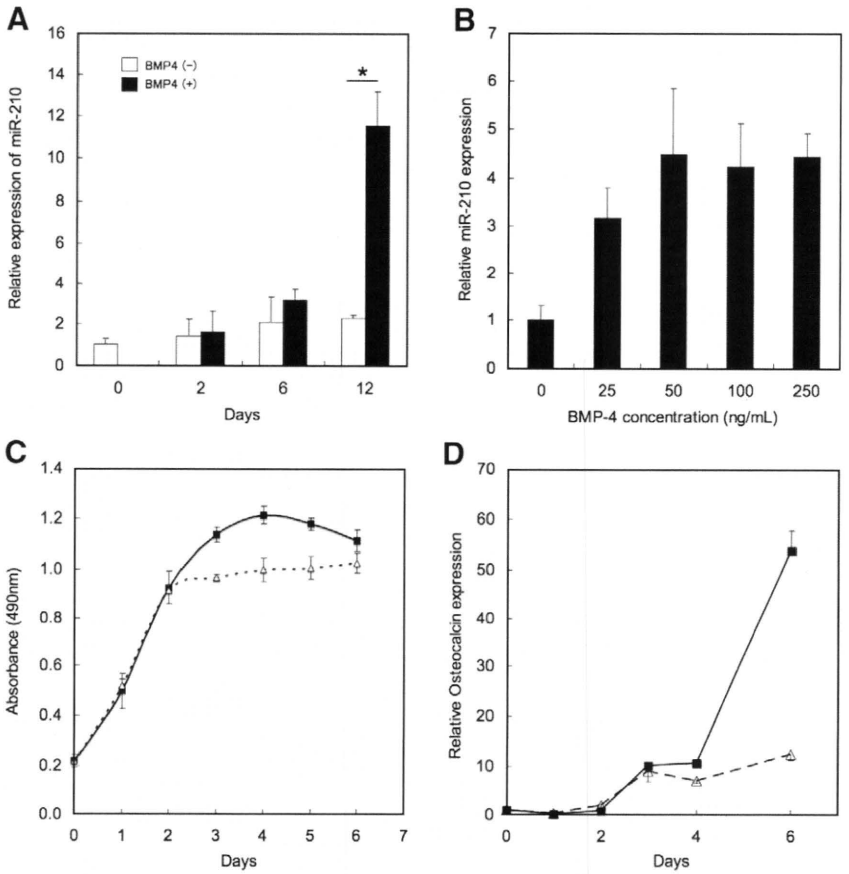

Fig. 1. Relative expression of endogenous miR-210 during osteoblastic differentiation of ST2 cells. (A) After plating ST2 cells, BMP-4 was added at a final concentration of 100 ng/ml. Control cells were cultured without BMP-4. Cells were harvested at 2, 6 and 12 days after the addition of BMP-4, and the relative levels of endogenous miR-210 expression at each time point with and without the addition of BMP-4 compared with day 0 were measured by qRT-PCR. Solid bars and white bars indicate cells incubated with and without BMP-4, respectively. Asterisks indicate significant *t*-test results ($P < 0.01$). (B) BMP-4 was added in increasing amounts (25, 50, 100 and 250 ng/ml) for 6 days, and the expression levels of miR-210 was measured by qRT-PCR. (C and D) Characterization of cell proliferation and osteocalcin (OC) expression during osteoblastic differentiation of ST2 cells. BMP-4 was added at a concentration of 100 ng/ml, then cell proliferation assays (C) and measurement of osteocalcin expression by qRT-PCR (D) were performed. Solid squares and white triangles indicate cultures with and without BMP-4, respectively.

miR-210 is BMP-4 dose-dependent. To clarify the time-dependent profiles of cell proliferation and the expression levels of the osteoblastic marker OC, cell proliferation assays and qRT-PCR were performed, respectively. We found that proliferation stopped at 2–3 days after osteoblastic differentiation (Fig. 1C), and that the expression of OC began to increase at 4 days after osteoblastic differentiation (Fig. 1D).

3.2. Transfection of miR-210 promotes osteoblastic differentiation of ST2 cells

To determine whether miR-210 could influence osteoblastic differentiation, miR-210 and anti-miR-210 were individually transfected into ST2 cells and their effects were assessed by observing the ALP activity and expression level of OC transcripts. ALP staining and activity were increased following transfection of miR-210 compared with transfection of control RNA (Fig. 2A and B). OC expression was also increased following miR-210 transfection (Fig. 2C), suggesting that stimulation of miR-210 activity promoted osteoblastic differentiation of ST2 cells. By contrast, transfection of anti-miR-210 reduced the expression of OC (Fig. 2C). The expression levels of other osteoblastic markers, namely osterix and ALP, were also upregulated following transfection with miR-210 (Fig. 2D). These results indicate that miR-210 positively regulates the osteoblastic differentiation of ST2 cells. To examine whether the regulation of osteoblastic differentiation by miR-210 occurs in other osteoblast-like cells, similar experiments were performed using the mouse NRG cell line. We used this cell line because osteoblastic differentiation can be induced at a lower concentration of BMP-4 and within a shorter period compared with the ST2 cell line. As shown in Fig. 2E, the level of ALP activity was elevated when miR-210 was transfected into NRG cells, as observed in ST2 cells, indicating that the promotion of osteoblastic differentiation by miR-210 is not restricted to the ST2 cell line.

3.3. AcvR1b is a target of miR-210

More than 200 genes were predicted by PicTar or TargetScan to be potential target genes for miR-210 [17,18]. We focused on *AcvR1b* (Fig. 3A), which is a member of the BMP receptor family. In this family, activin-like kinase 2 (Alk2) is known to regulate BMP signal transduction, whereas AcvR1b regulates the TGF-β/activin signaling pathway [19]. We used a luciferase reporter assay system and transfection of sense and antisense strands of miR-210 to determine whether *AcvR1b* was a target for miR-210, and to investigate its effects on osteoblastic differentiation. To test whether the predicted target sequence in the *AcvR1b* gene was regulated by miR-210, we cloned the predicted target sequence downstream of the luciferase reporter gene and cotransfected it with miR-210 or anti-miR-210. The relative luciferase activity was reduced by 50% following cotransfection with miR-210 compared with transfection with control RNA (Fig. 3B). By contrast, transfection with anti-miR-210 slightly increased the luciferase activity, although the difference was not statistically significant. When the targeted sequence of AcvR1b was mutated, the reduction of the luciferase activity by miR-210 was impaired (Fig. 3C). These results suggest that miR-210 targets the predicted site in the *AcvR1b* gene.

