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# PTHrP isoforms have differing effect on chondrogenic differentiation and hypertrophy of mesenchymal stem cells

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ABSTRACT

While several isoforms of parathyroid hormone-related peptide (PTHrP) have been commercially available, the difference in their effect has not been widely studied. The purpose of this study was to determine which isoform most effectively promoted chondrogenesis and suppressed hypertrophy from mesenchymal stem cells (MSCs). MSCs isolated from fresh bone marrow were cultured in pellet in chondrogenic medium containing 5 ng/ml of transforming growth factor (TGF)-$\beta_3$. From day 14 of culture, subsets of pellets were additionally treated with one of the four PTHrP isoforms (1-34, 1-86, 7-34, and 107-139) at 100 nM. After a further 2 weeks of *in vitro* culture, pellets were harvested for analysis. PTHrPs 1-34 and 1-86 significantly decreased the DNA level ($p < 0.05$) while PTHrPs 7-34 and 107-139 significantly increased DNA level ($p < 0.05$) compared with the control treated with TGF-$\beta_3$ only. Glycosaminoglycan per DNA significantly increased when treated with PTHrPs 1-34 and 1-86 ($p < 0.05$) while it significantly decreased with PTHrPs 7-34 and 107-139 ($p < 0.05$). PTHrP 1-34 significantly increased the gene and protein expression of the chondrogenic marker COL2A1, and decreased those of hypertrophic markers COL10A1 and alkaline phosphatase while other isoforms showed inconsistent effects. All of PTHrP isoforms significantly suppressed the gene and protein expression of indian hedgehog ($p < 0.05$) while all isoforms except PTHrP 107-139 significantly reduced the gene and protein expression of patched 1 ($p < 0.05$). In conclusion, of several PTHrP isoforms, PTHrP 1-34 most significantly enhanced chondrogenesis and suppressed hypertrophy in MSCs, supporting its use for cartilage tissue engineering.


## 1. Introduction

Adult human mesenchymal stem cells (MSCs) are promising candidates for cartilage tissue engineering due to their excellent proliferation and differentiation capacity [1–4]. While the induction of chondrogenic differentiation from MSCs was demonstrated in various cell culture systems, several challenging problems were discovered during the process, including phenotypic instability and hypertrophy [5–7]. Hypertrophy is a natural phenomenon occurring in the endochondral ossification during the developmental process. It predestines chondrocytes for apoptosis and subsequent ossification. Chondrocytes of articular cartilage (AC) are spared from these changes, meaning that, to be functional, tissue-engineered AC should be exempted from these changes as well [8,9]. Under the *in vitro* pellet culture conditions, MSCs can be induced to undergo a differentiation program analogous to that observed during endochondral embryonic skeletal development [10]. Unfortunately, several authors demonstrated that the markers of hypertrophy such as type X collagen and alkaline phosphatase (ALP) appears early in the chondrogenesis from MSCs, posing a dilemma in the timely induction of chondrogenic differentiation of MSCs [5,8,11]. To solve the dilemma, it is an appealing idea to apply the knowledge obtained from studying developmental process to the *in vitro* chondrogenesis.

Strings of evidences have demonstrated that several signaling systems including fibroblast growth factor (FGF), transforming growth factor (TGF)-β/bone morphogenetic protein (BMP), and parathyroid hormone-related peptide (PTHrP)/indian hedgehog (IHH) interact to regulate chondrogenesis and the onset of hypertrophy in the developmental process [12–16]. Whereas FGF acts to maintain cell in a proliferative status and prevent premature chondrogenic differentiation, PTHrP inhibits chondrocyte maturation, playing a key role in regulating the rate of differentiation in growth plate [13,15]. PTHrP regulates endochondral bone development by maintaining the growth plate at a constant width [15]. PTHrP is a peptide hormone structurally related to parathyroid hormone (PTH) [17]. Both peptides have a strong homology in N-terminal region but differ in C-terminal region. PTHrP is synthesized in many tissues, in contrast to PTH which is produced only from parathyroid gland [18]. PTHrP is a product of a single gene, which gives rise to three initial translation products: PTHrP (1-139), PTHrP (1-141), and PTHrP (1-173) through alternative splicing [19]. They all contain the N-terminal signal sequence

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(1-36), mid-region domain (66-94), and the C-terminal domain (107-139). These domains act through their own different receptors [20,21]. The three initial PTHrP translation products undergo extensive posttranslational processing and give rise to a family of mature secretory forms of the peptide.

Utilizing the finding that PTHrP controls the maturation and hypertrophic changes of cartilage [16], several groups including us have demonstrated that PTHrP suppressed hypertrophy in the *in vitro* chondrogenesis from MSCs [22–24]. However, data are conflicting on whether PTHrP promotes chondrogenesis as well. While we had previously reported that PTHrP promoted chondrogenesis and suppressed hypertrophy [24], another study reported that PTHrP potently inhibited chondrogenic differentiation of MSCs [22].

In addition, with several isoforms of PTHrP commercially available, it remains unclear which one of them most effectively promotes chondrogenesis and suppresses hypertrophy as compared with other isoforms. While the 1-34 isoform has been most widely used, it is not known whether other isoforms would be more useful for MSC-based cartilage tissue engineering. So the purpose of this study was to determine if four PTHrP isoforms (1-34, 1-86, 7-36, and 107-139) similarly promote chondrogenesis and suppress hypertrophy from MSCs and, if not, which one is the most effective isoform.

