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Endogenous Bmp4 in myoblasts is required for myotube formation in C2C12 cells

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ABSTRACT

Background: Our previous study revealed the indispensable activity of endogenous bone morphogenetic protein (Bmp) prior to differentiation induction of C2C12 myoblasts for myogenesis. Here we investigated the Bmp isoform responsible for endogenous Bmp activity during differentiation and its role in myogenesis.
Methods: Gene expression of *Bmp4* during myogenesis was evaluated in C2C12 cells. Effects of inhibition of the Bmp pathway on myogenesis were examined. Cells expressing *Bmp4* and regulation of *Bmp4* expression in myoblasts were explored.
Results: The expression of *Bmp4* increased with the progression of myogenesis, although the extent of the increase after differentiation induction was smaller than that before the induction. Down-regulation of Bmp signal components including *Bmp4*, *Bmpr2*, and *Alk2/3* inhibited the emergence of positive cells for myosin heavy chain II. The treatments also decreased the Myogenin expression. Treatment with cytosine arabinoside decreased the expression of *Bmp4*. Also, *Bmp4* expression was also lower in isolated myotubes than in residual cells. Expression of *Rgm c* was higher in the myotube fraction. Transcription of *Bmp4* was repressed by the conditioned medium of mixed cells consisting of myoblasts and myotubes.
Conclusion: Bmp4 expressed in myoblasts has a positive role in myotube formation/maturation through myogenin expression. The presence of myotubes inhibits *Bmp4* expression in proliferating myoblasts through transcriptional regulation, although the expression is intrinsically increased with time of culture.
General significance: Taken previous results on involvement of Bmp in the commitment of osteoblasts and adipocytes with the present results together, Bmp may act as a general promoter of mesenchymal cell differentiation.

1. Introduction

Skeletal muscle is a highly specialized tissue composed of postmitotic, multinucleated muscle fibers, which contract to generate force and movement. Skeletal muscle is also critical to maintain metabolic health through glucose uptake and insulin sensitivity [1,2]. In addition, contracting skeletal muscle has been recently suggested to play a role as an endocrine organ producing various cytokines [3–5]. Skeletal muscle formation consists of a complex set of differentiation steps: commitment of mesenchymal stem cells to myoblast lineage cells, progression of differentiation with the expression of muscle-cell-specific proteins, and fusion of myoblasts into multinucleated myotubes. Myogenic differentiation is principally governed by activities of the MyoD family basic helix–loop–helix (bHLH) transcription factors, also known as myogenic regulatory factors (MRFs), i.e., Myod, Myf5, Myogenin and Mrf4. MRFs form a complex with E proteins, another class of bHLH transcription factors, such as E12 and E47, and stimulate the transcription of skeletal muscle-specific genes through binding to E-box (CANNTG) in the regulatory region [6–8]. To accomplish appropriate myogenesis, the activities of MRFs must be strictly regulated.

Bone morphogenetic proteins (Bmps), which were originally isolated as bone-inducing proteins [9], potently regulate various biological processes, including hematopoietic and neuronal development, iron metabolism, and vascular homeostasis [10]. The diverse effects of Bmp are mostly elicited through the phosphorylation and activation of Bmp-regulated Smad (BR-Smad), i.e., Smad1, Smad5 and Smad8, at the carboxyl-terminal serines [10]. Phosphorylated BR-Smad forms complexes with Smad4, which accumulate in the nucleus where they participate in transcriptional regulation of the target genes.

Previous studies revealed that Bmp acted as a negative regulator of myogenesis. Addition of Bmp to the culture medium of myogenic cells led to differentiation into osteoblast lineage cells [11–15]. This transdifferentiation was related to gene induction of the inhibitor of DNA binding 1 (Id1) [11]. Id1 heterodimerized with E proteins, which blocked the formation of active complex consisting of E proteins and Myod [16,17]. In addition, Id1 expression accelerated the degradation of myogenin [18]. As compared with the well-known effects of exogenous Bmp, information on the activity and role of endogenous Bmp is limited.