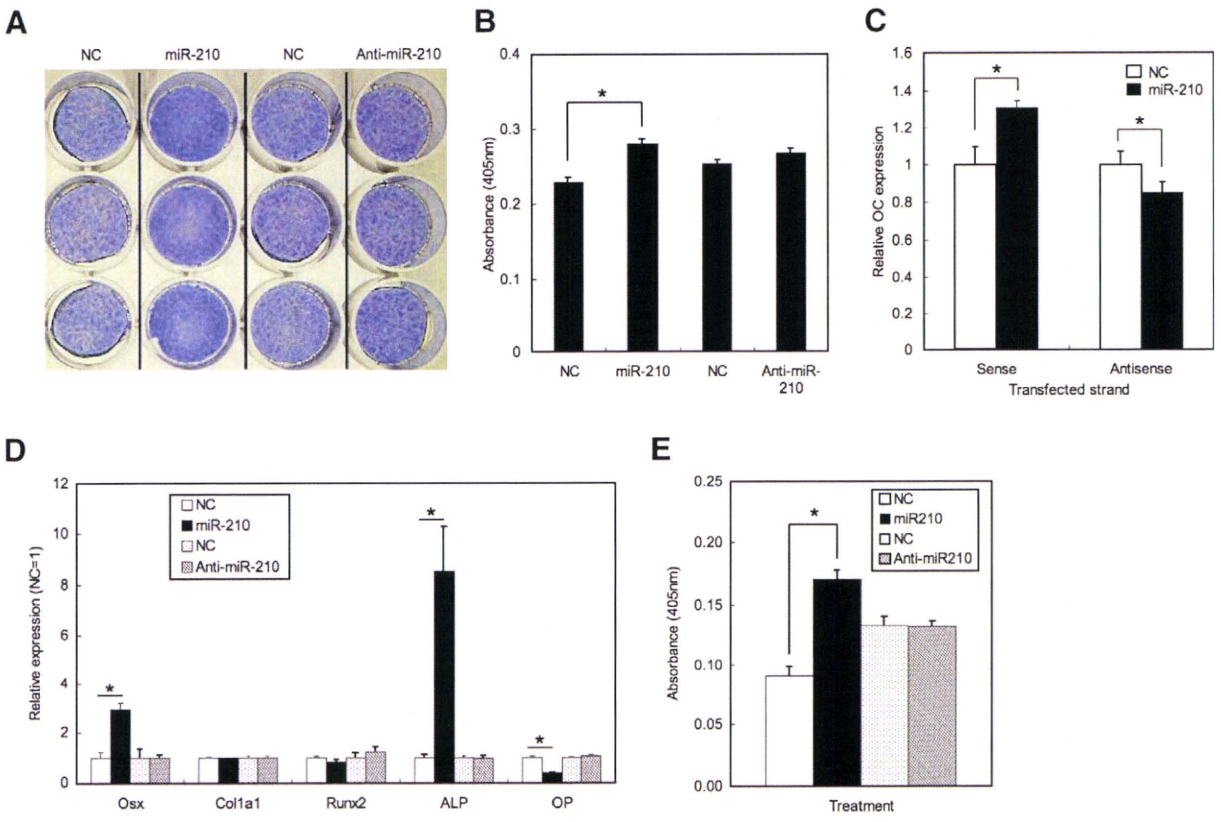

Fig. 2. Influences of transfections with miR-210 and anti-miR-210 on osteoblastic differentiation of ST2 cells. miR-210 (sense), anti-miR-210 (antisense) or a negative control (NC) RNA were transfected into ST2 cells and BMP-4 was added 4 h later. ALP staining was performed (A) and the ALP activity was measured (B), and compared with the NC values. (C) The relative expression of OC transcripts was measured after 5 days. (D) qRT-PCR analysis of other bone markers than OC in miR-210-transfected ST2 cells in which osteoblastic differentiation was induced by BMP-4. Osx: osterix; Col1a1: collagen, type 1, alpha 1; OP: osteopontin. (E) Measurement of ALP activity in miR-210-transfected NRG cells in which osteoblastic differentiation was induced by BMP-4 at 25 ng/ml for 4 days. Each experiment was performed in triplicate wells and on two independent days. Asterisks indicate significant *t*-test results ($P < 0.05$).

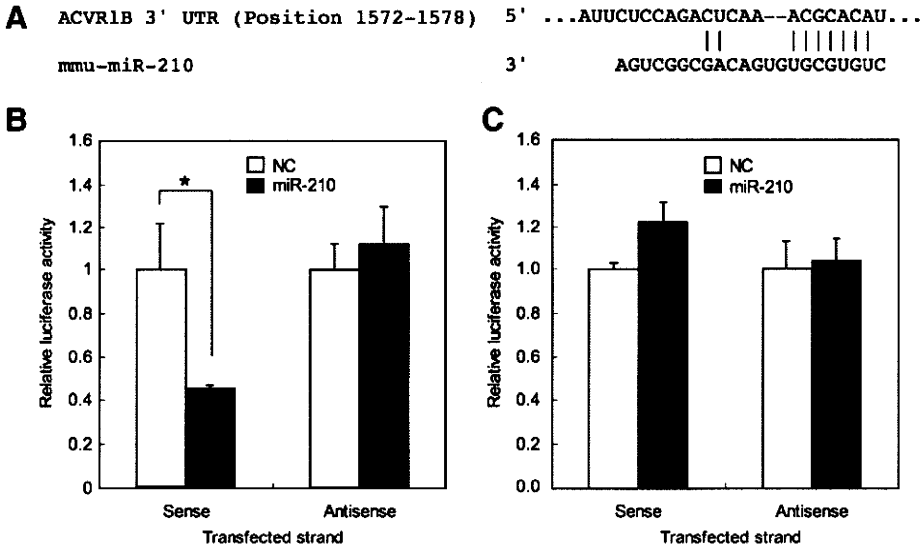

Fig. 3. Evaluation of targeting of *AcvR1b* by miR-210. (A) The region of the mouse *AcvR1b* mRNA 3′ UTR predicted to be targeted by miR-210 (TargetScan 4.1). (B and C) The relative activity of the luciferase reporter of the target sequence of miR-210 in the *AcvR1b* 3′ UTR after cotransfection of sense or antisense miR-210, compared with the transfection of a negative control (NC) RNA. Part of the *AcvR1b* 3′ UTR (the predicted target sequence for miR-210) (B) or its mutated sequence (C) was cloned into a luciferase vector and cotransfected with miR-210, anti-miR-210 or an NC RNA. The luciferase activity was measured at 2 days after transfection. Solid bars indicate transfection of miR-210 or anti-miR-210, and white bars indicate transfection of the NC miRNA. Asterisks indicate significant *t*-test results ($P < 0.05$).

3.4. Inhibition of AcvR1b by SB431542 promotes osteoblastic differentiation of ST2 cells

To further elucidate whether miR-210 promotes osteoblastic differentiation by inhibiting the signaling pathway of AcvR1b, we tried to use the chemical SB431542, which is a specific inhibitor for receptors of TGF-β/activin signaling [19,20]. Following addition of SB431542, ALP activity and OC expression were both elevated (Fig. 4A and B). These results suggest that osteoblastic differentiation of ST2 cells is promoted by downregulation of the TGF-β/activin signaling pathway. Taken together, the inhibition of TGF-β/activin signaling by either miR-210 or SB431542 promotes osteoblastic differentiation of ST2 cells.

