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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)-β_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-β_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 2.5×10^5 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-β_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

Table 1
Primers used for real-time PCR.

Gene	Sequences (5′–3′)	Accession No.	Product size (bp)
COL2A1	F-AACCAGATTGAGAGCATCCG R-ACCTTCATGGCGTCCAAG	NM_033150	149
COL10A1	F-ACGATACCAAATGCCCACAG R-GTACCTTGCTCTCCTCTTACTG	NM_000493	114
ALP	F-GACAAGAAGCCCTTCACTGC R-AGACTGCGCCTGGTAGTTGT	NM_000478	142
IHH	F-ATGAAGGCAAGATCGCTCG R-GATAGCCAGCGAGTTCAGG	NM_002181	149
PTCH1	F-TCTTGGTGTTGGTGTGGATG R-ATTGCTGATGGACGTGAGG	NM_001083607	145
GAPDH	F-CACATGGCCTCCAAGGAGTAA R-GTACATGACAAGGTGCGGCTC	NM_002046	143

blocked for 1 h in 5% nonfat dry milk in PBS containing 0.05% Tween 20 (PBST). Thereafter, the membranes were incubated overnight at 4 °C with primary antibody against collagen types II (Millipore, Billerica, MA; 1/500), collagen types X (Abcam; 1/1000), ALP (Abcam; 1/1000), IHH (Abcam, Cambridge, UK; 1/2000), PTCH1 (Abcam; 1/500), and β-actin (Cell Signaling Technology Inc., Danvers, MA; 1/1000). Next, membranes were washed three times with PBST, then incubated with horseradish peroxidase labeled anti-mouse or anti-rabbit IgG (Abcam; 1:3000) for 1 h at room temperature. After a further washing step, bands were visualized by using ECL + Plus™ Western blotting system (GE Healthcare Biosciences, Piscataway, NJ).

2.6. Histological and immunohistochemical analyses

After 4 weeks of culture, pellets were fixed in 4% paraformaldehyde solution for 3 h, dehydrated with 100% ethanol, washed with xylene, and embedded in paraffin. Four micrometer thick sections were cut from the paraffin blocks and coated on glass slides. Safranin-O staining for proteoglycan and immunohistochemistry for ALP was then performed. For Safranin-O staining, sections were deparaffinized with xylene and ethanol, aqueous Safranin-O (0.1%) was applied for 5 min, and sections were washed with distilled water. For immunohistochemistry, we used Dakocytomation LSAB2 System horseradish peroxidase (HRP) kits (DAKO, Hamburg, Germany). Tissue sections were deparaffinized, treated with Pepsin Soluble® (Fine Life Science, Seoul, Korea) for 15 min and washed in a 1× wash buffer (DAKO). Sections were incubated for 5 min with a peroxidase-blocking solution, and reacted with mouse ALP primary antibody (1.0 mg/ml: Abcam) diluted 1:100 in goat serum overnight at 4 °C. After three washes in 1× wash buffer, sections were incubated with HRP-labeled anti-mouse goat secondary antibody (DAKO) for 1 h, extensively washed, reacted with substrate buffer and diaminobenzidine (DAB) chromogen (DAKO, 50:1) for 10 min, and then mounted.

2.7. Statistical analysis

Descriptive statistics were used to determine group means and standard deviations. All p values were calculated using Mann Whitney U-test. A value of $p < 0.05$ was considered significant.

