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CCAAT/Enhancer Binding Protein Homologous Protein (DDIT3) Induces Osteoblastic Cell Differentiation

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CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP/DDIT3), a member of the C/EBP family of transcription factors, plays a role in cell survival and differentiation. CHOP/DDIT3 binds to C/EBPs to form heterodimers that do not bind to consensus *Cebp* sequences, acting as a dominant-negative inhibitor. CHOP/DDIT3 blocks adipogenesis, and we postulated it could induce osteoblastogenesis. We investigated the effects of constitutive CHOP/DDIT3 overexpression in murine ST-2 stromal cells transduced with retroviral vectors. ST-2 cells differentiated toward osteoblasts, and CHOP/DDIT3 accelerated and enhanced the appearance of mineralized nodules, and the expression of osteocalcin and alkaline phosphatase mRNAs, particularly in the presence of bone morphogenetic protein-2. CHOP/DDIT3 overexpression opposed adipogenesis, and did not cause substantial changes in cell number. CHOP/DDIT3 overexpression did not modify C/EBPα or -β mRNA levels but decreased C/EBPδ after 24 d of culture. Electrophoretic mobility shift and supershift assays demonstrated that overexpression of CHOP/DDIT3 decreased the binding of C/EBPs to their consensus sequence by interacting with C/EBPα and -β, confirming its dominant-negative role. In addition, CHOP/DDIT3 enhanced bone morphogenetic protein-2/Smad signaling. In conclusion, CHOP/DDIT3 enhances osteoblastic differentiation of stromal cells, in part by interacting with C/EBPα and -β and also by enhancing Smad signaling. (*Endocrinology* 145: 1952–1960, 2004)

CCAAT/ENHANCER BINDING PROTEIN (C/EBP)-ζ also known as C/EBP homologous protein (CHOP), growth arrest and DNA damage-inducible gene 153, and DNA damage inducible transcript 3 (DDIT3) is a member of the C/EBP family of transcription factors and plays a role in cell proliferation and differentiation (1–4). Six members of the C/EBP family have been characterized: α, β, δ, γ, ϵ, and ζ (5, 6). C/EBP proteins contain a highly conserved DNA-binding domain and a leucine dimerization domain and can form homo- and heterodimers that bind to similar sequence motifs. C/EBPs act as regulators of gene expression by either direct DNA binding or interacting with other transcriptional activators, including peroxisome proliferator-activated receptor (PPAR)-γ2 and runt-related transcription factor-2/core binding factor-1 (7, 8). CHOP/DDIT3 heterodimerizes with other C/EBPs, but the presence of two proline residues in the DNA-binding region disrupts its helical structure and prevents dimer binding to classic *Cebp* consensus DNA sequences (8). For the most part, CHOP/DDIT3 binds to other C/EBPs, forming heterodimers and serving as a dominant-negative inhibitor (9). However, CHOP/DDIT3 heterodimers can bind to other DNA sequences and also associate to activator protein (AP)-1 nuclear protein complexes by a tethering mechanism, resulting in modulation of their transcriptional activity (10).

Bone marrow stroma contain pluripotential cells with the potential to differentiate into various cells of the mesenchymal lineage including osteoblasts and adipocytes (11, 12). The ultimate cellular phenotype depends on extracellular and intracellular signals. C/EBPs are critical for adipocyte differentiation and maturation (13–16). In 3T3-L1 preadipocytes, C/EBPβ and-δ induce C/EBPα, resulting in transcriptional activation of PPARγ2 and adipocyte maturation (17–20). Recently we demonstrated differential expression of C/EBP mRNA levels during stromal cell differentiation and the enhanced expression of C/EBPβ and -δ by cortisol, a glucocorticoid known to induce adipogenesis and suppress osteoblastogenesis (21, 22). We also demonstrated that the inhibitory effects of cortisol on the transcription of IGF 1 were regulated by C/EBPs (22). In ST-2 stromal cells, CHOP/DDIT3 transcripts accumulate as the cells differentiate and express the osteoblastic phenotype and are higher in cells exposed to bone morphogenetic protein (BMP)-2, a factor that induces their differentiation toward the osteoblastic pathway (21, 23). These observations would suggest that, whereas C/EBPα, -β, and -δ are essential for adipogenesis, CHOP/DDIT3 could play a role in osteoblastogenesis either directly or, by binding adipogenic C/EBPs, indirectly favoring the osteoblastic differentiation pathway.

The intent of this study was to investigate the effects of CHOP/DDIT3 on stromal cell differentiation and function. For this purpose, we used replication incompetent retrovirus to create ST-2 stromal cell lines overexpressing CHOP/DDIT3 under the control of a constitutively active promoter and determined their phenotype during differentiation, using culture conditions that would favor osteoblastogenesis.

Abbreviations: AP, Activator protein; APA, alkaline phosphatase activity; BMP, bone morphogenetic protein; C/EBP, CCAAT/enhancer binding protein; CHOP, C/EBP homologous protein; DDIT3, DNA damage inducible transcript 3; FBS, fetal bovine serum; PPAR, peroxisome proliferator-activated receptor; Smad, phosphorylated mothers against decapentaplegic.

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Materials and Methods

Retroviral vectors and packaging cell lines

A 605-bp DNA fragment containing the coding region of the murine *Chop/Ddit3* gene, with *myc* cDNA sequences cloned in frame at the 5′ terminus and inserted into the retroviral vector pSRa, was kindly provided by D. Ron (New York University School of Medicine, New York, NY) (4). The vector contains a Moloney murine leukemia virus 5′ long-terminal repeat to drive the packaging signal and the constitutive expression of CHOP/DDIT3, and an simian virus 40 promoter to drive a neomycin resistance gene for positive selection. PT 67 packaging cells (Clontech, Palo Alto, CA) were grown to 70–80% density in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA), and transfected with pSRa and pSRa *Chop/Ddit3* using Transfast reagent (Promega, Madison, WI), in accordance with the manufacturer's instructions. After transfection, cells were selected for neomycin resistance, and the retrovirus-containing conditioned medium was harvested, filtered through a 0.45-μm membrane, and used for the transduction of ST-2 cells.

ST-2 stromal cell cultures

ST-2 cells, cloned stromal cells isolated from bone marrow of BC8 mice, kindly provided by S. Harris (University of Missouri at Kansas City, Kansas City, KS), were grown in a humidified 5% CO_2 incubator at 37 C in α-MEM (Invitrogen), supplemented with 10% FBS (Atlanta Biologicals) (24, 25). ST-2 cells were transduced with pSRa vector or pSRa *Chop/Ddit3* by replacing the culture medium with retrovirus containing medium from the transfected PT 67 packaging cells in the presence of 8 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO), and incubation for 16–18 h at 37 C. The culture medium was replaced with fresh α-MEM medium and cells were grown, trypsinized, replated, and selected for neomycin resistance. Two distinct ST-2 cell lines overexpressing CHOP/DDIT3 were created.

