

# Chondrogenic Differentiation of Clonal Mouse Embryonic Cell Line ATDC5 In Vitro: Differentiation-dependent Gene Expression of Parathyroid Hormone (PTH)/PTH-related Peptide Receptor

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**Abstract.** The regulatory role of parathyroid hormone (PTH)/PTH-related peptide (PTHrP) signaling has been implicated in embryonic skeletal development. Here, we studied chondrogenic differentiation of the mouse embryonal carcinoma-derived clonal cell line ATDC5 as a model of chondrogenesis in the early stages of endochondral bone development. ATDC5 cells retain the properties of chondroprogenitor cells, and rapidly proliferate in the presence of 5% FBS. Insulin (10  $\mu$ g/ml) induced chondrogenic differentiation of the cells in a postconfluent phase through a cellular condensation process, resulting in the formation of cartilage nodules, as evidenced by expression of type II collagen and aggrecan genes. We found that differentiated cultures of ATDC5 cells abundantly expressed the high affinity receptor for PTH ( $M_r \sim 80$  kD;  $K_d = 3.9$  nM;  $3.2 \times 10^5$  sites/cell). The receptors on differentiated cells were functionally active, as evidenced by a

PTH-dependent activation of adenylate cyclase. Specific binding of PTH to cells markedly increased with the formation of cartilage nodules, while undifferentiated cells failed to show specific binding of PTH. Northern blot analysis indicated that expression of the PTH/PTHrP receptor gene became detectable at the early stage of chondrogenesis of ATDC5 cells, preceding induction of aggrecan gene expression. Expression of the PTH/PTHrP receptor gene was undetectable in undifferentiated cells. The level of PTH/PTHrP receptor mRNA was markedly elevated parallel to that of type II collagen mRNA. These lines of evidence suggest that the expression of functional PTH/PTHrP receptor is associated with the onset of chondrogenesis. In addition, activation of the receptor by exogenous PTH or PTHrP significantly interfered with cellular condensation and the subsequent formation of cartilage nodules, suggesting a novel site of PTHrP action.

IT has been suggested that when limb mesenchymal cells move away from the influence of the apical ectodermal ridge, their cAMP content increases, triggering chondrogenic differentiation (38). The response of limb mesenchymal cells to dibutyryl cAMP is a responsive property of the cells, which changes temporally and spatially during limb differentiation (57). Elevation of cAMP content of the subridge mesenchymal cells precludes the necessity of cells passing through a condensation phase before overt cartilage formation (38). Thus, the increase in intracellular cAMP is hypothesized to be a key cellular response to cellular condensation that is a prerequisite for limb chondrogenesis (46, 49).

We previously reported that parathyroid hormone (PTH)<sup>1</sup> stimulates sulfate incorporation into cartilage-characteristic proteoglycans followed by elevation of intracellular cAMP in primary growth plate chondrocytes (23, 59, 60). This stimulation of sulfate incorporation was mainly caused by elongation of glycosaminoglycan chains rather than increased synthesis of proteoglycan core protein (23). Chondrocytes respond to PTH through the adenylate cyclase-coupled surface receptor, whose molecular mass was determined to be  $\sim 80$  kD by chemical cross-linking (22). Cell-surface expression of the receptor is

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1. *Abbreviations used in this paper:* b, bovine; BMP, bone morphogenetic protein; EC, embryonal carcinoma; HSAB, *N*-hydroxysuccinimidyl-4-azidobenzoate; MIX, 1-methyl-3-isobutylxanthine; PKA, PKC, protein kinase A, protein kinase C; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide.

markedly upregulated by treatment with bone morphogenetic protein (BMP) in association with chondrocyte maturation (22). In a previous study, PTH stimulated proliferation of primary growth plate chondrocytes isolated from rabbit ribs (36). PTH shares a common receptor with PTH-related peptide (PTHrP), which was originally identified as a causative agent of humoral hypercalcemia of malignancy (30, 31, 55). Unlike PTH, the expression of PTHrP has been demonstrated in a variety of fetal and postnatal tissues in normal animals (8, 50). The tissue distribution of PTHrP and its receptor suggests that PTH/PTHrP may play a role as a paracrine regulator of cellular growth and differentiation, and that it is involved in epithelial-mesenchymal interactions during early embryogenesis (42, 62, 63). Transgenic mice that overexpress PTHrP provided evidence for its role in hair follicle development (64).

Mice homozygous for the PTHrP null mutation exhibited widespread abnormalities of endochondral bone development with no effect on intramembranous bone formation (4, 32). The absence of morphological abnormalities in other tissues indicates the critical importance of PTH/PTHrP signaling in embryonic chondrogenesis and the subsequent replacement of cartilage by bone. Mechanisms regulating chondrogenic differentiation have been elucidated mainly by using mesenchymal cells isolated from chick limb buds. One of this paper's authors (T. Atsumi) previously established a chondrogenic clonal cell line, ATDC5, isolated from mouse embryonal carcinoma (EC) cells (5). Assuming a fibroblastic shape, ATDC5 cells rapidly proliferate to form a confluent monolayer until growth ceases as a result of contact inhibition. Supplementation of culture medium with insulin, however, induces typical cellular condensation before overt chondrogenesis. The high frequency of conversion of cells to chondrocytes enabled us to readily monitor the differentiation stages of the cells in molecular and biochemical terms. Taking advantage of inductive chondrogenesis *in vitro*, we correlated establishment of the PTH/PTHrP-signaling system with the initial stage of endochondral bone formation. Moreover, the potential role of PTH/PTHrP actions on cellular condensation is also discussed.

## Materials and Methods

### Cell Lines and Culture Conditions

ATDC5 cells were cultured in the maintenance medium consisting of a 1:1 mixture of DME and Ham's F-12 medium (Flow Laboratories, Irvine, U.K.) containing 5% FBS (GIBCO BRL, Gaithersburg, MD), 10  $\mu$ g/ml human transferrin (Boehringer Mannheim GmbH, Mannheim, Germany), and  $3 \times 10^{-8}$  M sodium selenite (Sigma Chemical Co., St. Louis, MO), as previously described (5). Inoculum size of the cells was  $2 \times 10^4$  cells/well in a 24-multiwell plate,  $4 \times 10^4$  cells/well in a 12-multiwell plate, or  $6 \times 10^4$  cells/well in a 6-multiwell plate (Corning, Corning, NY). For induction of chondrogenesis, the cells were cultured in maintenance medium supplemented with 10  $\mu$ g/ml bovine insulin (Wako Pure Chemical, Osaka, Japan); we refer to this medium as differentiation medium. Cells were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The medium was replaced every other day. In some experiments, the cells were cultured in maintenance medium supplemented with 300 ng/ml human recombinant insulin-like growth factor-I (rhIGF-I (GIBCO BRL)) instead of insulin.

To determine the growth curve, ATDC5 cells were inoculated in 24-multiwell plates and cultured in maintenance medium or differentiation medium containing insulin (10  $\mu$ g/ml). The cells were then rinsed with

PBS twice, treated with trypsin, and counted with a hemocytometer. On day 10 of culture or later, the number of cells was corrected according to the DNA content of the culture, since differentiated ATDC5 cell aggregates that formed failed to dissociate by trypsinization. Determination of the DNA content was performed by the method of Johnson-Wint (27).

### Receptor Binding of [<sup>125</sup>I]PTH(1-34)

ATDC5 cells were grown in 24-multiwell plates. [Nle<sup>8</sup>, Nle<sup>18</sup>, Tyr<sup>34</sup>]bovine PTH-(1-34) amide (bPTH(1-34); Bachem, Torrance, CA) was labeled by the chloramine-T method as previously described (55). The resultant [<sup>125</sup>I]-labeled bPTH(1-34) ([<sup>125</sup>I] bPTH(1-34); 2,200 Ci/mmol) was purified by reverse-phase HPLC on a  $\mu$ Bondasphere C18 column (Waters, Milford, MA). To monitor the appearance of specific binding sites, ATDC5 cells were cultured in differentiation medium. After the indicated times in culture, the cells were rinsed with binding buffer containing 50 mM Tris-HCl (pH 7.7), 100 mM NaCl, 5 mM KCl, 2 mM CaCl<sub>2</sub>, 5% heat-inactivated horse serum and then incubated at 15°C for 4 h in the same buffer containing [<sup>125</sup>I]bPTH(1-34) (17 pM) (54). Cells were then rinsed five times with ice-cold binding buffer, and the cell-associated radioactivity was counted after cell lysis with 0.2 M NaOH. Nonspecific binding was assessed by the addition of 1  $\mu$ M bPTH(1-34). For saturation analysis, [<sup>125</sup>I]bPTH(1-34) with a low specific activity (220 Ci/mmol) was prepared by iodination of bPTH(1-34) with a mixture of Na<sup>127</sup>I and Na<sup>125</sup>I at a molar ratio of 9:1 (54). For saturation studies, cells were incubated with varying concentrations of [<sup>125</sup>I]bPTH(1-34) with a low specific activity for 4 h at 15°C under conditions otherwise identical to those described above. Equilibrium binding data from the saturation experiments were analyzed by the method of Scatchard.

