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Identification of FAZF as a Novel BMP2-Induced Transcription Factor During Osteoblastic Differentiation

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Abstract Bone morphogenetic protein 2 (BMP2) is a key factor in the regulation of osteoblastic differentiation; however, its downstream mediators are not fully understood. Previously, we identified and characterized transcription factor promyelocytic leukemia zinc finger protein (PLZF), composed of an N-terminal BTB/POZ and C-terminal zinc finger motifs, as an upstream factor of CBFA1 (Runx2/core-binding factor 1). PLZF was induced in an osteoblastic differentiation medium, but was not induced by BMP2. Here, we report the identification of transcription factor fanconi anemia zinc finger protein (FAZF), which is closely related to PLZF. FAZF was induced by BMP2 in human mesenchymal stem cells (hMSCs). In addition to the full-length FAZF, we also identified alternatively spliced mRNAs in which the C-terminal zinc finger motifs were deleted (designated BTB/POZ-only FAZF). Both the full-length and BTB/POZ-only FAZF mRNAs were equally expressed in BMP2-treated hMSCs. The full-length FAZF was exclusively detected in the nucleus, whereas the BTB/POZ-only FAZF protein was localized in the cytoplasm of the transfected cells. The full-length FAZF, but not the BTB/POZ-only FAZF, increased the expression of osteoblastic differentiation markers, including CBFA1, collagen 1A1, osteocalcin, and alkaline phosphatase in C2C12 cells. In conclusion, both FAZF and PLZF differentially participate in the regulation of osteoblastic differentiation via the BMP2 and CBFA1 signaling pathways, respectively. J. Cell. Biochem. 101: 147–154, 2007.

Key words: FAZF; BMP2; osteoblastic differentiation

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor β (TGFβ) superfamily [see review in Chen et al., 2004]. Signal transduction studies have revealed that Smad1, 5, and 8 are downstream mediators of type I and II BMP receptors and play a critical role in BMP signal transduction. Phosphorylated Smad1, 5, and 8 proteins form a complex with Smad4 and are then translocated into the nucleus. This signaling axis regulates the master regulators of bone cell differentiation, such as CBFA1 (Runx2/core-binding factor 1) and its downstream target osterix [Ducy et al., 1997; Nakashima et al., 2002]. Recently, we characterized the roles of promyelotic leukemia zinc finger (PLZF) in the regulation of osteoblastic differentiation of human mesenchymal stem cells (hMSCs) and C2C12 cells [Ikeda et al., 2005]. In C2C12 cells, the overexpression of PLZF increased the expression of CBFA1. On the other hand, the overexpression of CBFA1 did not affect the expression of PLZF. These findings indicate that PLZF plays important roles in early osteoblastic differentiation as an upstream regulator of CBFA1. Interestingly, PLZF nullizygous mice exhibited a limb development defect involving all proximal cartilage condensations in the hindlimb [Barna et al., 2005].

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Among BMPs, preclinical and clinical studies have shown that BMP2 can be utilized in various therapeutic interventions for bone defects, non-union fractures, spinal fusion, osteoporosis, and root canal surgery as well as for craniofacial and tooth regeneration [Nakashima and Reddi, 2003]. BMP2 signaling is regulated at different molecular levels, but the molecular events downstream of BMP2 signaling that result in tissue specific gene expression and limb or skeletal development have been only partially elucidated [Johnson and Tabin, 1997]. Indeed, the expression of PLZF was unaffected by the addition of BMP2 [Ikeda et al., 2005]. Fanconi anemia zinc finger (FAZF), a member of the BTB/POZ family of transcriptional regulator proteins [Stogios et al., 2005], has the highest known degree of homology to the PLZF protein and has been shown to bind to both PLZF and Fanconi anemia complementation group C (FANCC), the protein that is defective in patients with bone marrow failure syndrome [Hoatlin et al., 1999]. FAZF is expressed at high levels in the early stages of differentiation but declines during subsequent differentiation into erythroid and myeloid lineages, and the enforced expression of FAZF is accompanied by accumulation in the G_1 phase of the cell cycle, followed later by apoptosis [Dai et al., 2002]. These results suggest an essential role for FAZF during the proliferative stages of primitive hematopoietic progenitors; however, its precise roles in other forms of cell differentiation remain unknown.

