Bcl-2 Lies Downstream of Parathyroid Hormone–related Peptide in a Signaling Pathway That Regulates Chondrocyte Maturation during Skeletal Development

Michael Amling,*‡§ Lynn Neff,*‡ Sakae Tanaka,*‡ Daisuke Inoue,*‡ Keisuke Kuida,‖ Eleanor Weir,¶ William M. Philbrick,¶ Arthur E. Broadus,¶** and Roland Baron*‡

*Department of Cell Biology, ‡Department of Orthopaedics, ¶Department of Internal Medicine, **Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut, 06510; §Department of Bone Pathology, Hamburg University School of Medicine, 20246 Hamburg, Germany; and ‖Tokyo Metropolitan Institute of Medical Science, Tokyo 113, Japan

Abstract. Parathyroid hormone–related peptide (PTHrP) appears to play a major role in skeletal development. Targeted disruption of the PTHrP gene in mice causes skeletal dysplasia with accelerated chondrocyte maturation (Amizuka, N., H. Warshawsky, J.E. Henderson, D. Goltzman, and A.C. Karaplis. 1994. *J. Cell Biol.* 126:1611–1623; Karaplis, A.C., A. Luz, J. Glowacki, R.T. Bronson, V.L.J. Tybulewicz, H.M. Kronenberg, and R.C. Mulligan. 1994. *Genes Dev.* 8: 277–289). A constitutively active mutant PTH/PTHrP receptor has been found in Jansen-type human metaphyseal chondrodysplasia, a disease characterized by delayed skeletal maturation (Schipani, E., K. Kruse, and H. Jüppner. 1995. *Science (Wash. DC).* 268:98–100). The molecular mechanisms by which PTHrP af-

fects this developmental program remain, however, poorly understood. We report here that PTHrP increases the expression of Bcl-2, a protein that controls programmed cell death in several cell types, in growth plate chondrocytes both in vitro and in vivo, leading to delays in their maturation towards hypertrophy and apoptotic cell death. Consequently, overexpression of PTHrP under the control of the collagen II promoter in transgenic mice resulted in marked delays in skeletal development. As anticipated from these results, deletion of the gene encoding Bcl-2 leads to accelerated maturation of chondrocytes and shortening of long bones. Thus, Bcl-2 lies downstream of PTHrP in a pathway that controls chondrocyte maturation and skeletal development.

P ARATHYROID hormone–related peptide (PTHrP)[1] was initially isolated from human carcinomas (Strewler et al., 1987; Suva et al., 1987; Mangin et al., 1988) and is responsible for the humoral hypercalcemia associated with various malignancies. PTHrP is structurally related to parathyroid hormone (PTH), a hormone of major importance in calcium metabolism. Both peptides share 8 of 13 NH_2-terminal residues and bind to and activate the same G-protein–coupled PTH/PTHrP receptor (Jüppner et al., 1991). Unlike PTH, however, PTHrP does not circulate in appreciable amounts in normal subjects but is instead, widely expressed in fetal and adult tissues, where it is thought to regulate cell differentiation, cell pro-

liferation, and organogenesis as a paracrine or autocrine-soluble factor (Goltzman et al., 1989; Broadus and Stewart, 1994; Wysolmerski et al., 1994). In this context, PTHrP is a mediator of cellular growth and differentiation (Amizuka et al., 1994; Karaplis et al., 1994) and is involved in mesenchymal–epithelial interactions in several tissues (Hardy, 1992; Van de Stolpe et al., 1993; Wysolmerski et al., 1994).

The critical role played by PTHrP and its receptor in skeletal development has recently been emphasized by gene deletion experiments in mice and by a natural mutation in humans. Mice homozygous for PTHrP gene ablation exhibit skeletal deformities that are due to a decrease in proliferation and the accelerated differentiation of chondrocytes in the developing skeleton (Amizuka et al., 1994; Karaplis et al., 1994). At the other end of the spectrum, striking skeletal deformities are observed in Jansen's metaphyseal chondrodysplasia, a human form of short-limbed dwarfism with delayed endochondral maturation (Jansen, 1934). This has been recently attributed to a single heterozygous nucleotide exchange in exon M2 of the

1. *Abbreviations used in this paper*: col II, collagen type II; PTH, parathyroid hormone; PTHrP, parathyroid hormone–related peptide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

gene encoding for the PTH/PTHrP receptor, resulting in a constitutively active mutant PTH/PTHrP receptor (Schipani et al., 1995). Although these findings clearly establish that PTHrP plays a regulatory role in the process of endochondral ossification, the precise mechanism by which PTHrP affects skeletal development is not known.

During endochondral ossification, the chondrocytes present in the early cartilaginous model, and later in the growth plate, first proliferate and then progressively differentiate into mature hypertrophic chondrocytes. Once fully differentiated, these hypertrophic cells participate in the mineralization of the cartilaginous matrix and undergo cell death. In normal bone development, this is followed by, and may be the necessary signal for, the local recruitment of blood vessels and osteoclasts into the zone of provisional mineralization. This leads to the progressive replacement of cartilage by bone, the homing of the hematopoietic bone marrow, and ultimately, longitudinal bone growth. In bone, osteoblasts and growth plate chondrocytes express PTH/PTHrP receptors and secrete PTHrP, suggesting the existence of autocrine/paracrine regulatory loops (Jüppner et al., 1988; Amizuka et al., 1994). The latter appear to be essential for normal chondrocyte maturation and/or endochondral bone formation, as shown by the various transgenic models and natural mutation discussed above (Amizuka et al., 1994; Karaplis et al., 1994; Schipani et al., 1995).

Apoptosis has been proposed as the mechanism responsible for the death of chondrocytes during endochondral bone formation (Farnum and Wilsman, 1989; Lewinson and Silbermann, 1992). In several cell types, apoptosis is regulated by the ratio of expression of the cell death inhibitor, Bcl-2, and the cell death inducer, Bax. Bcl-2 is the founding member of an emerging family of proteins whose function involves the regulation of programmed cell death (Vaux et al., 1988; Korsmeyer, 1992). Bax is another Bcl-2 family member which forms heterodimers with Bcl-2 and, when overexpressed, counters the anti-apoptotic effect of Bcl-2, causing accelerated cell death. Within a cell, it is the ratio of Bcl-2 to Bax that determines whether a cell dies or not. It has been shown that apoptosis is repressed when half or more of the endogenous Bax is heterodimerized with Bcl-2 (Oltvai et al., 1993; Miyashita et al., 1994; Yin et al., 1994; Sedlak et al., 1995).