Binding components for bPTH(1-34) were covalently cross-linked with [<sup>125</sup>I]bPTH(1-34) photochemically as previously described (55). Briefly, ATDC5 cells cultured for 3 wk were rinsed once with binding buffer, and incubated in the same buffer containing 800 pM [<sup>125</sup>I]bPTH(1-34) in the presence or absence of 1  $\mu$ M bPTH(1-34) for 4 h at 15°C. Cells were rinsed three times with ice-cold PBS, reincubated at 4°C with PBS, and then reacted in the dark with 0.5 mM *N*-hydroxysuccinimidyl-4-azidobenzoate (HSAB; Pierce, Rockford, IL) for 10 min followed by photolysis for 20 min at 4°C. The cells were mixed with SDS sampling buffer and heated at 80°C for 20 min. Aliquots were subjected to SDS-PAGE analysis on 5–15% linear gradient gels and subsequent autoradiography.

### Measurement of Adenylate Cyclase Activity in Response to PTH(1-34)

Stimulation of cAMP accumulation in ATDC5 cells was assayed by a modification of the method described previously (55). Briefly, ATDC5 cells were plated in 24-multiwell plates. Cells were rinsed once with Ham's F-12 medium (Nissui, Tokyo, Japan) containing 25 mM Hepes (pH 7.65, 22°C) and 0.1% (wt/vol) heat-inactivated BSA, and then incubated for 30 min at room temperature in the above medium containing 0.5 mM 1-methyl-3-isobutylxanthine (MIX; Sigma) and various concentrations of bPTH(1-34). The reaction was terminated by addition of ice-cold HCl to a final concentration of 0.1 M. The samples were boiled for 3 min to extract cAMP and then diluted with distilled water. Total cAMP in the cell layer and medium was determined by radioimmunoassay as previously described (55).

### RNA Extraction and Hybridization Analysis

ATDC5 cells were inoculated in six-multiwell plates and cultured in differentiation medium. Total RNA was prepared from the cultures by a single-step method according to Chomczynski and Sacchi (11). Total RNA (20  $\mu$ g) was denatured with 6% formaldehyde, separated by 1% agarose electrophoresis, and transferred on Nytran membranes (Schleicher & Schuell, Dassel, Germany). Hybridization was performed overnight at 42°C with an appropriate probe (10<sup>6</sup> cpm/ml) in solutions containing 50% formamide, 6 $\times$  SSPE (2 $\times$  SSPE contains 0.3 M NaCl, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM EDTA at pH7.4), 0.2% BSA, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.1% SDS, and 100  $\mu$ g/ml denatured salmon sperm DNA. Hybridization probes were prepared by the random-primer method with a BcaBEST labeling kit (Takara, Otsu, Japan) using the appropriate cDNA fragments: a 1.4-kb EcoRI fragment of pKT1180 (34) as a probe for  $\alpha$ 1(II) collagen mRNA; a 1.4-kb EcoRI fragment of p1310 (13) as a probe for aggrecan mRNA; and a 2.2-kb EcoRI-XhoI fragment of pCDNA1R15B (3) as a probe for PTH/PTHrP receptor mRNA. The filters were washed

twice for 15 min at 55°C in 0.1 × SSPE and 0.1% SDS, and exposed to X-OMAT film (Eastman Kodak Co., Rochester, NY) at -80°C with a Cronex lightening plus intensifying screen (DuPont, Boston, MA). For slot-blot hybridization analysis, RNA (4 or 1 µg) was isolated and denatured with 6% formaldehyde and applied to Nytran membranes with a Schleicher & Schuell Manifold II slot-blot apparatus. Quantification was performed by scanning densitometry of the autoradiographs with a Hoefer GS-300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA).

### Reverse Transcription PCR

First-strand cDNA was synthesized using SuperScript II RNase H<sup>-</sup> reverse transcriptase (GIBCO BRL) with total RNA extracted from ATDC5 cells according to the manufacturer's instructions. Briefly, purified total RNA (2.5 µg) was incubated at 42°C for 60 min with a mixture of 100 U of SuperScript II RNase H<sup>-</sup> reverse transcriptase; 2.5 µM of oligo-d(T)<sub>16</sub> primer (Perkin Elmer Cetus Corp., Norwalk, CT); 3 mM MgCl<sub>2</sub>; 50 mM Tris-HCl (pH 8.3); 75 mM KCl; 1 mM each of dCTP, dTTP, dGTP, and dATP; 5 mM DTT in a volume of 10 µl. Aliquots of one tenth of the cDNA were used to amplify types I and II collagen genes by PCR (1). A primer set specific for the mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene (Clontech, Palo Alto, CA) was used as a positive control for amplification of cDNA. The expected size of the amplified DNA fragment was 983 bp. The PCR conditions were 94°C for 30 s, 60°C for 1 min, 72°C for 1 min for 20 cycles, and final extension at 72°C for 5 min. Primers specific for the pro-α2(I) collagen gene were 5'-GAACG-GTCCACGATTGCATG-3' (forward direction) and 5'-GGCATGT-TGCTAGGCACGAAG-3' (reverse direction) (1). Primers for the pro-α1(II) collagen gene were 5'-CACACTGGTAAGTGGGGCAAGACCG-3' (forward direction) and 5'-GGATTGTGTTTTCAGGGTTCGGG-3' (reverse direction) (1). The distances between the forward and reverse primers for pro-α2(I) and pro-α1(II) collagen genes were 167 and 172 bp, respectively. Aliquots (5 µl) of the PCR reaction were resolved on 4% NuSieve 3:1 agarose gels (FMC BioProducts, Rockland, ME) along with molecular size markers, and the amplified products were stained with ethidium bromide. The amplified PCR products were subcloned into pCRII (Invitrogen, San Diego, CA), and their sequences were confirmed with a Prism 310 genetic analyzer (Applied Biosystems, Inc., Foster City, CA).

### Alcian Blue Staining

ATDC5 cells were plated in 12-multiwell plates and cultured in differentiation medium. At each time point, cells were rinsed with PBS and fixed with 95% methanol for 20 min. They were then stained with 0.1% Alcian blue 8GS (Fluka, Buchs, Switzerland) in 0.1 M HCl overnight.

### Treatment of Cells with PTH Analogues and PTHrP

To assess the effects of PTH and PTHrP on chondrogenesis, ATDC5 cells were plated in 12-multiwell plates. When the cells reached confluency in differentiation medium on day 3, cultures were treated with the following test samples: bPTH(1-34), [Tyr<sup>34</sup>]bovine PTH-(7-34) amide (bPTH(7-34); Peptide Institute, Osaka, Japan) and human PTHrP(1-141) (hPTHrP(1-141); a generous gift from Dr. T.J. Martin [St. Vincent's Institute of Medical Research, Fitzroy, Victoria, Australia]). The cells were treated with these agents for another 12 d, with replacement of the medium every other day. Effects on cellular condensation and differentiation were monitored by phase-contrast microscopy and Alcian blue staining of the cells. Secretion of endogenous PTHrP by the cells was examined by determining the PTHrP level in the medium conditioned for 3 d using an Allegro PTHrP immunoassay kit (Nicholas Institute, San Juan Capistrano, CA).