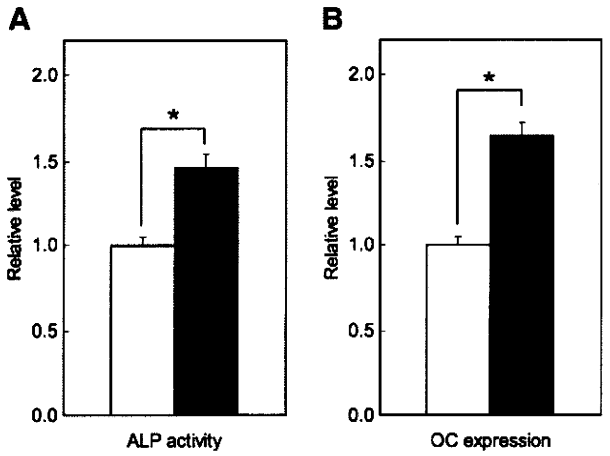

Fig. 4. Inhibition of TGF-β/activin signaling by SB431542 promotes osteoblastic differentiation. The culture medium was changed to fresh medium containing SB431542, an inhibitor of TGF-β/activin signaling, at 4 h and 3 days after plating of ST2 cells. The relative ALP activity (A) and relative expression of OC transcripts (B) compared with mock vector treatment were measured on day 6. Solid and white bars indicate cultures with and without SB431542, respectively. Asterisks indicate significant *t*-test results ($P < 0.05$).

4. Discussion

Osteoblastic differentiation of mesenchymal stem cells can be strongly induced by BMPs [21]. The involvement of miRNAs in various cellular events suggests that they could also have a regulatory function in osteoblast differentiation. However, the regulatory mechanisms of miRNAs in osteoblast proliferation and differentiation are currently poorly understood. We recently reported that miR-125b downregulated osteoblastic differentiation through inhibition of cell proliferation [16]. In that study, in vitro cell proliferation was inhibited when miR-125b was transfected, and osteoblastic differentiation was inhibited owing to the low density of the differentiating cells. That study was the first to demonstrate a function of miRNAs in regulating osteoblastic differentiation. miR-133 and miR-135 have subsequently been reported to inhibit BMP-2-induced osteoblastic differentiation by directly targeting the genes for *Runx2* and *Smad5* [22]. However, the miRNAs studied to date all act as inhibitors of osteoblastic differentiation, and no positive regulation by miRNAs has been reported.

The miRNA array data showed that miR-210 was upregulated during osteoblastic differentiation. Furthermore, the present results showed that miR-210 positively regulated osteoblastic differentiation of mouse mesenchymal ST2 cells. A recent study found that miR-210 was induced by hypoxia and involved in cell cycle regulation through targeting of E2F transcription factor 3 [23,24]. These results suggest that miR-210 could be involved in cell proliferation in cancer and under hypoxic conditions. However, the effects of miR-210 on cell differentiation have not been reported.

The results of the present study indicate that miR-210 was able to positively regulate osteoblastic differentiation via targeting of the TGF-β/activin signaling pathway. AcvR1b is a type I receptor which, together with the type II receptor ActRII, transmits signals from activin. When activated, this receptor is known to transmit signals to the receptor-regulated Smads (R-smad) Smad2 and 3, but not Smad1, 5 or 8, through their phosphorylation, resulting in the transcription of genes that function as inhibitory regulators of proliferation. BMP signals, however, are transmitted via other receptors, such as AcvR1 (Alk2), Bmpr1a (Alk3) and Bmpr1b (Alk6) and their signals are transmitted to Smad1, 5 and 8, thereby

initiating osteoblastic differentiation. It has been reported that Smad2/3 and Smad1/5/8 signaling can interfere with each other by competitive binding to a co-Smad, Smad4 [19]. Inhibition of Smad2/3 signaling is known to activate Smad1/5/8 signaling, resulting in acceleration of osteoblastic differentiation [19,20]. MiRanda and TargetScan both predicted that *Acvr1b* is a target gene for miR-210 in mice and humans. Therefore, we considered the possibility that the promotion of osteoblastic differentiation associated with the stimulation of miR-210 activity arose through its inhibition of *AcvR1b*. Regulation of the predicted 3′ UTR region of *AcvR1b* by miR-210 was demonstrated by luciferase reporter assays, and inhibition of *AcvR1b* was associated with increased osteoblastic differentiation in our culture system using the ST2 bone marrow-derived cell line. Thus the promotion of osteoblast differentiation by miR-210 could be explained by inhibition of the TGF-β/activin signaling pathway, through targeting of *AcvR1b*. Our results could provide an important step toward resolving bone disorders, such as osteoporosis. Elucidation of the roles of miRNAs in osteoblast regulation in vivo could help to clarify the mechanisms of bone metabolism and turnover.

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DRAGON, a GPI-anchored membrane protein, inhibits BMP signaling in C2C12 myoblasts

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Bone morphogenetic proteins (BMPs) induce osteoblastic differentiation of myoblasts via binding to cell surface receptors. Repulsive guidance molecules (RGMs) have been identified as BMP co-receptors. We report here that DRAGON/RGMb, a member of the RGM family, suppressed BMP signaling in C2C12 myoblasts via a novel mechanism. All RGMs were expressed in C2C12 cells that were differentiated into myocytes and osteoblastic cells, but RGMc was not detected in immature cells. In C2C12 cells, only DRAGON suppressed ALP and Id1 promoter activities induced by BMP-4 or by constitutively activated BMP type I receptors. This inhibition by DRAGON was dependent on the secretory form of the von Willbrand factor type D domain. DRAGON even suppressed BMP signaling induced by constitutively activated Smad1. Over-expression of neogenin did not alter the inhibitory capacity of DRAGON. Taken together, these findings indicate that DRAGON may be an inhibitor of BMP signaling in C2C12 myoblasts. We also suggest that a novel molecule(s) expressed on the cell membrane may mediate the signal transduction of DRAGON in order to suppress BMP signaling in C2C12 myoblasts.

Introduction

Bone morphogenetic proteins (BMPs) were first isolated from demineralized bone matrix and identified as factors responsible for inducing ectopic bone formation in muscular tissues (Urist 1965; Wozney *et al.* 1988). BMPs are responsible not only for artificial ectopic bone formation but also for physiological skeletal development (Thomas *et al.* 1997). BMP signaling is initiated when a ligand binds to complexes of type I and type II serine/threonine kinase receptors (Miyazono *et al.* 2005; Katagiri *et al.* 2008). The BMP-bound type II receptor phosphorylates the intracellular "GS" domain of the type I receptor. The activated type I receptor then phosphorylates Smad1/5/8 transcription factors in the cytoplasm. The phosphorylated Smad1/5/8 form transcriptionally active complexes with Smad4, translocate into the nucleus and bind to the regulatory elements of target genes (including Id1, which encodes an inhibitor of myogenesis) (Afrakhte *et al.* 1998; Katagiri *et al.* 2008). Among the type I receptors, substitution mutations of the specific conserved residues in the GS domains activate intracellular signaling without the binding of ligands or type II receptors (Wieser *et al.* 1995; Akiyama *et al.* 1997; Fujii *et al.* 1999; Aoki *et al.* 2001). Recently, we found that substitution of the phosphorylation site at the carboxyl terminus in Smad1 also activated downstream signaling events, including Id1 expression and osteoblastic differentiation, without phosphorylation by BMP type I receptors (Nojima & Katagiri, unpublished observation).