The temporal–spatial distribution of Bcl-2 suggests that it serves to regulate apoptotic cell death during embryonic development and in adulthood. Bcl-2 is widely expressed among fetal tissues, with substantial levels present in the developing limb bud (Veis Novack and Korsmeyer, 1994), and apoptosis is now recognized as an important process in organogenesis and development. In the adult, Bcl-2 expression is limited to renewing stem cell populations, such as those found in hematopoietic lineages, complex differentiating epithelia, and glandular epithelia, and to long-lived postmitotic cell populations (Hockenbery et al., 1991). Several observations led us to hypothesize that PTHrP and Bcl-2 might participate in the same regulatory pathways during skeletal development: (*a*) the temporal–spatial similarities of the expression patterns of PTHrP (Lee et al., 1995) and Bcl-2 (Veis Novack and Korsmeyer, 1994); and the characteristic changes in endochondral bone formation due to either the absence of PTHrP (Amizuka et al., 1994) or the constitutive activation of its receptor (Schipani et al., 1995).

We report here that Bcl-2 and Bax are expressed in chondrocytes in vivo and show a characteristic distribution within the developing growth plate, which is consistent with their role in regulating chondrocyte programmed cell death. Furthermore, we provide evidence that Bcl-2 is a direct player and not just a bystander in skeletal development, since accelerated endochondral bone maturation occurs in *bcl-2* knockout mice, leading to a phenotype paralleling that of the PTHrP knockout mice (Amizuka et al., 1994). Finally, we present in vitro and in vivo evidence, using transgenic mice with overexpression of PTHrP targeted to chondrocytes, that Bcl-2 is downstream of PTHrP in a signaling pathway that is required for normal skeletal development.

Materials and Methods

Confocal Immunofluorescence Analysis

The antibodies used in these experiments include a rabbit anti-mouse collagen type X antibody (dilution 1:100) generously provided by Dr. B. Olsen (Harvard University, Cambridge, MA), polyclonal antibodies against mouse Bcl-2 (dilution 1:200) obtained from PharMingen (San Diego, CA), and Bax (dilution 1:200) obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The fluorescein-conjugated secondary antibodies (dilution 1:100) and the normal goat serum were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). For immunofluorescence analysis, cultured cells plated on glass coverslips, 6 μm frozen sections, and 6 μm paraffin embedded sections of 6-d-old mice [(Procollagen Ia type II) col II-PTHrP transgenic mice and control littermates] were used. Mice were perfused via the heart with paraformaldehyde (2%), lysine (0.75 M), and sodium periodate (0.01 M) (PLP), for 5 min. The long bones were dissected out and fixed in PLP for an additional 4 h at 4°C, washed in PBS for 1 h, and infiltrated overnight with 40% sucrose in PBS. The tissue was subsequently quick-frozen or embedded in paraffin. The cultured cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature and washed in PBS. Further processing of tissue sections and isolated cells was identical. For collagen X staining, the samples were preincubated with bovine hyaluronidase in PBS (1 mg/ml) for 45 min at 37°C. All subsequent incubations were performed at room temperature with PBS containing 0.05% saponin, 0.1% BSA, and 5% normal goat serum (NGS). The samples were incubated in PBS–saponin–BSA–NGS for 30 min to block nonspecific binding, and then for 2 h with the primary antibody. The samples were washed in PBS–saponin–BSA and incubated with secondary antibody for 1 h in the dark. After washing in PBS, the samples were mounted in FluorSave fluorescent mounting media (Calbiochem-Novabiochem Corp., La Jolla, CA). Samples were examined with a scanning laser confocal microscope (Axiovert 10, Zeiss, Inc., Thornwood, NY and MRC 600 confocal imaging system; Bio-Rad Laboratories, Richmond, CA) with a krypton/argon laser using an optical slice thickness of 1–2 μm. Computer images were collected on optical memory discs and were computer enhanced with the Adobe Photoshop program.

In situ Analysis of Apoptotic Cells

Terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick end-labeling (TUNEL) was performed using a molecular biological–histochemical system, ApopTag kit (Oncor, Gaithersburg, MD) for specific staining of DNA fragmentation and apoptotic bodies (Gavrieli et al., 1992). Cells exhibiting DNA fragmentation and containing apoptotic bodies, thereby morphologically consistent with apoptosis, are referred to as apoptotic cells. TdT, which catalyzes a template-independent addition of deoxyribonucleotide to 3′-OH ends of DNA, was used to incorporate digoxigenin-conjugated dUTP to the ends of DNA fragments. Briefly, after digesting protein in quick-frozen bone sections (control mice and col II-PTHrP transgenic mice) with 10 μg/ml proteinase K at room temperature and then quenching endogenous peroxidase activity with 1% H_2O_2 in PBS, slides were placed in equilibration buffer, and then in terminal deoxynucleotidyl transferase solution, followed by stop/wash buffer. The signal

of TUNEL was then detected by an anti-digoxigenin antibody conjugated with peroxidase, a reporter enzyme that catalytically generates a brown-colored product from the chromogenic substrate diaminobenzidine. Because processing in paraffin may increase the number of apparent apoptoses, we used cryosections. Controls for specificity of labeling included positive control slides prepared by nicking DNA with DNAse I (Boehringer Mannheim Biochemicals) (Arends et al., 1990), and negative control sections were prepared by substituting distilled water for TdT enzyme. The slides were washed, dried, and mounted in Permount media.