Recently, we revealed the importance of endogenous Bmp activity in undifferentiated myoblasts for myogenesis in a C2C12 myoblast

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differentiation model [19]; in this model, mononucleated myoblasts are fused to multinucleated myotubes upon reduced serum or serum starvation [20]. Endogenous Bmp activity, which was monitored by the phosphorylation level of BR-Smad, was higher in the stage prior to differentiation induction, and inhibition of the Bmp pathway prior to differentiation induction down-regulated the expression of Myf5 and Myod, leading to impaired myotube formation [19]. The role of endogenous Bmp sharply contrasted to previous knowledge on the action of exogenously treated Bmp for myogenesis [11–15]; however, the level of phosphorylated BR-Smad remained after differentiation induction, although it was lower than before differentiation. This situation encouraged us to explore further the significance of endogenous Bmp activity during myotube differentiation. The present study reveals that *Bmp4* expression in proliferating myoblasts after differentiation induction is required for the progression of myogenesis to maintain *Myogenin* expression, and that the expression is negatively regulated by the presence of myotubes.

2. Materials and methods

2.1. Materials

The following reagents were purchased: recombinant Bmp4 was from R&D Systems (Minneapolis, MN, USA); cytosine arabinoside (cytosine-1-β-D(+)-arabinofuranocide) was from Wako (Tokyo, Japan); rabbit polyclonal antibody against Myogenin (ab11986), rabbit monoclonal antibody against Smad1 (ab33902) and β-actin (AC-15) were from Abcam (Cambridge, MA, USA); mouse monoclonal antibody against myosin heavy chain (MyHC) (MY-32) was from Sigma (St. Louis, MO, USA); rabbit polyclonal antibody against phospho-Smad1 (Ser463/Ser465) / Smad5 (Ser463/Ser465)/ Smad8 (Ser426/Ser428) (#9511) was from Cell Signaling Technology (Danvers, MA, USA); Alexa Fluoro 488 was from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture

C2C12 myoblasts were cultured in growth medium, i.e., Dulbecco's modified Eagle's medium (DMEM) with heat-inactivated 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C under a humidified 5% CO_2 atmosphere. To induce differentiation from myoblasts to myotubes, the medium was replaced at confluence (day 0) with differentiation medium consisting of DMEM with 2% horse serum supplemented the antibiotics. Alternatively, the differentiation was induced by culture with serum-free DMEM supplemented the antibiotics to collect conditioned medium. To examine the effects of removing proliferating myoblasts, cytosine arabinoside was added to the culture medium at the final concentration of 10 μM or 20 μM for days 4 to 7 or days 11 to 14, and cells were recovered on day 7 or day 14, respectively. To isolate myotubes, cells on day 10 were trypsinized for a short time under the microscope until detachment of multinucleated cells (~2 min), followed by centrifugation to obtain a myotube-rich fraction. Collection of conditioned medium was done for days 4–6, days 6–8 and days 8–10. The conditioned medium was concentrated, and the solvent was changed to 20 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM PMSF, 1% aprotinin by means of Centriprep-10 (Millipore, Bedford, MA), and protein concentrations were measured by the bicinchoninic acid method [21].