To analyze the phenotypic impact of CHOP/DDIT3, untransfected ST-2 cells and cells transduced with pSRa vector or pSRa *Chop/Ddit3* were plated at a density of 10^4 cells/cm^2 and cultured in α-MEM supplemented with 10% FBS until reaching confluence (2–3 d). At confluence (experimental d 0), ST-2 cells were transferred to α-MEM containing 100 μg/ml ascorbic acid, 5 mM β-glycerophosphate (Sigma), and 10% FBS (Atlanta Biologicals or Hyclone, Logan, UT) and cultured for an additional period of 3–24 d. Serum lots were selected in preliminary experiments for their properties to favor the differentiation of ST-2 and primary stromal cells toward the osteoblastic phenotype. Cells were cultured in the presence or absence of recombinant human BMP-2 (a gift from Genetics Institute, Cambridge, MA) or cortisol at 1 μM (Sigma). To analyze the impact of CHOP/DDIT3 on the adipogenic potential of ST-2 stromal cells, vector and *Chop/Ddit3* transduced cells were grown to confluence and transferred to αMEM containing an adipogenic cocktail consisting of dexamethasone 1 μM, insulin 100 nM, and 3-isobutyl-1-methylxanthine 0.5 mM (Sigma) and cultured for 3–24 d in the presence of ascorbic acid and in the absence of β-glycerophosphate (4). In all experiments, cells were refed with fresh medium containing control or test solutions every 3–4 d.

Cytochemical analysis and alkaline phosphatase activity

To estimate changes in culture mineralization, ST-2 cells were washed with PBS, fixed with 3.7% formaldehyde, and stained with 2% alizarin red (Sigma) (21, 26), and the number of mineralized nodules/78.5 mm^2 area of the culture well were counted. To estimate the levels of cellular fat, ST-2 cells were air dried for 1 h and stained with 0.5% Oil Red O in 60% isopropanol (Sigma) for 30–60 min (21, 27). To quantify changes in cellular fat, cells were extracted with 100% isopropanol, and the amount of Oil Red O incorporated was estimated colorimetrically at an absorbance of 525 nm. To assess morphological changes associated with apoptosis, cells were fixed with 2% glutaraldehyde and stained with acridine orange at 4 μg/ml (both from Sigma). Cells were examined by fluorescence microscopy, using 510/540-nm excitation filters, for nuclear condensation and fragmentation (28, 29). The number of apoptotic cells/field was counted in 30 fields/well at a ×300 magnification and averaged. Alkaline phosphatase activity (APA) was determined in 0.5% Triton X-100 cell extracts by hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol and measured by spectroscopy at 410 nm after 15–30 min of incubation according to manufacturer's instructions (Sigma). Data are expressed as picomoles of *p*-nitrophenol released per minute per microgram protein. Total protein content was determined in cell extracts by the DC protein assay in accordance with manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA).

Cellular DNA and cell number

To estimate DNA content, the CyQuant cell proliferator assay kit (Molecular Probes, Eugene, OR) was used in trypsynized ST-2 cells in accordance with the manufacturer's instructions. Total Cellular DNA was estimated by fluorometry at 490/520 nm and comparison with a DNA standard curve. Data are expressed in nanograms of DNA/culture well. To estimate the number of viable cells, mitochondrial dehydrogenase activity was estimated using the CellTiter 96 AQueous One cell proliferation assay (Promega, Madison, WI) in accordance with the manufacturer's instructions. Metabolically active cells were estimated by their ability to reduce the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5(-3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) to a formazan product, which was measured at an absorbance of 490 nm. Data are expressed in arbitrary units of absorbance at 490 nm.

Northern blot analysis

Total cellular RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) per the manufacturer's instructions. RNA was quantitated by spectrometry, and equal amounts of RNA were loaded on a formaldehyde agarose gel after denaturation. The gel was stained with ethidium bromide to visualize ribosomal RNA and confirm equal RNA loading of the experimental samples. The RNA was blotted onto GeneScreen Plus charged nylon (PerkinElmer, Norwalk, CT) and uniformity of transfer confirmed by revisualization of ethidium bromide-stained ribosomal RNA. A 600-bp murine *Chop/Ddit3* cDNA (provided by D. Ron), a 1.8-kb rat *Cebpa* cDNA, 1.5-kb rat *Cebpb* cDNA, and 1.0-kb rat *Cebpd* (all three provided by S. L. McKnight, Tularik, Inc., San Francisco, CA), a 2.5-kb rat alkaline phosphatase cDNA (Merck & Co., West Point, PA), 500-bp rat osteocalcin genomic DNA (J. Lian, University of Massachusetts School of Medicine, Worcester, MA), 900-bp human *Pparg2* cDNA, 800-bp murine adipsin cDNA, and 752-bp murine 18S ribosomal RNA (all three from American Type Culture Collection, Manassas, VA) were purified by agarose gel electrophoresis (2, 4, 30, 31). DNAs were labeled with [α-^{32}P]-deoxy-CTP (50 μCi at a specific activity of 3000 Ci/mmol; PerkinElmer) using Ready-To-Go DNA labeling beads ([α-^{32}P]-deoxy-CTP) kit (Amersham Pharmacia Biotech, Piscataway, NJ) in accordance with the manufacturer's instructions. Hybridizations were carried out at 42 C for 16–72 h, followed by two posthybridization washes at room temperature for 15 min in 1× saline sodium citrate, and a wash at 65 C for 20–30 min in 0.5× or 1× saline sodium citrate. The bound radioactive material was visualized by autoradiography on Kodak X-AR5 film (Eastman Kodak, Rochester, NY), employing Cronex Lightning Plus (PerkinElmer) or Biomax MS (Eastman Kodak) intensifying screens. Relative hybridization levels were determined by densitometry. Northern analyses shown are representative of three to five samples.