Cell Cultures

Chondrocytes were isolated from the resting zone of the proximal tibia and distal femur of 10-d–old mice. Growth plates were cleaned of perichondrium; the reserve zone was dissected by a transverse cut through the calcification zone and separated from the epiphysis under a dissecting microscope. Pooled growth plates from 10 to 12 animals were predigested for 30 min in a 0.1% solution of clostridial collagenase Ia (Sigma Chemical Co., St. Louis, MO) in culture media (Ham F-12/DME 1:1, 10% FBS, 1% penicillin/streptomycin [PS], 50 μg/ml L-ascorbic acid, 100 μg/ml sodium pyruvate) in a shaker at room temperature. Cartilage fragments were then washed twice in culture media and subsequently digested for 3 h, in a 0.2% solution of collagenase in culture media, in an incubator shaker at 37°C. The solution containing the isolated cells was filtered (through 50 μm mesh), and cells were recovered by centrifugation (1,500 rpm, 4°C, 5 min). Cells were resuspended in culture medium at a final concentration of 300,000 cells/ml. Cells were cultured as high-density monolayers in 24-well dishes (150,000 cells/well) (Falcon Labware, Oxnar, CA) and in 60-mm dishes (10^6 cells/dish) for protein extraction. Medium was changed every other day after the third day. PTH 1-34 and PTHrP 1-37 (both at a final concentration of 10^{-7} M) or forskolin (final concentration 50 μM) were added with fresh media every other day. Mouse primary osteoblastic cells were obtained from 3-d–old mouse calvaria and cultured in αMEM containing 10% FBS and 1% PS. The human renal carcinoma cell line (CAKI) was cultured in RPMI containing 10% FBS and 1% PS. All cultures were maintained at 37°C, gassed with 95% air/5% CO_2.

Western Blot Analysis

All extraction procedures were performed at 4°C. Cells were washed once with ice-cold PBS, and cell lysates were generated with ice-cold RIPA buffer (10% mM Tris-HCl, pH 7.2, 158 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (0.1 mM PMSF, 1 μM pepstatin, and 1 μM leupeptin) which disrupt Bcl-2/Bax interactions. RIPA extracts were prepared by sonication and subsequent centrifugation at 12,000 g for 10 min. The protein was eluted by boiling in sample buffer (62.5 mM Tris–HCl, pH 6.9, 2 mM EDTA, 3% SDS, 3.75% glycerol, and 180 mM β-mercaptoethanol) for 2 min. Western blotting was performed using the enhanced chemiluminescence Western blotting detection reagents (Amersham Corp., Arlington Heights, IL) according to the conditions recommended by the supplier. Briefly, samples (chondrocytes and osteoblasts, 200 μg/lane, kidney cells, 100 μg/lane) were analyzed by 12.5% SDS-PAGE followed by electrophoretic transfer of the proteins to nitrocellulose filters. Filters were first blocked in TBST (20 mM Tris, pH 7.6, 134 mM NaCl, 0.1% Tween-20) containing 5% Carnation nonfat dry milk for 2 h and then incubated with the rabbit anti–Bcl-2 N-19 (Santa Cruz Biotech.) polyclonal antibody (1:200 in TBST) for 6 h at 4°C. After three sequential 15 min washes in TBST, the filters were incubated with anti–rabbit peroxidase-conjugated secondary antibody (1:20,000 in TBST) for 1 h at room temperature and then again washed as described above. For human kidney cells, filters were first incubated with 5% BSA in TBST, to block nonspecific binding, and then with monoclonal mouse anti–human Bcl-2 124 (dilution 1:200 in TBST) (DAKO Corp., Carpinteria, CA); anti–mouse peroxidase-conjugated secondary antibody was used as described above. Bound protein was detected by enhanced chemiluminescence reaction. The blots were then stripped of antibody and reprobed with anti-actin monoclonal antibody (Boehringer Mannheim Biochemicals, 1 μg/ml) to confirm that equal amounts of protein were loaded in each corresponding treated and untreated lane.

Generation and Analysis of col II-PTHrP Transgenic Mice

Transgenic mice were generated as previously described (Weir et al., 1996). Briefly, a 568-bp human PTHrP cDNA fragment encoding the 1-141 isoform was inserted into a 6.5-kb segment of the mouse procollagen α1 type II (col II) promoter region (obtained from S. Garofolo, Shriners Hospital, Portland, OR and B. de Crombrugghe, M.D. Anderson Cancer Center, Houston, TX) at a site within the first intron. A consensus splice acceptor (courtesy of P. Soriano, Fred Hutchinson Cancer Research Center, Seattle, WA) was included in the col II construct at the 5' end of the insertion point to create an artificial exon and thus circumvent alternative splicing. The initiation codon in exon 1 of the procollagen gene was inactivated to allow translation to start within the cDNA sequences. Also, a 2.2-kb segment of the human growth hormone gene was added downstream of the cDNA to provide termination/polyadenylation signals and to increase expression efficiency. The growth hormone coding sequences are not translated.

For histology, col II-PTHrP transgenic mice and control littermates (6 d of age) were perfused via the heart with PLP for 5 min. Metatarsals were dissected out and fixed by 3.7% paraformaldehyde for 12 h at 4°C, embed-
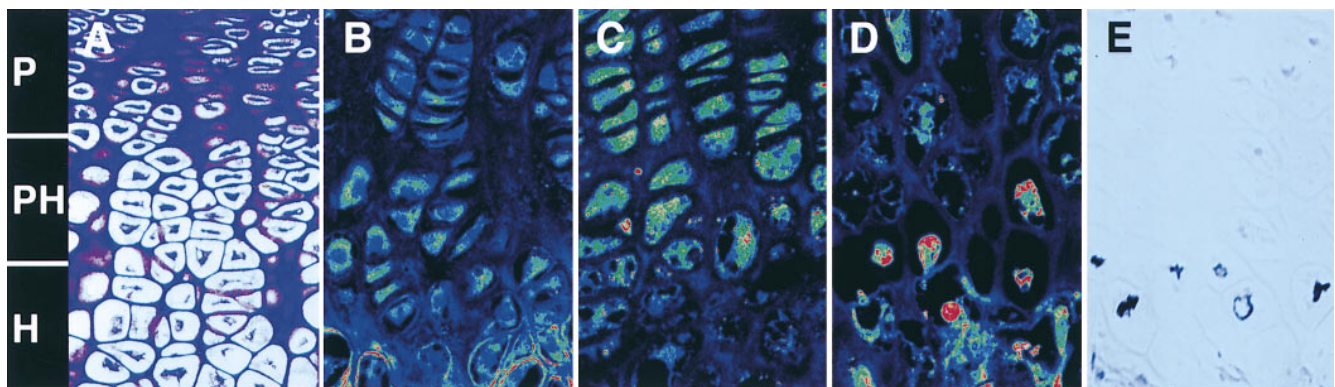

