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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

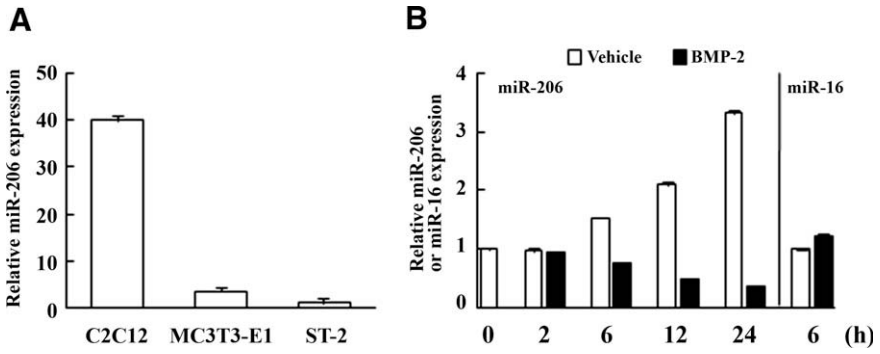

Fig. 1. Expression of miR-206 and the down-regulation of expression by bone morphogenetic protein (BMP)-2. (A) C2C12 cells, MC3T3-E1 cells and ST-2 cells were harvested at confluence, and total RNA was extracted from the cells. The endogenous expression level of miR-206 was analyzed. The expression levels 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.

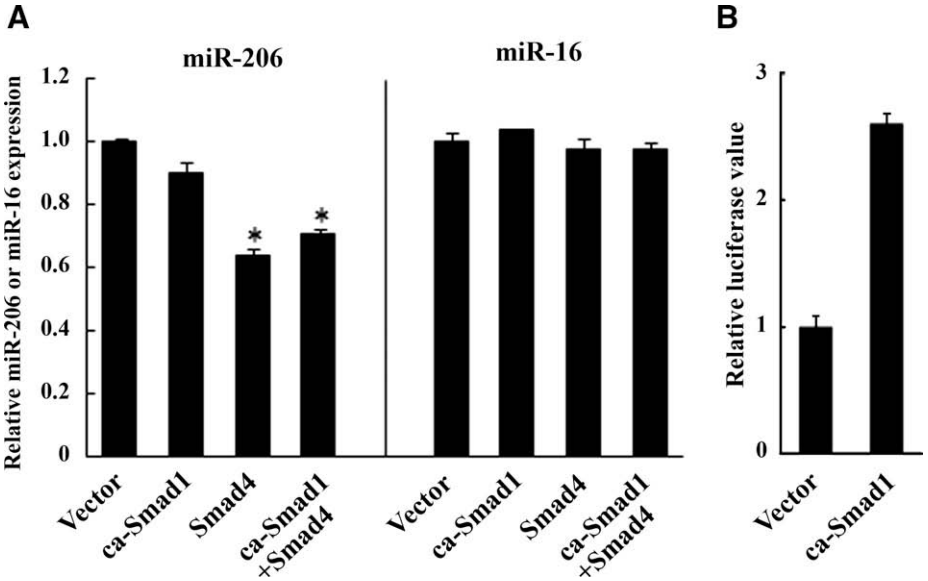

Fig. 2. Regulation of miR-206 expression by constitutively-active Smad1 (ca-Smad1) and Smad4 in C2C12 cells. Cells were plated and cultured with 10% FBS. After an overnight incubation, the cells were transiently transfected with 100 ng/ml of the expression construct for ca-Smad1 or Smad4 or an empty 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-