Western immunoblot analysis

To determine the expression of CHOP/DDIT3 or phosphorylated mothers against decapentaplegic (Smad) 1/5/8, transduced ST-2 stromal cells were washed with PBS. For CHOP/DDIT3 determinations, cells were suspended in 10 mM HEPES/KOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$ 0.5 mM dithiothreitol buffer, allowed to swell on ice for 15 min, and lysed with 10% Nonidet P-40 (Sigma) (32). CHOP/DDIT3 was determined in the nuclear pellet obtained by centrifugation. The pellet was resuspended in HEPES/KOH buffer in the presence of protease inhibitors at 4 C, incubated for 30 min and centrifuged, and the supernatant was stored at −70 C. For Smad determinations, cells were suspended in TNE lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1% P40, and 1 mM EDTA] in the presence of protease and phosphatase inhibitors as described (33, 34). Phosphorylated Smads were determined in the total cellular extract. Protein concentrations were determined by DC

protein assay, and 30 μg of nuclear protein for CHOP/DDIT3 or 100 μg of total cellular protein for phosphorylated Smads were resuspended in Laemmli sample buffer and fractionated by gel electrophoresis in 12% polyacrylamide gels under reducing conditions (35). Proteins were transferred to Immobilon P membranes (Millipore, Bedford, MA) and blocked with 3% BSA. To determine CHOP/DDIT3 expression, membranes were exposed to a murine monoclonal antibody raised against CHOP/DDIT3 or Myc (both from Santa Cruz Biotechnology, Santa Cruz, CA) in 1% BSA overnight. To determine phosphorylated Smads, membranes were exposed to a 1:1000 dilution of a polyclonal antibody, raised against the synthetic sequence KKKNPISSVS, which recognizes Smad 1, 5, and 8 phosphorylated at the last two serine residues [provided by C. H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) and P. ten Dijke (The Netherlands Cancer Institute, Amsterdam, The Netherlands)] (33, 34). Blots were exposed to goat antimouse or antirabbit IgG antiserum conjugated to horseradish peroxidase and developed with a chemiluminescence detection reagent (PerkinElmer).

EMSA

For gel shift assays, nuclear extracts from ST-2 cells transduced with vector or *Chop/Ddit3* were prepared as described for Western blots. Synthetic oligonucleotides containing a consensus *Cebp* binding sequence (5′-TGCAGATTGCGCAATCTGCA-3′) and its mutant (5′-TGCAGACTAGTCTCTGCA-3′), in which the mutated bases are underlined, were obtained from Santa Cruz Biotechnology. Synthetic double-stranded oligonucleotides were labeled with [γ-^{32}]-ATP using T_4 polynucleotide kinase. Nuclear extracts and labeled oligonucleotides were incubated for 20 min at room temperature in 10 mM Tris buffer (pH 7.5) containing 1 μg of poly (dI-dC) (22, 36). The specificity of binding was determined by the addition of homologous or mutated unlabeled synthetic oligonucleotides in 100-fold excess. DNA-protein complexes were resolved on nondenaturing, nonreducing 4 or 7% polyacrylamide gels, and the complexes were visualized by autoradiography. For gel supershift assays, labeled oligonucleotides were incubated with nuclear extracts and polyclonal antibodies to C/EBPα, -β, and -δ (all from Santa Cruz Biotechnology) for 20 min at room temperature and overnight at 4 C before electrophoretic separation (22).

Transient transfections

To determine changes in BMP-2 signaling, a construct containing 12 copies of a Smad 1 response element, linked to the osteocalcin basal promoter, and cloned upstream of a luciferase reporter gene (12× SBE-OC-pGL3, provided by M. Zhang, University of Texas Health Sciences Center, San Antonio, TX) was tested in transient transfection experiments (37). To determine changes in β-catenin transactivating activity, a pTOP-FLASH reporter construct containing three copies of the lymphoid enhancer binding factor 1/T-cell transcription factor 4 (Tcf-4) binding sequences, CCTTTGATC, or its mutant, pFOP-FLASH, cloned upstream of a minimal c-*fos* promoter and a luciferase-encoding gene (provided by J. Kitajewski, Columbia University, New York, NY) were tested in transient transfections (38). ST-2 stromal cells were cultured to 70% confluence and transiently transfected with the indicated constructs using FuGene 6 (3 μl FuGene/2 μg DNA) according to the manufacturer's instructions (Roche, Indianapolis, IN). Cotransfections with a construct containing the CMV promoter directing the expression of the β-galactosidase gene (Clontech) were used to control for transfection efficiency. Cells were exposed to the FuGene-DNA mix for 16 h, transferred to serum-containing medium, then either harvested in a reporter lysis buffer (Promega) 48 h later (for pTOP-FLASH and pFOP-FLASH constructs) or serum deprived overnight and treated (or not) with BMP-2 for 6 or 24 h, and harvested. Luciferase and β-galactosidase activities were measured using an Optocomp luminometer (MGM Instruments, Hamden, CT) as previously described (39). Luciferase activity was corrected for β-galactosidase activity to control for transfection efficiency.

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined by ANOVA.

Results

To investigate the impact of CHOP/DDIT3 overexpression on stromal cell differentiation, ST-2 stromal cells transduced with pSRa *Chop/Ddit3* were cultured and compared with untransduced cells and cells transduced with pSRa vector. Two cell lines transduced with pSRa *Chop/Ddit3* expressed transcripts of approximately 4.0 kb, derived from the 5′ long-terminal repeat promoter, whereas control cultures did not (Fig. 1). Both vector and *Chop/Ddit3* transduced cultures expressed 0.8 kb endogenous CHOP/DDIT3 mRNA, and the level of expression was higher in *Chop/Ddit3* transduced cells, suggesting possible autoregulation. Western blot analysis documented expression of *Myc*-tagged CHOP/DDIT3 only in CHOP/DDIT3-overexpressing cells and higher expression of immunoreactive CHOP/DDIT3 in overexpressing cells when using antibodies to native CHOP/DDIT3 (Fig. 1). The two CHOP/DDIT3 overexpressing cell lines tested displayed a similar phenotype, and the phenotype of wild-type cells and cells transduced with pSRa vector was not different. Consequently, the effect of pSRa *Chop/Ddit3* was compared with the phenotype of cells transduced with pSRa vector.