Figure 1. Expression of Bcl-2 and Bax in endochondral bone formation. (*A*) The normal growth plate shows a typical zonal structure of differentiating chondrocytes. Proliferating chondrocytes (*P*) progressively enlarge to prehypertrophic chondrocytes (*PH*), and further differentiate into hypertrophic chondrocytes (*H*). (*B*) Age-matched growthplates were labeled by immunofluorescence for collagen type X, which serves as a specific marker in the chondrocyte differentiation process. Intracellular expression of collagen X is characteristic of prehypertrophic chondrocytes, whereas the dense, pericellular matrix labeling shown in red is found solely in the hypertrophic zone. (*C*) Highest levels of Bcl-2 protein are detected in late proliferative and prehypertrophic chondrocytes, whereas, (*D*), hypertrophic chondrocytes exhibit an increased Bax protein expression. Age-matched frozen sections were labeled for DNA fragmentation by TUNEL. (*E*) Nick end labeling of DNA fragments was observed only in fully differentiated hypertrophic chondrocytes, at the level where resorption of cartilage and replacement by bone is taking place.
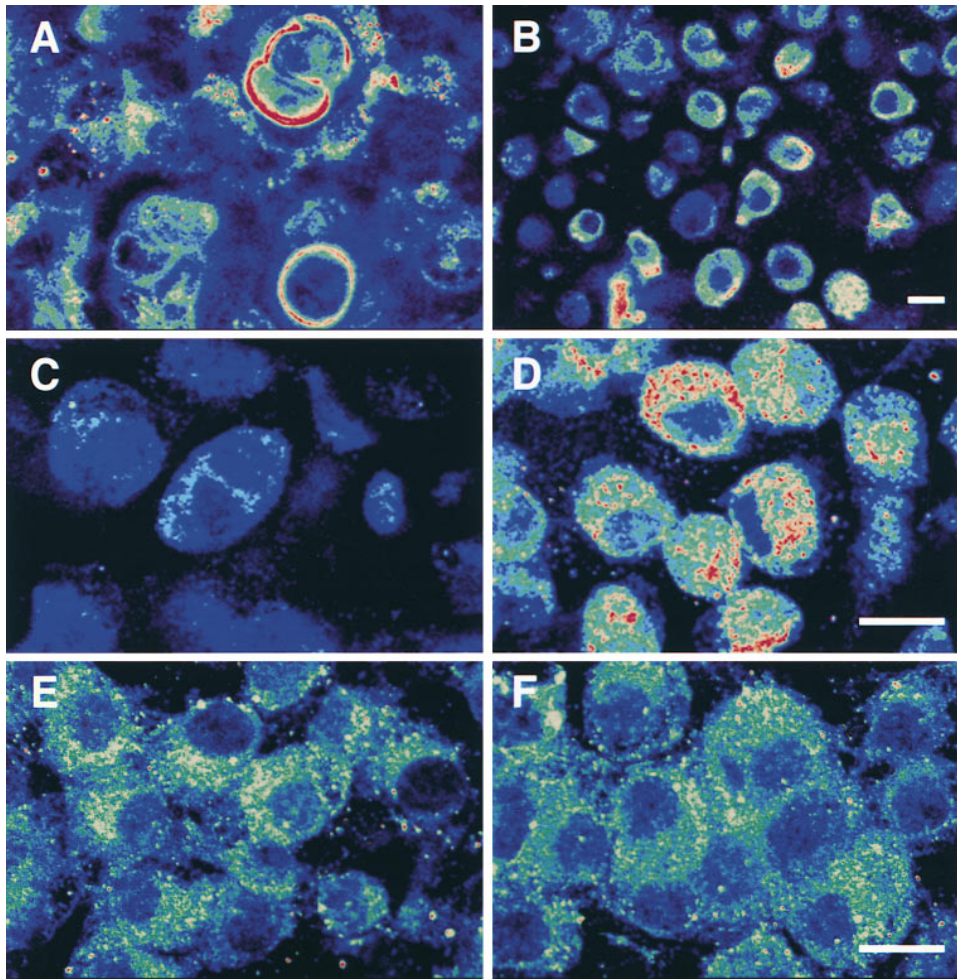
Figure 2. PTHrP increases Bcl-2 expression in chondrocytes in vitro. (*A*) Murine chondrocytes, cultured for 24 d as high-density monolayers, differentiated in vitro into hypertrophic chondrocytes with the typical extracellular matrix pattern of collagen X (compare to Fig. 1 *B*). (*B*) In contrast, treatment of these cultures with PTHrP 1-37 resulted in an inhibition of terminal differentiation and an accumulation of prehypertrophic chondrocytes, as confirmed by the pathognomonic intracellular collagen X expression. After 12 d in culture, a time at which treated and untreated cultures show no apparent morphological differences, (*C*) the untreated chondrocytes show only low cytoplasmic Bcl-2 protein expression, whereas, (*D*), a marked increase in Bcl-2 expression is visible after PTHrP treatment. In contrast, the same treatment had no detectable effect on the level of Bax protein expression (*E*, control; *F*, treated). Bars, 10 μm.

ded in epon, and 2 μm sections were prepared on a ultramicrotome (Reichert Scientific Instruments, Buffalo, NY). Sections were stained with toluidine blue and evaluated on a Reichert microscope (UnivaR, C. Reichert, Austria). Immunohistochemical analysis and TUNEL analysis were done as stated above for confocal immunfluorescence analysis and in situ analysis of apoptotic cells, respectively.

Generation and Histology of bcl-2 Knockout Mice

Generation of germ line mutant mice carrying ablated *bcl-2* coding regions was previously described (Nakayama et al., 1993; 1994). Offspring from heterozygote intercrosses were genotyped by PCR and by Southern blot analysis, as previously reported (Nakayama et al., 1993; 1994). After radiological analysis of the skeleton, histomorphometric analysis was performed on undecalcified, processed bones of neonate, 6-d, and 60-d–old knockout mice and control littermates (Hahn et al., 1991). Three samples each were analyzed, and statistical analysis was performed using Student's *t* test.

