

# Chondrocyte Terminal Differentiation, Apoptosis, and Type X Collagen Expression Are Downregulated by Parathyroid Hormone

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## ABSTRACT

Parathyroid hormone (PTH) regulates calcium and phosphate homeostasis through the endocrine system. Parathyroid hormone-related peptide (PTHrP) is a heterogeneous polypeptide with sequence homology to PTH in its first 13 amino acid residues. Both bind and activate a common receptor, the type 1 PTH/PTHrP receptor (PTH1R). Activation of this G-protein-coupled receptor by PTHrP has been shown to regulate chondrogenesis in a manner that attenuates chondrocyte hypertrophy. Here, we report the dose-response ( $10^{-7}$  to  $10^{-15}$  M) effects of PTH on chondrogenesis using an avian sternal organ culture model. PTH increased cartilaginous tissue length and downregulated the deposition of type X collagen and its mRNA expression. In addition, PTH increased chondrocyte cell diameter in prehypertrophic and proliferative regions while decreasing chondrocyte apoptosis in the hypertrophic zone. In conclusion, these experiments demonstrate that PTH regulates cartilage growth, chondrocytic apoptosis, deposition of type X collagen protein, and expression of type X collagen mRNA. Type X collagen mRNA expression was downregulated by PTH in this organ culture model, but cell size, another marker for terminal differentiation, increased. © 2004 Wiley-Liss, Inc.

**Key words:** hyaline cartilage; apoptosis; parathyroid hormone; parathyroid hormone-related peptide; chondrogenesis; parathyroid hormone receptor; type X collagen

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Parathyroid hormone (PTH) is one of the hormones and growth factors that regulate hyaline cartilage development. Classically, PTH is an 84-amino acid protein that regulates calcium and phosphate homeostasis primarily through actions on specific receptors in kidney and bone (Juppner et al., 1991; Juppner and Schipani, 1996; Bro and Olgaard, 1997). Native 1-84 PTH and 1-34 PTH fragments stimulate adenylate cyclase through a G-protein-coupled receptor (GPCR) with equal potency (Abou-Samra et al., 1992; Friedlander and Amiel, 1994; Blind et al., 1995; Bisello et al., 1996; Bro and Olgaard, 1997). PTH-related peptide (PTHrP) is a heterogeneous polypeptide having limited sequence homology to PTH in its amino

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terminal 13 residues. While limited in their homology, PTH and PTHrP can bind to and activate the same GPCR (Abou-Samra et al., 1989; Orloff et al., 1989; Amizuka et al., 2000a, 2000b). These receptors have been localized on embryonic chick and rabbit cartilage cells (Kioke et al., 1990). In chondrocytes, ligand binding of the type 1 PTH/PTHrP common receptor (PTH1R) activates several second-messenger systems (Abou-Samra et al., 1994; Azarani et al., 1996; Suda, 1997; Serra et al., 1999). PTH has a stimulatory effect on proliferation of chondroprogenitor cells and inhibits collagen and matrix synthesis in avian epiphyseal cartilage via cAMP-dependent pathways (Pines et al., 1990). In addition to PTH1R, another receptor, PTH2R, has been identified and characterized that selectively recognizes PTH and not PTHrP (Usdin et al., 1995; Behar et al., 1996).

PTHrP has been shown to repress the rate of growth plate cartilage differentiation (Vortkamp et al., 1996; Amizuka et al., 2000a). Receptor gene knockout studies have shown that PTH1R binding is required for normal chondrocyte differentiation and bone development, resulting in abnormal avian limb bud development when expression of the PTH/PTHrP receptor was repressed in prehypertrophic chondrocytes (Lanske et al., 1996; Amizuka et al., 2000b; Karaplis, 2001). Current research suggests that there is stage-dependent activation of the PTH/PTHrP receptor in developing cartilage, specifically that PTHrP functions to repress terminal differentiation, holding chondrocytes in a prehypertrophic stage. In addition, PTH1R activation appears to modulate rates of apoptosis in cartilage. One study reports decreased apoptosis and increased cell death inhibitor concentrations (bcl-2 upon activation of the PTH1R) (Amling et al., 1997, 1998; Hirsch et al., 1997), and another study observed mixed results reporting that both PTH and PTHrP can have both pro- and antiapoptotic effects in mesenchymal cells (Chen et al., 2002).

Our goal was to examine the effects of PTH on hyaline cartilage chondrocyte terminal differentiation. A serum-free avian organ culture model that recapitulates chondrogenesis to the hypertrophic stage (Hirsch and Svoboda, 1998) was used to study the following hypotheses. First, we tested the hypothesis that PTH1R activation delays terminal differentiation as measured by type X collagen deposition into the interstitial matrix. Subsequently, we asked if type X mRNA levels were changed through PTH1R activation.

Second, we asked whether during chondrogenesis there is a critical time for PTH activation of the PTH1 receptor. PTH treatment on the rate of growth was evaluated by measuring the length of the sterna daily. Experiments were conducted to determine if a temporal window of PTH exposure could maximize changes in sternal length and type X collagen deposition. We tested the theory that chondrogenesis may change with respect to developmental stage by using an effective concentration of PTH at separate time points. Finally, as PTH appeared to stimulate sternal longitudinal growth, we investigated whether this increase in sternal size was the consequence of decreased apoptosis or increased cell size.

