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## A Novel Role for GADD45 $\beta$ as a Mediator of MMP-13 Gene Expression during Chondrocyte Terminal Differentiation<sup>\*,S</sup>

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### Abstract

The growth arrest and DNA damage-inducible 45 $\beta$  (GADD45 $\beta$ ) gene product has been implicated in the stress response, cell cycle arrest, and apoptosis. Here we demonstrated the unexpected expression of GADD45 $\beta$  in the embryonic growth plate and uncovered its novel role as an essential mediator of matrix metalloproteinase-13 (MMP-13) expression during terminal chondrocyte differentiation. We identified GADD45 $\beta$  as a prominent early response gene induced by bone morphogenetic protein-2 (BMP-2) through a Smad1/Runx2-dependent pathway. Because this pathway is involved in skeletal development, we examined mouse embryonic growth plates, and we observed expression of Gadd45 $\beta$  mRNA coincident with Runx2 protein in pre-hypertrophic chondrocytes, whereas GADD45 $\beta$  protein was localized prominently in the nucleus in late stage hypertrophic chondrocytes where *Mmp-13* mRNA was expressed. In Gadd45 $\beta$ <sup>-/-</sup> mouse embryos, defective mineralization and decreased bone growth accompanied deficient *Mmp-13* and *Col10a1* gene expression in the hypertrophic zone. Transduction of small interfering

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RNA-GADD45 $\beta$  in epiphyseal chondrocytes *in vitro* blocked terminal differentiation and the associated expression of *Mmp-13* and *Col10a1* mRNA *in vitro*. Finally, GADD45 $\beta$  stimulated *MMP-13* promoter activity in chondrocytes through the JNK-mediated phosphorylation of JunD, partnered with Fra2, in synergy with Runx2. These observations indicated that GADD45 $\beta$  plays an essential role during chondrocyte terminal differentiation.

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Growth arrest and DNA damage-inducible (GADD)<sup>4</sup> 45 $\beta$  is a member of the GADD45 family of small (18 kDa) proteins, also including GADD45 $\alpha$  and GADD45 $\gamma$ . The GADD45 family is known to be associated with cell growth control, apoptotic cell death, and the cellular response to DNA damage (1, 2). Initially, GADD45 $\beta$ , encoded by *MyD118*, was identified as a myeloid differentiation primary response gene activated by IL-6 in murine myeloid leukemia cells upon induction of terminal differentiation (1, 3). More recently, GADD45 $\beta$ , which is induced by TGF- $\beta$  in a SMAD-dependent manner, has been identified as a positive regulator of TGF- $\beta$ -induced apoptosis (4). Although GADD45 $\alpha$  has been identified on DNA microarrays as prominently expressed genes in chondrocytes from adult articular cartilage and in chondrosarcoma or immortalized chondrocyte cell lines (5, 6), a role for GADD45 family members, including GADD45 $\beta$ , during cartilage development has not been reported previously.

Formation of the vertebrate skeleton through endochondrial ossification, involving progressive differentiation of proliferating chondrocytes to growth-arrested hypertrophic cells, is one of the most complex processes in biology. In the embryonic or postnatal growth plate, terminal chondrocyte differentiation occurs during conversion of cartilage to a vascularized tissue that supports matrix remodeling, cartilage calcification, and recruitment of osteogenic precursors. Cascades of growth and differentiation factors act through positive and negative signaling kinases and transcription factors to tightly control this process. Bone morphogenetic proteins (BMPs), which were identified originally as molecules that induce ectopic endochondral ossification (7), set the stage for bone morphogenesis by initiating chondrogenesis and by regulating chondrocyte maturation and terminal differentiation to the hypertrophic phenotype (8–11). The actions of the BMPs are determined by spatial and temporal differences in the distribution of BMP-binding proteins, such as noggin and chordin, BMP receptors, and the associated signal-transducing acceptor proteins, Smads 1 and 5, and inhibitory Smads 6 and 7.

BMP-2, -4, -6, and -7 are known to stimulate the expression of markers of the hypertrophic phenotype, including type X collagen and alkaline phosphatase (11, 12), and these responses to BMPs in the growth plate are restricted to pre-hypertrophic chondrocytes derived from regions where endochondral bone will form (13). Previous studies have demonstrated that BMP-induced Smad1 and interactions with the Runt domain transcription factor, Runx2, or Cbfa1, are important for chondrocyte hypertrophy (14–16). Runx2 is required for bone formation (17, 18), serving as a positive regulatory factor in chondrocyte maturation to the hypertrophic phenotype (15). Matrix metalloproteinase (MMP)-13, a downstream target of Runx2, is expressed by terminal hypertrophic chondrocytes during endochondral ossification (19–22). Recent evidence indicates that MMP-13 deficiency results in significant interstitial collagen accumulation leading to the delay of endochondral ossification in the growth plate (23, 24). Although the induction of MMP-13 expression by

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<sup>4</sup>The abbreviations used are: GADD45 $\beta$ , growth arrest and DNA damage-inducible 45 $\beta$ ; BMP, bone morphogenetic protein; MMP, matrix metalloproteinase; EMSA, electrophoretic mobility shift assays; JNK, c-Jun N-terminal kinase; siRNA, small interfering RNA; EGF, epidermal growth factor; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; bFGF, basic fibroblast growth factor; IL, interleukin; TGF, transforming growth factor; TNF, tumor necrosis factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IHC, immunohistochemistry; GFP, green fluorescent protein; ECM, extracellular matrix; WT, wild type; dpc, days post-conception; KD, knockdown; ERK, extracellular signal-regulated kinase.

cytokines in chondrocytes (25) and by parathyroid hormone-related protein in osteoblasts (26, 27) is known to involve cooperation between AP-1 and Runx2 transcription factors, the exact molecular mechanism of MMP-13 induction in hypertrophic chondrocytes remains unclear. Runx2 is expressed mainly in prehypertrophic and less in late hypertrophic chondrocytes (28, 29), whereas MMP-13 is expressed only in the late hypertrophic zone (30, 31). This spatial discrepancy suggests that unknown intermediate molecules may synchronize with Runx2 to regulate *Mmp-13* gene expression.

During the course of a study to identify the BMP-2-induced early genes that might be involved in signaling and transcriptional regulation in human chondrocytes, we identified *GADD45 $\beta$*  as one of the most highly induced genes. We further showed specific induction of *GADD45 $\beta$* , but not *GADD45 $\alpha$*  and *GADD45 $\gamma$* , by BMP-2, but not by EGF, FGF-2, or IGF-I, by a mechanism involving Smad1-dependent signaling and synergism with Runx2. Because BMP-2 is an important regulator of skeletal development, we examined mouse embryos and demonstrated for the first time that *Gadd45 $\beta$*  mRNA is expressed by prehypertrophic chondrocytes coincident with the Runx2 protein, whereas *GADD45 $\beta$*  protein accumulates in hypertrophic chondrocytes. Analysis of *Gadd45 $\beta$* <sup>-/-</sup> mouse embryos showed defective mineralization and decreased bone growth accompanied by decreased levels of *Mmp-13* and *Col10a1* mRNA in hypertrophic chondrocytes. In addition, lentiviral expression of siRNA-*GADD45 $\beta$*  blocked *Mmp-13* gene expression during hypertrophic differentiation of epiphyseal chondrocytes *in vitro*. Furthermore, we show that *GADD45 $\beta$*  induces AP-1 transcriptional activity through JNK-mediated phosphorylation of JunD partnered with Fra2 and stimulates *Mmp-13* promoter activity in synergism with Runx2. These results indicate that *GADD45 $\beta$*  has a critical role in mediating matrix remodeling during the final stages of chondrocyte terminal differentiation.

## MATERIALS AND METHODS

### Cell Culture

The immortalized human chondrocyte cell line, C-28/I2, was cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1/1, v/v; Invitrogen) containing 10% fetal calf serum (FCS) (BioWhittaker), as described previously (32, 33). For experiments with growth factors, subconfluent cultures were changed to medium containing 1% Nutridoma-SP (Roche Applied Science) for 24 h prior to incubation in the presence of growth factors. Primary chondrocytes were isolated from human articular cartilage, obtained from intact regions of femoral condyles at the time of total knee replacement surgery, and cultured for 7–10 days in DMEM/Ham's F-12 containing 10% FCS. ATDC5 cells were grown in DMEM/Ham's F-12 containing 5% FCS, 10  $\mu$ g/ml human insulin, 10  $\mu$ g/ml human transferrin, and 10  $\mu$ g/ml selenious acid, as described (34). After incubation in the presence of growth factors for 1 h, cells were harvested for RNA isolation. Three-dimensional pellet cultures of murine rib growth plate chondrocytes were prepared by the method of Ballock and Reddi (35). Chondrocytes from the ventral parts of rib cartilage of 2-day-old C57BL/6 mice were cultured in monolayer for 1 day followed by infection with lentiviral vector and culture for 2 days. Using trypsin/EDTA, the cells were resuspended at  $1.6 \times 10^5$  cells/ml, pelleted at 1 ml per tube in 15-ml conical polypropylene tubes by centrifugation at 1,000 rpm for 5 min at 4 °C, and cultured at 37 °C for periods of up to 21 days.

### RNA Isolation and Microarray Analysis

Total RNA was isolated using RNase-free DNase and RNeasy mini kit (Qiagen). Transcriptional profiling was performed at the Genomics Center of the Beth Israel Deaconess Medical Center on HU133A Affymetrix GeneChips containing 22,283 genes, according to protocols supplied by Affymetrix, with 8  $\mu$ g of total RNA per sample. For each

sample, 20  $\mu\text{g}$  of fragmented cDNA was hybridized with a pre-equilibrated HU133A Affymetrix chip, washed, stained, and scanned in the HP ChipScanner (Affymetrix Inc., Santa Clara, CA), as described (36).