Results

Localization of Bcl-2 and Bax in Normal Growth Plates

Bcl-2 protein is expressed in chondrocytes throughout the growth plate, with highest levels in late proliferative and prehypertrophic chondrocytes (characterized by their intracellular expression of collagen type X), and markedly decreased levels in late hypertrophic chondrocytes (characterized by their morphology and pathognomonic, pericellular, ring-like secretion of collagen type X) (Fig. 1, *A–C*).

The opposite pattern was observed for Bax protein expression, with undetectable levels in proliferative cells and a progressive increase towards hypertrophic chondrocytes (Fig. 1 *D*). Thus, within the growth plate, the ratio of Bcl-2 to Bax progressively decreases in favor of Bax, and fully differentiated (hypertrophic) chondrocytes die in an apoptotic manner, as confirmed by nick end-labeling of DNA fragments by TUNEL (Gavrieli et al., 1992) (Fig. 1 *E*).

PTHrP Increases Bcl-2 Expression in Chondrocytes In Vitro

Treatment of cultured murine growth plate chondrocytes with the NH$_2$-terminal fragments PTHrP 1-37 (Figs. 2 and 3) or PTH 1-34 (data not shown) resulted in an increase in cAMP production and a marked increase in Bcl-2 expression. By comparing pixel intensity of Bcl-2 immunolabeling, differences in Bcl-2 levels in treated and untreated chondrocyte cultures were clearly detectable, with a twofold increase in treated culture, as early as 3 d after starting PTHrP treatment, i.e., before the appearance of hypertrophic cells (data not shown). In parallel, in longer cultures (12 or 24 d), we observed an inhibition of chondrocyte differentiation, as determined by reduced expression of alkaline phosphatase and matrix calcification (data not shown); a marked decrease in the number of hypertrophic cells; and an accumulation of prehypertrophic chondrocytes

(Fig. 2, *A* and *B*). This increase in Bcl-2 expression was found to be cAMP dependent and was mimicked in these cultures by the addition of Forskolin (data not shown). Bax expression was lower and was not affected by PTH or PTHrP treatment (Fig. 2 *E*). Thus, in vitro treatment of chondrocytes with PTH or PTHrP results in a shift of the Bcl-2/Bax ratio in favor of Bcl-2, a change that delays terminal differentiation, prolongs chondrocyte survival, and leads to the accumulation of cells in their prehypertrophic stage.

As shown in Fig. 3, the effects of PTHrP on Bcl-2 expression were found to be restricted to chondrocytes, since treating both primary osteoblasts and a kidney cell line, that expresses the PTH/PTHrP receptor, with PTH or PTHrP failed to alter Bcl-2 levels in these other cell types, despite the fact that they responded to PTH/PTHrP with the expected increase in cAMP.

Targeted Overexpression of PTHrP to Chondrocytes In Vivo Leads to Increased Bcl-2 Expression and Delayed Skeletal Development

Targeted overexpression of PTHrP to chondrocytes, using the collagen type II promoter in transgenic mice, leads to a profound delay in endochondral bone formation with Jansen's metaphyseal dysplasia–like skeletal abnormalities (Weir et al., 1996). As shown on Fig. 4, this also leads to a marked increase in Bcl-2 expression in prehypertrophic chondrocytes, with no detectable change in Bax levels. At 6 d of age, the metatarsals of col II–PTHrP transgenic animals were still at the cartilaginous model stage with an accumulation of prehypertrophic chondrocytes, thereby mimicking the effects of PTHrP on chondrocytes in culture. These cells contained intracellular collagen type X (data not shown). Hypertrophic or apoptotic chondrocytes were not detectable, as further confirmed by the absence of ex-
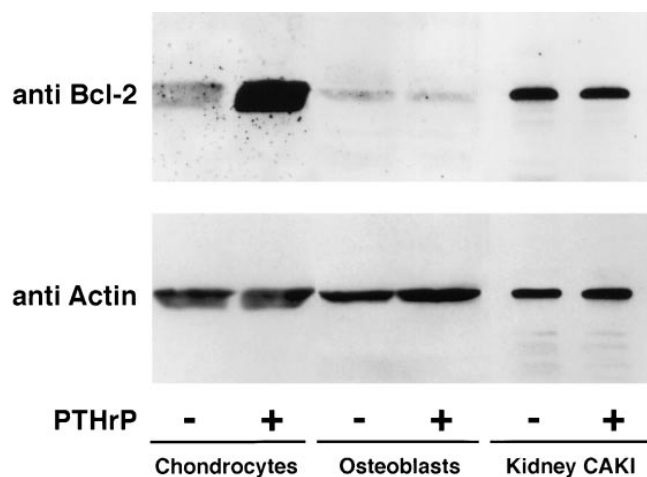

Figure 3. PTHrP effect on Bcl-2 is restricted to chondrocytes. Western blotting analysis revealed that treatment of murine chondrocytes in vitro (cultured for 12 d) with PTHrP 1-37 resulted in a marked increase in Bcl-2 expression. In contrast, the same treatment had no detectable effect on Bcl-2 levels in primary osteoblasts, obtained from 3-d–old mouse calvaria, and a kidney cell line (*CAKI*), both of which express the PTH/PTHrP receptor. The blots were then stripped of antibody and reprobed with anti-actin antibody to confirm that equal amounts of protein were loaded in each corresponding treated and untreated lane.

tracellular collagen type X rings and signal from TUNEL analysis (data not shown). In contrast with control littermates, the formation of bone or bone marrow was not yet apparent in the transgenic animals (Fig. 4).

Accelerated Endochondral Bone Formation in bcl-2 Knockout Mice

To determine whether Bcl-2 is directly involved in endochondral bone formation and whether its characteristic expression pattern within the growth plate is related to a functional role in regulating chondrocyte terminal differentiation, we analyzed the skeleton in *bcl-2* knockout mice. We found that *bcl-2* knockout mice exhibit premature chondrocyte maturation and terminal differentiation. Consequently, the program of endochondral bone formation is significantly accelerated, leading to earlier vascular invasion, replacement of cartilage by bone, and homing of bone marrow cells, as compared to control littermates (Fig. 5). Furthermore, *bcl-2* knockout mice exhibit a reduction in the growth plate thickness, mostly due to a decrease in the height of proliferative zone, and ultimately a significant decrease (15%–20%) in overall bone length. These data demonstrate that Bcl-2 is a direct player in the regulation of the endochondral bone formation and that *bcl-2* knockout mice display a skeletal phenotype paralleling that of PTHrP knockout mice, though far less pronounced (Fig. 5).