## Results

### Cell Growth and Chondrogenesis of ATDC5 Cells

A clonal chondrogenic cell line, ATDC5, was established from a differentiated culture of EC-derived AT805 cells on the basis of its chondrogenic potential (5). As shown in Fig. 1, ATDC5 cells were inoculated into 24-multiwell plates and maintained in DME/F-12 supplemented with 10 µg/ml human transferrin, 3 × 10<sup>-8</sup> M sodium selenite, and

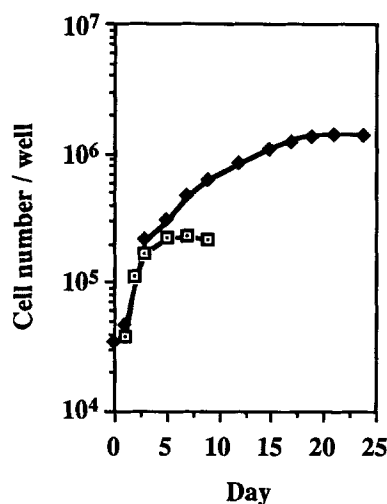
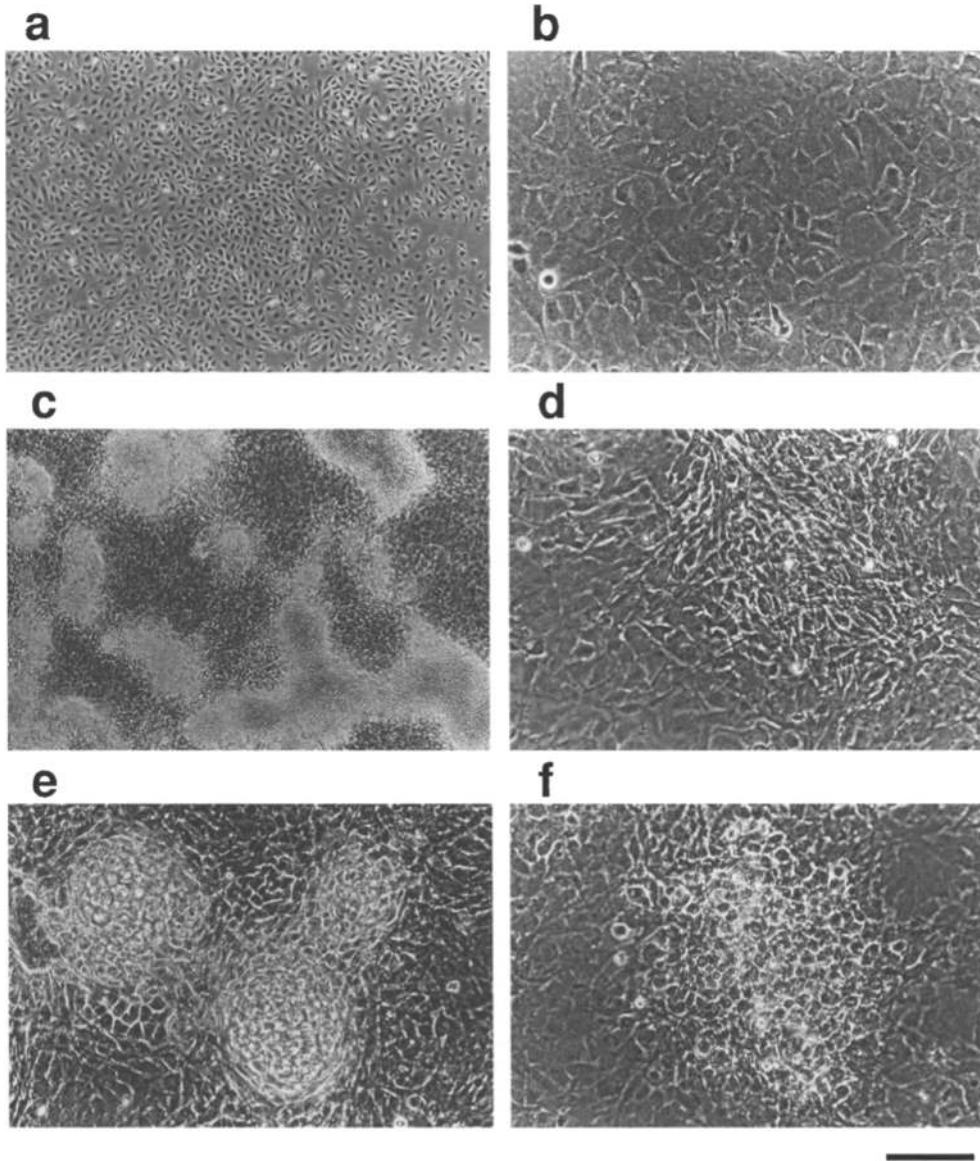


Figure 1. Growth curves of ATDC5 cells. Cells were cultured in 24-multiwell plates in maintenance medium in the absence of insulin (□) or in the presence of 10 µg/ml insulin (◆). Total cell numbers in culture were determined for three wells at each time point, as described in Materials and Methods. Values are the means of three wells. SEM were so small that they are hidden by the symbols. The figure represents one of two independent experiments with similar results.

5% FBS (the maintenance medium). ATDC5 cells rapidly proliferated with a short doubling time of 16 h (Fig. 1). The cells exhibited an elongated fibroblastic morphology at the subconfluent stage (Fig. 2 a). On day 3 of culture, the cells stopped growing at confluence to form a monolayer in which they remained undifferentiated with a fibroblastic morphology (Fig. 2 b). Under these conditions, saturation cell density was ~2.5 × 10<sup>5</sup> cells/well (1.41 × 10<sup>5</sup> cells/cm<sup>2</sup>). No Alcian blue-positive cartilage nodules appeared in these cultures.

Alternatively, the cells were grown in differentiation medium containing 10 µg/ml insulin. Insulin did not significantly affect cell doubling time (Fig. 1) or the morphology of the cells in the growth phase. Upon attainment of confluency, the cells similarly ceased to grow by contact inhibition. Within the three days after confluency, however, the culture reentered the growth state with a longer cell doubling time to form numerous nodular structures by day 21 (Fig. 2 c). A transient condensation of cells with an elongated spindle-like morphology (Fig. 2 d) preceded formation of the nodules, as seen in mesenchymal condensation in limb bud chondrogenesis (14). The nodular structures formed were composed of proliferating cells with a round morphology (Fig. 2 e). Logarithmic growth of cells in the postconfluent phase occurred for ~4 d, from days 6-10 (Fig. 1). The doubling time of the cells during this period was ~48 h, consistent with what was reported previously (5). IGF-I (300 ng/ml) also induced cellular condensation and the subsequent formation of cartilage nodules (Fig. 2 f). However, IGF-I was not as effective as insulin for chondrogenic induction of the cells. As shown in Fig. 3, Alcian blue-positive cartilage nodules were first detectable between days 6 and 10, and they increased in size. Then the apparent growth rate of the culture decreased, probably because a portion of the differentiated cells be-

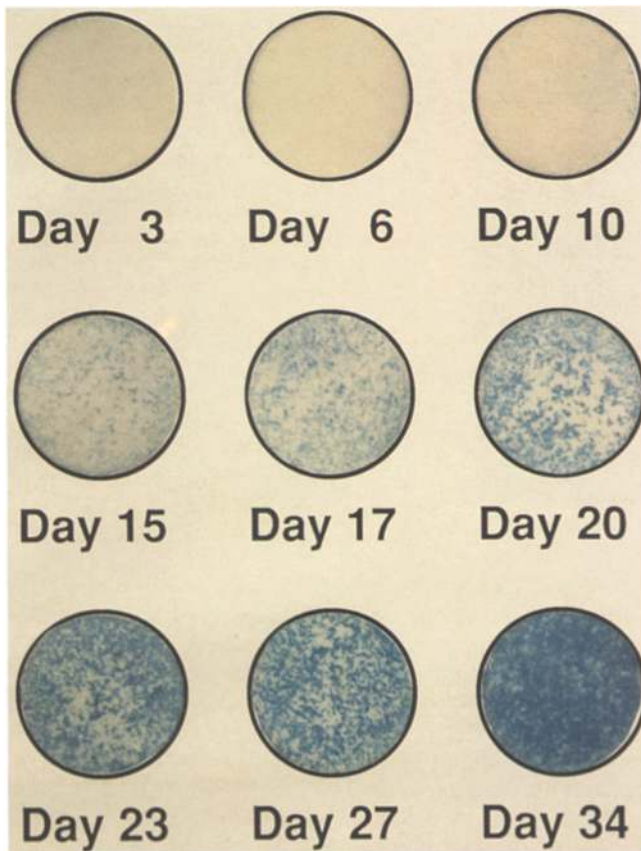


**Figure 2.** Phase-contrast micrographs of ATDC5 cells. Cells were cultured in maintenance medium in *a* and *b* or in differentiation medium in (*c*–*f*) in 24-multiwell plates. (*a*) Cells at the subconfluent stage on day 2 of culture. (*b*) Cells at confluency on day 3 are shown at a higher magnification. (*c*) Typical cartilage nodules were formed by day 21. Cells at the condensation stage on day 6 and at the growth stage of cartilage nodules on day 14 are shown in *d* and *e*, respectively. (*f*) The cells were cultured for 14 days in the presence of IGF-I (300 ng/ml) instead of insulin. Bar, 500  $\mu\text{m}$  for *a* and *c*, and 100  $\mu\text{m}$  for *b* and *d*–*f*.