In the present study, we focused on characterizing the roles of FAZF in BMP2-induced osteoblastic differentiation. The upregulation of FAZF, but not of PLZF mRNA expression, was observed during BMP2-induced osteoblastic differentiation. Moreover, an alternatively spliced form of FAZF, which lacked three C-terminal C2H2 zinc finger motifs, was also induced by BMP2 treatment but did not increase the expression of osteoblastic differentiation markers. These results suggest that FAZF activity may be linked to a transcriptional regulation pathway involved in BMP2-regulated osteoblastic differentiation.

MATERIALS AND METHODS

Plasmid Construction

Full-length (1–487 amino acid residues) and C-terminal zinc finger motifs-deleted FAZF (1–293 amino acid residues) cDNAs were amplified using PCR and the following primers: 5′-cggaattcgccgccatgtccctgccccccataagactgcccagc-3′, 5′-cccaagcttctgttcccgccagacctcctgccaggctcc-3′, and 5′-cccaagcttggtggtggaggaagaa-ggacaacagggaga-3′. Human Universal QUICK-Clone II (Clontech) was used as a cDNA template. The resulting PCR products were digested using *Eco*RI/*Hin*dIII and subcloned into a pCMV-Tag 4A vector (Stratagene), which contains a C-terminal Flag tag; sequencing analyses were then performed.

Cell Culture and Transfections

hMSCs, purchased from BioWhittaker (Walkersville, MD), and mouse pluripotent mesenchymal precursor cells (C2C12) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and an antibiotic/antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B) (Sigma). The osteogenic differentiation (OS) medium has been previously described [Ikeda et al., 2005]. Human recombinant BMP2 was purchased from Sigma. The C2C12 cells were transfected with pCMV-Tag 4A expression vector containing FAZF with lipofectamine 2000. After 48 h, the cells were harvested and subjected to RT-PCR or immunohistochemistry analyses.

RT-PCR

Total cellular RNA was extracted using a Trizol reagent according to the manufacturer's instructions. RT-PCR was performed using a SuperScript One-Step RT-PCR system (Invitrogen) with gene specific primers, according to the manufacturer's instruction. Reactions containing the total RNA (500 ng of each), 0.2 mM of dNTPs, 0.2 μM of each primer, an enzyme mixture composed of SuperScript II reverse transcriptase and Platinum *Taq* DNA polymerase, and a reaction buffer composed of 1.2 mM $MgSO_4$ were incubated at 50°C for 30 min and than at by 94°C for 2 min; PCR was then performed as follows: 30 cycles of 94°C for 15 sec, 55°C for 30 sec, and 70°C for 1 min. The primer sets for the RT-PCR reactions were designed based on human and mouse sequences in GenBank as follows: human FAZF, 5′-acaggcgcacttgcaacctgtg-3′ and 5′-gcatgtgcgcctgcatggaggc-3′; human FAZF (forward primer in exon 2 and reverse primer in exon 5),

5′-cagcctgccctgtggagcatcctgctgatg-3′ and 5′- gcacgcatagggccgagaccgagcaggagg-3′; human PLZF, 5′-tctcaaacgccacctgcgctcacat-3′ and 5′-cactggcagggcgaggcgccgttgt-3′; mouse CBFA1, 5′-acctctgacttctgcctctggc-3′ and 5′-atgctgacga-agtaccatagta-3′; mouse ALP, 5′-accttgactgtggttactgctg-3′ and 5′-gacgccgtgaagcaggtgtgcc-3′; mouse collagen1A1, 5′-cctggtgaatctggacgtgagg-3′ and 5′-gaccagagaagccacgatgacc-3′; mouse osteocalcin, 5′-aggtagtgaacagactccggcg-3′ and 5′-ctggtctgatagctcgtcacaa-3′; human GAPDH, 5′-agaacatcatccctgcctctactgg-3′ and 5′-aaaggtggaggagtgggtgtcgctg-3′; and mouse GAPDH, 5′-ccgcctggagaaacctgccaag-3′ and 5′-ggatagggcct-ctcttgctcag-3′.