Confirming previous observations, ST-2 cells exposed to ascorbic acid and β-glycerophosphate started to form detectable mineralized nodules after 21–24 d of culture, and this effect appeared earlier and was enhanced in the presence of BMP-2 (Fig. 2) (21). Overexpression of CHOP/DDIT3 enhanced osteoblastic differentiation. In the absence of BMP-2, CHOP/DDIT3 overexpression increased the formation of mineralized nodules after 15 and 24 d. In the presence of BMP-2, CHOP/DDIT3 accelerated and enhanced differentiation so that mineralized nodules appeared after 9 d of cul-

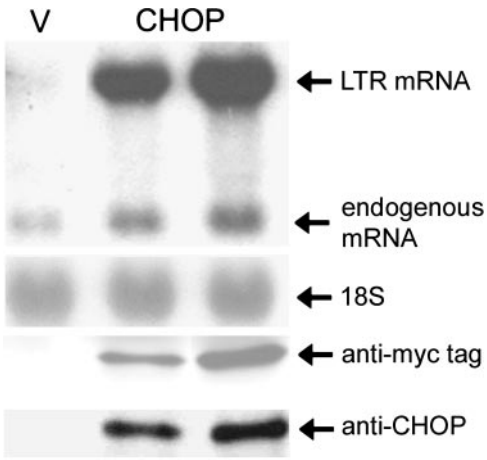

FIG. 1. Expression of CHOP/DDIT3 mRNA and protein by Northern and Western blot analyses in transduced ST-2 stromal cells. Total RNA (*upper panel*) was extracted from ST-2 cells transduced with pSRa vector (V) and the two cell lines transduced with pSRa *Chop/Ddit3* and cultured to confluence. RNA was resolved by gel electrophoresis, transferred to a nylon membrane, hybridized with [α-^{32}P]-labeled *Chop/Ddit3* and 18S cDNA, and visualized by autoradiography. For CHOP/DDIT3 determination (*lower panel*), nuclear extracts were obtained from ST-2 cells transduced with pSRa vector (V) or pSRa *Chop/Ddit3* and cultured to confluence. Then 30 μg of nuclear proteins were resolved by gel electrophoresis, transferred to an Immobilon P membrane, exposed to anti-Myc or anti-CHOP/DDIT3 and secondary antibodies, and visualized by fluorography.

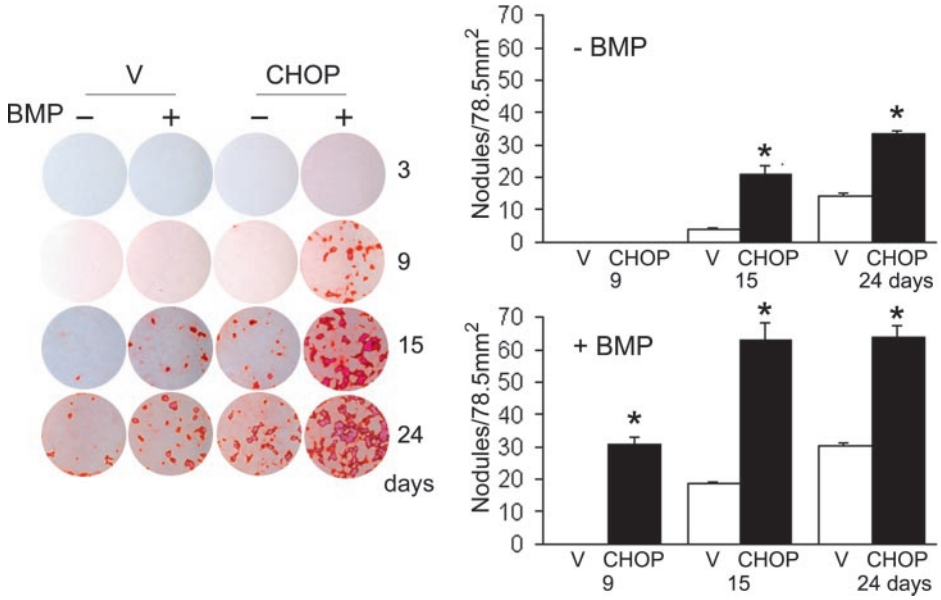

FIG. 2. Effect of CHOP/DDIT3 in the absence and presence of BMP-2 at 1 nM on the osteoblastic differentiation of ST-2 stromal cells. ST-2 cells transduced with pSRa vector (V) or pSRa *Chop/Ddit3* were cultured for 3–24 d after confluence and stained with alizarin red to detect mineralized nodule formation. Nodules were counted, avoiding the edge of the well in a uniform 78.5-mm^2 central area, and data are expressed as means ± SEM (n = 3) for the number of nodules/78.5 mm^2. *White bars* represent vector-transduced and *black bars* pSRa *Chop/Ddit3*-transduced cells. There was no difference in the phenotype between untransduced ST-2 cells (not shown) and cells transduced with pSRa vector. *, Significantly different, compared with vector, $P < 0.05$.

ture, and a greater number of nodules was observed in the CHOP/DDIT3-overexpressing cells.

To confirm that CHOP/DDIT3 induced osteoblastic differentiation, enzymatic assays and Northern blot analyses were performed to evaluate changes in alkaline phosphatase and osteocalcin expression. ST-2 cells exhibited low levels of APA in the initial phase of the culture, and APA increased as the culture progressed and the cells matured. In accordance with the acceleration of osteoblastic differentiation, pSRa *Chop/Ddit3*-transduced cells displayed higher levels of