3. Results

3.1. Effects on cell number and proteoglycan synthesis as demonstrated by DNA and GAG levels, and Safranin-O staining

While the application of PTHrPs 1-34 and 1-86 significantly decreased DNA level by 30% ($p < 0.05$) and 17% ($p < 0.05$) respectively, both PTHrPs 7-34 and 107-139 significantly increased DNA level by two-thirds ($p < 0.05$) compared with the control treated with TGF-β_3 only (Fig. 1A). In contrast, GAG per DNA significantly increased when treated with PTHrP 1-34 by 63% ($p < 0.05$) and by 8% ($p < 0.05$) with PTHrP 1-86 while it significantly decreased with PTHrPs 7-34 and 107-139 by a half ($p < 0.05$) and by one-third ($p < 0.05$) respectively, compared with when TGF-β solely was trea-

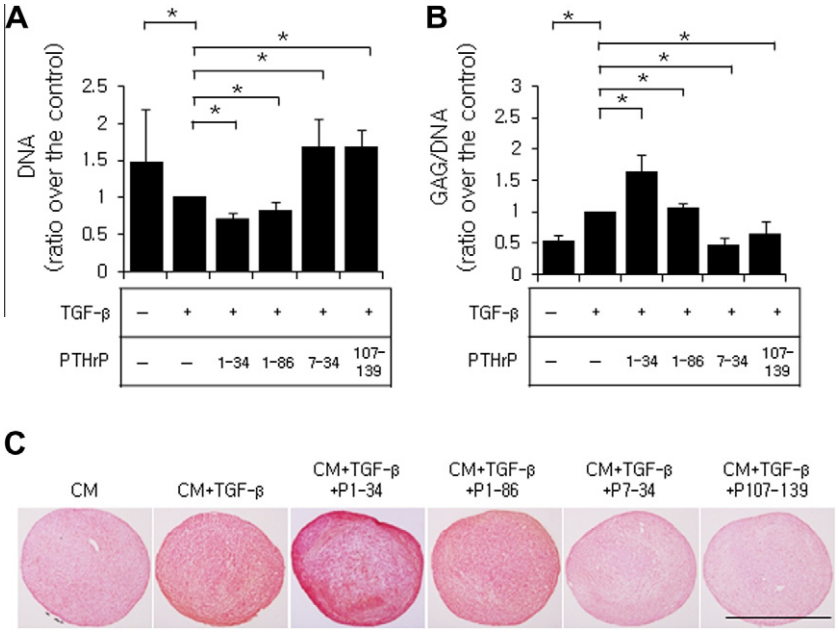

Fig. 1. DNA (A) and glycosaminoglycan (GAG) (B) amounts normalized versus DNA in pellets after 28 days of culture. Treatment with PTHrP started from the 14th day. The bars represent mean ± SD; $n = 4$, $^*p < 0.05$. (C) Safranin-O staining of hMSCs cultured in pellets for 4 weeks with the treatment of TGF-β (5 ng/ml) and PTHrP (100 nM); CM, MSC pellet treated with chondrogenic medium; CM + TGF-β, MSC pellet treated with chondrogenic medium containing TGF-β_3; CM + TGF-β + P, MSC pellet treated with chondrogenic medium containing TGF-β_3 and PTHrP isoforms. Scale bar = 0.5 mm.