Measurement of Alkaline Phosphatase (ALP) Activity

ALP activity was histochemically measured using staining kit No. 85L-3R (Sigma). Briefly, cells were fixed with citrate-buffered acetone for 30 sec followed by washing with deionized water for 45 sec; the cells were then incubated with an alkaline-dye mixture for 30 min at room temperature in the dark.

Immunofluorescent Staining

Cells cultured on cover slips were washed twice with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde for 20 min at room temperature. After washing three times with PBS, the cells were permeabilized with 0.3% Triton-X100 for 10 min at room temperature. After blocking with 3% skim milk in PBS for 30 min, the cells were incubated for 1 h with anti-Flag monoclonal antibody diluted 1:100 in PBS containing 3% skim milk. After washing three times with PBS, the cells were incubated for 20 min with Cy5- or Cy3-conjugated anti-mouse sheep IgG diluted 1:100 in PBS containing 3% skim milk. The cells were then washed three times with PBS, and the immunolocalizations patterns were examined using fluorescence microscopy (Olympus).

RESULTS

Identification of FAZF as a BMP2-Induced, but not an Osteogenic Differentiation (OS) Medium-Induced, Transcription Factor During Osteoblastic Differentiation

Previously, we identified PLZF as an OS medium (0.1 μM dexamethasone, 0.05 mM ascorbic acid-2-phosphate, and 10 mM β-glycerophosphate)-induced transcription factor that was active during osteoblastic differentiation [Ikeda et al., 2005]. We then asked whether FAZF, the most closely related known protein to PLZF [Lin et al., 1999], could be induced during osteoblastic differentiation. To answer this question, we first treated hMSCs with OS medium, since hMSCs differentiate into osteogenic cells in the presence of OS medium [Pittenger et al., 1999]. Treatment of the hMSCs with OS medium for 6 days greatly increased ALP activity (Fig. 1A). Total RNA extracted from the hMSCs on days 0 and 2 of the OS treatment was then subjected to RT-PCR analysis. Consequently, we observed that the mRNA expression of PLZF, but not of FAZF, was upregulated during the early stage of osteogenic differentiation (Fig. 1B).

Next, we tested whether BMP2 could induce FAZF mRNA expression in hMSCs. BMP2 is known to play important roles in bone formation and osteoblastic differentiation [Yamaguchi et al., 1991; Katagiri et al., 1994] and, more specifically, to induce CBFA1 expression [Lee et al., 2000]. Several studies have shown that BMP2 induces hMSCs to differentiate into osteoblastic cells [Katagiri et al., 1994; Lee et al., 2000]. In our assay, ALP activities were enhanced by BMP2 treatment in a

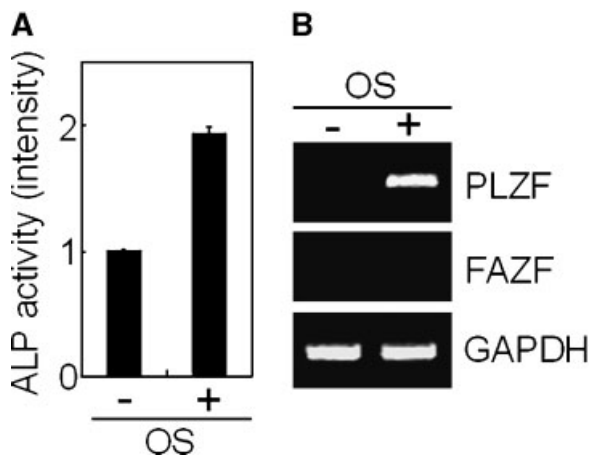

Fig. 1. Expression of PLZF and FAZF during the OS medium-induced osteoblastic differentiation of hMSCs. **A**: ALP activity in hMSCs. Cells were cultured in medium with or without OS for 6 days. ALP staining was performed as described in the Materials and Methods section, and the intensity was determined using an NIH imager. Experiments were performed at least three times; the mean and SD are shown. **B**: Expression of PLZF and FAZF in hMSCs after OS treatment. Cells were cultured for 48 h in the presence or absence of OS. Total RNA was extracted, and RT-PCR was performed using GAPDH as a standard.