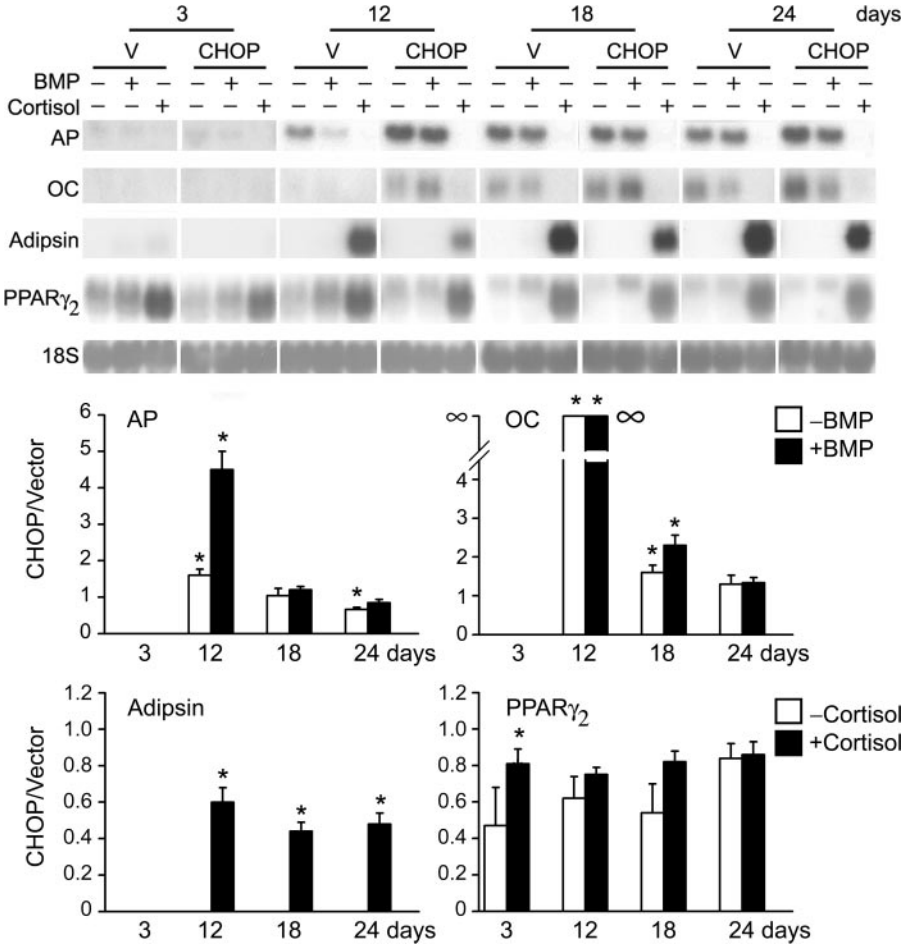

FIG. 3. Effect of CHOP/DDIT3 in the presence and absence of BMP-2 at 1 nM or cortisol at 1 μM on the differentiation of ST-2 stromal cells. ST-2 cells transduced with pSRa vector (V) or pSRa *Chop/Ddit3* were cultured for 3–24 d after confluence. Total RNA from vector and pSRa *Chop/Ddit3* transduced cells was subjected to Northern blot analysis and hybridized with [α-^{32}P]-labeled alkaline phosphatase (AP), osteocalcin (OC), adipsin, *pparg2*, and 18S DNAs. The *lower panels* represent fold changes of CHOP/DDIT3-overexpressing cultures/vector for the expression of alkaline phosphatase and osteocalcin mRNAs with (*black bars*) or without (*white bars*) BMP-2 or for the expression of adipsin and PPARγ2 mRNAs with (*black bars*) or without (*white bars*) cortisol, n = 3–5. Lack of bars represent undetectable transcripts in CHOP/DDIT3 and control cultures. Osteocalcin transcripts at 12 d were detectable in pSRa *Chop/Ddit3*-transduced but undetectable in vector-transduced cultures so that exact fold increase by CHOP/DDIT3 could not be determined and is labeled ∞. *, Significantly different, compared with vector, $P < 0.05$.

alkaline phosphatase in the initial 15 d of culture (data not shown), which were preceded by higher levels of alkaline phosphatase mRNA at 12 d of culture (Fig. 3) (21). Osteocalcin expression was detected after 12 d in CHOP/DDIT3 overexpressing cultures, whereas in control cultures it was detectable after 18 d, confirming earlier osteoblastic differentiation in CHOP/DDIT3-overexpressing cells. Changes in alkaline phosphatase and osteocalcin transcripts were of similar magnitude whether or not CHOP/DDIT3-overexpressing cells were cultured in the presence of BMP-2. CHOP/DDIT3 did not modify PPARγ2 expression, and the levels of adipsin mRNA were undetectable in control, BMP-2-treated, and CHOP/DDIT3-overexpressing cultures.

ST-2 stromal cells cultured in the presence of cortisol did not form mineralized nodules, and the levels of APA (not shown) and alkaline phosphatase and osteocalcin mRNA (Fig. 3) remained suppressed throughout the 24 d of culture. CHOP/DDIT3 did not reverse these effects of cortisol but opposed a modest effect of cortisol on adipocyte formation detected by Oil Red O staining of the cultures (not shown) and on adipsin mRNA levels (Fig. 3) (40). Consequently, CHOP/DDIT3-overexpressing ST-2 cells cultured in the presence of cortisol did not display phenotypic characteristics of either mature osteoblasts or adipocytes. To confirm that CHOP/DDIT3 inhibited adipogenesis, ST-2 stromal cells were cultured in the presence of an adipogenic cocktail. CHOP/DDIT3 overexpression reduced the effect of this cocktail on adipogenesis, as documented by a decrease in cellular fat content and PPARγ2 and adipsin mRNA levels (Fig. 4).

The changes in cell maturation caused by CHOP/DDIT3 were not associated with substantial changes in cell number or DNA content in the absence or presence of BMP-2 or cortisol (not shown). As the cells differentiated, there was an increase in apoptosis as defined by nuclear fragmentation and condensation detected in acridine orange-stained cultures. Consequently, it occurred earlier and was more apparent in cultures overexpressing CHOP/DDIT3, particularly in the presence of BMP-2 (not shown).

To assess possible mechanisms involved in the induction of osteoblast differentiation by CHOP/DDIT3, we examined its effects on the expression of C/EBPα, -β, and -δ mRNA levels. Cortisol increased C/EBPα, -β, and -δ mRNA levels (Fig. 5), whereas BMP had no effect (not shown). Overexpression of CHOP/DDIT3, in the absence or presence of BMP-2 or cortisol, did not cause consistent and significant changes in the levels of C/EBP transcripts, except for a decrease in C/EBPδ mRNA levels after 18 and 24 d of culture. Interactions and possible heterodimerization of CHOP/DDIT3 with C/EBPα, -β, and -δ were analyzed by electrophoretic mobility gel shift assay. Nuclear extracts from control and CHOP/DDIT3-overexpressing cells cultured for 3, 15, or 24 d bound to the *Cebp* consensus oligonucleotides, and the complex was displaced by unlabeled homologous, but not by mutated, oligonucleotide in excess (Fig. 6). Nuclear extracts from CHOP/DDIT3-overexpressing cells, cultured for 3, 15, or 24 d after confluence, exhibited decreased binding of nuclear proteins to the *Cebp* consensus sequence, although the decrease was small in cortisol-treated cultures (Fig. 6). These results suggest that the overexpressed CHOP/