gan to mature (Fig. 1). This postconfluent growth of cells, however, continued for 2 wk until day 21, and then ceased. On day 21, the total number of cells reached a maximum at  $1.6 \times 10^6$  cells/well ( $9.1 \times 10^5$  cells/cm<sup>2</sup>). Cells surrounding cartilage nodules retained their fibroblastic morphology and presumably did not grow during expansion of the nodules. Assuming that these cells were quiescent, 85% of the total cell population at the end of the growth stage of cartilage nodules was estimated to be differentiated chondrocytes. Even after cessation of postconfluent growth, the Alcian blue-positive area appeared to expand by accumulation of cartilage matrix resulting from the maturation of cells (Fig. 3).

Chondrogenic differentiation of ATDC5 cells was further characterized by expression of cartilage-characteristic extracellular matrix genes such as aggrecan (Fig. 4 *a*) and type II collagen (Fig. 4 *b*). Transcripts for these genes were undetectable in undifferentiated ATDC5 cells on day 2 (Fig. 4, *a* and *b*, lane 1). In contrast, these cartilage-characteristic transcripts became readily detectable in the differ-

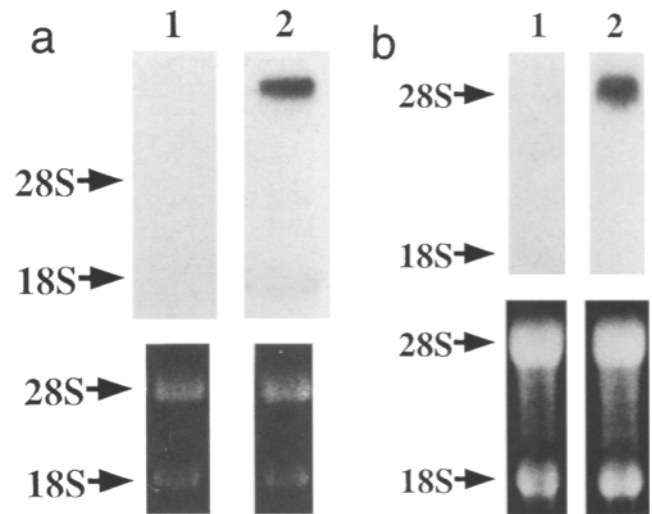
entiated cultures by day 14 (Fig. 4, *a* and *b*, lane 2). Expression of type I and type II collagen genes were compared by reverse transcription PCR under identical amplification conditions, using the specific primer sets described in Materials and Methods (Fig. 5) (1). A primer set specific for the mouse G3PDH gene was used as a positive control for amplification of cDNA. The amplified DNA fragments were verified by determination of their sequence. As shown in Fig. 5 *a*, undifferentiated ATDC5 cells on day 2 evidently expressed the type I collagen gene transcript, which remained detectable throughout the experimental period. In contrast, the type II collagen gene transcript was undetectable on day 2 (Fig. 5 *b*). It became barely detectable on day 10 and readily detectable on day 21, when cartilage nodules that had formed in culture ceased to grow. Analysis of cartilage nodules by transmission electron microscopy indicated that differentiated ATDC5 cells were surrounded by thin type II collagen fibrils ( $\sim 20$  nm in diameter) (20), but no thick fibrils characteristic of type I collagen were found (data not shown).



**Figure 3.** Formation of cartilage nodules in the presence of insulin. ATDC5 cells were cultured in differentiation medium in 12-multiwell plates. Cultures were stained with 0.1% Alcian blue.

#### Acquisition of PTH/PTHrP Responsiveness during Chondrogenic Differentiation of ATDC5 Cells

It has been established that rabbit growth plate chondrocytes express PTH/PTHrP receptors on their surface *in vitro* (15, 22). ATDC5 cells were plated in 24-multiwell plates, and they were maintained for 21 d in differentiation medium containing insulin (10  $\mu\text{g/ml}$ ) until the growth of cartilage nodules ceased. A saturation binding experiment was then carried out. Cells were incubated with incremental doses of [ $^{125}\text{I}$ ] bPTH(1-34) in the presence or absence of unlabeled bPTH(1-34). Available binding sites for the ligand were saturable, as shown in Fig. 6 *a*. Scatchard analysis indicated the presence of a single class of binding sites that exhibited an apparent dissociation constant of 3.9 nM and a binding capacity ( $B_{\text{max}}$ ) of  $3.2 \times 10^5$  sites/cell (Fig. 6 *a*; *inset*). Under the present experimental conditions,  $\sim 15\%$  of the cells in culture remained undifferentiated (Fig. 1). Assuming that undifferentiated cells do not express PTH/PTHrP receptors, the determined  $B_{\text{max}}$  value may be underestimated to some extent. Then we attempted to visualize the binding component on the differentiated cell culture by photochemical cross-linking. Cells were incubated with [ $^{125}\text{I}$ ]bPTH(1-34), extensively rinsed, photoreacted with HSAB, and then analyzed by autoradiography after SDS-PAGE (Fig. 6 *b*). The predominant binding component was identified as a diffuse 80-kD band. Labeling of

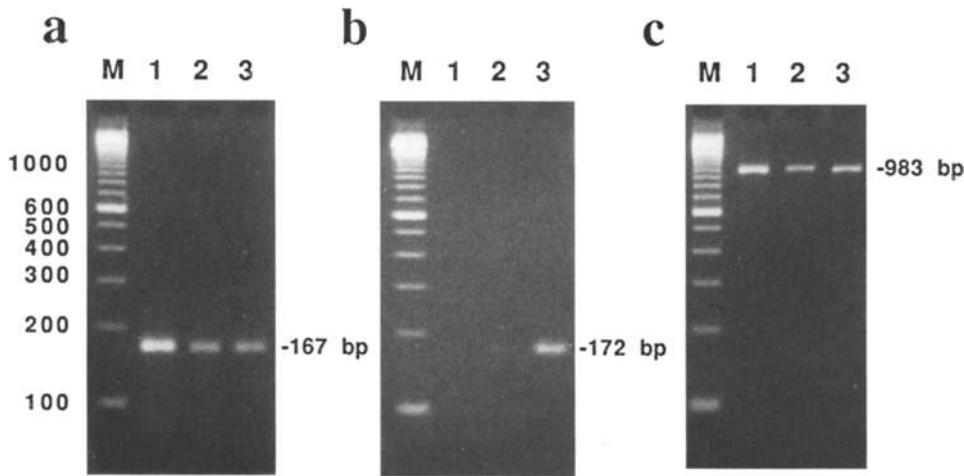


**Figure 4.** Northern blot analysis of aggrecan and type II collagen mRNAs in ATDC5 cells. Cells were grown in the differentiation medium in six-multiwell plates. Total RNA was isolated on day 2 (lane 1) and on day 14 (lane 2) of culture. RNA (20  $\mu\text{g}$ ) was loaded in each lane and then hybridized with rat aggrecan cDNA (*a*) or rat type II collagen cDNA (*b*). The membranes were exposed to film for 24 h in *a* and for 6 h in *b*. The positions of 28S and 18S ribosomal RNAs are indicated. The lower panels show ethidium bromide-stained gels.

the 80-kD band was completely abolished by unlabeled bPTH(1-34).