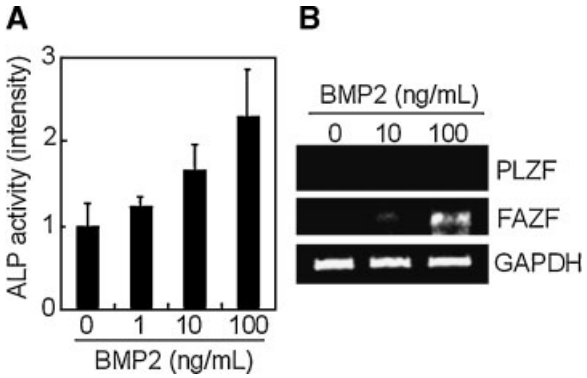

Fig. 2. Expression of PLZF and FAZF during BMP2-induced osteoblastic differentiation of hMSCs. **A**: hMSCs were cultured in the presence or absence of BMP2 (0–100 ng/ml). Six days later, ALP activity was determined by ALP staining, as described in Figure 1A. **B**: hMSCs were cultured in the presence or absence of BMP2 (10 or 100 ng/ml). Total RNA was extracted after 72 h of treatment, and the expression of PLZF, FAZF, and GAPDH mRNA was examined using RT-PCR.

dose-dependent manner (Fig. 2A). Surprisingly, BMP2 enhanced the expression of FAZF, but not of PLZF (Fig. 2B). These results suggest that PLZF and FAZF are differentially involved in the regulation of osteoblastic differentiation.

Full-Length FAZF, but not Zinc Finger Motifs-Deleted (BTB/POZ Domain-Only) FAZF, Increased the Expression of Osteoblastic Differentiation Markers

The predicted full-length FAZF cDNA (RefSeq no. NM_014383) is 1,960 base pairs long and encodes a 487-amino acid protein [Lin et al., 1999]. In the course of constructing an expression vector containing the full-length open reading frame FAZF cDNA, we accidentally identified two mRNA splicing variants (hereafter called variant 1 and 2). Sequencing analyses revealed that the novel cDNAs were derived from alternative splicing, resulting in out-of-frame transcripts (see Fig. 3A and discussion below). As illustrated in Figure 3B, FAZF shows a substantial degree of homology to PLZF throughout the entire region, except for the number of zinc finger motifs located in the C-terminus [Lin et al., 1999]. The full-length FAZF consisted of six exons, and exons 2–6 were translated into wild-type FAZF protein (Fig. 3C). FAZF has two characteristic domains, a BTB/POZ domain encoded by exon 2 and a region containing three zinc finger motifs encoded by exon 6. The BTB/POZ domain acts as a specific protein-protein interaction domain [Stogios et al., 2005]. On the other hand, the C-terminal PLZF-like C2H2 zinc fingers are known to bind to the TGTACAGTGT motif located at the upstream flanking sequence of the Aie1 gene [Tang et al., 2001]. An earlier report identified an alternatively spliced isoform that lacked exon 4 but transcribed the in-frame regions between exon $1+2+3$ and exon $5+6$, resulting in a 474-amino-acid that was virtually the same as the wild-type FAZF protein [Lin et al., 1999]. We called this protein wild-type 2, and the full-length FAZF protein will hereafter be referred to as wild-type 1 (Fig. 3C). On the other hand, exon 3 of variant 1 and exons $3+4$ of variant 2 are alternatively spliced-out (not transcribed into mRNAs), resulting in the appearance of stop codons in exons 4 and 5, respectively. The resulting proteins are 311- and 302-amino acids for variants 1 and 2, respectively. These variants lack the C-terminal zinc finger motifs but have a distinct C-terminal tail (Fig. 3A).

