Collagen-targeting parathyroid hormone-related peptide promotes collagen binding and *in vitro* chondrogenesis in bone marrow-derived MSCs

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Abstract. Parathyroid hormone-related peptide (PTHrP) is an important inductive factor during chondrogenesis of mesenchymal stem cells (MSCs). PTHrP induces chondrogenesis and suppresses hypertrophy, yet the lack of an efficient delivery system limits its use for cartilage tissue engineering in clinical application. In this study, a peptide of 7 amino acids was first used to engineer PTHrP to construct a collagen-targeting system. This peptide functioned as a collagen-binding domain (CBD) to specially target the PTHrP to collagen. ELISA assay was used to determine the collagen-binding ability of CBD-PTHrP. The effect of CBD-PTHrP on chondrogenesis was measured by an *in vitro* pellet assay in bone marrow-derived MSCs (BM-MSCs). As expected, the CBD peptide promoted the binding of CBD-PTHrP to collagen when compared to NAT-PTHrP. Furthermore, the recombinant protein CBD-PTHrP induced the expression of COL2A1 and Sox-9, inhibited the expression of COL1A1 at the mRNA and protein levels as effectively as NAT-PTHrP. Safranin-O and immunohistochemistry for collagen types I, II, X and Sox-9 generally paralleled qRT-PCR and western blotting findings with minor variations. In conclusion, our study demonstrated that CBD-PTHrP is a collagen-targeting system and promotes *in vitro* chondrogenesis in BM-MSCs. We suggest that this is an efficient delivery system for cartilage tissue engineering in clinical application.

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Introduction

Articular cartilage is a highly organized soft tissue. An articular cartilage defect is an area of damaged or missing cartilage (1). Although often caused by acute trauma, the defect may also occur as a result of osteoarthritis, osteonecrosis, osteochondritis dissecans and other pathologies (2). These degenerative joint diseases affect more than a third of the world population, and disorders of articular cartilage, in general, account for more than half of all chronic conditions in individuals aged 60 years and over (3). Therefore, optimized treatment strategies for articular cartilage lesions are of a high socio-economic importance. Symptomatic cartilage defects require surgical treatment such as microfracture, pridie drilling and abrasion arthroplasty, autologous chondrocyte transplantation, and the transfer of both autologous or allogeneic osteochondral transplants (4). Although the development of surgical techniques in cartilage has been extensively investigated (5,6), there is currently no surgical method available for cartilage injuries that can prevent its early onset (7). Tissue engineering offers promising new approaches that have the potential to provide such therapies.

Mesenchymal stem cells (MSCs), which can be easily isolated in a non-invasive and abundant manner from various tissues such as the bone marrow, bone, adipose tissue, muscle, synovium, periosteum, perichondrium and many adult tissues (8), are capable of self-renewal and differentiation into a variety of cell lineages, including chondrocytes, osteoblasts and adipocytes (9,10). MSCs have been identified in healthy and diseased cartilage, and appear to retain at least some potential to regenerate cartilage *in vivo* (11,12). Due to these advantages, MSCs are attractive targets for manipulation in the goal of cartilage regeneration.

Chondrocyte proliferation and differentiation toward hypertrophy are the main challenges for cartilage regeneration from MSCs (13,14). Various factors for chondrogenesis of MSCs have been developed, including parathyroid hormone-related protein (PTHrP) (15). PTHrP, first identified as a factor involved in humoral hypocalcemia of malignancy (16), maintains the function of proliferating chondrocytes and inhibits chondrocyte differentiation toward hypertrophy in the growth plate through the PTHrP-Indian hedgehog (IHH) axis (17,18). This anti-hypertrophic activity has been shown

to result from binding of the N-terminus of PTHrP to its cell surface receptor (PTH1R), activating Sox9 (19). PTHrP also stimulates proliferation of endochondral chondrocytes and inhibits apoptosis, partly via induction of Bcl-2 (20). Therefore, PTHrP may be a therapeutic factor in the production of MSC-derived tissue-engineered cartilage for use in cartilage repair.