## MATERIALS AND METHODS

### Organ Culture and Cartilage Preparation

White Leghorn chicken eggs were obtained from SPAFAS (Norwich, CT) and Texas A&M Poultry Depart-

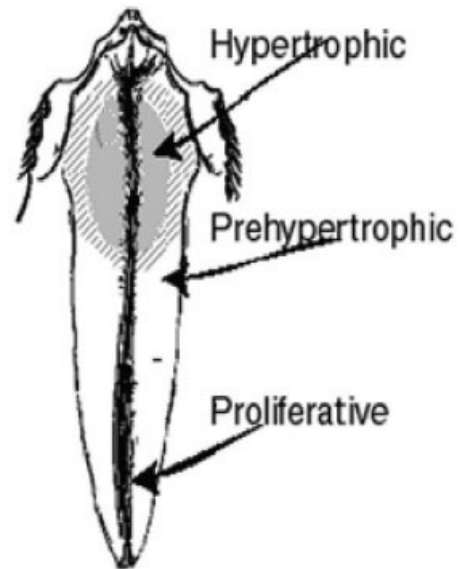


Fig. 1. A schematic diagram of the embryonic chicken sternum. This cartilage structure is characterized by a central spine region (keel) and two lateral plates. The areas of the three growth zones, hypertrophic, prehypertrophic, and proliferative, are illustrated.

ment. Day 14 sterna (Fig. 1) were dissected free of all tissue and perichondrial membranes under sterile conditions in Ham's F-12 medium containing 1% penicillin and streptomycin (PS), 1% antibiotic/antimycotic (Ab/Am), and 1% nonessential amino acids (NEAA) (Hirsch and Svoboda, 1998). The sterna were cultured for 8 days at 37°C with 95% air/5% CO<sub>2</sub> in a humid environment in sterile tissue culture chamber slides (Nunc) containing Ham's F-12 medium, 1% PS, 1% Ab/Am, 1% NEAA, 10<sup>-11</sup> M dexamethasone (Sigma Chemical, St. Louis, MO), 60 ng/ml insulin (Sigma), 10<sup>-11</sup> M triiodothyronine (T3; Collaborative Research), and 100 mg/ml ascorbic acid (Quarto et al., 1992). Length of cartilage samples were measured daily by placing each sternum on a flat sterile surface and calibrating (mm) a set of sterile calipers to both ends of the sternum.

Chick sterna have two lateral plates with a perpendicular medial spine or keel (Fig. 1) (Hirsch and Svoboda, 1998). After culturing, the spine and lateral plates were removed longitudinally along the medial keel, maintaining cephalic-caudal and medial-lateral orientations. On the final culture day, sterna were measured and immediately processed for immunohistochemistry, in situ TUNEL assays, or RNA extractions for RT-PCR.

### PTH Dose Study

To determine the effects of PTH on chondrocyte development, sterna were cultured for 8 days in varying concentrations of PTH (bovine fragment 1-34) ranging from 10<sup>-15</sup> to 10<sup>-7</sup> M at 10-fold dilutions in control media. Each group contained three sterna and the experiments were performed three times. Culture media were changed and sterna were measured daily. On the 8th day, the sterna were measured, then immediately processed for immunohistochemistry of the terminal differentiation marker, type X collagen. As 10<sup>-7</sup> M PTH (or 100 nM) appeared to

have the most significant downstream effect on terminal differentiation, that dose was used for additional experiments to determine the effect of PTH on growth.

### Immunohistochemistry

Sterna that were processed for type X immunohistochemistry were cut into three pieces (lateral plates and keel) and incubated for 1.5–3 hr in 0.1% testicular hyaluronidase (280 U/mg; Sigma) and washed in phosphate-buffered saline (PBS) to unmask the antigenic epitope (Schmid and Linsenmayer, 1985b). Tissues were then fixed in freshly prepared 4% paraformaldehyde for 15 min, rinsed in PBS, and blocked with 10% normal goat serum (NGS; Life Technologies). The tissues were incubated in the type X collagen monoclonal primary antibody (Schmid and Linsenmayer, 1985b), diluted 1:50 in 3% NGS overnight at room temperature on a rotary shaker. The primary antibody was visualized using FITC-conjugated affinity pure goat antimouse IgG secondary antibody (H+L chains; Jackson ImmunoResearch), diluted 1:50 in 3% NGS and incubated at room temperature overnight. Following rinses in PBS, the sterna keel and lateral plates were mounted in antifade mounting media (slowfade; Molecular Probes) on glass slides with nail polish spacers, coverslipped, and viewed on a Leica confocal laser scanning microscope (CLSM) equipped with an argon ion laser with a power output of 2–50 mW, two photomultiplier tubes, and narrow band filters for double-labeling experiments as described previously (Hirsch et al., 1997). This whole mount procedure allowed mapping the different regions (Fig. 1) in a single specimen. The type X collagen antibody (Schmid and Linsenmayer, 1985b) was a gift from Dr. Thomas Linsenmayer. The epitope on the type X collagen molecule is located within the triple-helical domain, 19 nm from the carboxy-terminal domain.