Discussion

The present study suggests that PTHrP, a major player in chondrocyte maturation and endochondral bone formation, exerts its effect on skeletal development via a pathway involving Bcl-2 expression and alterations in chondrocyte maturation and apoptotic cell death.

Bcl-2 is expressed in chondrocytes throughout the growth plate, with highest levels in late proliferative and prehypertrophic chondrocytes, and markedly decreased levels in late hypertrophic chondrocytes, a pattern of expression similar to that of PTHrP (Amizuka et al., 1994). The opposite pattern was observed for Bax expression, with undetectable levels in proliferative cells and a progressive increase towards hypertrophic chondrocytes. Thus, within the growth plate, the ratio of Bcl-2 to Bax progressively decreases in chondrocytes in favor of Bax. This should result in the apoptotic death (Oltvai et al., 1993) of fully differentiated (hypertrophic) chondrocytes, as confirmed by morphology and nick end-labeling of DNA fragments by TUNEL and previously suggested by others (Farnum and Wilsman, 1989; Lewinson and Silbermann, 1992; Henderson et al., 1995).

The presence of short ears and short and deformed limbs in the *bcl-2* knockout mice (Nakayama et al., 1993; Nakayama et al., 1994; Veis et al., 1993) also suggests a functional role for Bcl-2 in chondrocyte maturation. To directly study the role of Bcl-2 in chondrocyte differentiation, we analyzed the skeletal phenotype in *bcl-2* knockout. We found that despite the existence of nine family members and efficient redundancies, the pathway of endochondral bone formation is significantly altered in *bcl-2* knockout mice. A marked reduction in growth plate thick-