Taking advantage of the inductive chondrogenesis of ATDC5 cells, we examined the differentiation-dependent expression of PTH/PTHrP receptors by monitoring the specific binding of [ $^{125}\text{I}$ ]bPTH(1-34) (Fig. 7). Cells were maintained in differentiation medium containing 10  $\mu\text{g/ml}$  insulin, and were incubated with [ $^{125}\text{I}$ ]bPTH(1-34) in the absence or presence of unlabeled bPTH(1-34). No specific binding was detectable on day 4, at which time no cartilage nodules were observed. Only 2 d later, however, the specific binding of [ $^{125}\text{I}$ ]bPTH(1-34) was clearly detectable; it amounted to 3% on day 6, when cartilage nodules were first seen. In association with the formation and growth of cartilage nodules, the specific binding increased markedly and reached a maximum of 11% of the added radiolabeled ligand on day 21. Nonspecific binding remained reasonably low (from 1.0 to 1.5%) during the entire experimental period. These results suggest that the expression of cell-surface receptors for PTH is closely associated with chondrogenic differentiation of the cells.

The PTH/PTHrP receptor acts through activation of the coupled adenylate cyclase (30). The functionality of the PTH/PTHrP receptor on differentiated ATDC5 cells was monitored by the ligand-dependent activation of adenylate cyclase on the basis of a unit number of cells in the culture. As shown in Fig. 8, differentiated cells exhibited a bPTH(1-34)-dependent activation of adenylate cyclase. The enzyme activity was detectable in response to  $\leq 10$  pM bPTH(1-34). The half effective dose  $\text{ED}_{50}$  of bPTH(1-34) was  $\sim 0.2$  nM. Cells at the initial stage of chondrogenesis revealed only marginal activation of the enzyme on day 4. These lines of evidence suggest that acquisition of PTH/



**Figure 5.** Detection of type I collagen mRNA in ATDC5 cells by reverse transcription PCR, in comparison to type II collagen mRNA. Cells were grown in differentiation medium in six-multiwell plates. Total RNA was isolated on day 2 (lane 1), day 10 (lane 2), and day 21 (lane 3) from the cells grown in differentiation medium. Reverse-transcribed cDNA was amplified by PCR. Aliquots (5  $\mu$ l) of the PCR products were resolved on 4% agarose gels alongside markers (M). The primer pairs used were specific for the following target sequences: type I collagen in *a*; type II collagen in *b*; G3PDH in *c*.

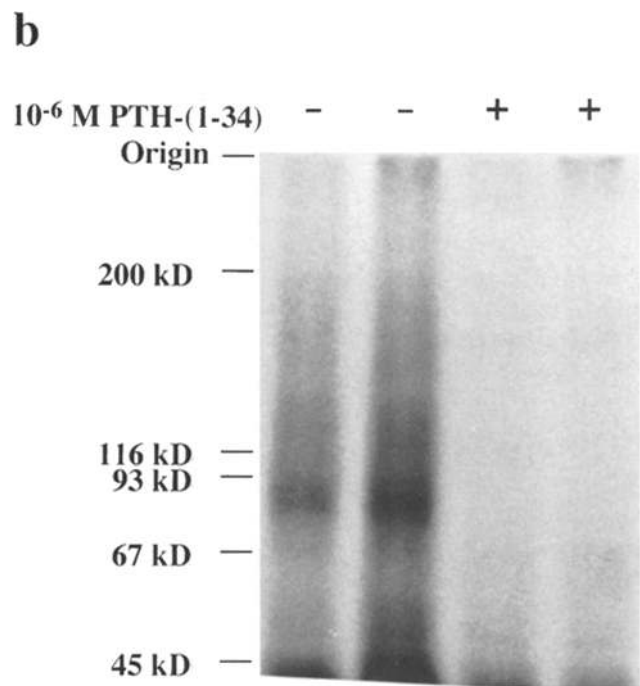
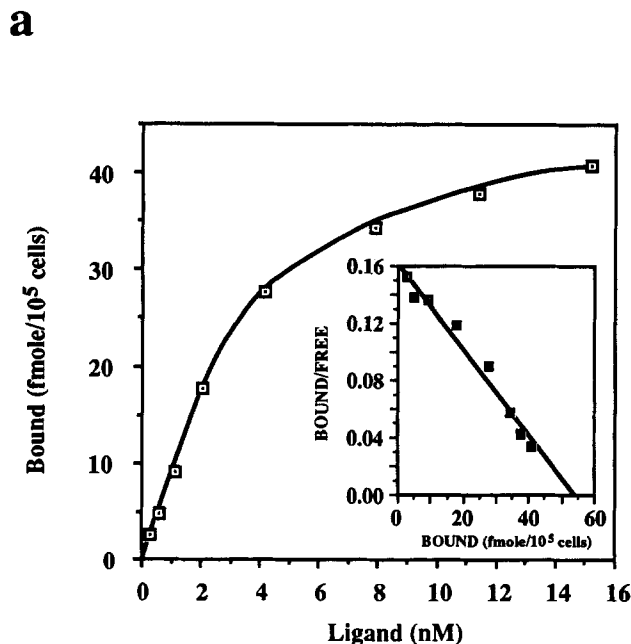
PTHrP responsiveness is closely associated with the onset of chondrogenesis.

#### Induction of the PTH/PTHrP Receptor Gene during Chondrogenesis of ATDC5 Cells

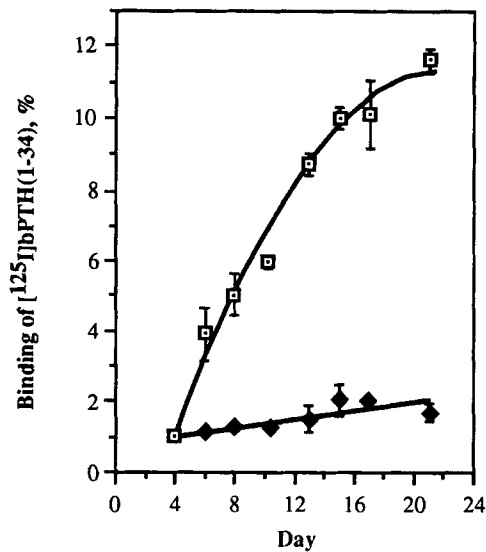
We studied the expression of a PTH/PTHrP mRNA in ATDC5 cells by Northern blotting. The rat PTH/PTHrP receptor cDNA probe was hybridized to total RNA (20  $\mu$ g) from cells at different stages of differentiation (Fig. 9 *a*). As shown in lane 1, there was no detectable hybridization to

RNA extracted from undifferentiated cultures of ATDC5 cells on day 2. In contrast, on day 7, when the formation of cartilage nodules was observed in culture, the 2.3–2.5-kb PTH/PTHrP receptor transcript was clearly detectable (Fig. 9 *a*; lane 2). The mRNA level increased markedly in parallel with the growth of cartilage nodules, and it reached a maximum by day 21, when cellular proliferation ceased (Fig. 9 *a*; lanes 3 and 4); then the mRNA level gradually decreased (Fig. 9 *a*; lanes 5 and 6).

The time course of changes in the PTH/PTHrP receptor mRNA level was determined in detail by slot blot analysis,



**Figure 6.** Cell-surface binding of [ $^{125}$ I]bPTH(1-34) to differentiated ATDC5 cells cultured in the presence of insulin. Cells were grown in differentiation medium for 21 d in 24-multiwell plates. (*a*) Equilibrium saturation binding of [ $^{125}$ I]bPTH(1-34) was determined in triplicate. Experiments were conducted twice with similar results. The inset shows Scatchard analysis of the same data. (*b*) PTH/PTHrP receptors on differentiated ATDC5 cells were visualized by photochemical cross-linking of [ $^{125}$ I]bPTH(1-34). Cells were incubated in the binding buffer containing [ $^{125}$ I]bPTH(1-34) in the absence (–) or presence (+) of unlabeled bPTH(1-34). Cells were then reacted with HSAB followed by photolysis. Experiments were carried out in duplicate.