### RT-PCR

Sterna ( $n = 16$ /treatment group) were cultured for 8 days in control media or supplemented with 100 nM PTH as described previously. Synthetic oligodeoxyribonucleotides were purchased from BioSynthesis (Dallas, TX) using sequences provided by Dr. Marion Gordon (Yamaguchi et al., 1991). PCR was performed using specific primers for chick type X collagen (5'-CCTGGCCAATCCACAATCCAGA-3' and 5'-TGAGCAAATAGGAAACCTGAGA-3'). The positive control for this RT-PCR was  $\beta$ -actin. Protocol for RT-PCR required RNA isolation and reverse transcription at 37°C for 90 min using MuLV transcriptase (Perkin Elmer). Each RT-Product was amplified in a thermocycler (Perkin Elmer) using AmpliTaq polymerase (Perkin Elmer), then separated on 2% agarose gels. Each amplification cycle for the type X primers was 30 sec at 95°C, 20 sec at 57°C, 15 sec at 72°C (repeated 29 times), followed by a 10-min extension at 72°C to increase annealing stability. A 100 bp ladder was used for product size evaluation (Perkin Elmer). These experiments were repeated three times with RNA from three experiments.

### Growth Study

**Continuous exposure to PTH.** Sterna were divided into two groups ( $n = 8$ ), controls and 100 nM PTH (bovine 1-34). The day the sterna were placed in culture was termed day 0. Media was changed daily and sternal length measurements were taken daily to determine overall

growth on both PTH-treated and control samples for 8 days (Table 1). In addition to overall growth, the rate of growth between groups was compared (Table 2). The experiment was repeated three times.

**Temporal exposure to PTH.** Sterna were divided into eight groups representing each day of culture. Each group received a one-time 24-hr treatment of 100 nM PTH (bovine 1-34) on different culture days, one group per culture day (Table 3). The day the sterna were placed in culture was termed day 0. Group 1 was treated on culture day 0, group 2 on day 1, and so forth, until group 8 received a PTH treatment on day 7. On all other culture days, the sterna were cultured in control media. Representative sterna were processed for immunohistochemistry after the 24-hr PTH exposure and the remaining sterna were processed, on either day 6 or day 8. Control sterna were harvested and processed on days 6 and 8. Sterna receiving a PTH treatment on day 6 were fixed on days 7 and 8. Sterna treated on day 7 were fixed on day 8. Sternal length measurements were taken daily to determine 24-hr growth on both PTH-treated and control samples. The experiment was repeated twice.

### Cell Size Determination

To evaluate if increase in cell size was associated with a change in sternal length, sternal keel and lateral plates were stained with phalloidin and cells were measured as described previously (Hirsch et al., 1997). Nuclei were stained with propidium iodide. Briefly, a grid overlay on the CLSM computer monitor was used to select areas and measure chondrocytes along their long and short axes at the surface, 10, 30, and 50  $\mu$ m optical depths into the cartilage. At least 45 chondrocytes from each sternal region (cephalic, middle, and caudal) in at least three different sterna were used to determine the average chondrocyte size.

An overall comparison of chondrocyte long-axis lengths was conducted with a three-way analysis of variance with depth, age, and region as between-subject variables. More specific comparisons were then obtained by two-way analyses of variance at each age level with depth and region as between-subject variables as described previously (Hirsch et al., 1997).  $P$  values were set at 0.05.

### Apoptosis

In situ nucleotide end labeling protocols (TUNEL) for the detection of internucleosomal breakdown of nuclear DNA were used (Gavrieli et al., 1992; Tilly, 1994; Hirsch et al., 1997). Briefly, the tissue samples (cultured sternal lateral plates) were fixed, permeated, rehydrated, and digested using proteinase K before incubation with biotin-dUTP and Tdt enzyme (Boehringer Mannheim).

Apoptotic nuclei were visualized by 10  $\mu$ g/ml avidin-FITC (Molecular Probes). All nuclear material was labeled by incubating samples in 0.5  $\mu$ g/ml propidium iodide (Molecular Probes) for 5 min. Sterna samples ( $n = 3$ /treatment group) were viewed on the CLSM as described previously. Negative controls were incubated in nucleotide end labeling mixture without probe, washed, detected with avidin-FITC, and viewed on the CLSM. Positive controls were incubated in 100  $\mu$ g/ml DNase 1 (Boehringer Mannheim) prior to nucleotide end labeling, hybridized, washed, double-labeled with avidin-FITC and propidium iodide, and viewed on the CLSM.

TABLE 1. Comparison of overall length\*

Culture day	0	1	2	3	4	5	6	7	8
PTH-treated	11.38 ± 0.21	14.9 ± 0.30 <sup>a</sup>	18.88 ± 0.47 <sup>a</sup>	21.88 ± 0.50 <sup>a</sup>	23.28 ± 0.43 <sup>a</sup>	23.72 ± 0.41 <sup>a</sup>	24.22 ± 0.34 <sup>a</sup>	24.56 ± 0.31 <sup>a</sup>	24.69 ± 0.28 <sup>a</sup>
Controls	11.31 ± 0.34	13.81 ± 0.30	16.81 ± 0.58	19.09 ± 0.45	20.031 ± 0.53	20.34 ± 0.46	20.41 ± 0.45	20.56 ± 0.46	20.60 ± 0.46

\*Values presented as mean of overall length (mm) ± standard error.

<sup>a</sup>Significant differences between PTH-treated sterna and controls ( $P < 0.05$ ).

All nuclei were stained with propidium iodide (red). Apoptotic nuclei were double-labeled and appeared yellow due to overlapping signals. Each field was counted for total number of nuclei and apoptotic nuclei. A ratio of apoptotic nuclei to total number of nuclei was calculated in each of three sternal regions: cephalic, middle, and caudal (Fig. 1). In each region, three counts were made in separate areas within the region at each of three optical depths: 10, 30, and 50 μm. This produced a total of nine counts per region per sample (n = 3). The ratios obtained from each field within each region were calculated and averaged, providing an average apoptotic ratio for each region.