The repulsive guidance molecule (RGM) family members RGMa, DRAGON/RGMb and RGMc/hemojuvelin/HEF2 are secretory glycosylphosphatidylinositol (GPI)-anchored membrane proteins that have been implicated in axonal guidance and neural tube formation during embryogenesis (Monnier *et al.* 2002; Niederkofler *et al.* 2004; Papanikolaou *et al.* 2004; Samad *et al.* 2004). They have significant sequence homology to one another (50–60% amino acid identity) (Papanikolaou *et al.* 2004;

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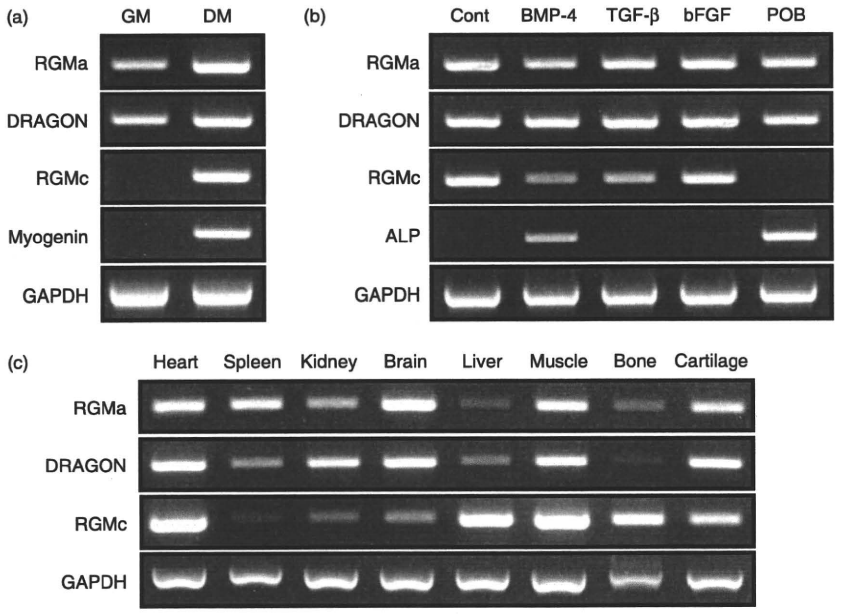

Figure 1 Expression levels of RGMs in C2C12 cells and various murine tissues. (a,b) RT-PCR analysis of RGM levels in C2C12 cells. C2C12 myoblasts were cultured with growth medium (GM) and differentiation medium (DM) for 7 days (a) or with DM in the presence of BMP-4 (100 ng/mL), TGF-β1 (5 ng/mL) or bFGF (20 ng/mL) for 3 days (b). Myogenin and ALP mRNA expression levels were used as markers of myogenic and osteoblastic differentiation, respectively. (c) RT-PCR analysis of RGMs in various murine tissues.

Babitt *et al.* 2005). All three proteins have a conserved von Willbrand factor (vWF) type D domain (Monnier *et al.* 2002), although its physiological function is still unknown. All of the RGM family members were found to act as co-receptors for the BMP subfamily. These proteins bind to BMP ligands as well as type I and type II BMP receptors, thereby enhancing BMP signaling (Babitt *et al.* 2005, 2006; Samad *et al.* 2005). A genetic mutation was found in the hemojuvelin gene in patients with juvenile hemochromatosis, a condition that is caused by the decreased expression of hepcidin, a key regulator of iron homeostasis in hepatocytes. Hemojuvelin enhanced the BMP-dependent up-regulation of hepcidin expression in hepatocytes (Papanikolaou *et al.* 2004; Babitt *et al.* 2006, 2007). Cells transfected with RGMa have been shown to utilize both BMPR-II and ActR-IIA for BMP-2 or BMP-4 signaling (Xia *et al.* 2007). However, to our knowledge, a physiological role for DRAGON, especially in bone metabolism, remains unestablished.

The ectopic bone-inducing activity of BMPs in muscle tissue is, at least in part, reflected in the *in vitro* culture of myoblasts, including mouse C2C12 cells. Treatment of these cells with BMPs inhibits the myogenic differentiation of myoblasts and converts their differentiation pathway into that of osteoblast lineage cells (Katagiri *et al.* 1994). Over-expression of a constitutively activated BMP type I receptor also induces similar differentiation conversions of myoblasts (Akiyama *et al.* 1997; Fujii *et al.* 1999; Aoki *et al.* 2001). We report here that DRAGON inhibits BMP signaling in C2C12 cells via a region that includes the secretory form of the vWF type D domain. DRAGON also inhibits the BMP signaling induced by a constitutively active form of Smad1. These findings indicate that a novel molecule(s) expressed on the cell membrane may mediate the signal transduction of DRAGON to suppress BMP signaling in C2C12 myoblasts.

Results

Expression levels of RGMs in C2C12 cells and mouse tissues

First, we examined the expression levels of RGM mRNAs in C2C12 myoblasts. RGMa and DRAGON were expressed in both growing and differentiated myocytes, but RGMc was detected only in myogenin-expressing differentiated cells (Fig. 1a). Although all DRAGON mRNAs were detected in C2C12 cells in the presence of BMP-4, TGF-β1 or bFGF, the expression levels of RGMc were decreased by BMP-4 (Fig. 1b). Primary osteoblasts expressed RGMa and DRAGON, but did not express RGMc (Fig. 1b), suggesting that osteoblastic differentiation induced by BMPs suppresses RGMc expression in C2C12 cells. RT-PCR analysis using RNAs prepared from various murine tissues indicated that RGMa and DRAGON were highly expressed in the heart, brain, muscle and cartilage (Fig. 1c). RGMc was strongly expressed in heart, liver, muscle, bone and cartilage (Fig. 1c). In bone tissue, RGMc may be expressed in osteocytes and/or bone marrow cells rather than