To substantiate the expression patterns of full-length and BTB/POZ-only FAZF during osteoblastic differentiation, we performed an RT-PCR analysis using mRNA from hMSCs treated with BMP2 (100 ng/ml). For this purpose, primers for exons 2 and 5 were designed (see Fig. 3C). This enabled us to distinguish mRNA species; namely, PCR amplification resulted in a 351-bp product for the full-length mRNA (wild-type 1, exon $2+3+4+5$), a 282-bp product for the wild-type 2 mRNA (exon $2+3+5$), a 277-bp product for variant 1 (exon $2+4+5$), and a 208-bp product for variant 2 (exon $2+5$). After three days of incubation with BMP2, two major bands smaller than 350-bp were equally amplified (Fig. 3D). Sequencing analyses showed that the faster and slower migrating bands corresponded to variant 2 (208-bp) and wild-type 2 (282-bp)/variant 1 (277-bp), respectively. These results suggested that BMP2 induced both wild-type FAZF and BTB/POZ-only FAZF during the BMP2-induced osteoblastic differentiation of hMSCs.

To determine the cellular localization of full-length and BTB/POZ-only FAZF, we transfected C2C12 cells with a control vector or an expression vector encoding full-length or BTB/POZ-only FAZF cDNA. After 48 h, the cells were fixed and visualized using fluorescence microscopy. Full-length FAZF was exclusively detected in the nucleus, whereas BTB/POZ-only FAZF protein was localized in the cytoplasm of