**Statistical Analysis**

An overall comparison of long-axis sternal growth during 24-hr periods was conducted with a one-way analysis of variance in the PTH dose-response and growth studies. An overall comparison of sternal growth rate was conducted with a one-way analysis of variance. An overall comparison of apoptotic ratios was conducted with a one-way analysis of variance in each region: cephalic, middle, and caudal. Experiments with a significant difference were further evaluated by a posteriori comparisons for simple main effects and the Tukey HSD method of pair comparisons among means (Dawson-Saunders and Trapp, 1994). *P* values were set at 0.05.

**RESULTS**

**PTH Decreased Type X Collagen Protein Deposition in a Dose-Dependent Manner**

Sterna cultured in control media had a continuous distribution of type X collagen arranged in filamentous bundles surrounding lacunae in the medial cephalic region of the sterna (Fig. 2A). Type X collagen staining intensity gradually decreased as PTH concentration increased. Sterna cultured in 10<sup>-15</sup> M PTH (Fig. 2B) had type X collagen staining intensity and distribution similar to controls (Fig. 2A). Sterna cultured in 10<sup>-11</sup> M PTH (Fig. 2C) had a decreased staining for type X collagen in the medial cephalic region (Fig. 2C). Sterna cultured in the highest PTH concentration tested (10<sup>-7</sup> M PTH or 100 nM) had very little type X collagen (Fig. 2D); immunohistochemical labeling in these sterna was very faint compared to controls. A PTH concentration of 100 nM PTH exhibits the greatest effective dose for inducing terminal differentiation in chondrocytes as measured by the amount of immuno-labeled type X collagen protein.

**PTH Inhibited Type X Collagen mRNA Expression During Chondrocyte Differentiation**

Sterna cultured with and without 100 nM PTH also had decreased type X collagen mRNA in RT-PCR analysis. The type X collagen RT-PCR product was a 493 base pair fragment from control sterna (Fig. 2E), whereas the PTH-treated sterna had no observable product. All RT-PCR procedure controls were included in the assay, including negative controls without RNA and positive controls using avian β-actin primers that produced a 318 base pair fragment (Fig. 2E).

**Continuous and Temporal Exposure to 100 nM PTH Increased Sternal Length**

The overall cumulative linear growth of the sterna treated with 100 nM PTH was significantly more than

**TABLE 2. Comparison of growth rates\***

Culture day	1	2	3	4	5	6	7	8
PTH-treated	3.50 ± 0.08 <sup>a</sup>	4.00 ± 0.12 <sup>a</sup>	3.00 ± 0.03 <sup>a</sup>	1.40 ± 0.07 <sup>a</sup>	0.44 ± 0.06	0.25 ± 0.04	0.34 ± 0.04	0.13 ± 0.05
Controls	2.50 ± 0.17	3.00 ± 0.22	2.28 ± 0.10	0.94 ± 0.08	0.31 ± 0.03	0.06 ± 0.02	0.16 ± 0.03	0.03 ± 0.02

\*Values presented as mean of overall length (mm) ± standard error.

<sup>a</sup>Significant differences between PTH-treated sterna and controls (*P* < 0.05).

**TABLE 3. Schedule for 24-hr PTH treatment and control sterna\***

Culture day		14-Day sterna									
		∅	1	2	3	4	5	6	7	8	
PTH treatment groups	Group 1 (n = 6)	X	_____XF	.....	.....	.....	.....	F	.....	F	
	Group 2 (n = 6)	X	.....X	_____XF	.....	.....	.....	F	.....	F	
	Group 3 (n = 6)	X	.....X	.....X	_____XF	.....	.....	F	.....	F	
	Group 4 (n = 6)	X	.....X	.....X	.....X	_____XF	.....	F	.....	F	
	Group 5 (n = 6)	X	.....X	.....X	.....X	.....X	_____XF	F	.....	F	
	Group 6 (n = 4)	X	.....X	.....X	.....X	.....X	.....X	_____XF	F	.....	F
	Group 7 (n = 4)	X	.....X	.....X	.....X	.....X	.....X	.....X	_____XF	F	
	Group 8 (n = 4)	X	.....X	.....X	.....X	.....X	.....X	.....X	.....X	_____XF	
Controls	n	X	.....X	.....X	.....X	.....X	.....X	.....X	.....X	X	
			40	34	28	22	16	12	8	4	

\*A diagrammatic protocol for the treatment and measurement schedule for PTH-treated and control sterna when looking at the temporal effects of PTH on chondrogenesis. Day 0 indicates the baseline when the cartilage tissue was extracted from 14-day chick embryos. Sternal length was measured (X) on a daily basis. On various days some sterna were measured, then fixed (XF) for further processing. PTH exposure to 100 nM PTH (bovine 1–34) is indicated by a solid line and incubation in control medium is indicated by a dotted line. The number of sterna (n) in the treatment group/day is noted at the bottom.

controls from days 3 to 7 (Fig. 3A, Table 1). The observed increased linear growth appeared to occur early in the culture period, during the prehypertrophic stage of chondrogenesis. Based on this result, we asked two questions: What is the effect on overall sternal growth rate to a continuous exposure to 100 nM PTH, and is there a specific temporal window when PTH has the maximum effect?