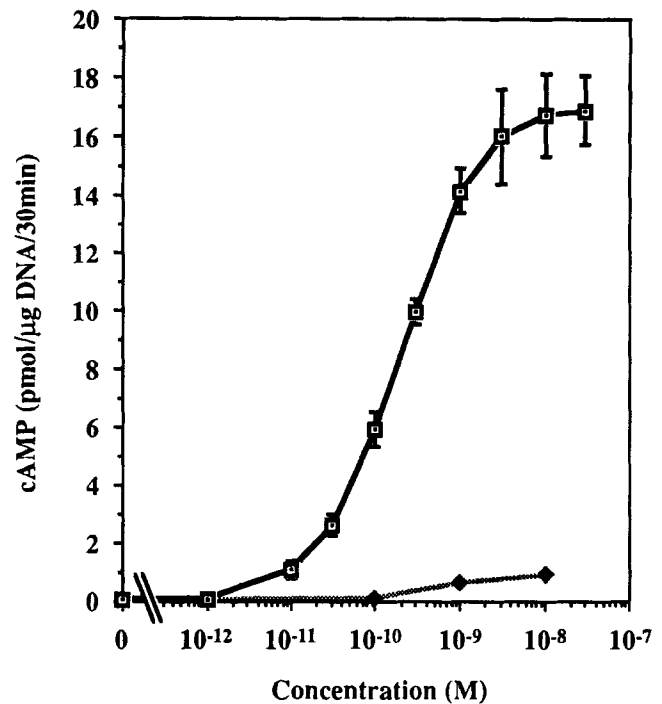
**Figure 7.** Time course of changes in binding capacity of ATDC5 cells to [<sup>125</sup>I]bPTH(1-34) during chondrogenic differentiation. Cells were grown in differentiation medium in 24-multiwell plates for the indicated periods. Cells were incubated with [<sup>125</sup>I]bPTH (1-34) (17 pM) in the absence (□) or presence (◆) of the corresponding unlabeled ligand (1 μM). The cell-associated radioactivity was counted. Each value represents the mean ± SEM in triplicate assays. The figure represents one of two independent experiments with similar results.

and was compared to that of type II collagen mRNA (Fig. 9 b). Expression of the genes was induced in parallel with the formation of cartilage nodules in culture. Densitometric analysis clearly indicated that inductive expression of the PTH/PTHrP receptor gene coincided with expression of the type II collagen gene in a logarithmic manner (Fig. 9 c). These results suggest that the expression of the PTH/PTHrP receptor gene in ATDC5 cells is closely associated with chondrogenic differentiation.

#### **Effect of PTH or PTHrP on Chondrogenic Differentiation of ATDC5 Cells**

To elucidate the functional role of PTH/PTHrP signaling in chondrogenesis, we studied the effects of PTH analogues on chondrogenic differentiation of ATDC5 cells (Fig. 10). Cells were grown to confluency in differentiation medium for the initial 3 d. The cells were then incubated for an additional 12 d with 10<sup>-8</sup> M bPTH(1-34) or hPTHrP(1-141), which activated adenylate cyclase to a maximal level. Both peptides completely inhibited cellular condensation and the subsequent formation of cartilage nodules in culture (Fig. 10, b and c). No Alcian blue-positive cartilage matrix accumulated in these cells in culture (data not shown). In contrast, treatment with bPTH(7-34) did not affect differentiation (Fig. 10 d).

Secretion of PTHrP, an endogenous ligand for the PTH/PTHrP receptor, from ATDC5 cells was examined. Undifferentiated cells were plated in 24-multiwell plates and grown in differentiation medium. Culture medium was replaced into fresh medium and conditioned for 3 d. Concentration of PTHrP in the 3-d conditioned medium was



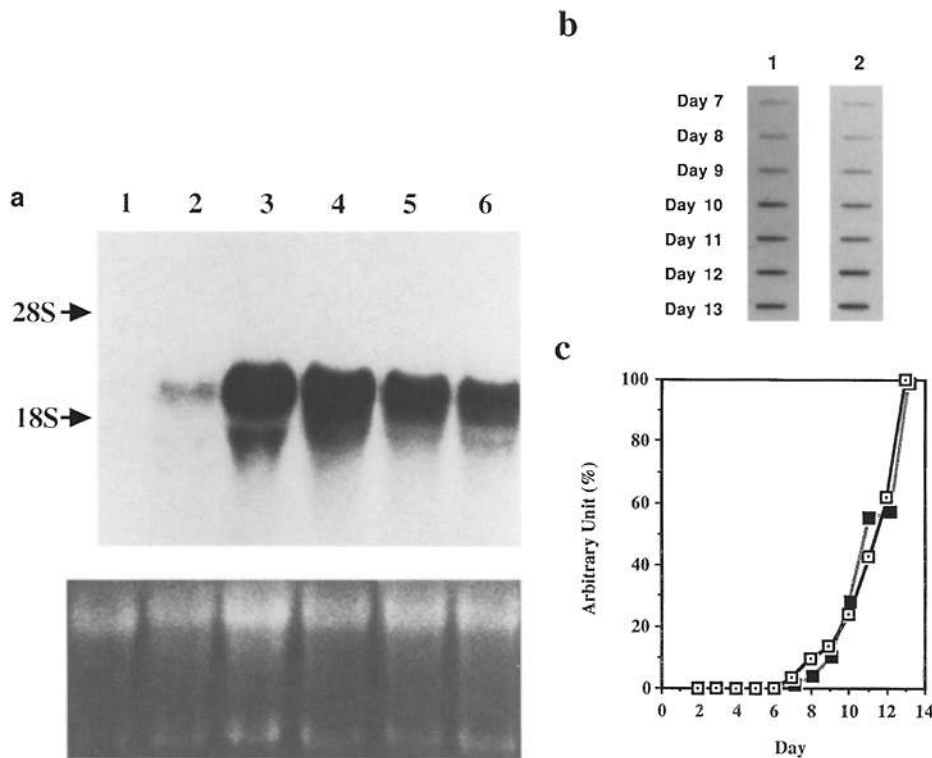
**Figure 8.** PTH-dependent adenylate cyclase activity in undifferentiated and differentiated ATDC5 cells. Cells were grown in a 24-multiwell plate for 4 d (◆) or for 21 d (□) in the differentiation medium. Cells were incubated for 30 min with 0.5 mM MIX and various doses of bPTH(1-34). The reaction was terminated by addition of ice-cold HCl. Production of cAMP was assessed by radioimmunoassay. Each value represents the mean ± SEM in triplicate assays. The figure represents one of two independent experiments with the similar results.

determined by radioimmunoassay. As shown in Table I, medium conditioned from days 2–5 contained a detectable level of PTHrP (4.4 pM). Concentration of PTHrP in the conditioned medium decreased as cell differentiation progressed, and it was below the detection limit (<0.1 pM) on day 52. Thus, ATDC5 cells, probably undifferentiated cells, secrete PTHrP into culture medium, but its concentration seemed too low to activate PTH/PTHrP receptor-coupled adenylate cyclase in the differentiated cells.

#### **Discussion**

Wherever bone is formed by replacement of a cartilaginous bone rudiment (endochondral pathway), this rudiment is preceded in development by the appearance of a cellular condensation within the mesenchyme or, in the case of the head, within the ectomesenchyme derived from the neural crest. Skeletal elements begin to become faintly discernible as the particular regions where cells become more densely packed (14). Once cells are in this condensation state, they begin to produce large amounts of cartilage-specific molecules such as type II collagen and aggrecan.

Cultured limb mesenchymal cells exhibit commitment and gene expression profiles in culture that closely resemble *in vivo* events (56). Their usefulness has been limited, however, by requirements for a high seeding density (39),



PTH/PTHrP receptor mRNA levels (□) and type II collagen (■) are compared. Each mRNA level was quantified by scanning densitometric analysis of the slot blot in *b*. Values are expressed as percentages of the highest hybridization intensity for each mRNA. Two independent experiments were performed and gave similar results.

**Figure 9.** Time course of changes in PTH/PTHrP receptor mRNA level during chondrogenic differentiation, compared to that for type II collagen. Cells were grown in differentiation medium in six-multiwell plates. (a) Northern blot analysis of PTH/PTHrP receptor mRNA is shown. Total RNA was isolated on day 2 (lane 1), day 7 (lane 2), day 14 (lane 3), day 21 (lane 4), day 24 (lane 5), and day 28 (lane 6). 20  $\mu$ g of total RNA was used per lane, and the membrane was exposed to film for 37 h. The positions of 28S and 18S ribosomal RNAs are indicated. The lower panel shows ethidium bromide staining of the gel. (b) RNA isolated on the indicated day of culture was analyzed by slot blotting. 4  $\mu$ g of total RNA was hybridized with rat PTH/PTHrP receptor cDNA probe, and the membrane was exposed to film for 8 d (lane 1). 1  $\mu$ g of total RNA was hybridized with rat type II collagen cDNA probe, and the membrane was exposed to film for 17 h (lane 2). (c) The time courses of changes in

e.g.,  $10^7$  cells/ml, and the availability of a homogeneous cell population. In a high density culture of chick stage 24 limb mesenchymal cells, chondrogenesis predominates with 60–85% of cells identifiable as chondrocytes, whereas muscle differentiation is maximal and chondrogenesis is totally lacking in low density culture (56). Thus, the molecular analysis of chondrogenesis awaits the establishment of a clonal cell line capable of chondrogenic differentiation *in vitro*. Cell lines such as RCJ3.1 and CFK2 exemplify the usefulness of a chondrogenic clonal cell line for the study of skeletal development (7, 21). These cell lines, however, were isolated from fetal calvariae, which do not develop via the endochondral pathway but via the intramembranous pathway of bone formation. EC cells are the stem cells of teratocarcinomas, and they show marked similarities to cells of the early embryo. They lose their malignancy as they differentiate. Teratocarcinoma cells are therefore a useful alternative to embryos for studying the processes of early mammalian development. Since there may be the potential limitations of EC cells, careful characterization of their phenotype is required.