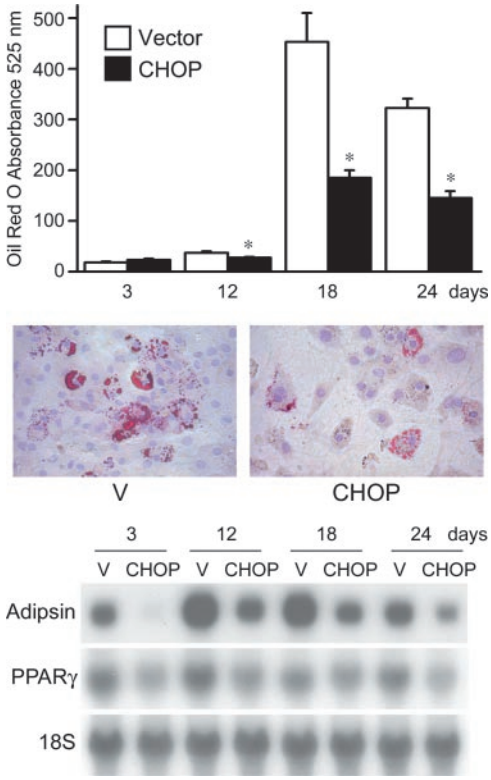

FIG. 4. Effect of CHOP/DDIT3 in the presence of an adipogenic cocktail consisting of dexamethasone 1 μM, insulin 100 nM, and isobutylmethylxanthine 0.5 mM on the differentiation of ST-2 stromal cells. ST-2 cells transduced with pSRa vector (V) or pSRa *Chop/Ddit3* were cultured for 3–24 d after confluence in the presence of the adipogenic cocktail, and Oil Red O-stained cells were extracted and quantitated (*upper panel*), and data are expressed as means ± SEM for vector (*white bars*) or CHOP/DDIT3 (*black bars*) overexpressing cultures; n = 6. *, Significantly different, compared with vector, $P < 0.05$. The *middle panel* represents a representative culture of vector (V) and *Chop/Ddit3*-transduced cells stained with Oil Red O after 18 d of culture in the presence of the adipogenic cocktail. In the *lower panel*, total RNA from vector and pSRa *Chop/Ddit3*-transduced cells, obtained after 3–24 d of culture in the presence of the adipogenic cocktail, was subjected to Northern blot analysis and hybridized with [α-^{32}P]-labeled adipsin, *pparg2,* and 18S DNAs.

DDIT3 interacts with endogenous C/EBPs and prevents their binding to the consensus *Cebp* DNA site. The identity of the proteins interacting with the *Cebp* probe was characterized further by supershift assays, which were performed by incubating the *Cebp* probe with nuclear extracts from control and test cells in the presence or absence of C/EBPα, -β, and -δ antibodies. C/EBPα and -β, but not -δ, antibodies decreased the abundance of the specific complex and caused a shift in binding complexes from control and CHOP/DDIT3-overexpressing cells, in the presence and absence of BMP-2 or cortisol (Fig. 7).

To investigate additional mechanisms involved in the effects of CHOP/DDIT3 on stromal cell differentiation, we explored whether it modified the canonical BMP/Smad or Wnt/β-catenin signaling pathways (37, 38, 41). CHOP/DDIT3 overexpression did not modify the basal activity of the BMP-responsive 12×SBE-Oc-pGL3 construct, but it amplified substantially the effect of BMP-2 on the activity of the construct so that the effect of BMP-2 at 1 nM after 24 h was

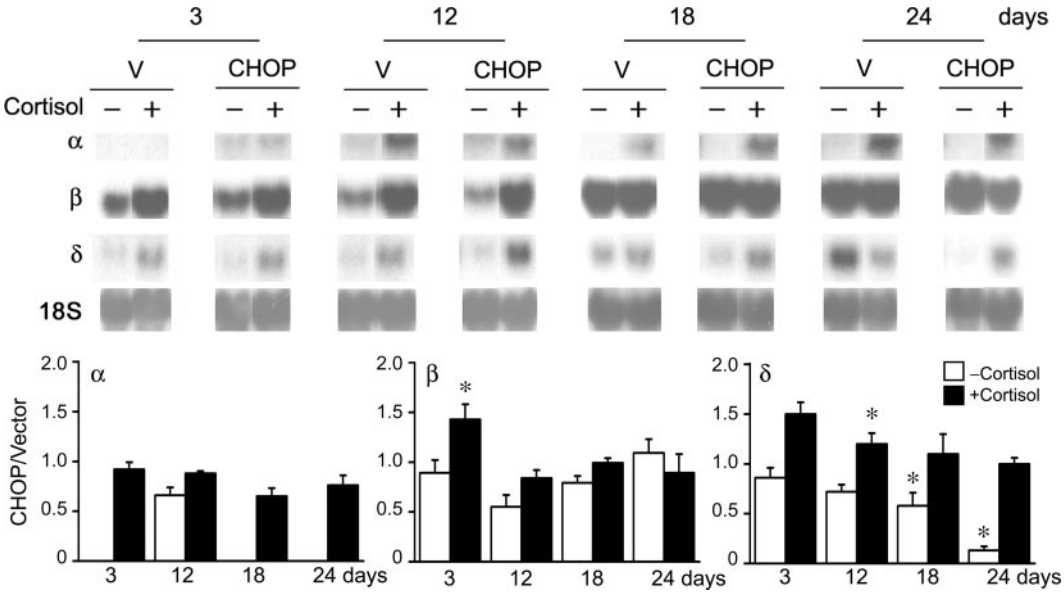

FIG. 5. Effect of CHOP/DDIT3, in the presence or absence of cortisol at 1 μM, on C/EBPα, -β, and -δ mRNA expression during the differentiation of ST-2 stromal cells. ST-2 cells transduced with pSRa vector (V) or pSRa *Chop/Ddit3* were cultured for 3–24 d after confluence. Total RNA from vector and pSRa *Chop/Ddit3*-transduced cells was subjected to Northern blot analysis and hybridized with [α-^{32}P]-labeled *Cebpa, -b,* and *-d* and 18S cDNAs. The *lower panel* represents fold changes of CHOP/DDIT3-overexpressing cultures/vector for the expression of C/EBPα, -β, and -δ mRNAs; n = 3–4. *White bars* represent cultures conducted in the absence of cortisol, *black bars* cultures treated with cortisol. *, Significantly different, compared with vector, $P < 0.05$.

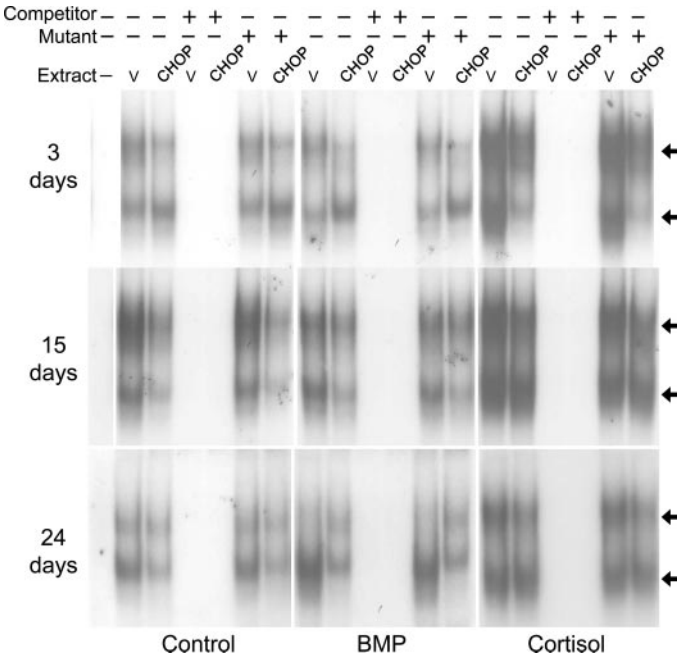

FIG. 6. EMSA revealing DNA-protein complexes, fractionated by PAGE in 7% gels and visualized by autoradiography. Nuclear extracts from ST-2 stromal cells transduced with pSRa vector (V) or pSRa *Chop/Ddit3* and cultured for 3, 15, or 24 d in the presence and absence of BMP-2 at 1 nM or cortisol at 1 μM were incubated with a [γ-^{32}P]-labeled 20-bp oligonucleotide containing a *Cebp* consensus sequence. Incubations were performed in the absence and presence of unlabeled *Cebp* cognate or mutant sequences. Specific CHOP/DDIT3-regulated complexes are indicated by the *arrows*.

