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Bmp4 expressed in preadipocytes is required for the onset of adipocyte differentiation

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ABSTRACT

We previously revealed that endogenous bone morphogenetic protein (Bmp) activity is required for lipid accumulation in 3T3-L1 adipocytes. The present study characterized the role of endogenous Bmp activity in preadipocytes. Endogenous Bmp activity was monitored by analyzing the level of phosphorylation of Smad1/5/8, downstream molecules in the Bmp pathway. Higher levels of phosphorylated Smad1/5/8 were detected in adipogenic cells but not in non-adipogenic cells prior to differentiation induction. The inhibition of the Bmp pathway during this period decreased the expression of *Pparγ2* and *C/ebpα*, which are transcription factors responsible for adipocyte differentiation. The expression of these transcription factors were also down-regulated by *Bmp4* knockdown. In addition, endogenous Bmp4 was required for the repression of *Intrleukin-11* expression. Endogenous Bmp4 in preadipocytes is indispensable for the onset of the adipogenic program, and may help to maintain the preadipocytic state during adipocyte differentiation.

1. Introduction

In addition to its role as the major energy-storage tissue, adipose tissue integrates a wide array of homeostatic processes by secreting various cytokines. Because excess and deficient levels body fat are related to various (patho-)physiological conditions, optimal body fat mass is essential for the maintenance of health [1]. The amount of adipose tissue is determined by the size and number of adipocytes. Adipocyte size largely reflects the amount of stored triglycerides, whereas adipocyte number is regulated by the commitment of mesenchymal stem cells in the adipocyte lineage and by the proliferation and differentiation of preadipocytes. Peroxisome proliferator-activated receptor γ (Pparγ, a nuclear hormone receptor, is the dominant regulator for adipocyte differentiation, and various molecules including growth factors and hormones affect adipogenesis through the modulation of Pparγ expression and activity [2].

Bone morphogenetic proteins (Bmps), members of the transforming growth factor-β (Tgf-β) family, regulate diverse physiological processes including adipose tissue formation [3]. The role of Bmps in the commitment of pluripotent stem cells to the adipocyte lineage (adipocyte progenitors) has been well established [4]. The addition of Bmp4 to the culture medium stimulated the adipocyte differentiation of multipotential C3H10T1/2 cells [5,6] and mouse embryonic fibroblasts [7]. A33 cells, a subline of C3H10T1/2 cells treated with the DNA methylation inhibitor 5-azacytidine, were committed to the adipocyte lineage and highly expressed and secreted Bmp4 [8]. In contrast, the roles of Bmps in the differentiation of preadipocytes into adipocytes are controversial; treatment with Bmp2 decreased insulin-induced lipid accumulation in 3T3-F442A preadipocytes [9], whereas Bmp7 stimulated the adipocyte differentiation of 3T3-L1 preadipocytes [10].

3T3-L1 preadipocytes are frequently used as a cell model for adipocyte differentiation. In a standard differentiation program, growth-arrested confluent 3T3-L1 preadipocytes are treated with the appropriate hormonal agents, which induce synchronous reentrance into the cell cycle and undergo at least 2 rounds of mitosis, referred to as mitotic clonal expansion. Subsequently, the preadipocytes exit the cell cycle and begin to differentiate into adipocytes [11]. We previously analyzed endogenous Bmp activity during the adipocyte differentiation of 3T3-L1 activity was higher before differentiation induction, and the inhibition of this activity decreased lipid accumulation [12]. These results suggest a novel activity of Bmp in preadipocytes, but the following points remained to be clarified: (1) the levels of endogenous Bmp activity in the other adipogenic and non-adipogenic cells, (2) the effects of exogenous Bmp after differentiation induction in 3T3-L1 cells, (3) the

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Table 1
Oligonucleotide PCR primers for RT-qPCR.

	Oligonucleotide		GenBank accession number
	5′-primer	3′-primer	
Actr2a	5′-CCCTCCTGTACTTGTTCCTACTCA-3′	5′-GCAATGGCTTCAACCCTAGT-3′	M65287
Actr2b	5′-GCTCAGCTCATGAACGACT-3′	5′-CTCTGCCACGACTGCTTGT-3′	M84120
Alk2	5′-AGGGCTCATCACCACCAAT-3′	5′-GCCACTTCCTGATGTACACG-3′	L15436
Alk3	5′-TGACCTGGGCCTAGCTGTTA-3′	5′-TTCAGGCTTTCATCCAGCA-3′	Z23154
Bmp4	5′-GAGGAGTTTCCATCACGAAGA-3′	5′-GCTCTGCCGAGGAGATCA-3′	NM_007554
Bmpr2	5′-TGGGAGGTGTTTATGAGGTGT-3′	5′-GAAAAGCCATCTGGTAATCTGG-3′	U78048
C/ebpα	5′-CAAGAACAGCAACGAGTACCG-3′	5′-GTCACTGGTCAACTCCAGCAC-3′	NM_007678
Fabp4	5′-AAGGTGAAGAGCATCATAACCCT-3′	5′-TCACGCCTTTCATAACACATTCC-3′	NM_024406
Hprt1	5′-TCCTCCTCAGACCGCTTTT-3′	5′-CCTGGTTCATCATCGCTAATC-3′	NM_013556
Interleukin-11	5′-CGCCGTTTACAGCTCTTGA-3′	5′-CAGGGGGATCACAGGTTG-3′	NM_008350
Lox	5′-CAGGCTGCACAATTTCACC-3′	5′-CAAACACCAGGTACGGCTTT-3′	NM_010728
Pparγ2	5′-TGCTGTTATGGGTGAAACTCTG-3′	5′-CTGTGTCAACCATGGTAATTTCTT-3′	NM_011146
Pref-1	5′-CGGGAAATTCTGCGAAATAG-3′	5′-TGTGCAGGAGCATTCGTACT-3′	NM_010052
Zfp423	5′-CGTGAAGTTCGAGAGTGCTG-3′	5′-GGCACTTGATACACTGGTACGTC-3′	NM_033327