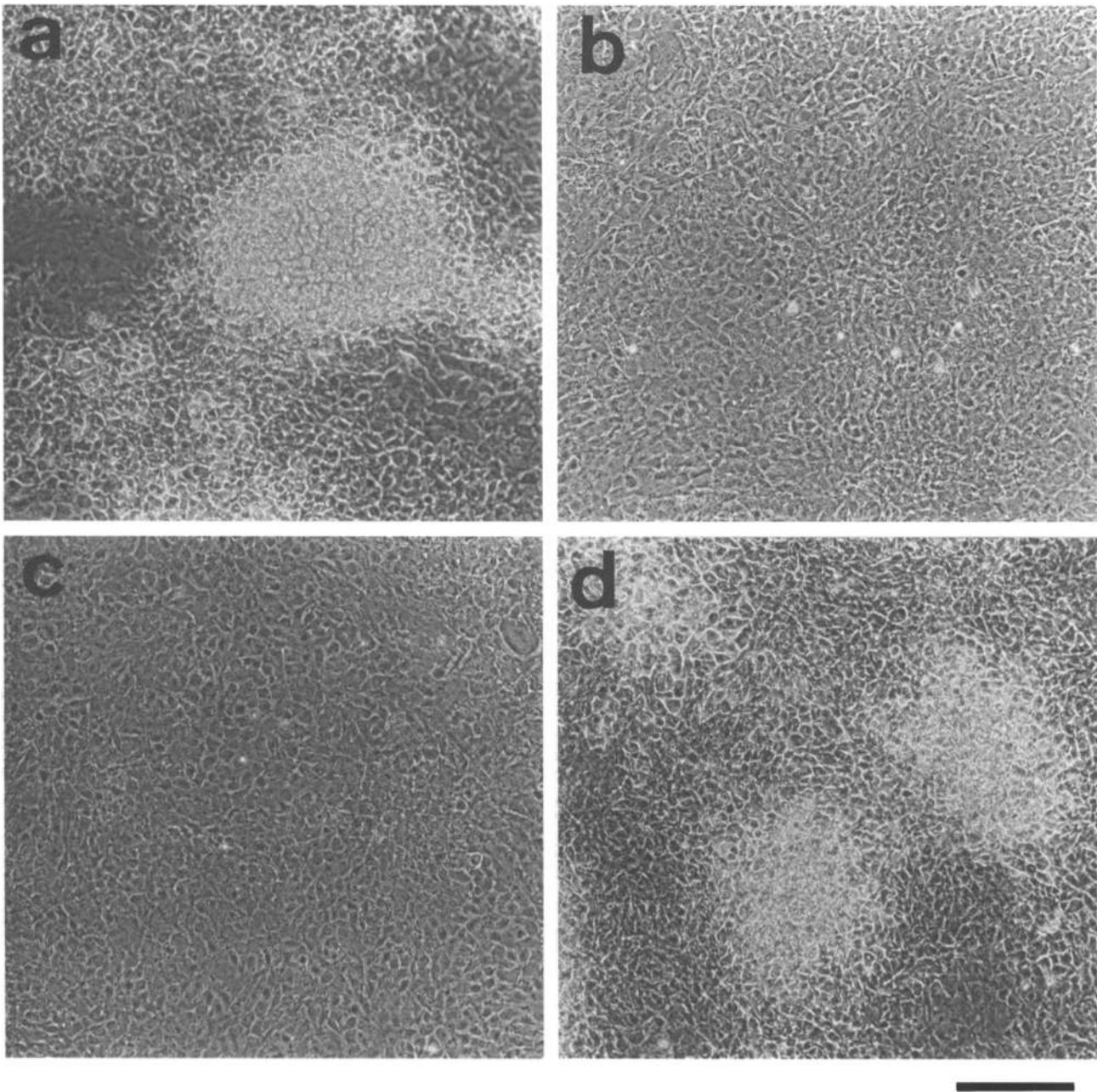
ATDC5 is a clonal cell line derived from the multipotent EC AT805, which grows *in vitro* without feeder cells (5). In the presence of insulin, ATDC5 cells differentiated into chondrocytes to form cartilage nodules at a very high frequency (Fig. 2 c), comparable to a high density micro-mass culture of chick limb bud cells (56). Taking on a fibroblastic morphology, ATDC5 cells expressed the type I collagen gene transcript (Fig. 5), and they initially proliferated with a short doubling time (16 h) irrespective of the presence of insulin. They exhibited contact inhibition at

confluence (Fig. 1). There was neither an accumulation of cartilage matrix (Fig. 3) nor expression of cartilage-specific transcripts (Fig. 4) at this stage. In the presence of insulin, there transiently appeared condensation areas in culture (Fig. 2 d), from which proliferating chondrocytes were generated to form cartilage nodules (Fig. 2 e). The second phase of growth was characterized by a longer cell-doubling time (48 h). The progressive expression of type II collagen mRNA was initiated along with condensation and the subsequent growth of nodules (Figs. 3 and 9).

In cultures of prechondrogenic mesenchymal cells from chick limb buds, there is a first phase in which all cells behave as fibroblasts, exhibiting contact inhibition (14). These cells rapidly proliferate at this stage and there is a considerable increase in cell-doubling time during chondrogenesis (19). Prechondrogenic mesenchymal cells express type I collagen mRNA, but no type II collagen mRNA is expressed. As the cells differentiate into chondrocytes through cellular condensation, they initiate the synthesis of type II collagen. Differentiated chondrocytes cease synthesizing type I collagen, while they still contain type I collagen transcripts (37). A marked increase in type II collagen mRNA level occurs coincidentally with the condensation stage in chondrogenesis. Thereafter, a continuous and progressive increase in the accumulation of type II collagen mRNA occurs (37). With respect to their chondrogenic potential, morphological appearance, growth behavior, and collagen gene expression, ATDC5 cells appear to retain the characteristics of chondrogenic precursor cells.

Cellular condensation is an important prerequisite for





**Figure 10.** Effects of PTH and PTHrP on chondrogenic differentiation. ATDC5 cells were grown to confluency in differentiation medium for the initial 3 d in 12-multiwell plates. The cells were then incubated for a further 12 d with no addition (a) or with  $10^{-8}$  M bPTH(1-34) (b),  $10^{-8}$  M hPTHrP(1-141) (c) or  $10^{-8}$  M bPTH(7-34) (d). Formation of cartilage nodules was examined under a phase-contrast microscope ( $\times 100$ ). Bar, 200  $\mu\text{m}$ .

initiation of chondrogenesis in mesenchymal cell culture. In the presence of insulin, the cell density of ATDC5 culture increased only by  $\leq 50\%$  during the postconfluent 3 d. This observation was compatible with an increase in packing cell density during chick limb bud condensation *in vivo* (14). At this stage, cells lose contact inhibition of movement and grow beyond confluence to produce two or three layers of cells. Recent reports have suggested that FGF signaling plays a role in support of the proliferation of limb bud mesenchyme and limb pattern formation (12, 44, 48, 53). There is no evidence of mitotic activity required for onset of condensation in chick limb chondrogenesis *in*

*vitro*. Cellular condensation is chiefly mediated by a change of cell motility and cell-cell interactions (40, 45). In fact, FGF-2 markedly stimulated the proliferation of confluent ATDC5 cells, but failed to initiate condensation (data not shown). Thus, the mitogenic action of insulin does not seem to be of primary importance for induction of condensation. Insulin probably affects cell morphology and motility during differentiation of ATDC5 cells.