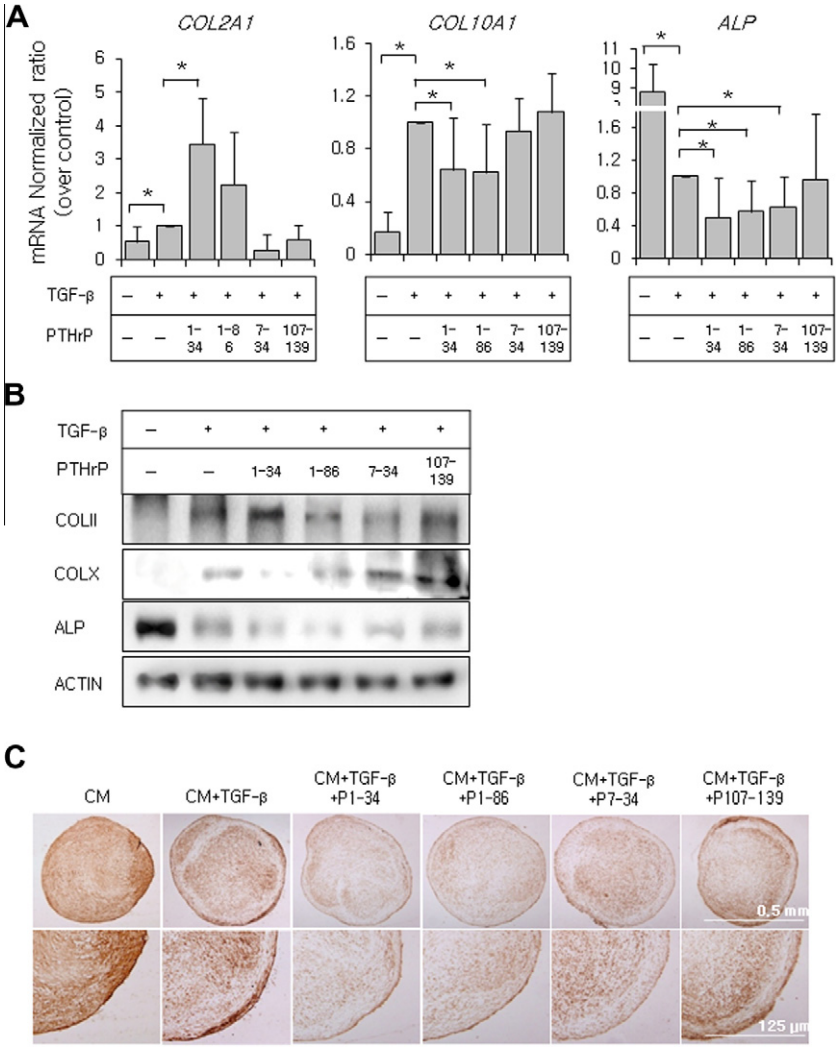

Fig. 2. Gene expression of COL2A1, COL10A1 and ALP in hMSCs cultured in pellets for 4 weeks with the treatment of PTHrP isoforms (A). The results are shown as the ratio over the GAPDH control. The bars represent mean ± SD; $n = 4$, $^{*}p < 0.05$. Protein expression after 4 weeks with the treatment of PTHrP isoforms (B). Chondrogenic marker (type II collagen), and hypertrophic markers (type X collagen and ALP) of chondrocyte were detected using Western blotting. ALP was also detected by immunohistochemistry (C).

ted (Fig. 1B). Safranin-O staining of pellets also showed that the proteoglycan synthesis was greatest when treated with PTHrP 1-34, while PTHrPs 7-34 and 107-139 rather decreased the proteoglycan synthesis (Fig. 1C).

3.2. Effects on chondrogenic marker (COL2A1) and hypertrophic markers (COL10A1 and ALP)

We then further evaluated the effect of the various isoforms of PTHrP on chondrogenic differentiation by investigating the gene and protein expression of COL2A1, and on hypertrophy by investigating COL10A1 and ALP. Treatment with PTHrP 1-34 significantly increased the gene expression of COL2A1 by 3.4-fold ($p < 0.05$) compared with control treated with TGF-β_3 only. While PTHrP 1-86 rather increased COL2A1 gene expression and PTHrPs 7-37 and 107-139 decreased it, the difference was not statistically significant ($p > 0.05$). On the other hand, treatment with PTHrPs 1-34 and 1-86 significantly reduced the gene expression of hypertrophic marker COL10A1 by 40% ($p < 0.05$), while PTHrPs 7-37 and 107-139 did not affect COL10A1 gene expression ($p > 0.05$). ALP, another marker of hypertrophy, was significantly decreased by PTHrPs 1-34, 1-86 and 7-34 ($p < 0.05$) while not affected by PTHrP 107-139 ($p > 0.05$) (Fig. 2A).