Microarray data pre-processing was carried out by assigning each sample a quality measure based on criteria such as 3'-5' ratios, strength of hybridization, and image analysis. The samples that passed these *a priori* quality control criteria were analyzed using dChip software (37), by which smoothing spline normalization was applied prior to obtaining model-based gene expression indices or signal values. When comparing two groups of samples to identify the genes enriched in a given phenotype, we used the lower confidence bound of the fold change between the experiment and the base line. If the 90% lower confidence bound of the fold change between the experiment and the base line was above 1.2, the corresponding gene was considered to be differentially expressed (38).

### Real Time PCR

For each sample, cDNA was generated as described previously (33, 39). Amplifications were carried out using SYBR Green I-based real time PCR on the MJ Research DNA Engine Opticon™ Continuous Fluorescence Detection System (MJ Research Inc., Waltham, MA), as described previously (40).

### Immunoprecipitation and Western Blotting Analysis

After incubation without or with BMP-2 at 100 ng/ml for 1 h, the C-28/I2 cells were collected by scraping, and total protein was extracted with 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor mixture (Roche Applied Science). The cell lysates were analyzed on Western blots using antibody against total Smad1 or phospho-Smad1/5/8 (Cell Signaling). For immunoprecipitations, the capture protein for the Runx2 antibody (PEBP2aA, Santa Cruz Biotechnology) was coated at 200  $\mu\text{l}$ /well in a 96-well flat bottom, high binding microplate (Costar Corp., Cambridge, MA) at a final protein concentration of 2.4  $\mu\text{g}/\text{ml}$  in 50 mM carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. The plate was washed with phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBST), blocked by applying 200  $\mu\text{l}$  of 1% bovine serum albumin in PBST to each well, and incubated at room temperature for 1 h. Plates were washed three times with PBST. Cell lysate (1.1 mg of protein) was applied to each well, incubated at room temperature for 4 h, and washed three times with PBST. The bound protein was eluted with 1 $\times$ SDS sample buffer and carefully loaded on a Tris-glycine SDS-10% polyacrylamide gel followed by Western blotting with rabbit anti-phosphoserine antibody (Zymed Laboratories Inc.) or the total Smad1 antibody (Cell Signaling).

### Immunohistochemistry (IHC) and in Situ Hybridization

Hind limbs were collected from C57BL/6 mouse embryos fixed for 24 h in 4% paraformaldehyde. Limbs were embedded in paraffin, and 5- $\mu\text{m}$  sections were obtained. Tissue sections were deparaffinized in xylene and rehydrated through an ethanol series for use in either IHC or *in situ* hybridization. For IHC, tissue sections were subjected to microwave antigen retrieval in 10 mM EDTA (pH 7.5) at 93 °C for 7 min and allowed to cool for at least 2 h. Sections were blocked with normal horse serum (for GADD45 $\beta$  IHC) or normal swine serum (for Runx2 IHC). IHC for GADD45 $\beta$  was performed as described previously (41), using goat polyclonal anti-GADD45 $\beta$  (sc-8776, Santa Cruz Biotechnology; 1:400 dilution, 0.5  $\mu\text{g}/\text{ml}$  final concentration), rabbit biotinylated anti-goat IgG (Sigma), and the Vectastain Elite ABC kit (Vector Laboratories, Inc.). Anti-Runx2 antibody (PEBP2aA, sc-10758, Santa Cruz Biotechnology; 1:25 dilution, 8  $\mu\text{g}/\text{ml}$  final concentration), swine biotinylated anti-rabbit F(ab)<sub>2</sub>, and streptavidin/horseradish peroxidase-conjugated (DAKO,

Denmark) were used to detect Runx2 by IHC. Sections were counterstained with hematoxylin. For negative controls, normal goat IgG (sc-2028) and normal rabbit IgG (sc-2027) were used in place of the primary antibodies against GADD45 $\beta$  and Runx2, respectively. We optimized the staining methods for detection of cytoplasmic and nuclear staining with each antibody by testing both microwave and enzyme retrieval of each antigen.

For *in situ* hybridization, a 391-bp fragment of human GADD45 $\beta$  cDNA (sense, 5'-CTGGTTGTTGCCCCGGCTTTCTTC-3'; anti-sense, 5'-CGCGGTGGAGGAGCTTTTGGTG-3') and a 1006-bp fragment of mouse MMP-13 cDNA (sense, 5'-CATCCACATGGTTGGGAAGTTCTG-3'; antisense, 5'-CATTACAGCTATCCTGGCCACCTTC-3') were obtained by reverse transcription-PCR and subcloned in the pCR II vector (Invitrogen). Plasmids were linearized using either HindIII or NotI for GADD45 $\beta$  and either KpnI or XhoI for MMP-13, respectively. The linearized plasmids served as templates for riboprobe synthesis using either T7 or SP6 RNA polymerases and digoxigenin-UTP RNA labeling nucleotide mix (Roche Applied Science) according to the manufacturer's instructions. The cDNA encoding type X collagen was a kind gift of Dr. Bjorn Olsen, Harvard Medical School, Boston. Hybridization was performed according to the method described previously (42).

### Analysis of Gadd45 $\beta$ <sup>-/-</sup> Mice

Gadd45 $\beta$ <sup>-/-</sup> mice were generated on the C57BL/6 background, as described previously (43). Briefly, the targeting vector was constructed to replace exons 3 and 4, encoding the region crucial for interaction with MTK-1/MEKK4 (44), with the neomycin resistance cassette. Gadd45 $\beta$ <sup>-/-</sup> embryos and wild type littermates at 14.5, 15.5, 16.5, and 17.5 days post-conception (dpc), generated with the approval of the University of Pittsburgh Institutional Animal Care and Use Committee, were fixed in 4% paraformaldehyde/phosphate-buffered saline overnight at 4 °C and stored in 70% ethanol at room temperature. Whole embryos were stained with Alcian Blue and Alizarin Red. Long bones were sectioned and stained with toluidine blue or von Kossa or analyzed by *in situ* hybridization as described above.

### Plasmids

The human GADD45 $\beta$  promoter fragment spanning -1604 to +141 was prepared by PCR using human genomic DNA (Clontech) as template. The PCRs were carried out using the *Pfu Turbo* DNA polymerase (Stratagene), and the products were subcloned in the pGL2-basic vector (Promega, Madison WI) and verified by DNA sequencing. Smad1, Smad4, and alk6 expression vectors were gifts from Dr. Xu Cao (University of Alabama). The Runx2 and  $\Delta$ Runx2 expression vectors were described previously (45). The pcDNA3-GADD45 $\beta$ -FLAG plasmid was generated as described previously (40). The pAP-1 Luc plasmid was obtained from Stratagene. The MMP-13 promoter sequence spanning -1007/+26 bp was prepared by PCR using a pCAT-MMP-13 promoter construct<sup>5</sup> as a template, and cloned in the pGL2-B vector in the XmaI and XhoI sites. Expression vectors for JunD, JunB, Fra1, and Fra2 were kindly provided by Dr. Paul R. Dobner (University of Massachusetts Medical Center, Worcester, MA). The dominant negative JunD was described previously (46).

### Transient Transfections and Luciferase Assay

Transient transfection experiments were performed in C-28/I2 cells and ATDC5 cells using Lipofectamine Plus (Invitrogen), as described (33, 40), but in 12-well plates. Cells were plated at 100,000 cells per well and transfected with 300 ng of pGL2-promoter plasmid and 10–100 ng of expression plasmid. Luciferase activity was measured 24 h later using the

<sup>5</sup>M. Rahman, unpublished data.

dual-luciferase reporter assay system (Promega). Cotransfection with pRL-TK *Renilla* luciferase control vector was used for controlling transfection efficiency. Cotransfections of the promoter-less pGL2-B vector with expression plasmids did not significantly increase activity compared with promoter-containing vectors. This is in contrast to a study showing that 1  $\mu$ g of Runx2 expression vector increased luciferase activity when cotransfected with 1  $\mu$ g of promoter-less pGL2 or pGL3 vector in cells plated in 6-well plates (47). Transfections were performed in triplicate, and experiments were repeated three times with different plasmid preparations with similar results.

### Knockdown of GADD45 $\beta$ Expression by siRNA

Two approaches were used. First, chemically synthesized complementary RNA oligonucleotides (siRNA-*GADD45 $\beta$*  sense-strand sequence, 5'-AAGTTGATGAATGTGGACCCA) were annealed, deprotected, and desalted as recommended by the manufacturer (Qiagen). A total of 50  $\mu$ M RNA duplexes was transfected into cells using TKO transfection reagent (Mirus, Madison, WI) and tested for specificity and efficiency, as described previously (40). Second, a lentiviral vector containing siRNA-*GADD45 $\beta$*  was constructed using single 83-mer oligonucleotides containing an XbaI site at the 5' end, an intermediate short spacer, and a partial sequence of the H1-RNA promoter at the 3' end, as described previously (40). Standard PCR procedures (Advantage 2 PCR kit, Clontech) were performed using specific siRNA oligonucleotides and T3 primer plus pSuper-like plasmid as template to provide H1-mediated siRNA cassettes with an additional XbaI site at the 3' end (48). PCR products were purified (Qiagen), digested with XbaI, and cloned into the 3'-long terminal repeat NheI site of a CMV-GFP lentiviral vector as described (48). The LV-siRNAGFP construct (control) was kindly donated by Dr. Oded Singer (Salk Institute). The following siRNA oligonucleotide for *GADD45 $\beta$*  was used, 5'-CTGTCTAGACAAAAAGTTGATGAATGTGGACCCA<sub>tctctttaa</sub>TGGGTCCACATTCATCAACGGGGATCTGTGGTCTCATACA-3'. Vesicular stomatitis virus G envelope protein-pseudotyped lentiviruses were prepared and purified as described previously (48–50). Vector concentrations were analyzed by immunocapture p24 *gag* enzyme-linked immunosorbent assay (Alliance, PerkinElmer Life Sciences) (49). The C-28/I2, ATDC5, or murine epiphyseal chondrocytes were plated in monolayer at 25,000 cells/cm<sup>2</sup> for 1 or 2 days, followed by infection with LV-siRNA-GFP or LV-siRNA-*GADD45 $\beta$*  at a multiplicity of infection of 10. In tests for specificity and efficiency, cells were transfected with GFP-FLAG and *GADD45 $\beta$* -FLAG. Cells infected with LV-siRNA-GFP showed no staining for GFP, whereas GFP staining was not affected in cells infected with LV-siRNA-*GADD45 $\beta$* . On Western blots, transfected *GADD45 $\beta$* -FLAG was detected on Western blots using anti-FLAG antibody in cells infected with LV-siRNA-GFP, but not in cells infected with LV-siRNA-*GADD45 $\beta$* . (see supplemental Fig. S1).