## 2. Materials and methods

### 2.1. Human MSC sample collection, isolation and cultivation

The bone marrow samples used to isolate mesenchymal stem cells (BMSCs) were obtained from four patients (mean age: 50 years, range: 37–64 years) undergoing total hip replacement due to osteoarthritis. Informed consent was obtained from all donors. BMSCs were isolated from fresh bone marrow samples, and then expanded as described previously [5,25].

### 2.2. Induction of in vitro chondrogenic differentiation

For *in vitro* investigation, pellets were formed as described here. The cell suspension was aliquoted into 15 mL polypropylene centrifuge tubes, and spun in a bench top centrifuge at 500 g for 10 min. Tubes were incubated in 5% $CO_2$ atmosphere for up to 4 weeks. Caps of tubes were loosened in order to allow air exchange. The medium was changed every third day. To induce chondrogenesis, *in vitro* pellet cultures were carried out using $2.5 \times 10^5$ MSCs at passage 3–5 in Dulbecco's modified Eagle's medium/F-12 supplemented with 1% insulin–transferrin–selenium, $10^{-7}$ M dexamethasone, 50 μM ascorbate-2-phosphate, 50 μM L-proline, and 1 mM sodium pyruvate. The starting concentrations used for the growth factors, 5 ng/mL of TGF-$\beta_3$, were on our previous findings; both published and unpublished [25,26]. From day 14 of culture, subsets of pellets were additionally treated with 100 nM of PTHrP isoform 1-34, 7-34, 107-139 (BACHEM, Bubendorf, Switzerland) and 1-86 (BioVision, Milpitas, CA). After a further 2 weeks of *in vitro* culture in their respective media, pellets were harvested for analysis.

### 2.3. DNA quantitation and glycosaminoglycan (GAG) contents analysis

Cell pellets were digested for 2 h in cell lysis buffer containing proteinase K of a GeneAll Tissue SV mini Kit (GeneAll, Seoul, Korea) at 56 °C. Genomic DNA from each pellet was prepared according to the manufacturer's instruction. DNA contents were determined using the Quanti-iT™ dsDNA BR assay kit and Qubit fluorometer (Invitrogen, Carlsbad, CA). For analysis of GAG contents, pellets were digested in papain buffer at 60 °C for 2 h and then transferred to 1.5 mL microcentrifuge tubes. For 1,9-dimethylmethylene blue (DMMB) assays, 50 μL of each sample was added to a total volume of 100 μL with appropriate buffer. GAG production was determined using a Blyscan kit (Biocolor, Carrickfergus, Northern Ireland). This assay is based on the specific binding of the cationic dye 1,9-DMMB to the sulfated GAG (s-GAG) chains of proteoglycans and protein-free s-GAG chains. The procedure was carried out according to the manufacturer's instructions. Briefly, standard solutions (0, 1.0, 2.0, 3.0, and 5.0 μg of chondroitin-4-sulfate in 100 μL) and test samples (100 μL) were mixed with 300 μL of Blyscan dye reagent for 30 min at room temperature. s-GAG–dye complex was recovered by centrifugation for 20 min at 15,000 rpm and pellets were resuspended in 300 μL of dissociation buffer. Absorbances were measured at 656 nm in a Spectra max plus 384 apparatus (Molecular Devices, Sunnyvale, CA). GAG contents were expressed as micrograms of GAG per microgram of DNA.

### 2.4. Reverse transcription and real-time polymerase chain reaction (PCR) analysis

The total RNA was isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction and quantified using the Quant-iT™ RNA assay kit and Qubit Fluorometer system (Invitrogen). Isolated RNA samples were converted to cDNA using a PrimeScript™ 1st strand cDNA synthesis kit (Takara Bio., Shiga, Japan). All PCR reactions were performed on the LightCycler 480 system® (Roche Diagnostics, Mannheim, Germany) in a standard 15 μL reaction volume. The expressions of the following genes were examined: collagen type II (COL2A1), collagen type X (COL10A1), alkaline phosphatase (ALP), Indian hedgehog (IHH), and patched 1 (PTCH1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. The primers used for amplification are listed in Table 1. After polymerase activation (95 °C for 15 s), 45 amplification cycles were run (5 s denaturation at 95 °C, 15 s annealing at 60 °C and 15 s extension at 72 °C). Melt-curve analysis was performed immediately after the amplification protocol using the following conditions: 5 s at 95 °C (holding time on reaching temperature), 1 min at 65 °C, and 1 s at 97 °C. The temperature change rate was 20 °C/s (except for the final step, during which the temperature change rate was 0.1 °C/s). The peak melting temperatures obtained were considered to be those of the specific amplified products. To guarantee the reliability of the results obtained, all samples were processed in triplicate. Each assay was performed using positive and negative controls. The threshold cycle ($C_t$) value of each gene was measured for each reverse transcript sample. The $C_t$ value of GAPDH was used as an endogenous reference for normalization purposes (User bulletin #2 Applied Biosystems, Roche Molecular System, Alameda, CA). The values thus obtained were normalized versus the negative control, and expressed as fold changes.

### 2.5. Western blotting

For total protein extraction, pellets were twice washed with cold phosphate buffered saline (PBS) and homogenized by grinding in liquid nitrogen and incubated with lysis buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 1% (v/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 50 mM NaF, 1 mM $Na_3VO_4$, 50 mg/mL phenylmethanesulfonylfluoride (PMSF), 1 mg/mL leupeptin, and 1 mg/mL pepstatin] for 30 min on ice, and centrifuged at 15,000 rpm for 20 min at 4 °C. Proteins were separated by 8% SDS–polyacrylamide gel electrophoresis (SDS–PAGE), blotted and probed using the appropriate antibody. Proteins were electroblotted onto a polyvinylidene fluoride (PVDF) membrane and