identities of the molecule(s) responsible for the higher Bmp activity prior to differentiation induction in 3T3-L1 cells, and (4) the possible target process(es) involved in the progression of the adipogenic program. In this study, we explored these unknowns to understand the precise role of Bmp during adipogenesis.

2. Materials and methods

2.1. Materials

The following reagents were purchased: dorsomorphin (compound C: 6-[4-(2-piperidin-1-yl-ethoxy)phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a] pyrimidine) was from Calbiochem (La Jolla, CA, USA);LDN-193189 was from Stemgent (San Diego, CA, USA);purified Tgf-β1 was from Becton Dickinson (Franklin Lakes, NJ, USA);recombinant Activin A, Bmp2 and Bmp4 were from R & D Systems (Minneapolis, MN, USA);rabbit polyclonal antibody against phospho-Smad1 (Ser463/Ser465)/Smad5 (Ser463/Ser465)/Smad8 (Ser426/Ser428) (#9511) and rabbit polyclonal antibody against phospho-Smad2 (Ser465/Ser467) were from Cell Signaling Technology (Danvers, MA, USA);and rabbit monoclonal antibody against Smad1 (ab33902), rabbit polyclonal antibody against Smad2 (ab63576), and mouse monoclonal antibody against and β-actin (AC-15) were from Abcam (Cambridge, MA, USA).

2.2. Cell culture

3T3-L1 preadipocytes were cultured and differentiated as described previously [12]. Bovine stromal–vascular (SV) cells were isolated from perirenal adipose tissues as follows. The tissues from 28- to 32-month-old Japanese Black steers were digested by with type I collagenase (1 mg/ml) in Hank's balanced salt solution containing penicillin (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (250 ng/ml) and subsequently filtered through a 250 μm nylon mesh filter to remove undigested tissue fragments and debris. After centrifugation, the cell pellet consisting of SV cells containing preadipocytes was washed with growth medium, i.e., Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, ascorbic acid phosphate magnesium salt (100 μM) and antibiotics, followed by resuspension in the growth medium containing 10% DMSO and storage in liquid nitrogen. The adipocyte differentiation of the SV cells was induced as described previously [13]. Two days after reaching confluence (day 0), cells were cultured in the growth medium in the presence of differentiation inducers (3-isobutyl-1-methylxanthine (0.5 mM), dexamethasone (0.25 μM) and insulin (10 μg/ml)) for 2 days, followed by culture with insulin (5 μg/ml) in the growth medium. To investigate the effects of dorsomorphin and LDN-193189 on adipocyte differentiation, dorsomorphin (10 μM) or LDN-193189 (100 nM) dissolved in DMSO was added to the culture medium for the indicated period; treatments with the concentration of the inhibitors effectively blocked Bmp-induced phosphorylation of Smad1 and transcriptional activation of a Bmp-responsive gene Id1 [14,15]. An equal concentration of DMSO (0.1%) was used as a control. Lipid accumulation was examined by Oil Red O staining on day 8. The images were obtained by scanning stained wells (GT-9400UF;EPSON, Tokyo, Japan). Subsequently, dye was extracted with 2-propanol. The absorbance of the solution was measured at 510 nm for quantification [12].

2.3. Western blotting

To examine changes in phosphorylated Smad1/5/8 expression over time, cells were recovered in 200 mM phosphate buffer, pH 7.4, 2 M NaCl, 2 mM Na3VO4, 1 mM PMSF and 1% aprotinin and then lysed by ultrasonication. DNA content was measured by the method of Labarca and Paigen [16]. Samples containing equal amounts of DNA were subjected to SDS–PAGE, followed by immunoblotting as described previously [17]. The reacted proteins were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare) according to the manufacturer's protocol. After stripping the antibodies and the detection reagents, the membranes were reprobed with anti-Smad1 antibody or anti-β-actin antibody.

To examine effects of protein kinase inhibitors on ligand-induced Smad phosphorylation, cells were cultured in medium with 0.2% FBS for 4 h, treated with vehicle (DMSO), dorsomorphin (5 μM) or LDN-193189 (100 nM) with 0.2% FBS for 20 min, and subsequently stimulated with Tgf-β1 (200 pM), Activin A (4 nM) or Bmp2 (4 nM) in the presence of the kinase inhibitor for 1 h.

2.4. RT-qPCR

RNA isolation and RT-qPCR were performed as described previously [12]. The oligonucleotide primers for RT-qPCR are presented in Table 1. The C_t value was determined, and the abundances of gene transcripts were calculated from the C_t value using *Hprt1* as the normalization gene.

2.5. Double-stranded RNA transfection

Double-stranded RNAs (dsRNAs) targeting the expression of *Bmp4* and *green fluorescent protein* (*GFP*) were synthesized by BONAC corporation (Kurume, Japan). The coding sequence of the