### EMSA and Supershift Assay

Whole cell extracts were made from uninfected, LV-siRNA-GFP-infected (GFP KD), or LV-siRNA-*GADD45 $\beta$* -infected (*GADD45 $\beta$*  KD) C-28/I2 cells, and DNA binding reactions and EMSAs were performed using a <sup>32</sup>P-labeled oligonucleotide containing the AP-1 consensus (Santa Cruz Biotechnology), as described (33, 46). Extracts from DU145 cells were used as a positive control for activated AP-1 (data not shown) (46). For supershift analysis, the cell extracts were preincubated with specific antibodies against different members of the AP-1 family (Santa Cruz Biotechnology) for 20 min before the incubation with labeled probe for an additional 20 min (46).

## Analysis of Protein Kinases

To examine protein kinase activities, the C-28/I2 cells without or with siRNA-*GADD45 $\beta$*  were transfected with pcDNA3 expression vectors encoding control-FLAG or *GADD45 $\beta$* -FLAG. After transfection, the cells were cultured for 12 h with DMEM/F-12, 1% Nutridoma, followed by addition of BMP-2 (100 ng/ml) and further incubation for 12 h before collection of total cell lysates. Western blots were performed using antibodies against JunD (Santa Cruz Biotechnology) and phospho-c-Jun (Ser-73) (Cell Signaling) according to the manufacturers' protocols. PD98059 (10  $\mu$ M), SB203580 (10  $\mu$ M), or SP600125 (100  $\mu$ M) was added to cultures 1 h before BMP-2. JNK, ERK and p38 kinase activities were measured by using the SAPK/JNK, p44/42, and p38 MAPK assay kit (Cell Signaling) according to the manufacturer's protocol.

## RESULTS

### Specific Up-regulation of *GADD45 $\beta$* by BMP-2 in Human Chondrocytes

To characterize the genes involved in the early response to BMP-2 in chondrocytes, we performed oligonucleotide microarray-based gene expression profiling using RNA isolated from the immortalized human chondrocyte cell line, C-28/I2, after treatment with BMP-2 for 1 h. BMP-2 induced distinct patterns of coordinated changes in a wide range of transcripts, which encode cell cycle-related and anti-apoptotic molecules and transcription factors that are known to regulate growth and survival cascades. Identification of highly expressed genes induced by BMP-2 (more than 2 times >control) yielded 249 transcript variants. Actin-related protein 2/3 complex subunit 4 was the most highly expressed gene stimulated by BMP-2 (18.4 times). Analysis of gene ontology molecular function revealed 31 transcription factors, including ZNF238 (7.5 times), JunD (5.3 times), JunB (4 times), ID3 (4 times), and DLX2 (3.7 times) and 11 kinases, such as phosphatidylinositol 3-kinase regulatory subunit 3 (7.5 times), AKT2 (6.5 times), TK 2 (4 times), and AXL (3 times), which were induced by BMP-2 after 1 h. A complete set of the microarray data is provided as supplemental material. *GADD45 $\beta$* , which was among the most highly up-regulated BMP-2-induced genes, was detected by all three representative probe sets present on the Affymetrix GeneChip Human Genome U133A, whereas *GADD45 $\alpha$*  and *GADD45 $\gamma$*  transcripts were not altered by the treatment with BMP-2 (TABLE ONE). Real time PCR corroborated the microarray data showing that BMP-2 at concentrations of 50 and 100 ng increased *GADD45 $\beta$*  mRNA (Fig. 1A). Other growth and differentiation factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF)-I, had no effect (Fig. 1A), indicating the specificity of the BMP-2 response. Real time PCR also confirmed that BMP-2 had no effect on *GADD45 $\alpha$*  or *GADD45 $\gamma$*  mRNA (Fig. 1, B and C). Furthermore, the peak of induction was 1 h after the addition of BMP-2 with a gradual decline thereafter, such that there was no difference between treated and untreated at 24 h (Fig. 1D). Finally, the BMP-2-induced increase in *GADD45 $\beta$*  mRNA was confirmed in primary human articular chondrocytes (Fig. 1E).

### Induction of *GADD45 $\beta$* Promoter Activity by BMP-2 in Chondrocytes Requires Signaling by Smad1 Interacting with Runx2

Because BMP-2 is known to regulate gene expression by Runx2-mediated recruitment of Smad 1 to subnuclear sites (51, 52), we examined *GADD45 $\beta$*  promoter activity in the C-28/I2 cells after BMP-2 treatment to determine the consequence of the direct cooperation between Runx2 and Smad1 in this model. After transfection of a luciferase reporter vector driven by the *GADD45 $\beta$*  promoter sequence spanning -1604 to +141 bp, BMP-2 increased promoter activity by around 2-fold (Fig. 2A). Overexpression of *alk6*, the BMP type IB receptor, activated *GADD45 $\beta$*  promoter activity to a similar extent as BMP-2. Cotransfection with Smad1 or Runx2 alone further enhanced the BMP-2-induced activation of the

*GADD45 $\beta$*  promoter. In contrast, Smad6, which is known to repress transcriptional activity by competing with Smad4 for binding to Smad1, and  $\Delta$ Runx2 inhibited *GADD45 $\beta$*  promoter activation by Smad1 and Runx2, respectively (Fig. 2A). To confirm the Runx2-Smad1 interaction in this model, we examined Smad1 and Runx2 phosphorylation in C-28/I2 chondrocytes treated with BMP-2. As shown in Fig. 2, B and C, both Runx2 and Smad1 were phosphorylated after treatment with BMP-2 for 1 h. The Runx2 interaction with Smad1 was further investigated by coimmunoprecipitation of total cell lysates with anti-Runx2 and Western blotting with anti-Smad1. As shown in Fig. 2C, Western blotting with anti-Smad1 detected a single BMP-2-induced band after immunoprecipitation with anti-Runx2. Together, these data indicate that the induction of *GADD45 $\beta$*  by BMP-2 in chondrocytes is mediated by Smad1-dependent signaling and that Runx2 contributes to the full induction.

### Expression of *GADD45 $\beta$* in the Mouse Embryonic Growth Plate

Because Runx2 is expressed in the growth plate as a major transcriptional regulator of chondrocyte terminal differentiation, we asked whether *GADD45 $\beta$*  is also expressed during skeletal development. Therefore, we performed *in situ* hybridization and immunohistochemical assays in the mouse embryonic limb. We detected expression of *Gadd45 $\beta$*  mRNA at 14.5 (data not shown) and 15.5 dpc (Fig. 3, A and B) primarily in prehypertrophic chondrocytes, where Runx2 protein was localized most prominently in the nucleus (Fig. 3C). On the other hand, immunoreactive *GADD45 $\beta$*  protein was detected most strongly in the nuclei of intact hypertrophic chondrocytes (Fig. 3, D, F, and J). Runx2 persisted through this region into the late hypertrophic zone of the femur (Fig. 3C), where *Mmp-13* mRNA was also detected (Fig. 3, H and K). As shown in Fig. 3 (H and K), *Mmp-13* mRNA colocalized with nuclear *GADD45 $\beta$*  protein in the late hypertrophic zone of the tibia. These data demonstrate that *Gadd45 $\beta$*  mRNA is induced in prehypertrophic chondrocytes coincident with prominent nuclear Runx2 protein, whereas *GADD45 $\beta$*  protein accumulates in the nuclei of hypertrophic chondrocytes in the region destined to undergo ossification, where *Mmp-13* mRNA is also expressed.

### Defective Mineralization and Decreased Bone Growth Accompany Decreased *Mmp-13* mRNA Expression in Hypertrophic Chondrocytes of the *Gadd45 $\beta$ <sup>-/-</sup>* Mouse Embryo

To determine whether *GADD45 $\beta$*  has a crucial function in hypertrophic chondrocytes during endochondral ossification, we performed histological analysis of embryonic limbs from *Gadd45 $\beta$ <sup>-/-</sup>* mice. At 15.5 dpc, the tibiae showed the first signs of the formation of a primary ossification center in wild type (WT) mice (Fig. 4A). Mineralization of the extracellular matrix (ECM) in WT mice was clearly identified by von Kossa staining (Fig. 4B). *Mmp-13* mRNA-expressing hypertrophic chondrocytes were distributed throughout this zone (Fig. 4, C and G). On the other hand in *Gadd45 $\beta$ <sup>-/-</sup>* mice, the primary ossification center was decreased in size (Fig. 4D). Mineralization of the ECM was defective, as shown by the decreased von Kossa staining (Fig. 4E), but persisted in the surrounding bone collar, and the number and distribution of *Mmp-13* mRNA-expressing hypertrophic chondrocytes were markedly diminished (Fig. 4, F and H).