IGF-I has been implicated in early limb development and chondrogenesis (18). We demonstrated that IGF-I also induces cellular condensation and formation of cartilage nodules in cultures of ATDC5 cells. Thus, it is possi-

**Table I. Secretion of Endogenous PTHrP by ATDC5 Cells during the Course of Differentiation In Vitro: Concentration in the Conditioned Medium**

Samples	Concentration of PTHrP
	<i>pmol/liter</i>
Fresh differentiation medium alone	<0.1
Conditioned medium (days 2–5)	4.43 ± 0.88
Conditioned medium (days 7–10)	3.13 ± 0.32
Conditioned medium (days 20–23)	2.38 ± 0.03
Conditioned medium (days 35–38)	0.58 ± 0.22
Conditioned medium (days 52–55)	<0.1

ATDC5 cells were cultured in differentiation medium in 12-multiwell plates, as described in Materials and Methods. Culture medium was replaced with fresh differentiation medium on the days indicated, which was then conditioned for 3 d. Concentration of PTHrP secreted was determined by radioimmunoassay. Values are means ± SEM in triplicate assays. Experiments were conducted twice with similar results.

ble that insulin acts through an IGF-I signaling pathway. IGF-I, however, was less effective than insulin for chondrogenic induction of the cells. Bassas and his co-workers reported the presence of insulin immunoactivity and bioactivity in early chick embryos (6). They also found insulin receptors as well as IGF-I receptors on plasma membranes isolated from limb buds (6). Telford and her co-workers demonstrated the presence of transcripts for insulin receptors and IGF-I receptors in early mouse embryos by reverse transcription PCR, and suggested a possibility that these embryos respond to exogenous (e.g., maternal) sources of insulin (61). Our preliminary experiments indicated that undifferentiated ATDC5 cells express transcripts for insulin receptors and IGF-I receptors (data not shown). Therefore, it is also possible that insulin induces differentiation of ATDC5 cells through an insulin-specific signaling pathway, although the physiological relevance of the action of insulin for chondrogenic induction remains to be elucidated.

There is growing evidence to suggest that BMPs stimulate differentiation of cells of the chondroblastic lineage as well as those of the osteoblastic lineage (10, 33, 47). Transcripts for BMP genes have been localized in mesenchymal condensations and the perichondrium in mouse embryos (28, 43). Recent studies on *brachypodism* and *short ear* mice implied that BMPs mediate cell–cell interactions during condensation and subsequent chondrogenesis (35, 58). Intriguingly, precondensing ATDC5 cells responded to exogenous BMP-2 to undergo chondrogenic differentiation without condensation (Shukunami, C., unpublished data). Thus, BMP-dependent serine/threonine kinase signaling may participate in the chondrogenesis of condensing ATDC5 cells. cAMP signaling, however, has also been implicated in limb chondrogenesis (49, 57). Its effect on cartilage differentiation in vitro was dependent on cell density (46). It is known that PTH stimulates phenotypic expression of differentiated chondrocytes via intracellular production of cAMP (23, 60). We previously demonstrated that cultured growth plate chondrocytes expressed 80-kD PTH/PTHrP receptors on the cell surface (22). It seems unlikely, however, that PTH, an endocrine hormone, acts as a natural ligand for the receptor in early chondrogenic development. Therefore, the physiological significance of PTH responsiveness was puzzling until the wide distribution and the early onset of PTHrP synthesis in embryos were observed (8, 62).

Differentiated ATDC5 cells expressed a large number of 80-kD PTH/PTHrP receptors on the cell surface, and these receptors were functionally active in stimulating adenylate cyclase (Figs. 6 and 8). The binding kinetics of [<sup>125</sup>I]bPTH(1-34) were compatible with previous findings in primary chondrocytes (Fig. 6). Only 10 pM bPTH(1-34) evidently stimulated production of cAMP. The expression of the PTH/PTHrP receptor transcript was readily detectable in differentiated ATDC5 cells by Northern blot analysis using total RNA (Fig. 9). On the other hand, precondensing undifferentiated ATDC5 cells exhibited no specific binding of PTH (Fig. 7). Elevation of binding capacity was correlated with progression of chondrogenic differentiation of cells. Capelhart (9) and Zull (65) described in their earlier reports that the appearance of PTH-sensitive adenylate cyclase was one of the earliest events in chick limb differentiation. It was unclear at the time, however, whether acquisition of PTH responsiveness occurred in cells of the chondrogenic or osteoblastic lineage. In the present study, we used a clonal cell line to demonstrate unequivocally that acquisition of PTH responsiveness is associated with chondrogenic differentiation. The expression of PTH/PTHrP receptor mRNA occurred in parallel with the induction of type II collagen mRNA, and it is one of the earliest events in chondrogenic differentiation (Fig. 9).

Segre and his co-workers suggested by *in situ* hybridization that expression of PTH/PTHrP receptor mRNA is high in maturing chondrocytes, and that PTHrP and PTH/PTHrP receptor genes are expressed in largely discrete but mostly neighboring areas in the developing bones in fetal rats (42). As shown in Table I, undifferentiated ATDC5 cells secreted PTHrP immunoreactivity into culture medium. PTHrP in the medium conditioned for 3 d reached a concentration of 4.4 pM. Although PTHrP in the conditioned medium was higher than that of normal human plasma, capacity of the cells for PTHrP production was lower than that of mammary epithelial cells that were isolated from lactating glands *in vitro* (16). Secretion of PTHrP decreased as differentiation of ATDC5 cells progressed. Since ATDC5 cells were maintained by replacing the medium every other day during the present study, the concentration of endogenous PTHrP cannot be higher than several picomolar. When the dose–response data (Fig. 8) are taken into consideration, it is unlikely that endogenous PTHrP activates PTH/PTHrP receptors that are expressed on the surface of ATDC5 cells in culture.

In contrast, when PTH/PTHrP receptors were continuously activated by bPTH(1-34) or hPTHrP(1-141) during differentiation of the cells, these agents inhibited the formation of cartilage nodules. There was no inhibitory effect by the treatment with bPTH(7-34). Upon interaction with the ligands, PTH/PTHrP receptors activate both the cAMP/protein kinase A (PKA) and the phospholipase C/protein kinase C (PKC) signal pathways (2, 25). Janulis (26) suggested that the phospholipase C/PKC pathway mediates PTH stimulation of renal proximal tubule 1,25-dihydroxyvitamin D<sub>3</sub> secretion. Because NH<sub>2</sub>-terminal amino acids of PTH fragments are required for adenylate cyclase activation (17), bPTH(7-34) binds to PTH/PTHrP receptors without activation of adenylate cyclase (24). Jouishomme (29) reported that the PKC activation domain lies within the 28–34 region of the PTH molecule. Fujimori and co-

workers (17) reported that bPTH(7-34) activates PKC with a relative potency of 51% of that of bPTH(1-34). These results suggest that activation of PTH/PTHrP receptors interferes with the early stages of chondrogenesis through the cAMP/PKA signaling pathway. Recently, Jüppner and his co-workers identified a single heterozygous nucleotide exchange in the PTH/PTHrP receptor gene of a patient with Jansen-type metaphyseal chondrodysplasia (51, 52). The nucleotide exchange causes a missense mutation in the PTH/PTHrP receptor. When the mutant receptor was expressed in COS-7 cells, ligand-independent constitutive cAMP accumulation was observed in the cells. Thus, it is possible that the interference of chondrogenesis by constitutive activation of the PTH/PTHrP receptors may account for the abnormal formation of endochondral bone in this rare form of short-limbed dwarfism.

Homozygous mice carrying the PTHrP null mutation exhibited widespread abnormalities of endochondral bone development. Despite the wide distribution of PTHrP and its receptor transcripts in the body, there were no morphological abnormalities in other tissues (4, 32). Animals homozygous for the null mutant of the PTH/PTHrP receptor displayed essentially the same abnormalities (41). These results suggest a specific importance of PTHrP signaling in endochondral bone formation. Since BMPs modulate PTHrP responsiveness of chondrocytes (22), chondrogenic differentiation must be coordinately regulated by BMP-dependent signaling and PTHrP-dependent signaling. Further analysis of PTHrP signaling in chondrogenesis is in progress, using the DNA transfection technique that produces perturbations in PTHrP signaling in ATDC5 cells. Thus, ATDC5 cells provide a valuable opportunity for the molecular analysis of early chondrogenesis.

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