We observed that all cultured sterna grew at an increased rate during the first days of culture. The growth rate in each 24-hr period was significantly different on days 1–4 between groups (Fig. 3B, Table 2). Specifically, the PTH treatment group had increased length compared to controls by a greater margin after each 24-hr interval (Fig. 3B, Table 2).

To answer the question of whether there was a maximal temporal effect occurring, experiments were designed to expose individual sterna (n ≥ 4) to a one-time dose of 100 nM PTH for one 24-hr period during one of the seven culture days (Table 3). Sterna exposed to 100 nM PTH during one of the first six culture days showed a significant increase in 24-hr rate of growth compared to controls (Fig. 3C, Table 4). Maximum enhanced growth occurred in PTH-treated sterna exposed on culture days 1 and 2. In both the PTH-treated sterna and controls, 24-hr growth generally slowed over the 8-day culture. Analysis of variance tests determined significant differences in 24-hr

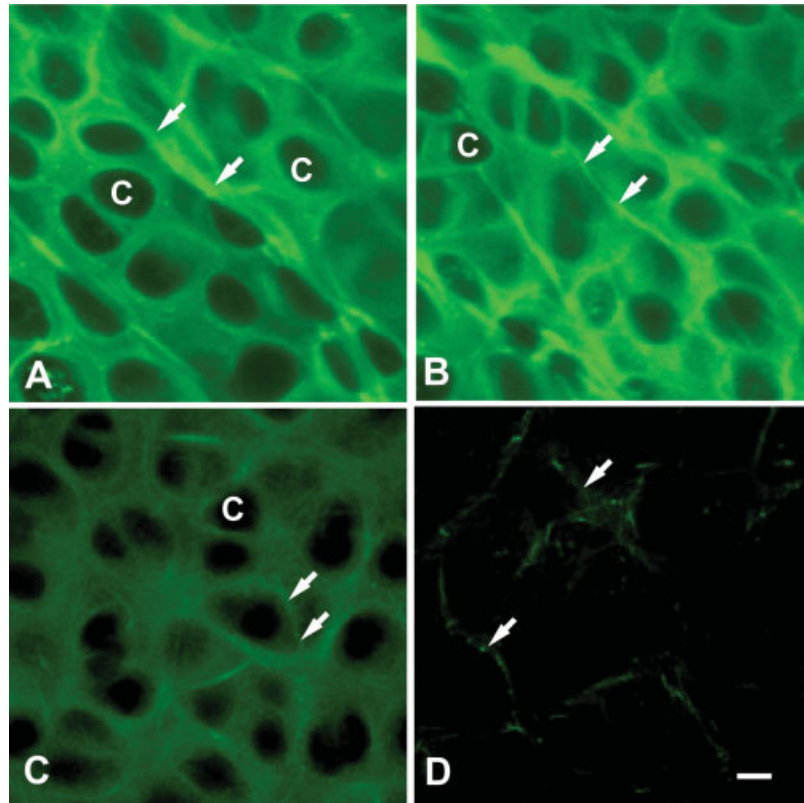
growth rate between PTH-treated sterna and controls on culture days 1 through 6 (Fig. 3C, Table 4), but not culture days 7 and 8. Type X collagen distribution in 100 nM PTH-treated sterna for any single 24-hr period was similar to controls on all culture days (data not shown). No type X collagen was observed in either the PTH-treated or the control sterna before culture day 6. Intensity and distribution of type X collagen after 8 days were similar between all 1-day PTH-treated sterna groups and controls.

**Cell Size**

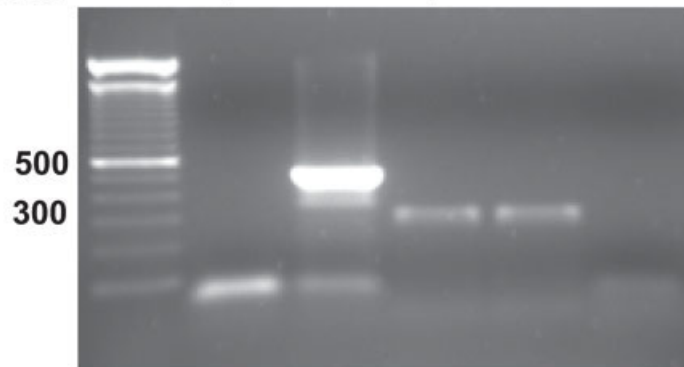
Data were collected at 10, 30, and 50 μm below the surface. Chondrocytes from sterna cultured with 10<sup>-15</sup> M PTH demonstrated no significant difference in cell diameter in any sternal region compared to sterna grown in control media (Table 5). In contrast, sterna grown in the presence of 10<sup>-11</sup> and 10<sup>-7</sup> M PTH contained larger cells in prehypertrophic and proliferative regions (Table 5). However, as the cell diameter increased in these regions, the nuclear diameter remained relatively unchanged in all regions.