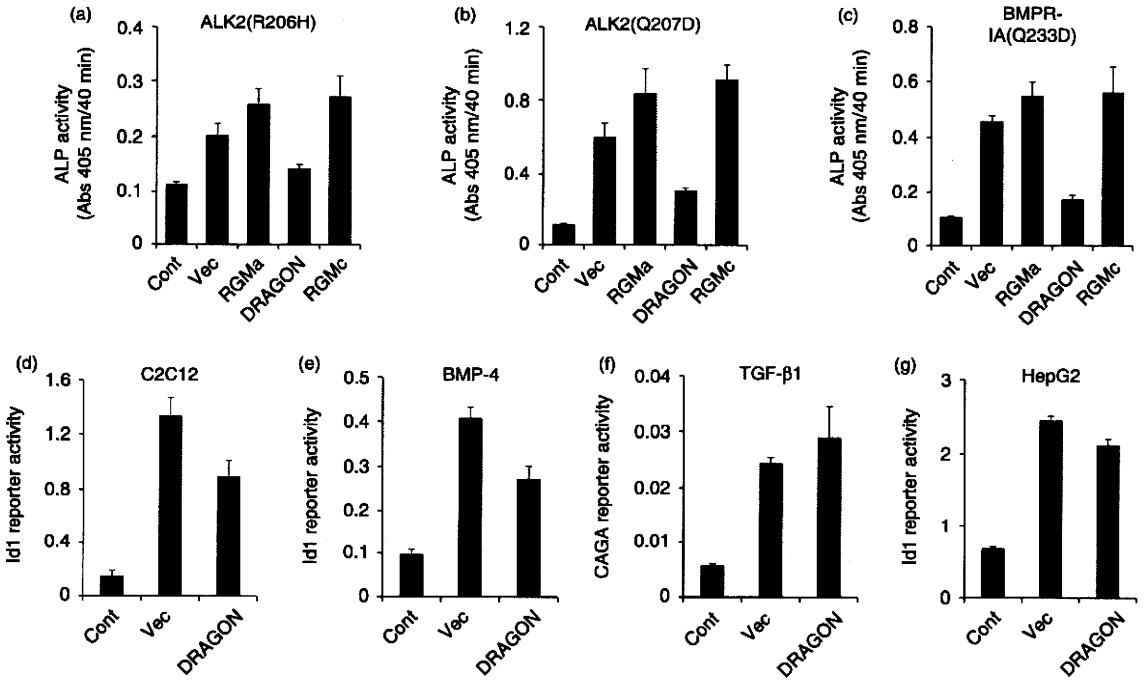

Figure 2 DRAGON inhibits BMP signaling in C2C12 cells. (a–c) Effects of the RGM family on BMP activity in C2C12 cells. C2C12 cells were co-transfected with ALK2(R206H) (a), ALK2(Q207D) (b) or BMPR-IA(Q233D) (c) and RGMa, DRAGON or RGMc in the presence of Smad1. ALP activity was determined on day 3. Data represent the mean ± SD ($n = 3$). (d,g) IdWT4F-luc was co-transfected with DRAGON or an empty vector into C2C12 (d) and HepG2 (g) cells. Luciferase activity was determined on day 2. Data represent the mean ± SD ($n = 3$). (e,f) C2C12 cells were co-transfected with DRAGON or an empty vector and the IdWT4F-luc (e) or CAGA-luc (f) reporter plasmid and then treated with 100 ng/mL of BMP-4 (e) or 5 ng/mL of TGF-β1 (f). Data represent the mean ± SD ($n = 3$).

osteoblasts because it was not expressed in primary osteoblasts (Fig. 1b,c).

DRAGON inhibits BMP signaling in C2C12 cells

Next, we examined the effect of RGMs on BMP-induced osteoblastic differentiation. A constitutively activated BMP receptor—ALK2(R206H), ALK2(Q207D) or BMPR-IA(Q233D)—was co-transfected with one of the RGMs into C2C12 cells, and as a result of that process we identified alkaline phosphatase (ALP) activity as a typical marker of osteoblastic differentiation. Unexpectedly, DRAGON suppressed ALP activity in all of the cultures that were examined (Fig. 2a–c). The suppression of BMP signaling by DRAGON was also confirmed in a luciferase assay using BMP-specific IdWT4F-luc in C2C12 cells (Fig. 2d,e). In contrast to BMP signaling, TGF-β signaling (as determined by CAGA-luc) was not suppressed by DRAGON in C2C12 cells (Fig. 2f). DRAGON showed a weaker inhibitory activity on IdWT4F-luc induced by ALK2(Q207D) in HepG2 cells compared with the inhibitory activity seen in C2C12 cells (Fig. 2d,g).

Deletion analysis of DRAGON to determine the inhibitory domain affecting BMP signaling

We generated Sol- and Cyt-DRAGON mutants, in which a GPI-anchor domain and a signal peptide, respectively, were deleted (Fig. 3a). The cellular localization of these DRAGON proteins was confirmed by immunohistochemical analysis in the presence and absence of detergent. Wild-type DRAGON, but not Sol- or Cyt-DRAGON, was detected on the cell membrane, even in the absence of detergent; Sol- and Cyt-DRAGON were detected only in the cytoplasm in the presence of detergent (Fig. 3b). ALP activity inhibition induced by ALK2(Q207D) was observed in the wild-type and Sol-DRAGON constructs, which may be secreted by a signal peptide. To investigate whether the signal peptide itself suppressed ALP activity, we further generated an additional mutant of DRAGON (Swap-DRAGON) in which the signal peptide was replaced with that of another protein (Fig. 3a). Swap-DRAGON was anchored on the cell membrane and suppressed ALP activity in a manner similar to that of wild-type DRAGON, indicating that the signal peptide of

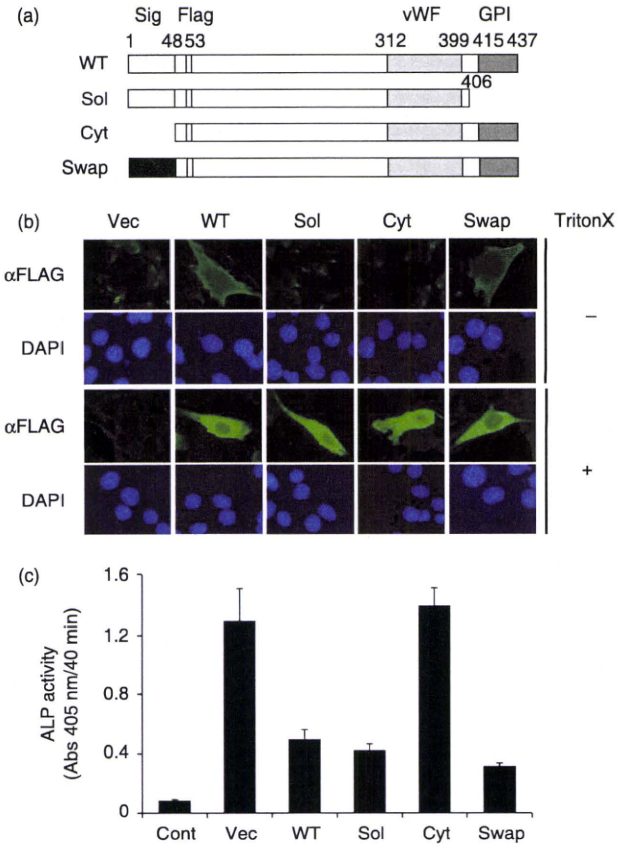

Figure 3 The secreted form of DRAGON inhibits BMP signaling. (a) Schematic diagram of DRAGON mutants. Location of a signal peptide (Sig), vWF type D domain (vWF), GPI-anchor domain (GPI) and FLAG-epitope tag (Flag) are indicated. (b) Cellular localization of DRAGON mutants in C2C12 cells. C2C12 cells were transfected with a wild-type (WT) form, a deleted form lacking the GPI-anchor domain (Sol), a deleted form lacking the signal peptide (Cyt) or a swapped signal peptide form (Swap) of the FLAG-tagged DRAGON construct. Cells were stained with an anti-FLAG antibody and DAPI in the absence and presence of Triton X-100. (c) C2C12 cells were co-transfected with one of the DRAGON mutants and ALK2(Q207D) in the presence of Smad1. ALP activity was determined on day 3. Data represent the mean ± SD ($n = 3$).