The defective mineralization and deficient *Mmp-13* mRNA expression at the site of the primary ossification center at 15 dpc preceded the decreased bone growth observed in *Gadd45 $\beta$ <sup>-/-</sup>* mouse embryos at 16.5 dpc (Fig. 5). A delay in mineralization, indicated by Alizarin Red staining was noted in the ribs of *Gadd45 $\beta$ <sup>-/-</sup>* embryos (arrows, Fig. 5A) and in both fore and hind limbs (Fig. 5, B and C). The hypertrophic zone of the tibia of the *Gadd45 $\beta$ <sup>-/-</sup>* embryo was decreased in length compared with WT (Fig. 5D). Decreased expression of type X collagen (*Col10a1*) mRNA, a specific marker of the hypertrophic phenotype (14), throughout the hypertrophic zone accompanied the markedly decreased *Mmp-13* mRNA expression in the late hypertrophic zone (Fig. 5E). These data suggest that



GADD45 $\beta$  is involved in terminal differentiation of hypertrophic chondrocytes and may contribute to *Mmp-13* gene regulation.

### Requirement of GADD45 $\beta$ for Expression of *Mmp-13* mRNA during Hypertrophic Chondrocyte Differentiation in Vitro

To confirm the results in Fig. 5 and to determine the extent of dependence of *Mmp-13* gene expression in hypertrophic chondrocytes upon GADD45 $\beta$ , we used murine rib growth plate chondrocytes in three-dimensional pellet culture, an established model in which terminal chondrocyte differentiation occurs spontaneously (35). In control GFP knockdown (KD) cultures infected with a lentiviral vector encoding siRNA-GFP, chondrocytes with hypertrophic morphology were present at days 14 and 21 and were surrounded by matrix that stained metachromatically with toluidine blue (*pink staining* in matrix in Fig. 6A), evidence of the early proteoglycan deposition that occurs in this model. Strong expression of *Mmp-13* and *Col10a1* mRNA was confirmed by *in situ* hybridization in the GFP KD cultures at day 21 (Fig. 6B). In contrast, GADD45 $\beta$  KD cells, which were infected with a lentivirus encoding siRNA-GADD45 $\beta$ , did not develop hypertrophic morphology or increase metachromatic staining with toluidine blue even after 21 days of culture (Fig. 6A). Furthermore, *in situ* hybridization analysis showed that the levels of *Mmp-13* mRNA, as well as *Col10a1* mRNA, specific marker of the hypertrophic phenotype, were decreased markedly in the GADD45 $\beta$  KD cells compared with the GFP KD cells (Fig. 6B). These results support the *in vivo* data, where GADD45 $\beta$  expression is restricted in a temporal-spatial manner, and suggest that GADD45 $\beta$  is an essential regulator of chondrocyte terminal differentiation that may influence, directly or indirectly, *Mmp-13* gene expression during hypertrophy.

### Regulation of AP-1 Activity by GADD45 $\beta$ in Chondrocytes

Because members of the AP-1 family of transcription factors are known to be involved in *MMP-13* gene regulation (25), we examined whether GADD45 $\beta$  could regulate AP-1 activity in chondrocytes. We used the C-28/I2 cell line to generate sufficient numbers of lentivirally transduced cells for reproducible transient expression assays and for nuclear extracts for EMSA. Cotransfection GADD45 $\beta$ -FLAG together with the *cis*-reporter plasmid containing AP-1-responsive elements resulted in concentration-dependent transactivation (2–3-fold increase) of the AP-1-dependent reporter (Fig. 7A). Transduction of siRNA-GADD45 $\beta$  decreased constitutive AP-1 activity by around 25% and blocked the GADD45 $\beta$ -FLAG-dependent activation to the control level (Fig. 7B). EMSAs were also performed using a radiolabeled probe containing the AP-1 consensus binding site and whole cell extracts from cells infected with lentiviral siRNA-GADD45 $\beta$  and transfected with GADD45 $\beta$ -FLAG. As shown in Fig. 7C, the low level of AP-1 binding activity was increased by overexpression of GADD45 $\beta$ . Overexpression of GADD45 $\beta$  further increased AP-1 binding activity in GFP KD cells, but not in GADD45 $\beta$  KD cells, which showed decreased AP-1 binding activity compared with GFP KD cells. The AP-1 binding activity due to endogenous GADD45 $\beta$  was also decreased in GADD45 $\beta$  KD cells in the absence of GADD45 $\beta$ -FLAG. These results correspond directly to the GADD45 $\beta$ -dependent AP-1 promoter activity shown in Fig. 7, A and B.

To characterize further the AP-1 binding activity in extracts from GADD45 $\beta$ -expressing chondrocytes, we performed supershift analyses using antibodies that recognize various members of the Jun and Fos families. JunD, JunB, Fra1, and Fra2 were identified as the predominant proteins present in the AP-1-binding complex (Fig. 7D). Because Jun proteins are known to bind to AP-1 sites as heterodimers with Fos proteins such as Fra1 or Fra2, we assessed the AP-1 reporter activity after cotransfection with JunD, JunB, Fra1, and Fra2 expression vectors alone and in various combinations (Fig. 7E). Each expression vector

alone was ineffective as an activator, whereas JunD/Fra1 and JunD/Fra2 increased AP-1 reporter activity by around 5-fold, and JunB/Fra1 and JunB/Fra2 increased activity by 2–2.5-fold. Most interestingly, cotransfection with GADD45 $\beta$  in the presence of JunD, which can form homodimers, increased AP-1 reporter activity by more than 4-fold, whereas the activity in the presence of each of the other factors alone or in combination increased 2-fold above that in control-FLAG cells (Fig. 7E). These results suggest that JunD is a major component of the AP-1 activity induced by GADD45 $\beta$  in chondrocytes.

### JNK Activity Is Necessary for JunD Activation by GADD45 $\beta$

To unravel the mechanism of JunD activation by GADD45 $\beta$ , we evaluated the phosphorylation status of JunD in GADD45 $\beta$ -expressing and GADD45 $\beta$  KD chondrocytes. We analyzed cell extracts from C-28/I2 cells for kinase activation using antibodies against phospho-c-Jun, phospho-Elk-1, and phospho-ATF-2, specific substrates for JNK, ERK, and p38, respectively. As shown in Fig. 8A, transduction of GADD45 $\beta$  induced JunD phosphorylation at serine 100, which is reported as a direct JNK target residue (53). The phosphorylation was completely blocked in the presence of the JNK inhibitor, SP600125, but not by the ERK inhibitor, PD98059, or by the p38 inhibitor, SB203580. Transduction of siRNA-GADD45 $\beta$  blocked the GADD45 $\beta$ -dependent JunD phosphorylation (Fig. 8A). JNK activity, detected as phospho-c-Jun, was clearly increased in GADD45 $\beta$ -expressing cells and siRNA-GADD45 $\beta$  blocked its activity, whereas ERK was activated only slightly by GADD45 $\beta$ , and p38 activity was not altered (Fig. 8B). These results are consistent with previous studies reporting that GADD45 family members are upstream activators of the JNK pathway (2, 54–56).

### JunD-dependent MMP-13 Promoter Activation by GADD45 $\beta$ in ATDC5 Cells and Synergism with Runx2

To determine the functional consequences of GADD45 $\beta$ -induced AP-1 transcriptional activity in hypertrophic chondrocytes, we investigated the response of the *MMP-13* promoter using ATDC5 cells, a well characterized chondrogenic cell line that undergoes terminal differentiation to the hypertrophic phenotype *in vitro* (34). Because *MMP-13* gene transcription is known to involve Runx2 and AP-1 (25–27), we performed cotransfection experiments using FLAG-GADD45 $\beta$ , Runx2, and JunD expression vectors in various combinations with the pGL2-*MMP-13* reporter vector containing promoter sequences spanning –1007 to +26 bp. As shown in Fig. 9A, overexpression of either GADD45 $\beta$  or Runx2 up-regulated *MMP-13* promoter activity by 2.5–3-fold. Cotransfection with both Runx2 and GADD45 $\beta$  expression vectors enhanced promoter activity by around 4-fold (Fig. 9A). Furthermore, transduction of siRNA-GADD45 $\beta$  reduced *MMP-13* promoter activity to 25% of the constitutive activity in ATDC5 cells (Fig. 9B).

To evaluate further the contribution of JunD in the GADD45 $\beta$ - and Runx2-dependent *MMP-13* promoter activation, we performed cotransfections with expression vectors encoding JunD or dominant negative JunD. Most strikingly, JunD mimicked the GADD45 $\beta$ -dependent up-regulation of the *MMP-13* promoter, enhancing the basal or Runx2-dependent activity by 2-fold (Fig. 9C), and dominant negative-JunD down-regulated the GADD45 $\beta$ -dependent activity (Fig. 9D). Similar results were obtained using the C-28/I2 cells, which express higher basal MMP-13 activity but with similar responses to transfected expression vectors. These results indicate that *Mmp-13* gene expression in terminally differentiating chondrocytes is predominantly a result of JunD activation by GADD45 $\beta$  in cooperation with Runx2.