**Apoptosis Decreased in PTH-Treated Sterna**

The greatest number of apoptotic cells was found in the cephalic hypertrophic region of control sterna (0.18–0.22;



Primer	Type X		Actin		C
	+	-	+	-	
PTH					



**E**

Fig. 2. PTH effects on type X collagen. Type X collagen was used as a marker for terminal differentiation. Type X collagen was deposited into the stroma surrounding hypertrophic chondrocytes (A) in the medial cephalic region as shown by indirect immunohistochemistry. Sterna cultured in a low concentration of PTH ( $10^{-15}$  M; B) had a normal distribution of type X collagen in the pericellular matrix surrounding the negative staining lacunae areas. Type X collagen was decreased in sterna cultured in  $10^{-11}$  M PTH (C) compared to controls and further decreased in sternal cultured in the presence of  $10^{-7}$  M (or 100 nM) PTH (D). All images were obtained with the same confocal microscope set-

tings on the same day to compare intensity. Cell lacunae (C) and large type X bundles are labeled (arrows). Scale bar = 10  $\mu$ m. E: RT-PCR was used to determine that sterna treated with PTH (100 nM)-inhibited mRNA expression of type X collagen. A 495 base pair fragment was produced from control sterna RNA, whereas PTH-treated tissues did not have the PCR product. Chicken actin primers produced the same size (300 base pairs) and quantity of actin product, demonstrating that the RNA was intact in both samples and that the PCR reaction was stable. Internal controls (C) for the RT-PCR kit were negative.

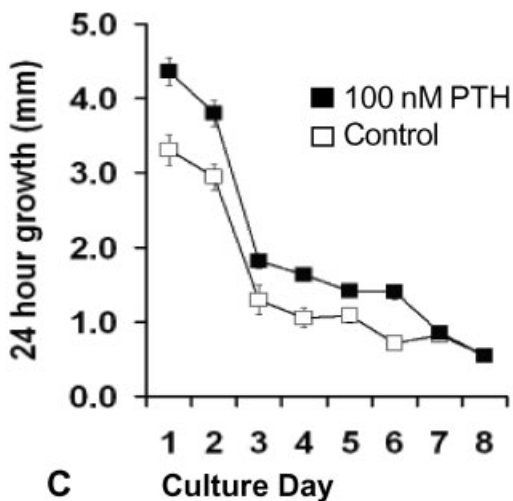
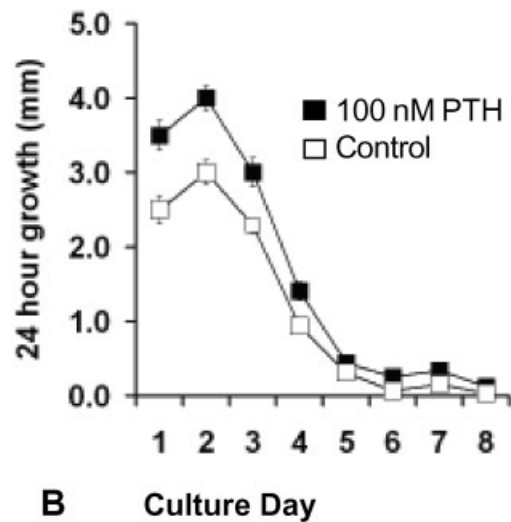
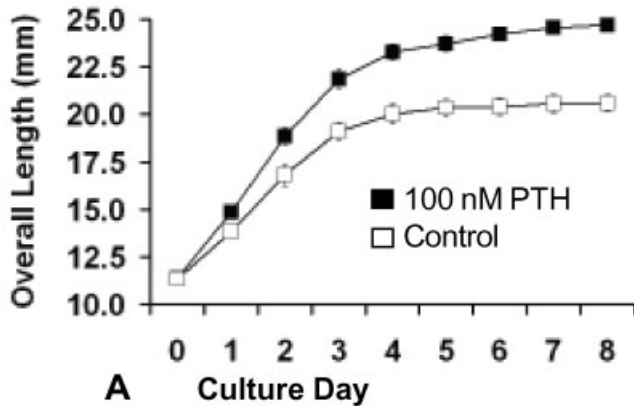


Fig. 4) as previously reported (Hirsch et al., 1997). The middle prehypertrophic (0.05) and caudal proliferative (0.10–0.15) regions of control sterna had significantly lower amounts of apoptotic cells than the cephalic region, and the caudal region had a significantly higher number of apoptotic cells than the middle region. These results suggest that there may be regional differences, but not tissue depth differences, within control sternum as three depths from each specimen were analyzed (10, 30, and 50  $\mu\text{m}$  from the surface). Sterna cultured in the presence of PTH had a dose-dependent decrease in apoptosis in the hypertrophic cells but did not change in prehypertrophic or proliferative cells (Fig. 4).

## DISCUSSION

The present study suggests that PTH has stimulatory effects on chondrogenesis during prehypertrophic stages of development compared to the hypertrophic stage, which begins after 6 days in our cultured sterna model. The PTH-stimulated sternal growth increase could be the result of several factors, including increased cell number, size, and/or matrix deposition or decreased cell death. Both the continuous and temporal window PTH treatments induced significant increases in longitudinal growth and daily growth rate for sterna exposed to PTH compared to controls. The largest change in sterna size was during the first 48 hr for both continually and temporally exposed sterna with or without the PTH treatment. This may be simply due to increased water retention and swelling. However, the PTH-treated sterna were significantly longer than controls, indicating that other factors such as increased cell size, cell number, or extracellular matrix deposition may be contributory to the significant change. Here we report that in the prehypertrophic and proliferative regions, chondrocyte diameter does increase. A major contributing factor to overall growth and cell size may be that an increased number of chondrocytes remained viable (Fig. 4). Therefore, the increased viability of the cephalic chondrocytes may contribute to the increased sternal length by attenuating the natural decrease in cell number during terminal differentiation via apoptosis. The PTH-exposed groups had significantly more growth per 24-hr period on each of the first six culture days compared to controls. These results are supported by other studies showing PTH is a potent stimulator of mitogenic activity in other tissues (Kioke et al.,