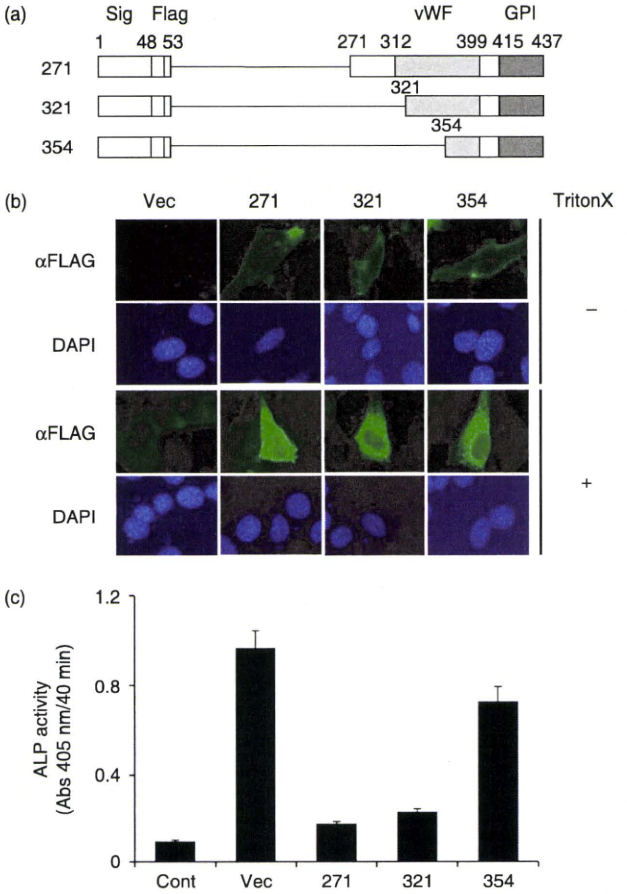

Figure 4 C-terminal region of DRAGON is important for BMP activity inhibition. (a) Schematic diagram of DRAGON deletion mutants. (b) Cellular localization of DRAGON mutants in C2C12 cells. C2C12 cells were transfected with one of the FLAG-tagged deletion DRAGON mutants and were stained with an anti-FLAG antibody and DAPI in the presence and absence of Triton X-100. (c) C2C12 cells were co-transfected with one of the DRAGON mutant constructs and ALK2(Q207D) in the presence of Smad1. ALP activity was determined on day 3. Data represent the mean ± SD ($n = 3$).

DRAGON was not involved in the inhibition of BMP signaling (Fig. 3b,c).

We further examined the inhibitory domain of DRAGON using the three deletion mutants 271, 321 and 354 (Fig. 4a). All of these deletion mutant DRAGON constructs were detected on the cell membrane in the absence of detergent (Fig. 4b). Both the 271 and 321 mutants showed the capacity to inhibit ALP activity, but mutant 354 had little suppressive effect on enzyme activity (Fig. 4c). Together, these findings indicate that a region including the vWF type D-like domain may play an important role in the inhibition of BMP signaling by DRAGON.

DRAGON inhibits BMP signaling independent of neogenin

Because the secretory form of DRAGON inhibited BMP signaling without a GPI-anchor domain (Figs 3, 4), we hypothesized that a cell surface molecule(s) may be involved in the inhibitory activity of DRAGON. Neogenin has been identified as a cell surface receptor for RGM family members (Matsunaga *et al.* 2004; Rajagopalan *et al.* 2004). Because neogenin was only weakly expressed in C2C12 cells, we cloned its cDNA and transiently over-expressed it in C2C12 cells (Fig. 5a, unpublished observation). Co-transfection of neogenin showed no effect on the ability

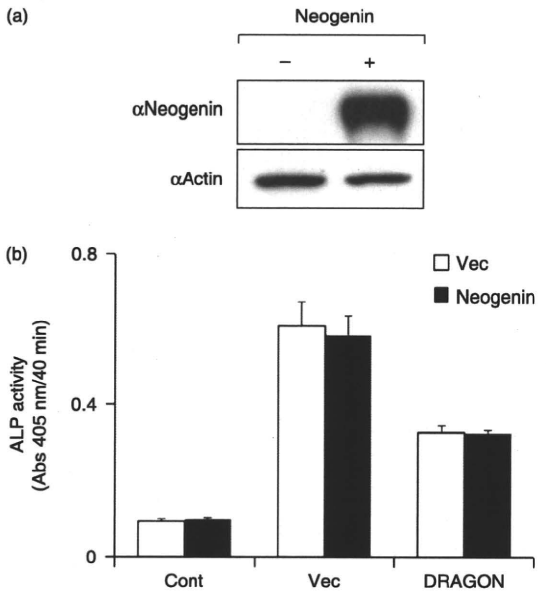

Figure 5 Inhibitory activity of DRAGON on BMP signaling is independent of neogenin. (a) Over-expression of neogenin in C2C12 cells. C2C12 cells were transfected with a neogenin expression vector, and protein levels were determined by immunoblots with anti-neogenin and anti-actin antibodies. (b) Neogenin did not affect the inhibitory activity of DRAGON on BMP signaling. C2C12 cells were co-transfected with ALK2(Q207D), DRAGON and Smad1 with or without neogenin. ALP activity was determined on day 3. Data represent the mean ± SD ($n = 3$).

of DRAGON to inhibit the ALP activity induced by ALK2(Q207D) (Fig. 5b).

DRAGON inhibits the transcriptional activity of Smads in C2C12 cells

Both wild-type and Sol-DRAGON decreased the expression levels of ALP and osterix mRNAs induced by ALK2(Q207D) in C2C12 cells, suggesting that DRAGON inhibited BMP signaling-induced osteoblastic differentiation (Fig. 6a). We examined the effect of DRAGON on phosphorylation levels of Myc-Smad1 and found that DRAGON did not significantly decrease phospho-Smad1 levels (Fig. 6b). Next, we asked whether a direct interaction between DRAGON and BMP receptor is involved in the inhibition process. Immunoprecipitation with immunoblotting analysis indicated that wild-type DRAGON, but not Sol-DRAGON, interacted with ALK2(Q207D) (Fig. 6c). Finally, we examined the effect of DRAGON on constitutively activated Smad1, which is able to induce downstream signal transduction cooperatively with Smad4 without phosphorylation by BMP receptors. Wild-type DRAGON, Sol-DRAGON and mutant 321 markedly inhibited the ALP activity and IdWT4F-luc activity induced by constitutively activated Smad1 and Smad4 (Fig. 6d,e). These results indicate that the inhibitory effect of DRAGON represses the transcriptional activity of Smad. Because both wild-type DRAGON and Sol-DRAGON suppressed ALK2 activity to a similar degree, we hypothesized that the interaction between DRAGON and BMP receptors may not be involved in this inhibition.

Discussion

The present study demonstrates that DRAGON, a member of the RGM family, inhibits BMP signaling in C2C12 myoblasts. The inhibitory domain of DRAGON was mapped around the vWF type D-like (but not GPI-anchor) domain. In our preliminary experiments, mouse vWF did not have any effect on BMP signaling in C2C12 cells, suggesting that a DRAGON-specific sequence in this domain may be involved in this inhibition. Although RGMs have been reported to interact with both BMP ligands and BMP receptors, DRAGON suppressed signaling induced by constitutively activated BMP receptors in the absence of BMP ligands. Moreover, secretory forms of DRAGON suppressed BMP signaling induced by constitutively activated Smad1. Taken together, our results suggest that DRAGON may bind to a novel molecule(s) expressed on the cell membrane to transduce inhibitory signaling on the transcriptional activity of Smads (Fig. 6f).