## DISCUSSION

In this study, we have highlighted a new function of GADD45 $\beta$ , a member of a group of stress-response proteins that are associated with growth arrest and cell survival. We initially identified GADD45 $\beta$  as a candidate target of BMP-2 using microarray analysis. Because BMP-2 is known to be involved in hypertrophic differentiation of chondrocytes, we asked whether GADD45 $\beta$  could play a role during endochondral ossification in the mouse embryo. Thus, we made the novel observation that *Gadd45 $\beta$*  mRNA is expressed by pre-hypertrophic chondrocytes in the mouse embryonic growth plate, where Runx2 is also detected, whereas GADD45 $\beta$  protein accumulates in the nuclei of chondrocytes of the hypertrophic zone, where Runx2 protein also persists, prominently in late hypertrophic chondrocytes expressing *Mmp13* mRNA prior to mineralization. In further studies, we found that *Gadd45 $\beta$* <sup>-/-</sup> mice embryos display defective mineralization accompanied with a decreased number of *Mmp-13*- and *Col10a1*-expressing hypertrophic chondrocytes with the consequence of decreased bone growth. By using models of chondrocyte hypertrophic differentiation *in vitro* and siRNA technology, we provide clear evidence to support an essential role for GADD45 $\beta$  in the regulation of *Mmp-13* gene expression. Finally, we reveal that GADD45 $\beta$ -mediated up-regulation of *MMP-13* promoter activity is because of an increase in AP-1 activity through the JNK/JunD pathway and that Runx2 cooperates with GADD45 $\beta$  and JunD to enhance this response.

The BMPs along with other growth and differentiation factors are responsible for initiating and directing cartilage morphogenesis in the embryonic limb and generally set the stage for bone morphogenesis (31, 57–60). We initiated this study by examining directly the global responses of chondrocytes to BMP-2 to profile on microarrays the early induced genes that are likely to play regulatory roles. Many of these gene products are already known as BMP-induced transcription factors and signaling kinases, including JunB, JunD, ID3, and DLX2 (61–64). A recent report indicates that phosphatidylinositol 3-kinase-AKT signaling is involved Runx2-induced chondrocyte and osteoblast differentiation (65). The markedly enhanced expression of GADD45 $\beta$  on these arrays and our recent findings on the differential roles of GADD45 family members in apoptosis and tumor growth (40) led us to ask whether GADD45 $\beta$  may play a role in chondrocyte differentiation. Thus, we report for the first time that *Gadd45 $\beta$*  mRNA is expressed by pre-hypertrophic chondrocytes and that the protein accumulates in hypertrophic chondrocytes in the mouse embryonic growth plate.

Runx2, or Cbfa1, belongs to the family of Runt domain genes, which play fundamental roles in organ development and cell differentiation (17), and together with SMAD proteins is involved in the regulation of phenotypic gene expression during osteogenesis (51). We demonstrate in our study that *GADD45 $\beta$*  promoter activity is regulated by Smad1-dependent signaling in cooperation with Runx2. Previous studies have focused on regulation by NF- $\kappa$ B of *GADD45 $\beta$*  gene expression, which is induced by interleukin (IL)-6, IL-1, TGF- $\beta$ , TNF- $\alpha$ , IL-18, and genotoxic and oxidative stress in various cell types (44, 66). Three  $\kappa$ B elements at positions -447/-438 bp, -426/-417 bp, and -377/-368 bp are responsive to the NF- $\kappa$ B subunit RelA (66). Yoo *et al.* (4) suggested the existence of a TGF- $\beta$ -responsive sequence within the *GADD45 $\beta$* -220/-1-bp promoter region, and potential SMAD enhancers have been identified in both the murine and human promoters (67). Recent studies suggest that both SMAD-dependent and -independent mechanisms could be involved in BMP-2-induced *GADD45 $\beta$*  expression (68, 69). Thus, analysis of the *GADD45 $\beta$*  promoter will require a more extensive study in our chondrocyte models.

Regarding the specific function of Runx2 during endochondral ossification, it serves as a positive regulatory factor in chondrocyte maturation to the hypertrophic phenotype (15, 19, 20) and is expressed in the adjacent perichondrium and in prehypertrophic chondrocytes but

less in late hypertrophic chondrocytes (28, 29), overlapping with *Col10a1* (70, 71). Our findings indicate that Runx2 plays a synergistic role in both the induction and activity of GADD45 $\beta$  by enhancing BMP-2-induced Smad1 signaling and AP-1-dependent *MMP-13* gene expression. The distinct spatial localization of *Gadd45 $\beta$*  mRNA compared with the accumulated intracellular protein in the late hypertrophic zone suggests that it acts in a temporal-spatial manner to regulate the duration of chondrocyte terminal differentiation.

In studies using *Mmp-13*<sup>-/-</sup> mice, MMP-13 deficiency results in significant interstitial collagen accumulation leading to the delay of endochondral ossification in the growth plate and increased length of the hypertrophic zone (23, 24). In *Gadd45 $\beta$* <sup>-/-</sup> mice, we observe defective mineralization at 15.5 and 16.5 dpc persisting at least until 18.5 dpc (data not shown), but decreased length of the hypertrophic zone. This discrepancy may be due to regulation by GADD45 $\beta$  of other activities in hypertrophic chondrocytes, but also the selective loss of MMP-13 in the hypertrophic zone of the *Gadd45 $\beta$* <sup>-/-</sup> growth plate appears to affect the late stage of terminal differentiation only. Furthermore, GADD45 $\beta$  is not the only determinant of the expression of MMP-13, which is expressed also in osteoblasts, osteoclasts, and periosteal cells below the inner periosteal region of ossification (72). Consistent with the persistent mineralization in the bone collar surrounding the *Gadd45 $\beta$* <sup>-/-</sup> growth plate, abundant tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like cells were present in the diaphyseal collar in *Gadd45 $\beta$* <sup>-/-</sup> mice.<sup>6</sup>

In contrast to *Mmp-13* knock-out mice but similar to the *Gadd45 $\beta$*  knock-out mice, both *Col10a1* knock-out mice and transgenic mice with a dominant interference *Col10a1* mutation have subtle growth plate phenotypes with compressed proliferative and hypertrophic zones and altered mineral deposition (73, 74). The decreased *Col10a1* expression because of GADD45 $\beta$  deficiency suggests that GADD45 $\beta$  plays a role in matrix remodeling at the late hypertrophic stage. The chondrodysplasia (cho/cho) mouse, which carries a loss of function mutation in the *Col11a1* gene, has wider long bones and shorter length possibly because type XI collagen influences the formation of type II collagen fibrils at early stages of chondrogenesis (75). Other ECM proteins, including osteocalcin and osteopontin, are known to play functional roles in cell-matrix adhesion during endochondral ossification. Thus, the tight regulation of matrix composition at different stages is required for normal development of long bones.

The dwarfism observed in human chondrodysplasias with *COL10A1* mutations involves skeletal elements that are under great mechanical stress because of disruption in the pericellular matrix in the hypertrophic zone, although a role for defective vascularization has been proposed (73). Several previous studies have focused on vascularization as a critical step in endochondral ossification. Removal of angiogenic stimuli by ablating vascular endothelial growth factor (VEGF) (76) or VEGF receptors (77) resulted in increased length of the hypertrophic zone. A similar phenotype was observed in *Mmp-9*<sup>-/-</sup> mice (78). These and other studies indicate that ECM remodeling is the dominant rate-limiting process for chondrocyte hypertrophy, angiogenesis, and osteoblast recruitment during endochondral ossification (24, 30). Whether GADD45 $\beta$  plays a role in coupling ECM remodeling and vascularization by regulating the expression of MMP-9 or VEGF, in addition to MMP-13, in hypertrophic chondrocytes will require further study.

Although our analyses of *Gadd45 $\beta$* <sup>-/-</sup> mice did not demonstrate complete suppression of hypertrophic differentiation itself, our finding that GADD45 $\beta$  silencing decreases the expression of *Mmp-13* as well as *Col10a1* during terminal differentiation *in vitro* supports a role of GADD45 $\beta$  as a crucial regulator in this process. *Col10a1* is also a Runx2-responsive

<sup>6</sup>K. Ijiri, N. Walsh, and M. B. Goldring, unpublished data.

gene in hypertrophic chondrocytes (14). Thus, GADD45 $\beta$  may be involved, directly or indirectly, in the regulation of other key processes in hypertrophic differentiation through cooperation with Runx2. GADD45 $\beta$  is associated with cell cycle G<sub>2</sub>-M arrest (79), suggesting that it may act as a switch to inhibit chondrocyte proliferation during the transition to hypertrophy. The remodeling of matrix proteins induces an alteration in the environmental stress experienced by hypertrophic chondrocytes (12, 30). Our data suggest that GADD45 $\beta$ , which is highly expressed in response to environmental stresses (44), is an essential stress-response protein during chondrocyte hypertrophy.

The process of chondrocyte death subsequent to terminal differentiation to hypertrophy is also one of the most complicated steps during endochondral ossification, but the mechanism remains unknown. The GADD45 family members are indispensably involved in the mechanism of cell survival and apoptosis in cancer cells (54). Recently, we reported that NF- $\kappa$ B-mediated repression of GADD45 $\alpha$  and GADD45 $\gamma$  is essential for the survival of cancer cells, but we found no relevance for GADD45 $\beta$  in cell survival and apoptosis (40). In fibroblasts, GADD45 $\beta$  is essential for JNK-mediated blockade of TNF- $\alpha$ -induced apoptosis (80). Although our present study does not address whether GADD45 $\beta$  is an anti-apoptotic or pro-apoptotic protein in chondrocytes, its intracellular localization in intact hypertrophic chondrocytes suggests a role as a survival factor that maintains expression of critical genes prior to apoptosis. The decreased length of the hypertrophic zone in *Gadd45 $\beta$ <sup>-/-</sup>* mice suggests that GADD45 $\beta$  may prolong survival of hypertrophic chondrocytes at later stages.