2.3. qRT-PCR

RNA isolation and qRT-PCR were carried out as previously described [22]. The following oligonucleotides were used as PCR primers: 5′-ACTCCCTTACGTCCATCGTG-3′ and 5′-CAGGACAGCCCCACTTAAAA-3′ for *Myogenin* (Genbank accession number: NM_031189.2), 5′-CAGCTACAAACCCAAGCAAG-3′ and 5′-AGGCATCCACGTTTGCTC-3′ for *Mrf4* (Genbank accession number: NM_008657.2), 5′-GCCTGGGCTTACCTCTCTATCAC-3′ and 5′-CTTCTCAGACTTCCGCAGGAA-3′ for *myosin heavy chain (Myhc) I* (Genbank accession number: BC158018), 5′-CCTGAGCAAGAAGCTGAGGA-3′ and 5′-GGTCATTCCACGCGTACAG-3′ for *Noggin* (Genbank accession number: NM_008711), 5′-ACCGCCCTACACCTAGTCTTC-3′ and 5′-CATACCCATCCATCCAGCTC-3′ for *repulsive guidance molecule (Rgm) a* (Genbank accession number: NM_177740.5), 5′-ACCTTTCGGTTCAAGTGACG-3′ and 5′-TCACAGCTTGGTACACCTTCTG-3′ for *Rgm b* (Genbank accession number: NM_178615.3), and 5′-GGCAATCATGGAGAAAGAGATG-3′ and 5′-TTTCTCTGGGTACTTCTGTGATGT-3′ for *Rgm c* (Genbank accession number: NM_027126.4). The other PCR primers used in this study were previously described [22,23]. The Ct value was determined, and the abundance of gene transcripts was calculated from the Ct value using *Hprt1* as the corrected gene.

2.4. Measurement of DNA content

To examine time-course changes in DNA content during myotube differentiation, recovered cells were resuspended in hypertonic buffer (200 mM phosphate buffer (pH 7.4), 2 M NaCl, 2 mM Na_3VO_4, 1 mM PMSF, 1% aprotinin), followed by lysis by ultrasonication. DNA content was measured by the method of Labarca and Paigen [24].

2.5. Double-stranded RNAi transfection

To target the expression of *Bmp4*, *Bmpr2*, *Alk2* or *Alk3* and green fluorescent protein (GFP) control, double-stranded (ds) RNAi of the respective genes was synthesized by Samchully Pharmaceuticals (Seoul, Korea). The coding sequences to inhibit the expression of *Bmp4*, four oligonucleotides, were designed: 5′-GUUGAAAAAUUAUCAGGAGAU-3′ (set 1), 5′-CUCCUGAUAAUUUUUCAACAC-3′ (set 1), and 5′-CAGACUAGUCCAUCACAAUGU-3′ (set 2), and 5′-AUUGUGAUGGACUAGUCUGGU-3′ (set 2). Two oligonucleotides of each set were mixed, and 1:1 mixtures of dsRNAi were prepared. The other dsRNAi used in this study were described previously [23]. Overall, 60 pmol dsRNAi in a 12-well plate was transfected using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocol.

2.6. Immunofluorescence staining

Immunofluorescence staining for Myhc was performed as described previously [19]. Nuclei were also stained by 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). The number of nuclei was calculated by Image J (http://rsbweb.nih.gov/ij/). Fusion index was calculated as a ratio of the number of nuclei incorporated in myotubes to the number of total nuclei [25].

2.7. Western blotting

To evaluate roles of endogenous Bmp activity in myogenin expression, dsRNAi for Bmp signal components was introduced in cells for 48 h. In addition, BR-Smad phosphorylation was evaluated in cells transfected with dsRNAi for *Bmp4* and treated with recombinant Bmp4. Western blotting was performed as described previously [19].

2.8. Reporter assays

The 5′-flanking regions of *Bmp4* (nucleotides (nt) −2372 to +115, relative to the transcriptional initiation site at +1) was isolated by PCR using mouse genomic DNA. The DNA fragment was cloned into a region upstream of the luciferase gene in pGL4-Basic (Promega, Madison, WI, USA) using the *Bgl* II and *Hind* III sites; it was verified by DNA sequencing. Luciferase-based reporter assays were conducted as described previously [23]. C2C12 myoblasts were seeded on 24-well plates, and transiently transfected with Bmp4-luc by use of Lipofectamine LTX reagent (Invitrogen). A *Renilla reniformis* luciferase vector