Neogenin, a homologue of deleted colorectal cancer (DCC) and the netrin-1 receptor, was a potential candidate for the DRAGON binding protein on the cell membrane because it has been shown to act as a high-affinity receptor for RGMs (Vielmetter *et al.* 1994; Keino-Masu *et al.* 1996; Stein *et al.* 2001). The interactions between RGMa and neogenin were involved in the regulation of neuronal survival (Wilson & Key 2006). Interestingly, the interaction with neogenin was required for RGMc release from muscle cells, and soluble RGMc played a critical role in the negative regulation of hepatic hepcidin expression through BMP signaling in hepatocytes (Zhang *et al.* 2007). However, neogenin over-expression did not affect the DRAGON activity in C2C12 cells. We noticed that the degree of BMP signaling inhibition by DRAGON is dependent on cell type; for example, HepG2 and other types of cells showed weaker activities than C2C12 cells. It is possible that the amount of novel molecule(s) expressed on the cell membrane may regulate the cell type-specific activity of DRAGON. In addition, DRAGON was expressed in bone, cartilage and primary osteoblasts. However, the expression of DRAGON was unaltered during osteoblastic differentiation in C2C12 cells. Thus, it is possible that the suppressive function of DRAGON depended

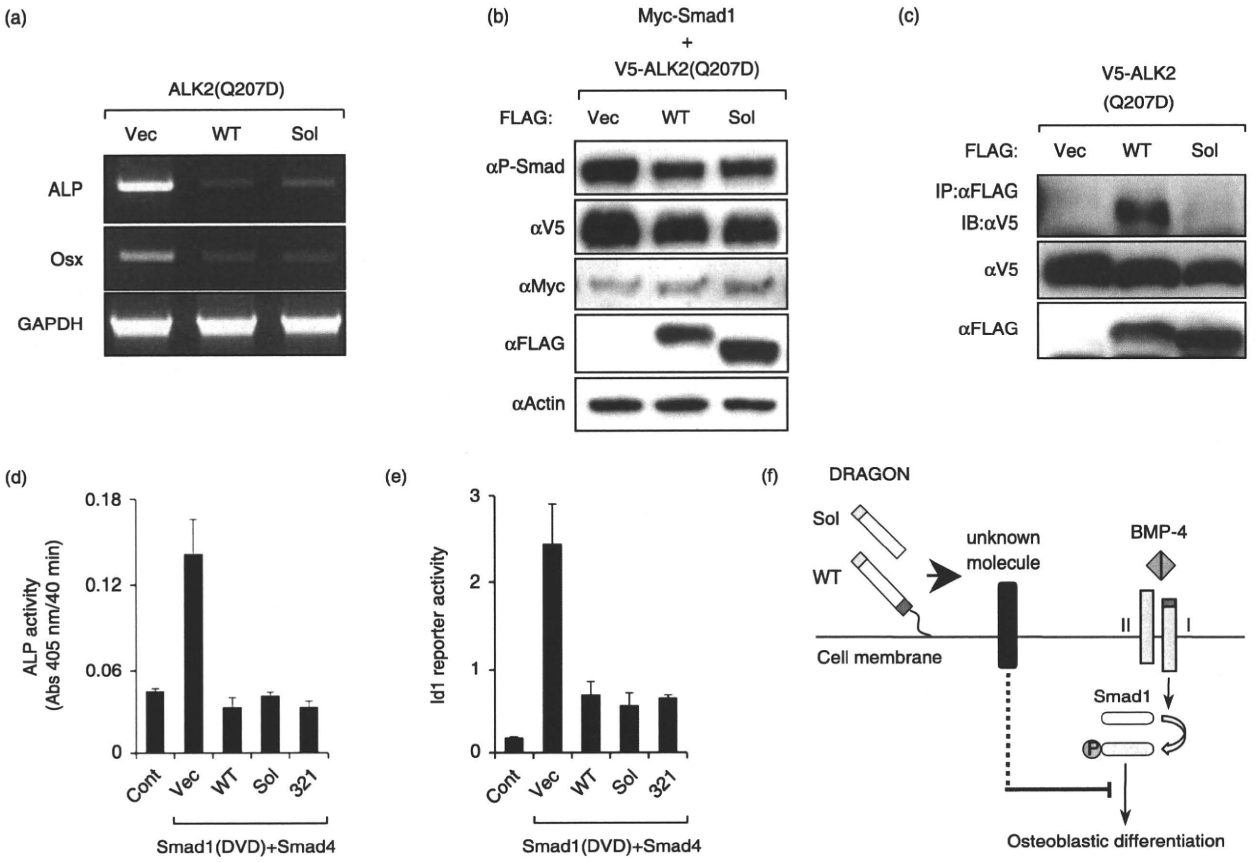

Figure 6 DRAGON inhibits the transcriptional activity of Smads independent of binding to the BMP receptor. (a) RT-PCR analysis of ALP and osterix in C2C12 cells. C2C12 cells were co-transfected with ALK2(Q207D) and an empty vector, wild-type or Sol-DRAGON in the presence of Smad1. ALP and osterix mRNA levels were determined by RT-PCR analysis on day 3. (b) Effect of DRAGON on the phosphorylation of Smad1. C2C12 cells were co-transfected with V5-ALK2(Q207D), Myc-Smad1 and FLAG-tagged wild-type, Sol-DRAGON or an empty vector. Whole cell lysates were immunoblotted with anti-phospho-Smad1/5/8, anti-V5, anti-Myc, anti-FLAG and anti-actin antibodies. (c) Interaction between DRAGON and ALK2(Q207D). C2C12 cells were co-transfected with V5-ALK2(Q207D) and FLAG-tagged wild-type, Sol-DRAGON or an empty vector. Whole cell lysates were immunoprecipitated with an anti-FLAG antibody and immunoblotted with an anti-V5 antibody. (d,e) DRAGON inhibits BMP activity directly induced by Smads. (d) C2C12 cells were co-transfected with constitutively activated Smad1 and Smad4 with wild-type, Sol- or mutant 321 DRAGON. ALP activity was determined on day 3. Data represent the mean ± SD ($n = 3$). (e) C2C12 cells were co-transfected with IdWT4F-luc, constitutively activated Smad1 and Smad4 with wild-type, Sol- or mutant 321 DRAGON. Luciferase activity was determined on day 2. Data represent the mean ± SD ($n = 3$). (f) A schematic diagram of the inhibitory mechanism of DRAGON in C2C12 cells. Secreted DRAGON with and without a GPI-anchor domain may interact with an unknown molecule(s) expressed on the cell membrane. This interaction may activate an intracellular signaling pathway to suppress the transcriptional activity of Smads and the osteoblastic differentiation of C2C12 myoblasts.

on the alteration of expression levels of a novel molecule(s). The identification of the DRAGON-binding molecule(s) on the cell membrane will shed light on the novel regulatory mechanisms associated with BMP signaling.

Recently, heterozygous mutations in the ACVR1 gene, which encodes the BMP type I receptor ALK2, were identified in familial and sporadic patients with FOP (Shore *et al.* 2006; Nakajima *et al.* 2007; Fukuda *et al.* 2009). These mutations cause an amino acid substitution in the intracellular ALK2 domain. We found that these ALK2 mutants were constitutively activated BMP receptors and that they cooperatively induced BMP signaling and osteoblastic differentiation with Smad1 and Smad5 (Fukuda *et al.* 2009). At the present time, no treatments are available to prevent heterotopic bone formation in FOP. We found in this study that DRAGON clearly inhibited the activity of ALK2(R206H), a typical mutation in FOP patients. Therefore, DRAGON may provide a novel concept for intracellular signal transduction inhibition in the design of novel drugs for the treatment of FOP.