AP-1 transcription factors, including c-Jun, JunB, and JunD, are substrates of the JNK signaling pathway (81), and GADD45 $\beta$  is known to activate the JNK pathway in response to environmental stress (44). This interaction activates MEKK4 kinase leading to MKK4 and, subsequently, JNK activation. However, GADD45 $\beta$  also mediates the NF- $\kappa$ B suppression of JNK signaling in the apoptotic response to TNF- $\alpha$  (54, 80). This controversy surrounding GADD45 $\beta$  and JNK has left open the question of how GADD45 $\beta$  contributes to cell differentiation. We applied a mass spectrometric assay (MALDI-TOF MS) to identify protein-protein interaction partners of GADD45 $\beta$  proteins *in vivo* resulting in identification of MEKK4, the upstream kinase of JNK, as an *in vivo* interactor of GADD45 $\beta$ ,<sup>7</sup> as reported previously (44). Several mitogen-activated protein kinase cascades are involved in BMP-induced signaling during skeletal development. The p38 pathway contributes to the initiation of chondrogenic cellular condensation (82), and ERK1/2 activation cross-interacts with BMP-2-induced signaling to regulate chondrogenesis in a positive manner (83). Our results indicate that chondrocytes also use the JNK/MEKK4 pathway during terminal differentiation.

Several genetic studies have provided insight into the role of AP-1 family members, including c-Fos, Fra-1, c-Fos, FosB, JunB, and ATF-2 in skeletal development *in vivo* (84–89). MMP-13 is also a target of c-Maf, which can form heterodimers with AP-1 family members and regulates the differentiation of hypertrophic chondrocytes (90). Our results show that GADD45 $\beta$ -induced AP-1/JunD activity is essential for MMP-13 expression in hypertrophic chondrocytes and are consistent with previous findings of functional interaction between AP-1 and Runx2 during parathyroid hormone-related protein-dependent MMP-13 expression in osteoblasts (26, 27). The nuclear localization of GADD45 $\beta$  in hypertrophic chondrocytes also suggests a direct mechanism by which AP-1 family members could regulate terminal differentiation.

In conclusion, our data demonstrate a novel role in chondrocyte hypertrophy for GADD45 $\beta$ , which has been implicated in the stress response and cell survival during terminal

<sup>7</sup>L. F. Zerbin, K. Ijiri, M. B. Goldring, and T. A. Libermann, unpublished data.

differentiation of other cell types. It appears to be a major BMP-2-responsive gene induced in chondrocytes by Smad1-dependent signaling in cooperation with Runx2, which immunolocalizes with *GADD45 $\beta$*  mRNA in the prehypertrophic zone of the embryonic growth plate. The intracellular accumulation of Gadd45 $\beta$  protein prominently in the nucleus in intact hypertrophic chondrocytes in the mouse embryonic growth plate and the consequences of *GADD45 $\beta$*  deficiency *in vivo* and *in vitro* indicate that Gadd45 $\beta$  is required for MMP-13 expression in the late hypertrophic zone where Runx2 is also expressed. Our observations that *GADD45 $\beta$*  can stimulate *MMP-13* gene transcription via the JNK/JunD pathway in synergy with Runx2 provides a new concept regarding a temporal and spatial link between BMP-2-induced pathways upstream and the expression of genes required at terminal stages. Together, our findings support a previously undiscovered role for *GADD45 $\beta$*  as a critical mediator of chondrocyte hypertrophy during endochondral ossification.

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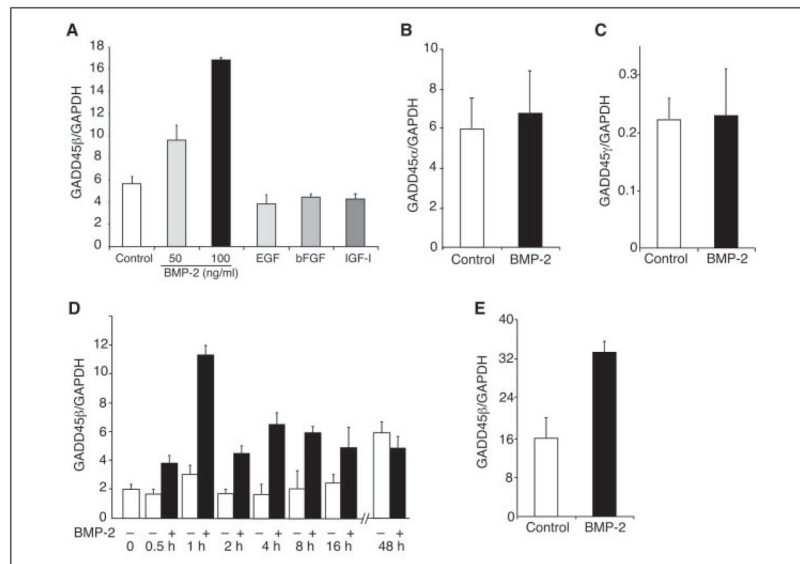
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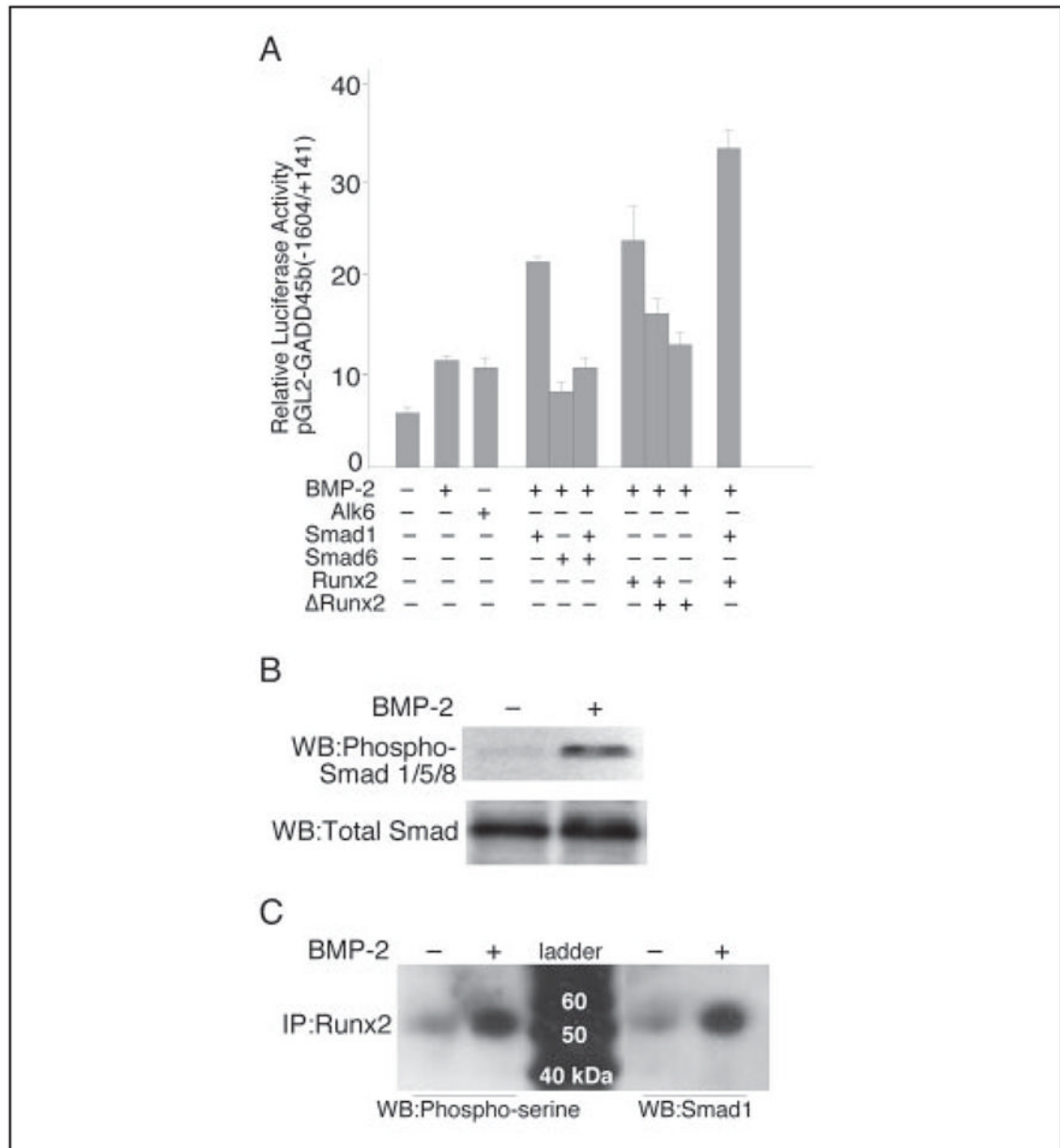
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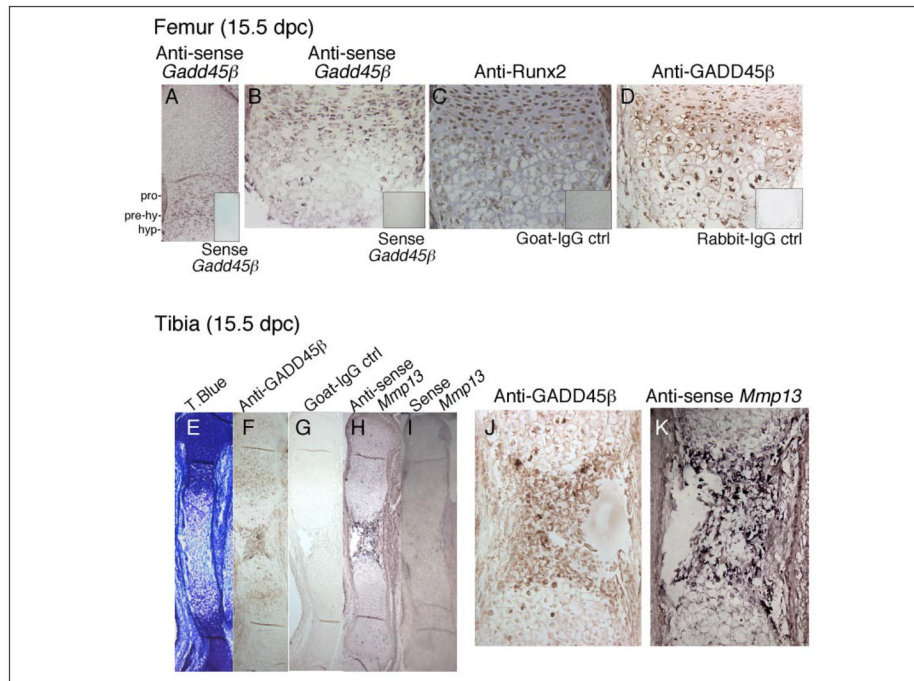