6-fold greater in CHOP/DDIT3-overexpressing compared with control cells (Fig. 8). Using an antibody that recognizes phosphorylated Smad 1, 5, and 8, BMP-2 induced these phosphorylated Smads maximally after 15 min, and the effect was sustained for 6 h (not shown). CHOP/DDIT3 overexpression did not modify the level of phosphorylated Smad 1/5/8 in control or BMP-2 (0.03–3 nM for 20 min)-treated cells. These results indicate that CHOP/DDIT3 did not activate Smad phosphorylation and that the transactivation of the 12× SBE-OcGL3 construct occurs by indirect mechanisms. In addition, CHOP/DDIT3 overexpression increased the activity of the Wnt/β-catenin pTOP-FLASH-responsive construct by 2.9 ± 0.08 ($P < 0.05$)-fold, whereas the activity of the mutant pFOP-FLASH was modestly increased by 1.7 ± 0.10 ($P < 0.05$)-fold.

Discussion

We used stromal cells to study phenotypic changes associated with osteoblastic cell differentiation under conditions of CHOP/DDIT3 overexpression. Confirming previous observations, ST-2 stromal cells, in the presence of BMP-2, differentiated toward the osteoblastic phenotype (21, 42). Overexpression of CHOP/DDIT3 accelerated osteoblastic differentiation, particularly in the presence of BMP-2, and opposed the differentiation of ST-2 cells toward an adipocytic pathway under the influence of cortisol and an adipogenic cocktail. The effects of CHOP/DDIT3 overexpression on stromal cell differentiation are similar to those of BMPs and are in contrast with the effects of glucocorticoids in ST-2 stromal cells because these steroids impair ST-2 stromal cell differentiation (21).

In the present studies, we demonstrated that CHOP/DDIT3 overexpression decreased C/EBPδ mRNA levels in ST-2 stromal cells and decreased the interaction of C/EBPα and -β with a *Cebp* consensus DNA binding site. Although one could postulate that no C/EBPδ interactions were detected by supershift assay because of the decrease in C/EBPδ

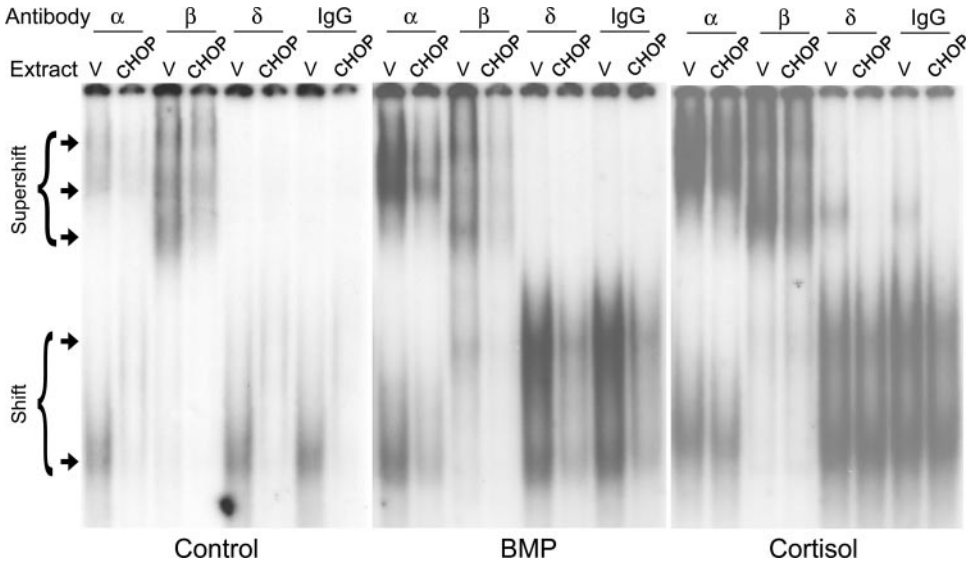

FIG. 7. EMSA revealing DNA-protein complexes, fractionated by PAGE in 4% gels and visualized by autoradiography. Nuclear extracts from ST-2 stromal cells transduced with pSRa vector (V) or pSRa *Chop/Ddit3* and cultured for 15 d in the presence and absence of BMP-2 at 1 nM or cortisol at 1 μM were incubated with a [γ-^{32}P]-labeled 20-bp oligonucleotide containing a *Cebp* consensus sequence. Incubations were performed in the absence and presence of C/EBPα-, -β-, and -δ-specific antibodies and nonspecific IgG antibodies. Shifted or supershifted complexes are indicated by the *arrows*.