Collagen is the ubiquitous component of the tight network of glycoproteins, collagen IV and proteoglycans in basement membranes (21), and is widely dispersed in the articular site (22). Therefore, collagen could be a potential target for PTHrP which could be retained and enriched at the injured site, enhancing the efficacy of cartilage regeneration. A polypeptide TKKTLRT named collagen-binding domain (CBD) peptide was derived from von Willebrand factor (vWF). Epidermal growth factor, transforming growth factor-1 and basic fibroblast growth factor have been added along with a collagen-binding peptide and the results showed that CBD could specifically bind to native collagen and the modified growth factors achieved better repair ability compared to the native growth factors at the same concentration (23-25). Here, we constructed a collagen-based PTHrP-targeting system, and the effect on chondrogenesis was tested by *in vitro* pellet assay in bone marrow-derived (BM)-MSCs.

Materials and methods

Isolation and expansion of BM-MSCs. MSCs were isolated from fresh bone marrow samples obtained from patients undergoing total hip replacement or iliac bone graft harvest, as described elsewhere (26,27). Briefly, cells were fractionated on a Ficoll-Paque Plus density-gradient (GE Healthcare), and the low-density cell fraction was washed and seeded in expansion medium consisting of high-glucose Dulbecco's modified Eagle's medium/F12 (DMEM/F12) and 10% FBS (Gibco-BRL, Carlsbad, CA, USA). Nonadherent material was removed after 24-48 h. For expansion, cells were replated at a density of $5x10^3$ cells/cm^2 and used at passage 3.

Engineering and preparation of NAT-PTHrP and CBD-PTHrP. Engineering and preparation of NAT-PTHrP and CBD-PTHrP were performed as previously described (28). Briefly, the gene of CBD together with the linker domain was synthesized and then inserted into pET-32a (Novage, USA). The vector was named pET-32a-CBD. A human PTHrP DNA encoding a mature form was inserted into the pET-32a and pET-32a-CBD vector, and the recombinant expression plasimd was named pET-CBD-PTHrP (with CBD) and pET-NAT-PTHrP (without CBD). They both contained a 6X His purification tag for purification and detection in the subsequent experiments. Both of the plasmids were transformed into *Escherichia coli* Rosetta (DE3) for expressing the protein. *E. coli* was induced with 0.2 mM isopropyl b-D-thiogalactopyranoside (IPTG) at 25˚C for 1 h. The recombinant fusion proteins were isolated as soluble bodies. The purity and yields of recombinant proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Collagen binding assay. The comparison of binding ability of these 2 factors to collagen was studied using modified solid phase binding assays as previously described (29). Collagen member (0.1 mg) (Zenghai Bio, Shandong, China) was neutralized and added to 96-well plates (1 mg/well), and washed 3 times. Wells were blocked with 200 μl bovine serum albumin (BSA) (2.5 mg/ml) in phosphate-buffered saline (PBS) plus 0.1% Tween-20 for 1 h at room temperature (RT). After washing the wells once with PBS, 50-μl aliquots of the recombinant proteins diluted in PBS were added for 1 h at 37˚C with a series of concentrations of 0.156-10 mM. Wells were washed 2 times with PBS, and 50-μl aliquots of mouse anti-polyhistidine monoclonal antibody (1:1,000 dilution) were added for 1 h at RT. After 3 washes as above, 100-μl aliquots of sheep anti-mouse-alkaline phosphatase antibody (1:10,000 dilutions) were added for 1 h at RT, followed by 3 washes as above. Bound proteins were detected with 100 μl/well 2 mg/ml p-nitrophenyl phosphate hexahydrate (p-NPP; Ameresco) in alkaline phosphatase buffer (100 mM Tris-HC, 100 mM NaCl, 10 mM $MgCl_2$, pH 9.6) for 10 min. The reactions were stopped with 0.2 M NaOH (100 μl/well). One hundred-microliter solutions were then transported to a new 96-well plate. The plate was read in an ELISA reader at a wavelength of 405 nm. All binding assays were carried out in duplicate, and values showed <15% difference for the same plate.

Induction of in vitro chondrogenesis. To induce chondrogenesis, *in vitro* pellet cultures were carried out using $2.5x10^5$ BM-MSCs at passage 3 in chondrogenic differentiation medium (Cyagen, USA). From the 14th day of culture, subsets of pellets were additionally treated with PTHrP (100 ng/ml) and CBD-PTHrP (100 ng/ml), and following 2 additional weeks of *in vitro* culture in their respective media, the pellets were harvested for analysis. For pellet cultures, 0.5 ml of the cell suspension was aliquoted into 15-ml polypropylene centrifuge tubes, and spun in a bench top centrifuge at 150 x g for 5 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 2 days.