Fig. 3. Sternal growth. **A**: Continuous exposure to PTH. Whole sterna were cultured with (solid squares) or without (open squares) 100 nM PTH (bovine 1-34) and measured daily for 8 days ( $n = 8$ /treatment group). The cumulative length of the PTH-treated sterna was significantly longer on culture days 3–7 (Table 1). **B**: Temporal exposure to PTH. The growth rate was determined for each 24-hr period (Table 2). The first 4 culture days exhibited an accelerated growth rate in both groups; however, the PTH-treated group had significantly higher growth rates. Sterna were exposed to PTH for a 24-hr interval on each culture day (Table 3). **C**: Growth rates were compared between controls (open squares) and 100 nM PTH (solid squares) for each day. Sternal length measurements were taken at the beginning and the end of each 24-hr treatment in 100 nM PTH and compared to controls (Table 4). Prior to exposure to PTH, all samples were incubated in control medium (Table 3). Growth rates in the PTH group were significantly higher than controls on culture days 1–6 ( $P < 0.05$ ), but not days 7–8 (Table 4).

**TABLE 4. 24-hr PTH treatment vs. controls: comparison of growth rates\***

Culture day	1	2	3	4	5	6	7	8
PTH-treated	4.37 ± 0.19 <sup>a</sup>	3.80 ± 0.17 <sup>a</sup>	1.82 ± 0.09 <sup>a</sup>	1.63 ± 0.08 <sup>a</sup>	1.42 ± 0.09 <sup>a</sup>	1.40 ± 0.10 <sup>a</sup>	0.85 ± 0.05	0.55 ± 0.05
Controls	3.31 ± 0.20	2.95 ± 0.17	1.29 ± 0.19	1.06 ± 0.12	1.08 ± 0.09	0.71 ± 0.06	0.83 ± 0.08	0.55 ± 0.05

\*Values presented as average growth in 24 hours (mm) ± standard error.

<sup>a</sup>Significant difference between PTH-treated sterna and controls (*P* < 0.05).

**TABLE 5. Cell and nuclear size\***

Treatment	Cephalic		Middle		Caudal	
	Cells ± SD	Nuclear size ± SD	Cells ± SD	Nuclear size ± SD	Cells ± SD	Nuclear size ± SD
Control	16.25 ± 1.23	6.32 ± 0.38	11.17 ± 1.03	5.92 ± 0.44	11.08 ± 0.81	5.85 ± 0.38
10 <sup>-15</sup> M PTH	15.92 ± 1.25	6.65 ± 0.44	10.69 ± 1.02	6.01 ± 0.57	10.00 ± 0.75	5.57 ± 0.41
10 <sup>-11</sup> M PTH	16.29 ± 1.04	6.72 ± 0.34	13.39 ± 1.60	5.91 ± 0.47	12.99 ± 1.38	6.07 ± 0.58
10 <sup>-7</sup> M PTH	16.76 ± 1.31	6.58 ± 0.46	14.60 ± 1.38	6.12 ± 0.51	12.56 ± 1.27	5.61 ± 0.45

\*Cell and nuclear size analysis was determined in sterna cultured in control media or PTH (1–34) (10<sup>-15</sup>, 10<sup>-11</sup>, and 10<sup>-7</sup>). Sternal chondrocytes cultured with 10<sup>-15</sup> PTH demonstrated no significant difference in cell diameter in any sternal region compared controls. Sterna grown in the presence of 10<sup>-11</sup> and 10<sup>-7</sup> PTH contained larger cells in prehypertrophic and proliferative regions. Both cell and nuclear size changed uniformly. No difference was observed when comparing cell and nuclear growth.

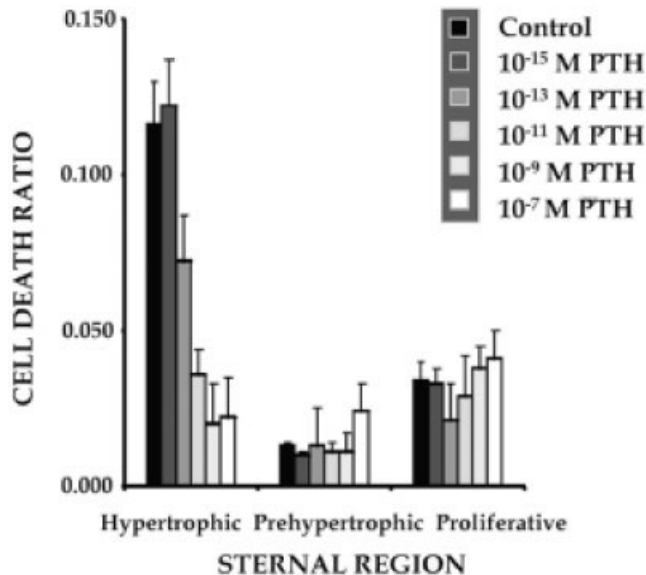


Fig. 4. PTH decreased apoptosis. Control hypertrophic chondrocytes have the highest apoptotic ratio. Sterna treated with 10<sup>-15</sup> M PTH were the same as control sterna; however, with 10<sup>-13</sup>–10<sup>-9</sup> M, there was a dose-dependent decrease in apoptosis. There was no difference between 10<sup>-9</sup> and 10<sup>-7</sup> M PTH treatments. Prehypertrophic sternal chondrocytes had the lowest overall cell death ratio and there was no significant difference between treatment groups. A slight increase of apoptosis was observed in the proliferative chondrocytes, probably as a result of mechanical insult when handling the sterna specimens, and PTH treatment had no effect on apoptotic ratios in this region.