**FIGURE 1. Specific up-regulation of *GADD45β* mRNA by BMP-2 in chondrocytes**  
 Monolayer cultures of the human chondrocyte cell line C-28/I2 were treated as indicated, and total RNA extracts were analyzed by real time PCR. Each value was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in the same sample and shown as the mean  $\pm$  S.D. of triplicate cultures. *A*, *GADD45β* mRNA levels were increased in cultures of the human chondrocyte cell line, C-28/I2, after treatment with BMP-2 for 1 h but not with EGF, bFGF, or IGF-I. *B* and *C*, incubation of C-28/I2 cells with BMP-2 for 1 h did not increase the levels of *GADD45α* or *GADD45γ* mRNA. *D*, time course of BMP-2-induced *GADD45β* mRNA in C-28/I2 cells showed transient induction with a peak at 1 h. *E*, treatment of primary human articular chondrocytes with BMP-2 for 1 h increased the levels of *GADD45β* mRNA by 2-fold.



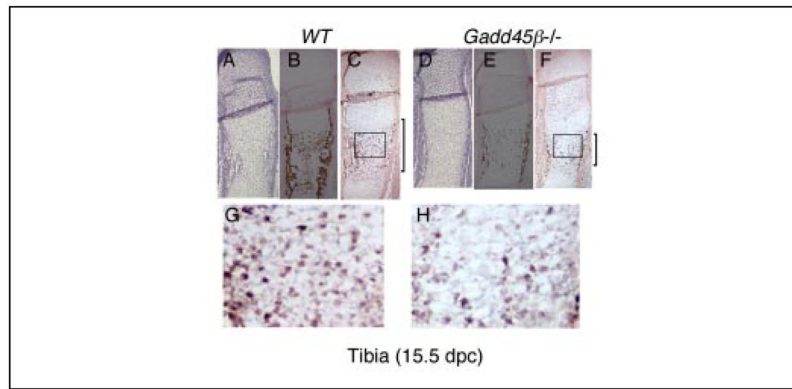
**FIGURE 2. The induction of GADD45 $\beta$  promoter activity by BMP-2 requires signaling by Smad1 interacting with Runx2**

*A*, the pGL2-GADD45 $\beta$  promoter (-1604/+141 bp) was cotransfected with expression vectors encoding the BMP type IB receptor (alk6), Smad1, or Smad6 alone or in combination, or Runx2 or  $\Delta$ Runx2 alone or in combination. The results show the means  $\pm$  S.D. of determinations of samples from three or more separate wells. *B*, the C-28/I2 cells were incubated without (-) or with (+) BMP-2 for 1 h, and total lysates were analyzed on Western blots (WB) using antibodies against phospho-Smad1/5/8 and total Smad1. *C*, total cell lysates were subjected to immunoprecipitation (IP) with anti-Runx2 and analyzed on Western blots using anti-phosphoserine or Smad1 antibody.

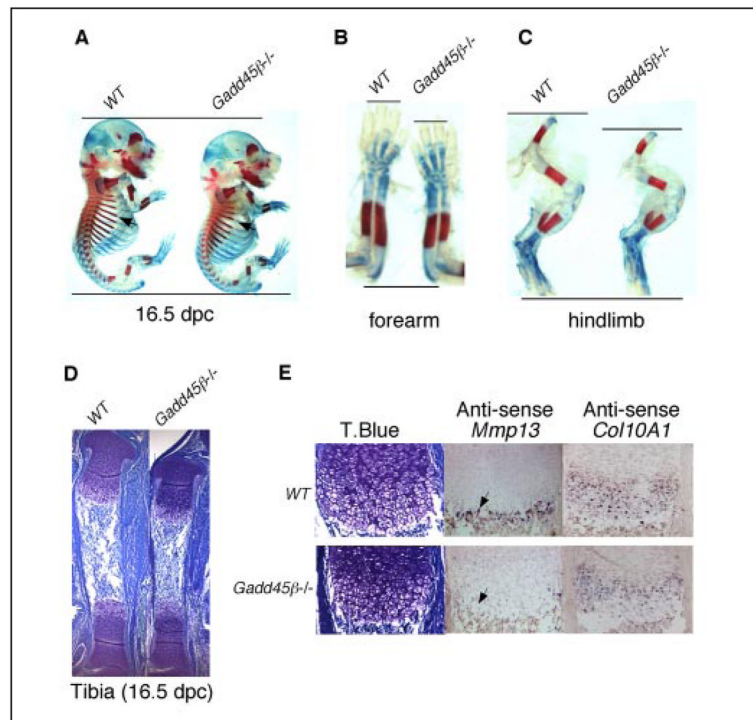


**FIGURE 3. Expression of *Gadd45β* mRNA together with Runx2 protein in prehypertrophic chondrocytes and intracellular GADD45β protein in the hypertrophic chondrocytes that express *Mmp-13* mRNA**

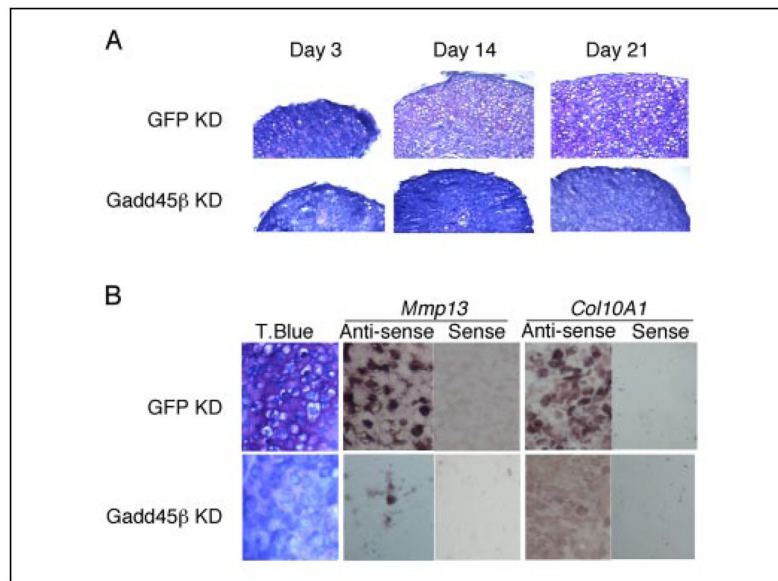
Adjacent longitudinal sections from mouse embryonic femurs or tibiae (15.5 dpc) were analyzed by immunohistochemistry and *in situ* hybridization to illustrate the expression of GADD45β in relation to Runx2 protein and *MMP-13* mRNA in proliferating (*pro*), prehypertrophic (*pre-hy*), and hypertrophic (*hyp*) zones indicated in A. Antisense *Gadd45β* (A and B) and anti-GADD45β antibody (D) were used to detect *Gadd45β* mRNA, and protein and anti-Runx2 antibody (C) detected Runx2 protein in mouse femurs. Sense *Gadd45β* (insets, A and B), normal goat IgG (inset, C), and normal rabbit IgG (inset, D) were applied as negative controls. Tibiae at 15.5 dpc were stained with toluidine blue (T. Blue, E). GADD45β protein was detected in late stage hypertrophic chondrocytes (F and J), in which *Mmp-13* mRNA was also detected using antisense *MMP-13* (H and K). Normal goat IgG (G) and sense *Mmp-13* (I) were applied as negative controls. Brown staining indicates immunodetectable protein, and purple staining indicates a positive signal using antisense probe. Original magnification: A, E–I, ×40; B–D, F, and G, ×200.



**FIGURE 4. Defective mineralization accompanies decreased *Mmp-13* mRNA expression in hypertrophic chondrocytes of *Gadd45 $\beta$ <sup>-/-</sup>* mouse embryos at 15 dpc**  
 Tibiae from WT and *Gadd45 $\beta$ <sup>-/-</sup>* mouse embryos at 15.5 dpc were examined by toluidine blue staining (A and D), von Kossa staining (B and E), and *in situ* hybridization for *Mmp-13* mRNA (C, F, G, and H). Original magnifications: A–F,  $\times 40$ ; G and H,  $\times 200$ .