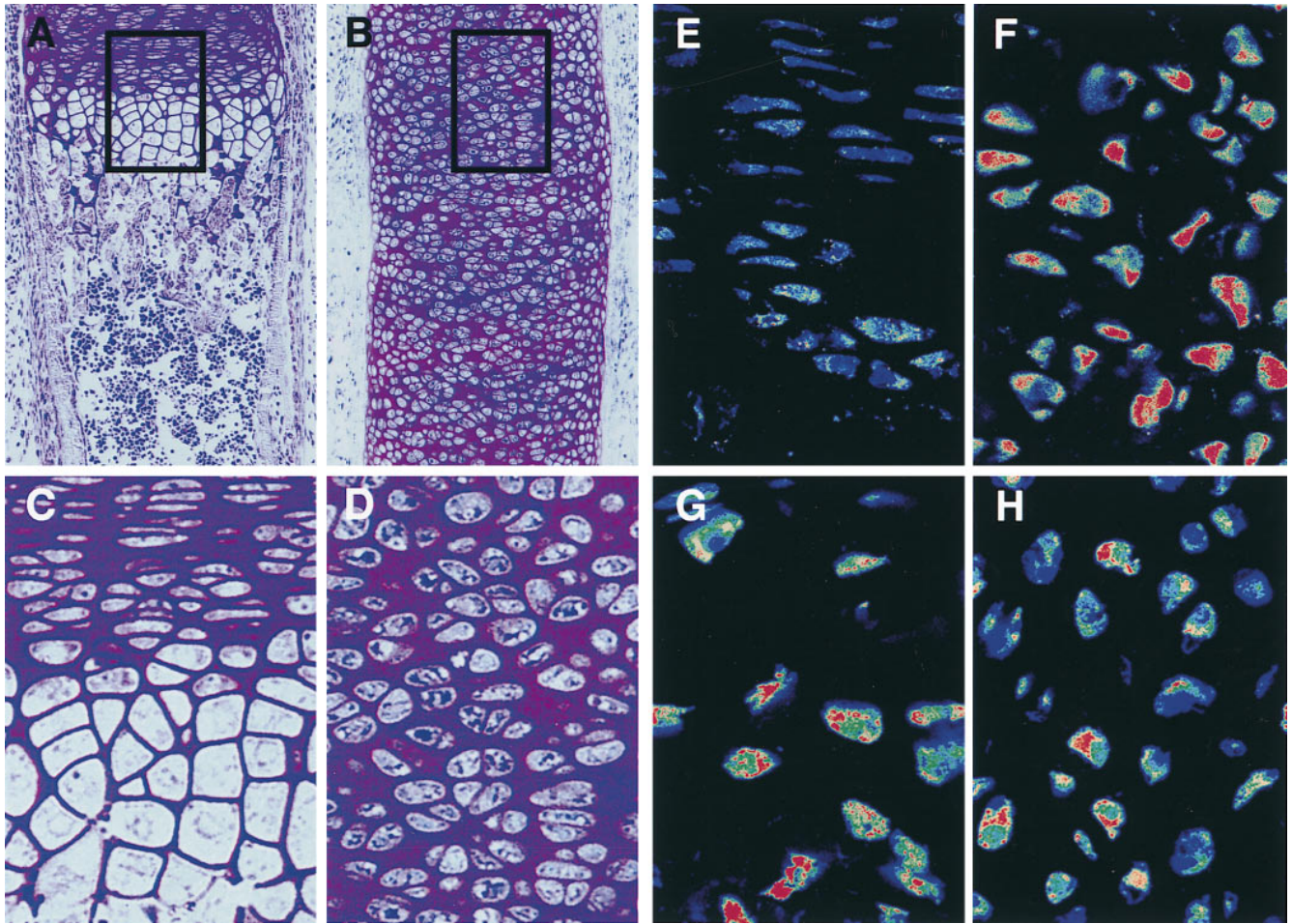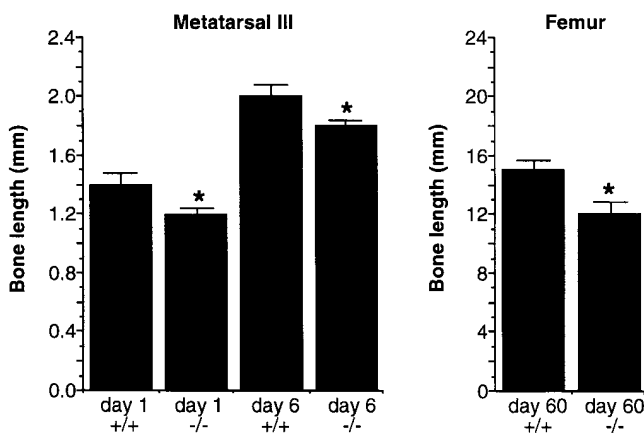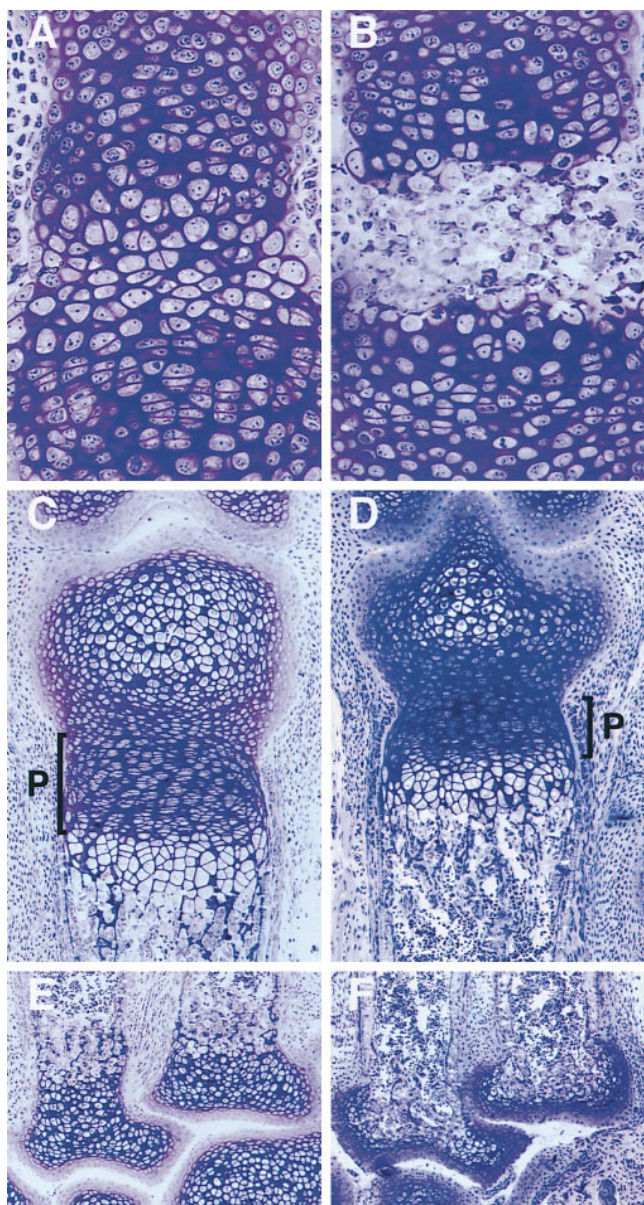
Figure 4. Targeted overexpression of PTHrP to chondrocytes in transgenic animals leads to increased Bcl-2 expression and delayed chondrocyte maturation. (*A*) Metatarsals of a normal 6-d–old mouse, showing the zonal structures of a growth plate, trabecular and cortical bone, and the presence of a marrow cavity. In contrast (*B*), the metatarsal of the col II–PTHrP transgenic littermate demonstrates a delay in chondrocyte differentiation, an accumulation of prehypertrophic chondrocytes (as confirmed by intracellular collagen X expression, data not shown), and the complete absence of bone formation. At a higher magnification of the insets in *A* and *B*, the progression of chondrocyte differentiation is visible in the normal growth plate: proliferating cells differentiate first into prehypertrophic cells, and after further enlargement, they finally die at the border to bone formation (compare to Fig. 1) (*C*). In contrast, the same region in the metatarsal of the col II–PTHrP transgenic mouse shows that the cells accumulate at the prehypertrophic stage (*D*). Frozen sections of the corresponding growth plate regions labeled for Bcl-2 and Bax by immunofluorescence demonstrate the normal pattern of Bcl-2 expression in the growth plate, with highest levels in the zone of prehypertrophic chondrocytes, (*E*). In contrast, in the col II–PTHrP transgenic animals, not only the number of chondrocytes expressing Bcl-2 but also the level of Bcl-2 expression are markedly increased (*F*); panels *G* and *H* demonstrate that Bax expression is not affected in the col II–transgenic animals (*H*) and is comparable to normal levels in prehypertrophic chondrocytes (*G*).