RNA isolated and real-time PCR analysis. RNA was isolated using the standard guanidine isothiocyanate TRIzol reagent (Invitrogen). Isolated RNA samples were converted to cDNA using Rotor-Gene SYBR Green RT-PCR kit (Qiagen) and oligo(dT) primers. All PCR reactions were performed using ABI system in standard 25-μl reaction volumes containing 5 μl RNA, 0.5 μl of 100 mM sense and 0.5 μl of 100 mM antisense primer, 12.5 μl Rotor-Gene SYBR-Green PCR Master Mix (Qiagen) and 6.5 μl RNA-free ddH_2O. The expression of the following genes were examined: collagen type I (COL1A1), collagen type II (COL2A1), collagen type X (COL10A1), Sox-9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a housekeeping gene. The primers used for amplification were: collagen type I, 5'-CCG CCGCTTCACCTACAGC-3' and 5'-TTTTGTATTCAA TCACTGTCTTGCC-3'; collagen type II, 5'-CCGAATAGC AGGTTCACGTACA-3' and 5'-CGATAACAGTCTTGC CCCACTT-3'; collagen type X, 5'-AAAGGCCCACTACCC AACAC-3' and 5'-CTTCCGTAGCCTGGTTTTCC-3'; Sox-9, 5'-CACACAGCTCACTCGACCTTG-3' and 5'-TTCGGT TATTTTTAGGATCATCTCG-3'.

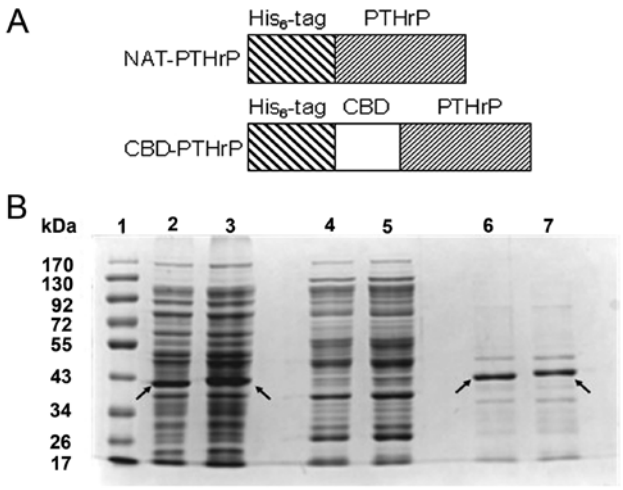

Figure 1. Expression and purification of NAT-PTHrP and CBD-PTHrP. (A) Scheme of protein expression constructs. (B) Expression of NAT-PTHrP and CBD-PTHrP soluble proteins as detected by SDS-PAGE. Lane 1, molecular weight markers; lanes 2, 4 and 6, total soluble proteins of *E. coli* DE3/pET-NAT-PTHrP after induction, penetration fluid and purified protein of NAT-PTHrP, respectively. Lanes 3, 5 and 7, total soluble proteins of *E. coli* DE3/pET-CBD-PTHrP after induction, penetration fluid and purified protein of CBD-PTHrP, respectively. The arrows indicate the bands of the target proteins.

Collagen extraction and western blotting. Three pellets were homogenized and subjected to pepsin digestion overnight at 4˚C (0.5 M acetic acid, 0.2 M NaCl and 2.5 mg/ml of pepsin). The pH was then adjusted to a neutral pH 7.0 with 1 M Tris Base prior to extraction of the collagens with 4.5 M NaCl (overnight at 4˚C). The following day, the extracted collagens were pelleted by centrifugation at 16,000 x g at 4˚C for 30 min and subsequently precipitated with 400 μl of precipitation buffer (0.4 M NaCl and 0.1 M Tris Base, pH 7.4) and 1,200 g of ethanol/sample for 4 h at -20˚C. The precipitated collagens were pelleted by centrifugation at 16,000 x g for 30 min at 4˚C and resolved in 50 μl of RIPA lysis buffer (1% Triton X-100, 150 mM NaCl and 50 mM Tris, pH 8.0). The proteins (20 μg) were separated by SDS-PAGE and electronically transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking, the membranes were incubated with the recommended dilution primary antibodies against collagen I, collagen II, collagen X, Sox-9 (Millipore) and GAPDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by incubation with peroxidase-conjugated secondary antibodies (Abcam, Cambridge, MA, USA). Peroxidase-labeled bands were visualized using an ECL kit (Millipore).