In conclusion, DRAGON inhibited the intracellular signaling of activated BMP receptors by suppressing the transcriptional activity of Smad in C2C12 cells. DRAGON may bind to a novel molecule(s) expressed on the cell surface to transduce the inhibitory signals of BMPs.

Experimental procedures

Cell culture, transfection, reporter assay and ALP assay

Mouse C2C12 myoblasts and human HepG2 hepatocytes were maintained as described (Katagiri *et al.* 1994; Babitt *et al.* 2006). Myogenic differentiation of C2C12 cells was induced by culturing the cells with differentiation medium (Dulbecco's modified Eagle's medium containing 2.5% fetal bovine serum) for 7 days. Cells were transfected with plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. BMP and TGF-β signaling were monitored using IdWT4F-luc and CAGA-luc reporter plasmids, respectively (Dennler *et al.* 1998; Katagiri *et al.* 2002). ALP activity was measured as described (Kodaira *et al.* 2006). In brief, cells were incubated with a substrate solution (0.1 M diethanolamine, 1 mM $MgCl_2$ and 10 mg/mL of *p*-nitrophenylphosphate). After appropriate incubation, reactions were terminated by adding 3 M NaOH; absorbance was measured at 405 nm.

Reverse transcription-PCR analysis and plasmid construction

Total RNAs were prepared using TRIzol Reagent (Invitrogen) and then reverse transcribed by SuperScript III reverse transcriptase (Invitrogen) in accordance with the manufacturer's instructions. PCR reactions were performed using Go-Taq (Promega, Madison, WI). The primer sets used were previously described (Zhao *et al.* 2006) as follows: 5′-CACGCTGGCTGCCTACTATGCTTTG-3′ (RGMa-U), 5′-TGGGCTGCATCCTGAGGCTGACTTG-3′ (RGMa-L), 5′-ACTCATAGACAACAATTACCTTTCG-3′ (DRAGON-U), 5′-GGGAAGAGTCTCTCACACACACATG (DRAGON-L), 5′-CTCCGGTGACCCCAACTTTACTGTG-3′ (RGMc-U) and 5′-CTGTCCCCGCTGTTTCCTTTGGCAC (RGMc-L). Each cDNA of the RGM family was cloned into a pcDEF3 expression vector and confirmed by DNA sequencing. A series of mutant DRAGON constructs was generated using a standard PCR technique.

Immunoblotting and immunohistochemistry

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 immunoprecipitation and immunoblotting as described previously (Fukuda *et al.* 2009). The following antibodies were used: anti-phosphorylated Smad1/5/8 antibody (Cell Signaling, Beverly, MA), anti-V5 antibody (Invitrogen), anti-FLAG antibody (Sigma, St Louis, MO), anti-Myc antibody and anti-actin antibody (SantaCruz, Santa Cruz, CA). The cells transfected with DRAGON mutants were fixed with formalin and stained with an anti-FLAG antibody and DAPI.

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Tumor Necrosis Factor α Represses Bone Morphogenetic Protein (BMP) Signaling by Interfering with the DNA Binding of Smads through the Activation of NF-κB*

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Bone morphogenetic proteins (BMPs) induce not only bone formation *in vivo* but also osteoblast differentiation of mesenchymal cells *in vitro*. Tumor necrosis factor α (TNFα) inhibits both osteoblast differentiation and bone formation induced by BMPs. However, the molecular mechanisms of these inhibitions remain unknown. In this study, we found that TNFα inhibited the alkaline phosphatase activity and markedly reduced BMP2- and Smad-induced reporter activity in MC3T3-E1 cells. TNFα had no effect on the phosphorylation of Smad1, Smad5, and Smad8 or on the nuclear translocation of the Smad1-Smad4 complex. In p65-deficient mouse embryonic fibroblasts, overexpression of p65, a subunit of NF-κB, inhibited BMP2- and Smad-induced reporter activity in a dose-dependent manner. Furthermore, this p65-mediated inhibition of BMP2- and Smad-responsive promoter activity was restored after inhibition of NF-κB by the overexpression of the dominant negative IκBα. Although TNFα failed to affect receptor-dependent formation of the Smad1-Smad4 complex, p65 associated with the complex. Chromatin immunoprecipitation and electrophoresis mobility shift assays revealed that TNFα suppressed the DNA binding of Smad proteins to the target gene. Importantly, the specific NF-κB inhibitor, BAY11-7082, abolished these phenomena. These results suggest that TNFα inhibits BMP signaling by interfering with the DNA binding of Smads through the activation of NF-κB.

Bone morphogenetic proteins (BMPs)[2] are members of the transforming growth factor β superfamily (TGF-β) that were originally identified by their ability to induce ectopic bone formation when implanted into muscle tissue (1, 2). BMP signaling is transduced by two types of transmembrane serine-threonine kinase receptor, type I and type II (3, 4). After type II receptors phosphorylate type I receptors in a ligand-dependent fashion, activated type I receptors phosphorylate downstream molecules in the cytoplasm. After BMP type I receptors phosphorylate Smad1, Smad5, and Smad8 (Smad1,5,8), the three Smads form heteromeric complexes with Smad4 and other transcription factors. These complexes translocate into the nucleus and activate the transcription of target genes, including Id1, which encodes an inhibitor of myogenesis (5). This unique and specific ability of BMPs should be useful for the development of bone regeneration. However, BMPs cannot generate enough of a clinical response to be used in bone regeneration (6–8). One possible reason might be that inflammatory cytokines inhibit bone formation and osteoblast differentiation induced by BMPs. For example, several lines of evidence have shown that tumor necrosis factor (TNF) α inhibits osteoblast differentiation in multiple models, including fetal calvaria, bone marrow stromal cells, and MC3T3-E1 cells (9–12).

TNFα is a non-glycosylated protein of 17 kDa, composed of 157 amino acids, that acts as a pleiotropic pro-inflammatory cytokine (13, 14). TNFα is produced primarily by activated macrophages but is also produced by a variety of other structural cell types, including fibroblasts, smooth muscle cells, and osteoblasts (13, 14). Biological responses of TNFα are mediated by the specific binding of either a type I or a type II receptor expressed on the surface of one of many cell types. The binding of TNFα to its receptors results in the activation of an inflammatory response that is classically mediated by a wide variety of pro-inflammatory cytokines, including interleukins, interferon-γ, and chemokines (13, 14). In addition, intracellular signal transduction generated by TNFα elicits a wide spectrum of other cellular responses. These responses include the modulation of the differentiation and proliferation of a variety of cell types and the induction of apoptosis via several signaling pathways, such as the meiosis-specific serine/threonine protein kinase (MEK) pathway, the extracellular signal-regulated kinase (ERK) pathway, the c-Jun N-terminal kinase (JNK) pathway, the p38 kinase pathway, and the NF-κB pathway (14).

Signal transduction in the TNFα pathway occurs partly through the activity of the NF-κB family of transcriptional fac-

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[2] The abbreviations used are: BMP, bone morphogenetic protein; TNFα, tumor necrosis factor α; NF-κB, nuclear factor κB; ALP, alkaline phosphatase; EMSA, electrophoretic mobility shift assay; BRE, BMP2-responsive element; MEF, mouse embryonic fibroblast cells.