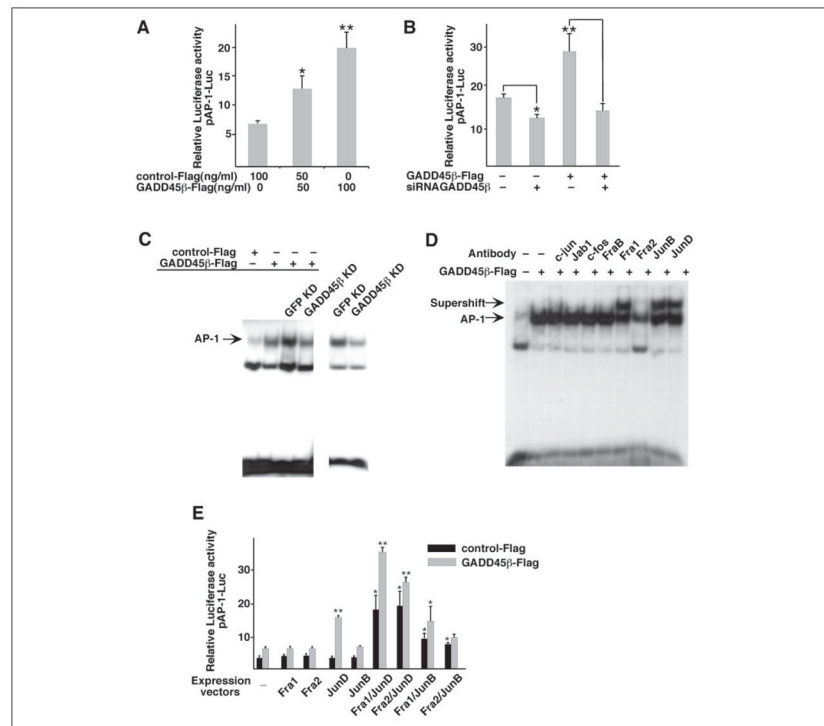


**FIGURE 5. *Gadd45 $\beta$* <sup>-/-</sup> mouse embryos at 16.5 dpc exhibit decreased bone growth associated with decreased levels of *Mmp-13* and *Col10a1* mRNA in hypertrophic chondrocytes**  
 Whole mounted embryos at 16.5 dpc were stained with Alcian Blue and Alizarin Red to show decreased cartilage matrix and mineralization in ribs (A, arrowheads), forearms (B), and hind limbs (C) of *Gadd45 $\beta$* <sup>-/-</sup> compared with WT mice. Adjacent longitudinal sections from the tibia at 16.5 dpc showed a smaller hypertrophic zone by toluidine blue (T.Blue) staining (D) accompanied by markedly decreased levels of *Mmp-13* mRNA (E, arrowhead) and *Col10a1* mRNA in *Gadd45 $\beta$* <sup>-/-</sup> mouse embryos compared with WT. Original magnifications: D,  $\times 40$ ; E,  $\times 200$ .



**FIGURE 6. GADD45 $\beta$  is required for hypertrophic chondrocyte differentiation *in vitro* and associated expression of *Mmp-13* and *Col10a1* mRNA**

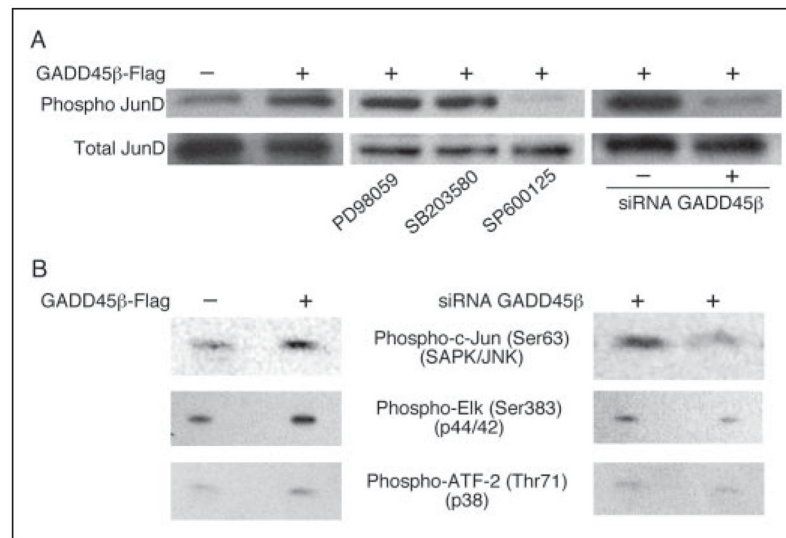
*A*, murine rib growth plate chondrocytes were infected with lentivirus encoding siRNA-GFP to generate GFP KD cells or with siRNA-GADD45 $\beta$  to generate GADD45 $\beta$  KD cells and cultured as three-dimensional pellets for 3, 14, and 21 days. Toluidine blue staining showed the progressive hypertrophy in cultures of GFP KD chondrocytes but not in the cultures of GADD45 $\beta$  KD chondrocytes. Magnification is  $\times 40$ . *B*, after 21 days of culture, toluidine blue (*T. Blue*) staining and the levels of *Col10a1* and *Mmp-13* mRNA, detected by *in situ* hybridization using antisense probes, were markedly decreased in GADD45 $\beta$  KD compared with GFP KD cells. Magnification is  $\times 200$ .



**FIGURE 7. GADD45 $\beta$  positively regulates AP-1 activity and JunD/Fra2 binding in chondrocytes**

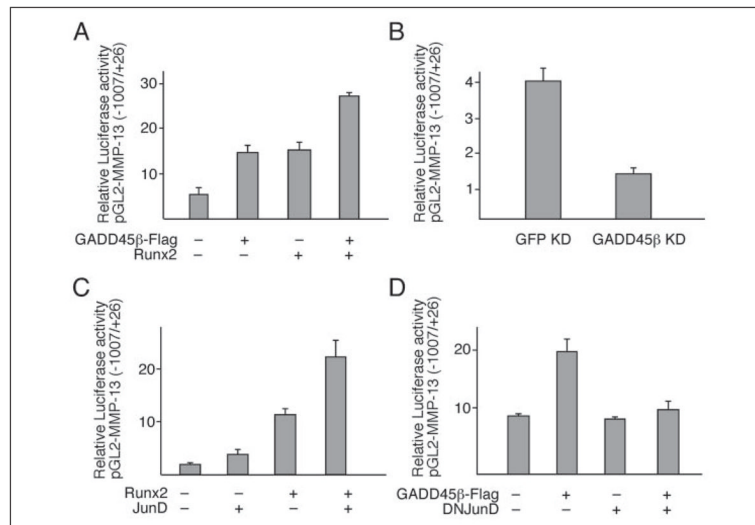
The AP-1-driven luciferase reporter vector (pAP-1-Luc) was cotransfected in C-28/I2 cells with the GADD45 $\beta$  expression vector (GADD45 $\beta$ -FLAG) at 50 and 100 ng/ml (A) or with GADD45 $\beta$ -FLAG alone or together with siRNA-GADD45 $\beta$  (B). C, extracts were prepared from uninfected C-28/I2 cells (1st and 2nd lanes), cells infected with lentiviral siRNA-GFP (GFP KD; 3rd and 5th lanes), or siRNA-GADD45 $\beta$  (GADD45 $\beta$  KD; 4th and 6th lanes). Cells were then transfected with GFP-FLAG (control) or GADD45 $\beta$ -FLAG, as indicated. AP-1 binding activity was examined by EMSA using the double-stranded AP-1 consensus oligonucleotide as labeled probe. The cells extracted for the 5th and 6th lanes (lower exposure of gel shift using probe labeled at different time) were not transfected to show that decreasing endogenous GADD45 $\beta$  also decreases AP-1 binding activity. D, supershift analysis of binding activities in GADD45 $\beta$ -expressing C-28/I2 cells was performed using antibodies against different AP-1 family members. Note that the Fra1, JunB, and JunD antibodies produce supershifts, whereas the Fra2 antibody produces a block shift. E, the C-28/I2 cells were cotransfected with pAP-1-Luc together with expression vectors encoding Fra1, Fra2, JunD, and JunB alone or in combination in the absence or presence of the GADD45 $\beta$ -FLAG. Luciferase activities are shown as means  $\pm$  S.D. of replicates from representative transfections. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; by analysis of variance with subsequent Dunnett test in multiple comparisons. Comparisons in E are with respective controls containing single expression vectors.





**FIGURE 8. JNK activity is necessary for JunD activation by GADD45 $\beta$**

*A*, extracts from C-28/I2 cells expressing GADD45 $\beta$ -FLAG or siRNA-GADD45 $\beta$  were analyzed by Western blotting using antibodies against phosphorylated (*phospho*) or total JunD. GADD45 $\beta$ -expressing cells were also treated with inhibitors of ERK (*PD98059*), p38 (*SB203580*), and JNK (*SP600125*) to examine the roles of the different kinases in JunD phosphorylation by GADD45 $\beta$ . *B*, kinase activities were analyzed on Western blots to show increased JNK (*phospho-c-Jun*) and ERK (*phospho-Elk*) activities in GADD45 $\beta$ -FLAG cells and decreased activities in cells expressing siRNA-GADD45 $\beta$ . Note that p38 activity (*phospho-ATF-2*) did not change.



**FIGURE 9. JunD-dependent MMP-13 promoter activation by GADD45β in cooperation with Runx2 in the ATDC5 chondrogenic cell line**

The ATDC5 cells were cotransfected with the pGL2-MMP-13 promoter (-1007 bp/+26 bp) construct and expression vectors encoding GADD45β and Runx2 (A), siRNA-GADD45β (B), Runx2 and JunD (C), or GADD45β and dominant negative (DN) JunD (D). The results show the means ± S.D. of determinations in triplicate wells from representative experiments. Empty expression vector controls or promoter-less pGL2-B did not induce or express any significant luciferase activity (not shown).

**TABLE ONE**

Microarray analysis of GADD45 family genes in chondrocytes treated with BMP-2. Subconfluent cultures of the immortalized human chondrocyte cell line, C-28/I2, were incubated for 1 h in the absence (control) or presence of 100 ng/ml of BMP-2. Total RNA was applied to the HU133A Affymetrix GeneChip.

Probe id	Gene	Control	BMP-2	FC	LCB	Rank
207574_s_at	GADD45 $\beta$	285.86	832.17	2.91	2.71	13
209304_x_at	GADD45 $\beta$	420.60	1051.55	2.5	2.26	31
209305_s_at	GADD45 $\beta$	266.40	668.53	2.51	2.2	37
203725_at	GADD45 $\alpha$	529.55	512.18	-1.03	-1	NA
204121_at	GADD45 $\gamma$	221.52	251.23	1.13	1.04	NA

Probe identification (id), Affymetrix identifier for separate probes on the microarray; FC, fold change between control and BMP-2-treated sample; LCB, lower confidence bound; NA, not applicable.