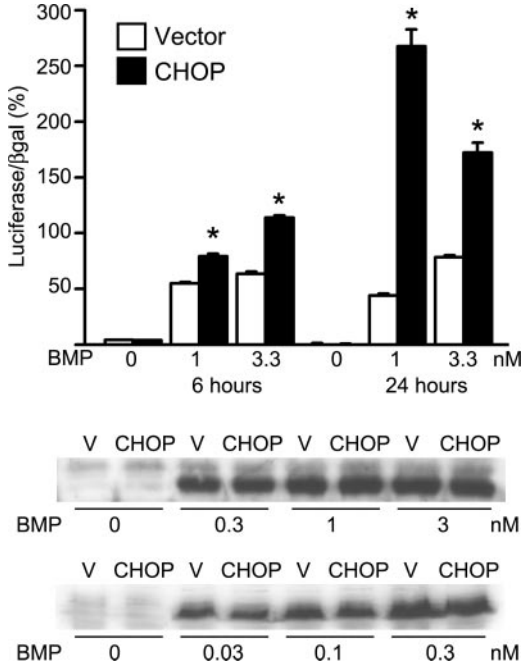

FIG. 8. Effect of CHOP/DDIT3 in the presence and absence of BMP-2 on the transactivation of a BMP-2/Smad-responsive construct (*upper panel*) and the phosphorylation of Smad 1/5/8 (*lower panel*). To test for effects on BMP signaling, subconfluent cultures of ST-2 cells transduced with *Chop/Ddit3* were transiently cotransfected with the BMP-responsive 12× SBE-Oc-pGL3 plasmid or pGL3 and a CMV β-galactosidase expression vector and treated with BMP-2 at the indicated doses for 6 or 24 h. Data shown are for luciferase activity/β-galactosidase activity for vector (*white bars*) or CHOP/DDIT3 (*black bars*)-overexpressing cultures, and are means ± SEM for six observations. Overexpression of CHOP/DDIT3 did not modify the activity of pGL3 plasmid without SBE-Oc sequences. *, Significantly different from vector, $P < 0.05$. To determine the effect on Smad phosphorylation, confluent cultures of ST-2 cells transduced with vector or *Chop/Ddit3* were serum deprived overnight and tested with BMP-2 at 0.03–0.3 nM (*lower blot*) and 0.3–3 nm (*upper blot*) for 20 min; resolved by gel electrophoresis; transferred to an Immobilon P membrane; exposed to an antibody that recognizes antiphosphorylated Smad 1, 5, and 8 and to an antirabbit IgG secondary antibody; and visualized by chemiluminescence and fluorography.

mRNA in CHOP/DDIT3 overexpressing cells, this is not a plausible explanation because no interactions were detected in control cultures, suggesting an alternate mechanism. It is possible that there is a limited amount of C/EBPδ protein in ST-2 cell cultures or that C/EBPδ-containing dimers have limited affinity for the sequence used. The results obtained are somewhat different from those observed in 3T3-L1 preadipocytes, in which CHOP/DDIT3 prevents adipogenesis by heterodimerizing with other C/EBPs, but it also reduces C/EBPα and -β mRNA levels (4). This is not surprising because the role and regulation of different C/EBPs varies among cell lines and models employed, and C/EBPs have complex cellular functions (43). It is possible that the differences in the effect of CHOP/DDIT3 on C/EBPα and -β mRNA levels could be due to differences in the CHOP/DDIT3 expression level in the ST-2 and 3T3L1 cell lines studied. CHOP/DDIT3 decreased but did not prevent the effect of cortisol on adipogenesis, possibly because in the early phases of the cultures, cortisol induced C/EBPα, -β, and -δ in excess of the binding capacity of the CHOP/DDIT3 being overexpressed. Another explanation for the observation is that glucocorticoids induce adipogenesis by C/EBP-dependent and -independent mechanisms, such as a direct induction of PPARγ2 transcripts, and this effect was not altered by CHOP/DDIT3 overexpression (27, 44).

Our data would suggest that the formation of heterodimers with C/EBPα and -β, which lack transactivating properties, could play a role in the osteoblastogenic effect of CHOP/DDIT3 but do not exclude other direct or indirect effects of CHOP/DDIT3 on osteoblastogenesis. Because C/EBPα and -β play a role in adipogenesis, one could postulate that the induction of osteoblastic differentiation by CHOP/DDIT3 is a simple default mechanism. This is not probable because CHOP/DDIT3 amplified the effect of BMP-2 on osteoblastic differentiation by sensitizing the Smad signaling pathway to the effects of BMP-2. This would suggest novel interactions between CHOP/DDIT3 and signaling Smads but does not exclude additional interactions between CHOP/DDIT3 and mitogen-activated kinases, also used by BMPs to signal in cells of the osteoblastic lineage (45).

It is important to note that CHOP/DDIT3 did not enhance the phosphorylation of BMP-dependent Smads using an antibody that recognizes phosphorylated Smad 1, 5, and 8, indicating that the transactivation on BMP-2/Smad-responsive promoter occurs by indirect mechanisms. This would be expected and is in accordance with the reported tethering of CHOP/DDIT3 with Fos/Jun proteins (10). It is possible that CHOP/DDIT3 stabilizes or enhances the interactions of phosphorylated Smad 1/5/8 with DNA recognition sequences or that it decreases the levels or activity of their inhibitors (23). We also provide evidence that CHOP/DDIT3 sensitized the canonical Wnt signaling pathway, suggesting an additional mechanism of action for the effect on osteoblastogenesis. CHOP/DDIT3 and Wnt/β-catenin have similar effects opposing adipogenesis and enhancing osteogenesis, and Wnt/β-catenin can interact with Smad signaling pathways (23, 41, 46).

Even though our studies reveal that CHOP/DDIT3 overexpression enhances the differentiation of osteoblastic cells, its ultimate function in skeletal cells has not been established. One of the functions of CHOP/DDIT3, like C/EBPγ and liver-enriched inhibitor protein, an isoform of C/EBPβ, is to act as a transdominant-negative inhibitor of C/EBPα, -β, and -δ (47, 48). However, unlike C/EBPγ and liver-enriched inhibitor protein, CHOP/DDIT3 has additional properties. In addition to its effects on Smad and Wnt signaling, CHOP/DDIT3 interacts with members of the Fos/Jun family of nuclear proteins by a tethering mechanism, suggesting that it could modulate genes expressed by osteoblasts regulated by AP-1 nuclear proteins (10). Members of the AP-1 family of transcription factors are differentially regulated during osteoblast differentiation, and CHOP/DDIT3 could modify their activity (49). In a variety of cellular systems, CHOP/DDIT3 is induced by cellular stress and amino acid deprivation, which results in apoptosis and arrested cellular growth (3, 50–53). In the present studies, we did not detect changes in the number of viable cells, attributable to CHOP/DDIT3 overexpression. However, CHOP/DDIT3 accelerated the appearance of apoptosis due to the acceleration of terminal stromal cell differentiation and mineralization (29). It is of interest that carbonic anhydrase is a CHOP/DDIT3-dependent gene induced by cellular stress and that overexpression of the oncogenic variant of CHOP/DDIT3, Fus/TLS-CHOP causes liposarcomas and scoliosis (54, 55). These observations would suggest a role for CHOP/DDIT3 in skeletal metabolism, but no skeletal phenotype has been reported in *Chop/Ddit3* null mice, and CHOP/DDIT3 overexpression in the bone microenvironment *in vivo* has not been examined (51). These studies are necessary to establish the role of CHOP/DDIT3 in skeletal homeostasis.

In conclusion, our studies demonstrate that CHOP/DDIT3 overexpression enhances osteoblastic differentiation in ST-2 stromal cells, a mechanism that may involve the formation of heterodimers with C/EBPα and -β and the sensitization of the BMP/Smad signaling pathway.

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