Protein expression confirmed by Western blotting generally followed the findings from qPCR, albeit with some variations. PTHrP 1-34 was the most effective isoform in elevating chondrogenic marker COL2A1 and lowering hypertrophic marker COL10A1 and ALP. PTHrP 1-86 treatment decreased ALP while not affecting COL2A1 and COL10A1. PTHrP 7-34 also did not affect COL2A1 and COL10A1 protein expression while decreasing ALP expression. PTHrP 107-139 did not affect COL2A1 and ALP protein expression while increasing COL10A1 expression (Fig. 2B). Immunohistochemistry for ALP matched the finding from Western blotting, with decreased expression by PTHrP 1-34, 1-86, and 7-34 being evident (Fig. 2C).

3.3. Effects on IHH and its signaling molecule

After confirming the effect of various PTHrP isoforms on the chondrogenesis and hypertrophy, we lastly investigated how the different isoforms affected IHH, the molecule which induces chondrocyte hypertrophy and is suppressed by PTHrP. PTCH1, the signaling molecule downstream of IHH, was also investigated. All of the PTHrP isoforms significantly suppressed the gene and protein expression of IHH ($p < 0.05$), while all isoforms except PTHrP 107-139 significantly reduced the gene and protein expression of PTCH1 ($p < 0.05$) (Fig. 3A and B).

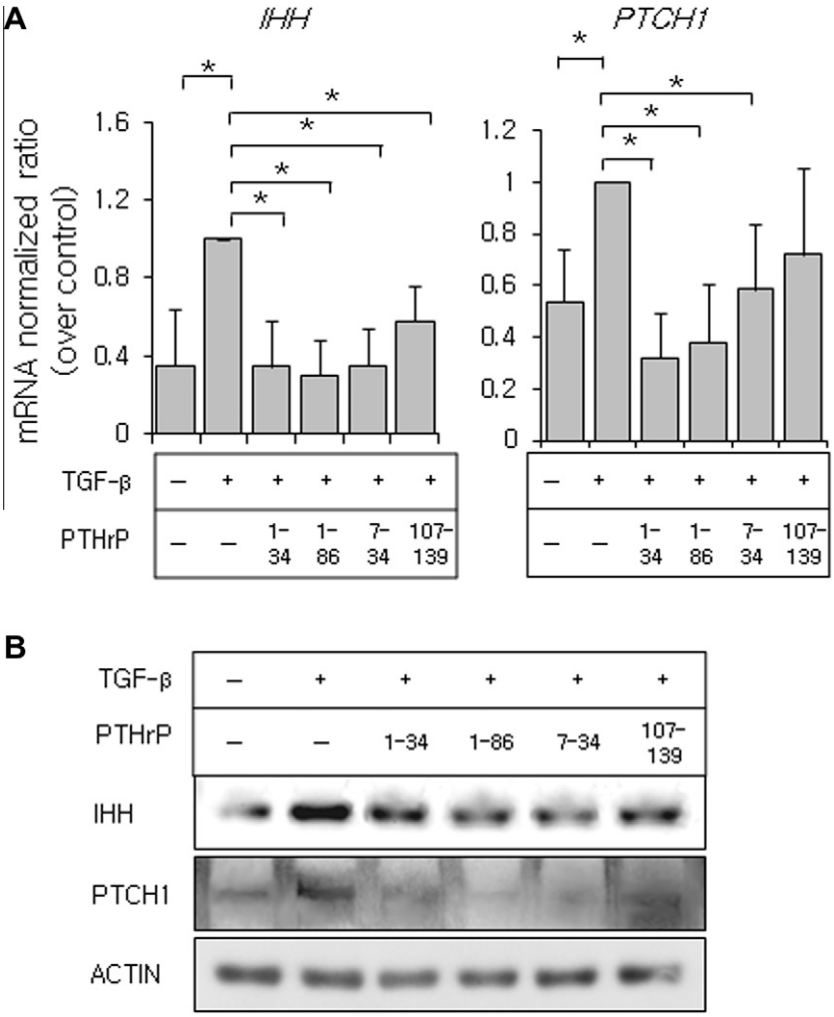

Fig. 3. Gene expression of hedgehog signal molecules in hMSCs cultured in pellets for 4 weeks with the treatment of PTHrP isoforms (A). The results are shown as the ratio over the GAPDH control. The bars represent mean ± SD; $n = 4$, $^{*}p < 0.05$. IHH and downstream signal, PTCH1 were detected using Western blotting (B).