ness, predominantly due to a shortening of the proliferative zone, and a significant decrease in overall bone length, provided evidence for accelerated chondrocyte differentiation. This firmly established that Bcl-2 is directly involved and required for normal skeletal development.

Since absence of PTHrP in the null mutant leads to accelerated chondrocyte differentiation in the growth plate (Amizuka et al., 1994; Karaplis et al., 1994), we and others (Amizuka et al., 1994; Henderson et al., 1995) hypothesized that this could be due to an increase in programmed cell death. To further test this hypothesis and to study the mechanisms by which PTHrP delays chondrocyte differentiation (Kato et al., 1990; Iwamoto et al., 1994; Klaus et al., 1994) and apoptotic cell death (Henderson et al., 1995), we first determined whether PTHrP could alter Bcl-2 expres-

sion in chondrocytes. We found that in vitro treatment of chondrocytes with PTH or PTHrP results in a shift of the Bcl-2/Bax ratio in favor of Bcl-2, a change that delays terminal differentiation and apoptosis of hypertrophic chondrocytes and leads to the accumulation of cells in their prehypertrophic stage.

Despite the fact that other cell types are known to express PTH/PTHrP receptors and to respond to PTHrP with an increase in cAMP, the only alterations reported in the PTHrP knockout mice, and in individuals with the receptor mutation, are chondrodysplasias. We consequently determined whether the effects of PTHrP on Bcl-2 expression in chondrocytes were cell-specific, by treating primary osteoblasts and a kidney cell line that expresses the PTH/PTHrP receptor with PTH or PTHrP. While responding to

Figure 5. Bcl-2 directly affects skeletal development and endochondral bone formation. *bcl-2* knockout mice exhibit accelerated chondrocyte differentiation. (*A*) While under normal conditions at day 1, the foot middle phalanx is still a cartilaginous model. (*B*) In *bcl-2* knockout littermates, vascular invasion, progressive replacement of cartilage by bone, and homing of bone marrow cells has already taken place. (*D*) This acceleration of chondrocyte differentiation leads to a reduction in the growth plate thickness, mostly in the proliferative zone (*P*), shown here for the distal metatarsal bone of a 6-d–old *bcl-2* knockout mouse as compared to a 6-d–old normal control (*C*). Even more striking is the almost complete loss of cartilage at the proximal end of the metatarsal bones (day 6) in the *bcl-2* −/− (*F*), while in the normal mice, the present zone of chondrocytes is still a resource of bone formation (*E*). Consequently, *bcl-2* knockout mice are markedly smaller than control littermates (*G*) (contact x-ray of 60-d–old mice; on the right *bcl-2* −/−, on the left normal control littermate), and the absence of Bcl-2 leads to a significant decrease (15%–20%) in overall bone length (*H*). Significant levels of $P < 0.05$, Student's *t* test, are indicated by asterisks.

PTH/PTHrP with the expected increase in cAMP production, this treatment had no detectable effect on Bcl-2 or Bax levels in these two cell types. We therefore concluded that the downstream effect of PTH/PTHrP on Bcl-2 ex-

pression is, at least among those cells that are known to express the PTH/PTHrP receptor, specific for chondrocytes. These findings are entirely consistent with the seemingly chondrocyte-specific abnormalities in the PTHrP knockout

mice (Amizuka et al., 1994; Karaplis et al., 1994), as well as in individuals with Jansen's-type metaphyseal chondrodysplasia (Schipani et al., 1995; Jüppner, 1996). This specificity may be due to the fact that in chondrocytes, Bcl-2 may alter an antioxidative pathway to repress cell death (Allsopp et al., 1993; Boise et al., 1993; Hockenbery et al., 1993; Kane et al., 1993), since chondrocytes have been shown to be resistant to various stresses, such as the withdrawal of growth factors or serum starvation, as long as antioxidants are also present (Tschan et al., 1990; Ishizaki et al., 1994). These cells literally commit suicide in response to nitric oxide (Blanco et al., 1995).

The most compelling evidence that the regulation of Bcl-2 in chondrocytes is a key mechanism by which PTHrP exerts its control on endochondral ossification during skeletal development, however, comes from our recent observations in vivo. Targeted overexpression of PTHrP to chondrocytes using the collagen type II promoter in transgenic mice leads to a profound delay in endochondral bone formation with Jansen's-like skeletal abnormalities (Weir et al., 1996). We show here that this targeted overexpression of PTHrP also induces a marked increase in Bcl-2 expression (fourfold increase) in prehypertrophic chondrocytes, with no detectable change in Bax levels. The observation that levels of Bcl-2 expression in chondrocytes of the col II-PTHrP transgenic animals were markedly higher than that of growth plate chondrocytes in control animals, further indicates that Bcl-2 lies downstream of PTHrP, rather than PTHrP directly inhibiting differentiation, and thereby keeping the cells at a stage intrinsically programmed to produce Bcl-2.

We therefore conclude that the apoptotic inhibitor, Bcl-2, is involved in the regulation of the programmed cell death of hypertrophic chondrocytes in the growth plate, an event that is critical for endochondral ossification and skeletal development. PTH/PTHrP increases the expression of Bcl-2 in chondrocytes in a cell-specific manner, thereby delaying their terminal differentiation and subsequent apoptosis, and regulating the maturation of the growth plate. We propose that this regulation may be the mechanism underlying the chondrodysplasias observed in the PTHrP knockout mice, in the mice with targeted overexpression of PTHrP in cartilage, and in human Jansen-type metaphyseal chondrodysplasia. These findings are entirely consistent with the alterations in endochondral bone formation reported here after manipulating Bcl-2 levels independently of PTHrP. Thus, as would be expected if one was lying downstream of the other, the changes in the *bcl-2* knockout mice are similar, though far less pronounced, to those in the PTHrP knockout mice, i.e., accelerated chondrocyte maturation. The fact that the *bcl-2* knockout mice show a phenotype which is less severe than that in the mice lacking PTHrP (Amizuka et al., 1994; Karaplis et al., 1994) is most likely due to the fact that the function of Bcl-2 during endochondral bone formation can be in part compensated for by redundant pathways, possibly involving other Bcl-2 family members. Although we were not able to detect Bcl-x in the normal growth plate, redundancy is a very likely explanation, since compensation of the absence of Bcl-2 expression by the other 9 Bcl-2 family members has been previously described in different cell systems (Reed, 1995; Han et al., 1996).

These observations directly establish the functional role of Bcl-2 in endochondral bone formation. However, it still remains to be determined whether the regulation of Bcl-2 expression by PTHrP is of relevance to tumorigenesis: if the upregulation of PTHrP, which is known to occur in several malignancies, affects the level of Bcl-2 expression, it might thereby increase the malignant potential of a tumor by repressing the death of tumor cells due to this autocrine/paracrine pathway. Preliminary data analyzing the coexpression of PTHrP and Bcl-2 in human chondrosarcomas (Pösl et al., 1996) further confirms the importance of the PTHrP/Bcl-2 pathway, at least in chondrogenic tumors, where the level of coexpression of PTHrP and Bcl-2 seems to be correlated with the degree of malignancy of the tumor. Thus, Bcl-2 is the first in a new category of proto-oncogenes that oppose apoptosis and extend cell survival rather than promote proliferation (Vaux et al., 1988; Korsmeyer, 1992). We show here that it may be involved in both physiological development and tumorigenesis of the skeleton.

Most recently, Indian hedgehog protein was found to be upstream of PTHrP in the control of chondrocyte differentiation (Lanske et al., 1996; Vortkamp et al., 1996). Our observations suggest that Bcl-2 acts downstream of PTHrP in the same pathway, slowing down chondrocyte maturation during normal skeletal development.

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