1990). Growth on days 7 and 8 of culture, however, showed no difference between PTH-exposed and control sterna, suggesting that chondrocytes in the prehypertrophic stage are more responsive to PTH.

Gene knockout studies have demonstrated that PTHrP and Indian hedgehog (Ihh) repress terminal chondrocyte differentiation in avian and murine models (Lanske et al.,

1996, 1999; Vortkamp et al., 1996; La Vail et al., 1997; Lanske and Kronenberg, 1998). The data suggest that proliferating chondrocytes express PTHrP receptors and Ihh protein as they begin to differentiate. It was theorized that Ihh in prehypertrophic chondrocytes acts on cells in the periarticular perichondrium to increase the production of PTHrP. PTHrP in turn binds to its receptor, located inside the prehypertrophic zone of the growing cartilage, and inhibits Ihh production through negative feedback. This summarily results in the downregulation of both PTHrP receptors and Ihh, and theoretically controlling cartilage growth through the complex process of terminal chondrocyte differentiation (Vortkamp, 2001). Recently, additional growth factors and receptors have been observed to modulate PTH1R-regulated chondrogenesis. TGF-β2 has sparked interest as a possible mediator within this negative feedback loop (Alvarez et al., 2002), as well as FGFR3 (Amizuka et al., 2000b).

Since PTH and PTHrP share the PTH1R receptor in many tissues (Juppner et al., 1991; Lanske and Kronenberg, 1998), it is reasonable that PTH ligand binding of the PTH1R may elicit the same negative feedback in prehypertrophic chondrocytes as PTHrP activation appears to do. In this study, the perichondrium was removed from sterna prior to culturing, allowing examination of PTH effects without interference of perichondrial PTHrP production. Current studies support cooperative involvement of the perichondrium and periosteum in regulation of endochondral cartilage growth (Di Nino et al., 2002). By removing the perichondrium, one endogenous component of the feedback loop should be controlled, namely, PTHrP.

Studies using isolated rabbit rib chondrocytes maintained in the presence of 10% serum in mass cultures suggest that during hypertrophy there was a decrease in PTH binding due to a decrease in the number and not the affinity of the PTH1R (Iwamoto et al., 1994). Work in our laboratory using Hirsch's serum-free whole organ culture model has shown that chondrocytes enter the hypertrophic stage and begin to express type X collagen at day 6 of the 8-day culture (Hirsch et al., 1997; Hirsch and Svoboda,

1998). These results are consistent with our findings that PTH may have stage-dependent stimulatory effects on terminal differentiation in chondrocytes. We have observed these effects through day 6, suggesting that PTH effects are more prominent during the prehypertrophic than the hypertrophic stage (Fig. 3). We hypothesize that this window of decreased PTH sensitivity may be caused by the downregulation of the PTH1R upon entering the hypertrophic stage. Further studies using an antibody to the PTH1R or tagged PTH would be valuable in determining the developmental time course of the PTH1R and its activity in hyaline cartilage.

Hypertrophy has been classified as the terminal stage of chondrocyte differentiation and a marker for maturation during endochondral bone development (LuValle et al., 1992). Hypertrophy is characterized by an increased cell volume of individual chondrocytes, matrix accommodation for the enlarged chondrocytes, production of type X collagen, and a concomitant decrease in the synthesis of collagen types II, IX, and XI (Schmid and Linsenmayer, 1985a, 1985b; Linsenmayer et al., 1991). In the chicken sternum, the cells actually increase in size before type X is expressed (Hirsch et al., 1996). The present study demonstrates that PTH1R binding by PTH affects hyaline cartilage by inhibiting terminal chondrocyte differentiation as measured by decreased type X collagen deposition and mRNA expression. As the PTH concentration was increased from  $10^{-15}$  to  $10^{-7}$  M at 10-fold intervals, there was a decrease in type X collagen secretion into the interstitial matrix. However, the cell size did not decrease (Table 5), indicating that type X collagen regulation is not directly connected to hypertrophy.

The inhibition of terminal differentiation by PTH was consistent with previously reported studies on cartilage from chick and rabbit that used PTHrP to activate the PTH1R (Iwamoto et al., 1994; Vortkamp et al., 1996). Our results suggest that PTH increases growth as demonstrated by increased overall sternal length. An increase in sternal growth and inhibition of terminal chondrocyte differentiation in PTH-treated sterna suggests that PTH may repress terminal differentiation by maintaining chondrocytes in a proliferative state. Proliferation experiments will be conducted in the future to determine the effects of PTH.

After observing the inhibition of terminal differentiation in the PTH-treated chondrocytes, we asked whether this inhibition occurred at protein translation or whether the inhibition was further upstream. This study suggests that it is possible that activation of the PTH1R has downstream effects that directly block nuclear transcription factors.

The present study presents several experimental protocols for studying the effect of PTH on hyaline cartilage. We have shown that PTH has a growth-stimulating effect on chondrocytes. In conclusion, we have shown that parathyroid hormone acts to repress hyaline cartilage terminal chondrocyte differentiation and type X collagen secretion and expression without altering cell size. Furthermore, PTH appears to protect the chondrocytes from apoptosis.

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