4. Discussion

We compared the effect of various isoforms of PTHrP on the chondrogenic differentiation and hypertrophy of MSCs. The overall results indicated that the 1-34 isoform most effectively promoted chondrogenesis and suppressed hypertrophy, with 1-86 isoform performing to a much lesser degree, while 7-34 and 107-139 isoforms were either ineffective or actually suppressed chondrogenesis compared with the control chondrogenic condition provided by 5 ng/mL of TGF-β_3 in the three-dimensional pellet culture.

Although MSC has been suggested as an attractive option for cartilage tissue engineering due to their proliferate capacity and availability from different cell sources [1–4], the qualities of cartilage induced from MSCs do not surpass those from chondrocytes. Prime causes are phenotypic instability and hypertrophy, which eventually leads to apoptosis or ossification [27,28]. Cues to solve the predicament were deduced from the observation of developmental processes [9]. Several groups including us have focused on PTHrP, which plays a key role in delaying maturation of chondrocytes in the growth plate [9,24,29,30]. We had reported that PTHrP promoted chondrogenesis and suppressed hypertrophy, as evidenced by gene expression studies and histological findings [24]. On the other hand, Kafiensch et al. observed the suppression of types I and X collagen without a change in type II collagen after treating chondrogenic cultures of human MSCs with PTHrP [29]. Also, the results of Weiss et al. showed that PTHrP inhibited the TGF-β-responsive COL2A1 and COL10A1 expression as a potent inhibitor for early and late chondrogenesis [22]. While these discrepant results could be attributed to individual difference of primary MSCs obtained from patients and the duration of treatment as well as dosage, the use of different isoforms may have been an influencing factor as well.

PTHrP is synthesized in various tissues and has different actions in each. Three parts of PTHrP: N-terminal domain PTHrP (1-36), mid-region domain (66-94), and the C-terminal domain; have their own receptors [20,31,32]. A previous report showed that the N-terminal and the C-terminal domains of PTHrP have different actions on human MSCs: the latter favoring adipogenesis while the former having a mild osteogenic effect [33]. Our results indicate that the isoforms that contain only the C-terminal domain (107-139) or only part of N-terminal domain (7-34) had no effect or rather suppressed chondrogenesis from MSCs.

It is of note that three isoforms of PTHrP, i.e., 1-34, 1-86 and 7-34 acted to decrease the gene and protein expression of IHH and its signaling molecule PTCH1, which was matched with decreased gene and protein expression of the hypertrophic marker ALP and to a lesser extent, COL10A1. It is suggested that the suppression of hypertrophy by PTHrP is mediated through the inhibition of IHH pathway in the *in vitro* chondrogenesis from MSCs. In contrast, the promotion of chondrogenesis as denoted by proteoglycan and

COL2A1 synthesis was not related to the decrease of IHH, meaning that PTHrP acts to enhance chondrogenesis via other pathways.

The strength of this study lies in the direct comparison of several PTHrP isoforms regarding the promotion of chondrogenesis and suppression of hypertrophy on MSCs from the same patients. While there were considerable individual variations, PTHrP 1-34 definitely suppressed hypertrophy while promoting chondrogenesis from MSCs and clearly was the most effective isoform for this purpose. The limitations are the limited number of samples and the absence of data using weight-based concentration.

In conclusion, of several isoforms of PTHrP, PTHrP 1-34 most significantly enhanced chondrogenesis and suppressed hypertrophy in MSCs, which supports its use for cartilage tissue engineering. Further investigations are warranted to confirm the results of this study using *in vivo* models.

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