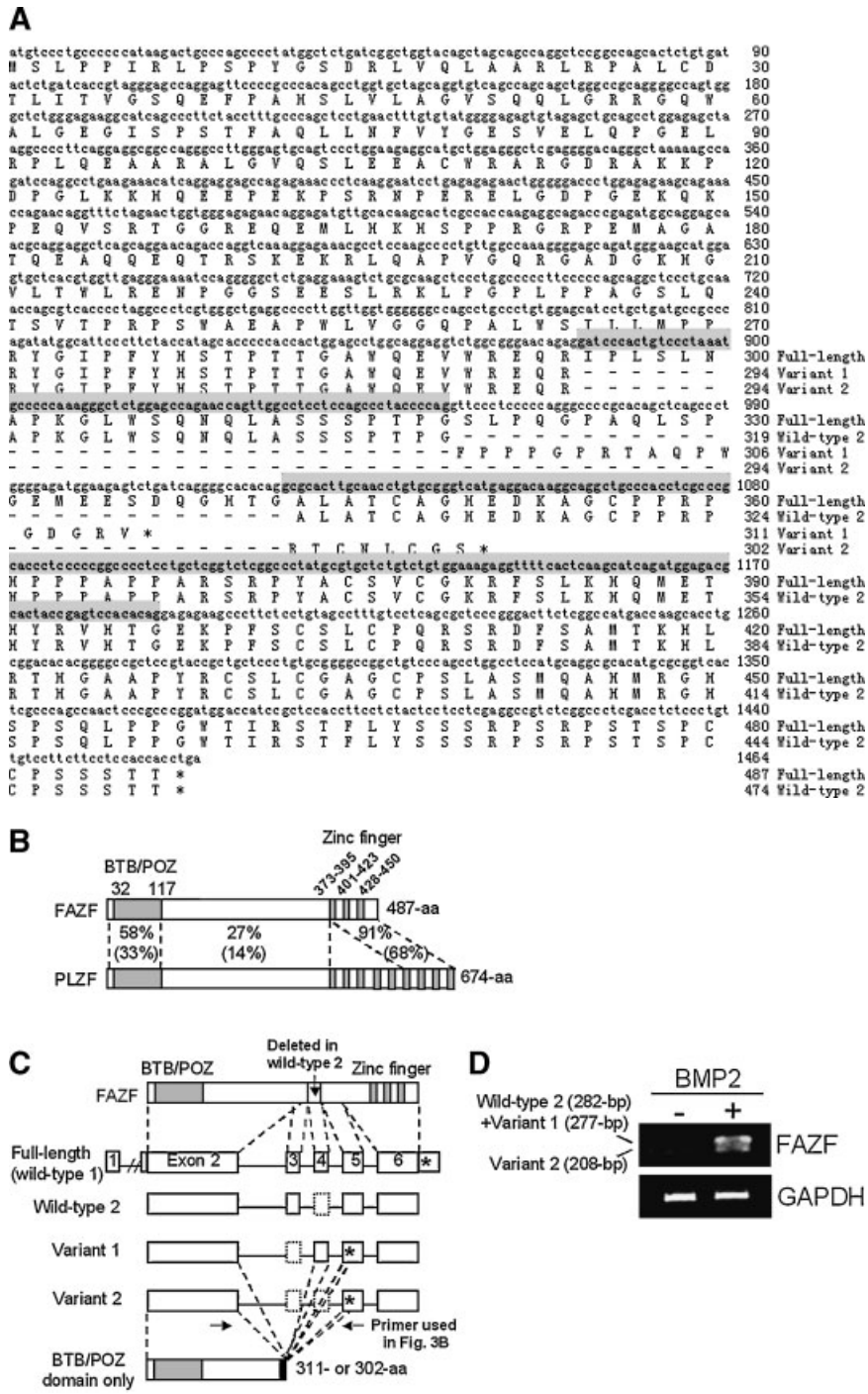

Fig. 3. Endogenous expression of full-length (wild-type) and C-terminal zinc finger motifs-deleted type (BTB/POZ-only) FAZF. **A**: Nucleotide sequences and the deduced amino acid sequences of human FAZF cDNA. The open reading frame (ORF) of full-length (wild-type 1), wild-type 2, variant 1 and 2 are shown in the one-letter amino acid code and aligned. To distinguish each exon (exons 2–6), the nucleotide sequences of exon 3 and 5 are indicated by the shaded boxes. Exon 1 is not included in the ORF. The asterisk indicates the stop codon. Nucleotide and amino acids numbers are shown on the right. **B**: Schematics of FAZF and PLZF proteins. The N-terminal BTB/POZ domain and the C-terminal three zinc finger motifs region (indicated by the gray box) are indicated. Amino acid homology (%) is shown for the different regions. The parentheses indicate amino acids similarities (%). **C**: Exon/intron structures and various isoforms of FAZF. Wild-type FAZF contains an N-terminal BTB/POZ domain and a C-terminal region containing three zinc finger motifs (indicated by the gray box). Exon 4 is deleted in-frame in FAZF wild-type 2. The full-length FAZF cDNA encodes a 487-amino acid protein encoded by exons 2–6. The BTB/POZ domain only FAZF proteins derived from variants 1 and 2, which are 311- and 302-amino acids, respectively, have different C-terminal tails (indicated by the black box). Variants 1 and 2 are shown where the boxes formed from dotted lines are spliced-out. The stop codon is indicated by an asterisk. The arrows indicate the position of RT-PCR primers used in Figure 3B. **D**: hMSCs were cultured in the presence or absence of 100 ng/ml of BMP2. The total RNA was extracted after 72 h of BMP2 treatment, and the expression patterns of wild-type or variant FAZF were verified by RT-PCR. PCR products were separated on 1.5% agarose gels and visualized using EtBr staining. The faster and slower migrating bands corresponded to variant 2 (208-bp) and wild-type 2 (282-bp)/variant 1 (277-bp), respectively. The GAPDH bands are shown as the loading control.