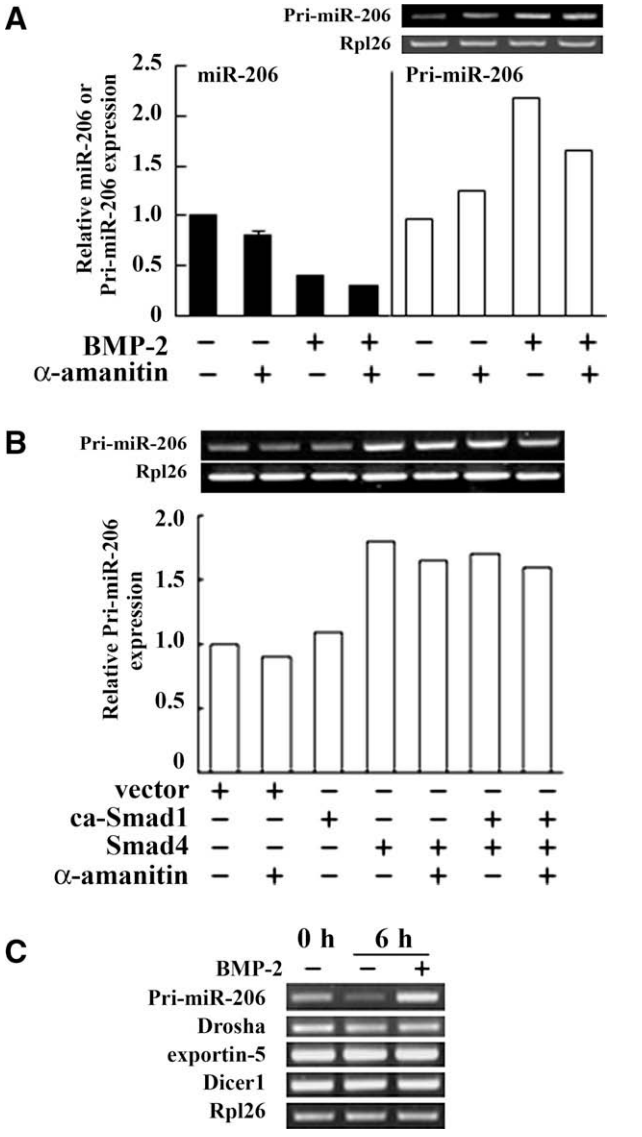

Fig. 3. Regulation of the expression of pri-miR-206 and Drosha, exportin-5 and Dicer1 mRNA by BMP-2. (A and C) C2C12 cells were plated and cultured with 10% FBS. After 24 h, the medium was replaced with fresh medium containing 2.5% FBS and α-amanitin (10 μg/ml) (+), BMP-2 (300 ng/ml) (+) or vehicle (−) 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

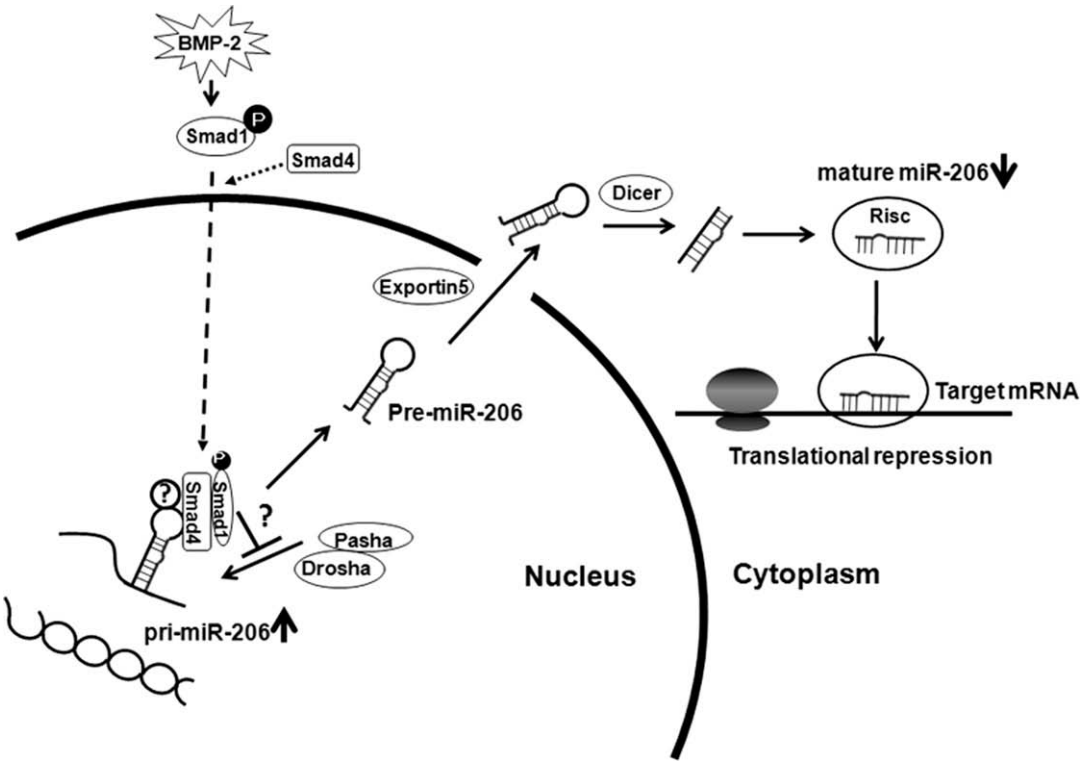

Fig. 4. Schematic model of the hypothetical involvement of BMP-2 and Smad1/4 in miR-206 maturation processes. BMP-2 or Smad1/4 (the intracellular signaling molecules for BMP-2) might inhibit the processing of pri-miR-206 into miR-206. Pri-miR-206 would then accumulate in these cells.

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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