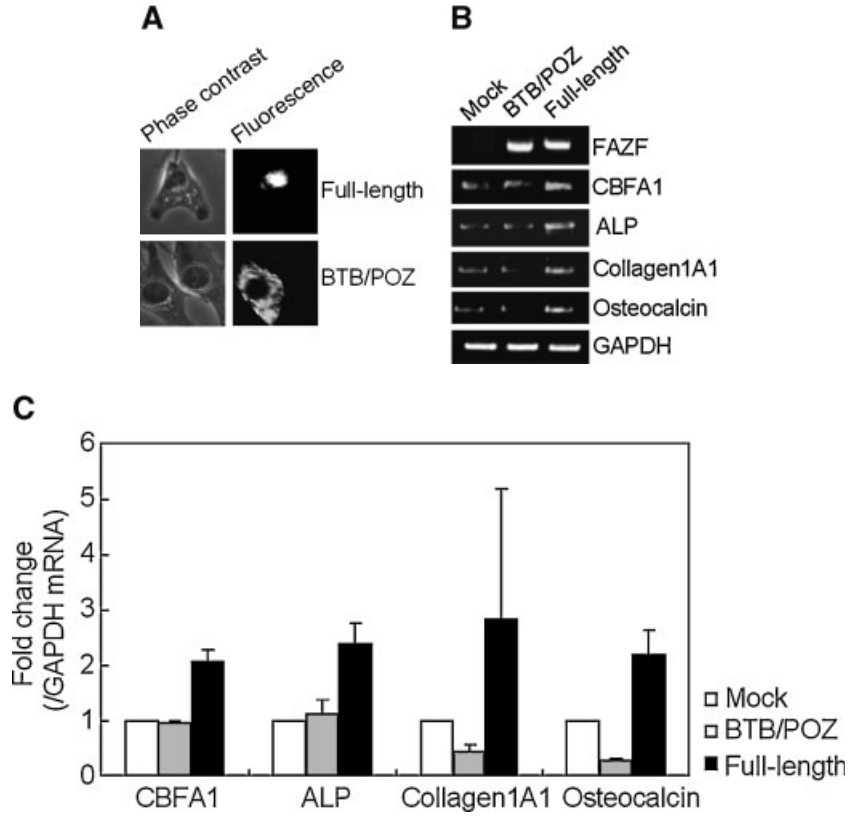

Fig. 4. BTB/POZ-only FAZF is distinct from full-length FAZF. **A**: Subcellular localization of FAZF. Full-length and BTB/POZ-only FAZF were transiently expressed in C2C12 cells, and the cells were immunostained. **B**: FAZF increased the expression of osteoblastic differentiation marker genes. C2C12 cells were transfected with a control vector (Mock) or an expression vector bearing full-length or BTB/POZ-only FAZF. After 48 h, RT-PCR was performed for the FAZF, CBFA1, ALP, collagen1A1, osteocalcin, and GAPDH mRNAs. **C**: Histogram analysis of expression changes of osteoblastic differentiation marker genes. The staining intensities were quantified using NIH image (http://www.scioncorp.com). Values represent the average of three independent experiments and are represented as relative-fold activation as compared to that of mock transfection taken as 1. Bars indicate the SD.

the transfected cells (Fig. 4A). To further elucidate the functional role of full-length and BTB/POZ-only FAZF during osteoblastic differentiation, C2C12 cells, a well-established mouse cell line capable of osteoblastic differentiation, were transfected with an expression vector bearing either the full-length or BTB/POZ-only FAZF cDNA, followed by RT-PCR. As shown in Figure 4B, the over-expression of full-length FAZF resulted in the elevated expression of CBFA1, ALP, collagen1A1, and osteocalcin, whereas BTB/POZ-only FAZF failed to induce the expression of osteoblastic differentiation markers. The GAPDH mRNA level was constant in each lane (Fig. 4B).

DISCUSSION

FAZF encodes a 487-amino acid protein containing a conserved N-terminal BTB/POZ interacting domain and three C-terminal Kruppel-like zinc-finger motifs [Hoatlin et al., 1999]. FAZF has been characterized as a transcriptional regulator that is important for the regulation of development, tissue-specific proliferation, and differentiation [Hoatlin et al., 1999; Dai et al., 2002]. In particular, FAZF has a high degree of similarity to PLZF, which was originally recognized as the fusion partner of the retinoic acid receptor α gene on chromosome 17 in a Chinese patient with acute promyelocytic leukemia and a translocation at t(11;17)(q23;21) [Chen et al., 1993]. More recently, PLZF was found to function upstream of CBFA1 in the context of osteoblastic differentiation [Ikeda et al., 2005]. In addition, PLZF nullizygous mice showed that PLZF is essential for axial skeleton patterning and normal limb development [Barna et al., 2005]. Because so little is known about the functions of FAZF, the

current work focused on the involvement of FAZF in pathways reminiscent of PLZF's unique effect on osteoblastic differentiation.

The present study revealed that FAZF is a BMP2-induced but not dexamethasone/ascorbic acid-2-phosphate/β-glycerophosphate (OS)-induced transcription factor during osteoblastic differentiation. During the osteogenic process, various extrinsic factors such as dexamethasone and cytokines regulate osteoblastic differentiation. Our results clearly suggest that the function of FAZF could be linked to a transcriptional regulation pathway involved in BMP2-regulated osteoblastic differentiation. In addition to PLZF, we demonstrated that FAZF is capable of inducing markers of gene expression that are associated with osteoblastic differentiation. Interestingly, the expression of FAZF, but not of PLZF, was increased by the addition of BMP2 to the culture medium of hMSCs. Conversely, an OS medium upregulated the PLZF, but not FAZF, mRNA expression in hMSCs. These results indicate that the differential involvement of PLZF and FAZF in the regulation of osteoblastic differentiation. From our results, distinct signaling pathways regulated by these two transcription factors during osteoblastic differentiation could be partially linked by CBFA1, and resulted in inducing the expression of osteoblastic differentiation marker genes.

We identified alternative splicing variants of FAZF that lacked the C-terminal zinc finger motifs. These types of transcripts were found to exert a dominant negative effect because the forced expression of C-terminal truncated BTB/POZ-only FAZF failed to induce the expression of marker genes of osteoblastic differentiation, including CBFA1, ALP, collagen 1A1, and osteocalcin in C2C12 cells. An immunofluoresence analysis of FAZF expression in this study indicated that full-length FAZF was expressed in the nuclear compartment, as previously reported [Hoatlin et al., 1999]; however, BTB/POZ-only FAZF was detected in the cytoplasmic compartment, suggesting that this form could associate with full-length FAZF or other BTB/POZ-containing proteins as a heterodimer and inhibit them from being tethered into the nucleus, where they normally function.

The diversity of FAZF proteins resulting from alternative splicing can be explained by the relatively short introns in the FAZF gene. For example, intron 1 is 1,489-bp, intron 2 is 249-bp, intron 3 is 108-bp, intron 4 is 125-bp, and intron 5 is 180-bp. More interestingly, the FAZF gene is closely linked to the MLL4 gene on 19q13.1, and this is reminiscent of the fact that the PLZF and MLL genes have both been mapped to 11q23 [Baysal et al., 1997]. Therefore, the two paralogous BTB/POZ domains and zinc finger motif-containing sequences may have arisen from a syntenic duplication during evolution [Zhang et al., 1999], suggesting that the two proteins not only have unique functional roles, but also share fundamental roles in biological processes, such as the regulation of gene expression.

FAZF mRNA and protein are detected in primary hematopoietic $CD34^+$ progenitor cells and increase during early proliferation; they are then downregulated during terminal differentiation in both erythroid and myeloid lineages [Dai et al., 2002]. These results, together with the present findings, suggest that FAZF influences the proliferation/differentiation status of specific cells. In conclusion, we showed that FAZF was upregulated in response to BMP2 in hMSCs and that it plays an important role in the upregulation of osteoblastic differentiation markers. BTB/POZ-only FAZF failed to induce osteoblastic differentiation markers. The biological impacts of FAZF in the context of osteoblastic differentiation should be addressed in the near future by means of RNA interference or DNA microarray-based global gene expression analyses.

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