Histological analysis. After 4 weeks of culture, pellets were fixed in 4% paraformaldehyde solution for 4 h, dehydrated with 100% ethanol, washed with xylene, and embedded in paraffin. Sections (4 μm) were cut from paraffin blocks and coated on APES-treated glass slides. Safranin-O staining for proteoglycan and immunohistochemistry for collagen types I, II, X and Sox-9 (Millipore) were then performed. For Safranin-O staining, sections were deparaffinized with xylene and ethanol, aqueous Safranin-O (0.1%) (Sigma, USA) was applied for 20 min, and then sections were washed with distilled water. For immunohistochemistry, sections were deparaffinized in xylene, treated with a graded series of alcohol [100, 95 and 80% ethanol/double-distilled H_2O (v/v)], and rehydrated in PBS (pH 7.4). Endogenous peroxide was blocked with 3% H_2O_2 for 10 min. After PBS washes, slides were blocked with 5% normal goat serum in PBS for 15 min at RT followed by incubation with primary anti-collagen I (1:100), anti-collagen II (1:500), anti-collagen X (1:400) or anti-Sox-9 (1:400) antibody in blocking solution overnight at 4˚C. All slides were subsequently incubated with a 1:200 dilution of biotin-conjugated goat anti-mouse, or goat anti-rabbit secondary antibody for 15 min at 37˚C and the streptavidin-biotin complex at 37˚C for 15 min. The immunoreaction was visualized using diaminobenzidine (DAB) peroxide solution, and cellular nuclei were counterstained with hematoxylin. All specimens were evaluated using an Olympus BX600 microscope and a Spot Fiex camera. Control samples exposed to the secondary antibody alone showed no specific staining.

Statistical analysis. Data are expressed as the means ± SD. Statistical analysis was performed using the Student's test for comparing 2 groups and by ANOVA for multiple group comparisons. $P<0.05$ was taken to indicate a statistically significant result. The Statistics Analysis System was used for all statistical analyses.

Results

CBD-PTHrP expression and purification. Western blotting showed that *E. coli* expressed the recombinant proteins CBD-PTHrP and NAT-PTHrP when induced by IPTG (Fig. 1). The total soluble protein was purified by 6X His purification tag, and the purified proteins were then diluted in PBS for subsequent experiments.

CBD-PTHrP specifically binds to collagen. The binding abilities of NAT-PTHrP and CBD-PTHrP to collagen were then studied *in vitro* through collagen-based ELISAs. As shown in Fig. 2A, at each concentration, the OD405 value in the CBD-PTHrP group was higher than that in the NAT-PTHrP group, suggesting that more proteins bound to collagen during the ELISA assay.

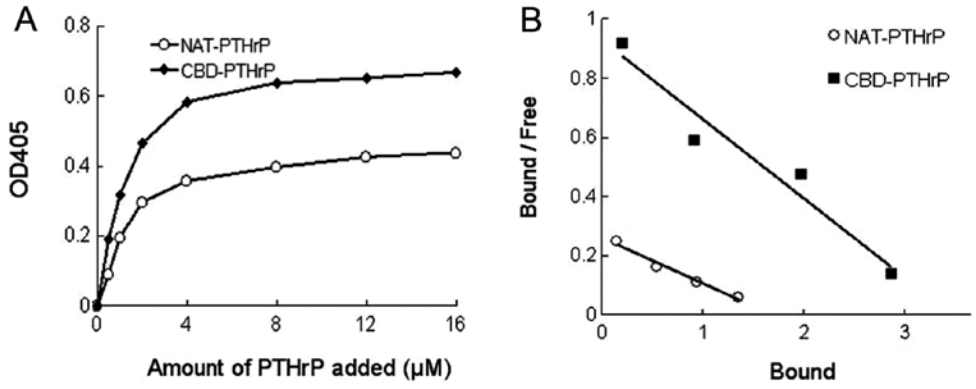

Figure 2. Binding curves of NAT-PTHrP and CBD-PTHrP to native collagen *in vitro*. (A) Different concentrations of the two types of PTHrP were added to collagen-coated 96 wells. Binding was measured by the color development at 405 nm after ELISA. (B) Data derived using Scatchard analysis to determine Kd values for collagen interaction with NAT-PTHrP and CBD-PTHrP. Slope of each line =-1/Kd.

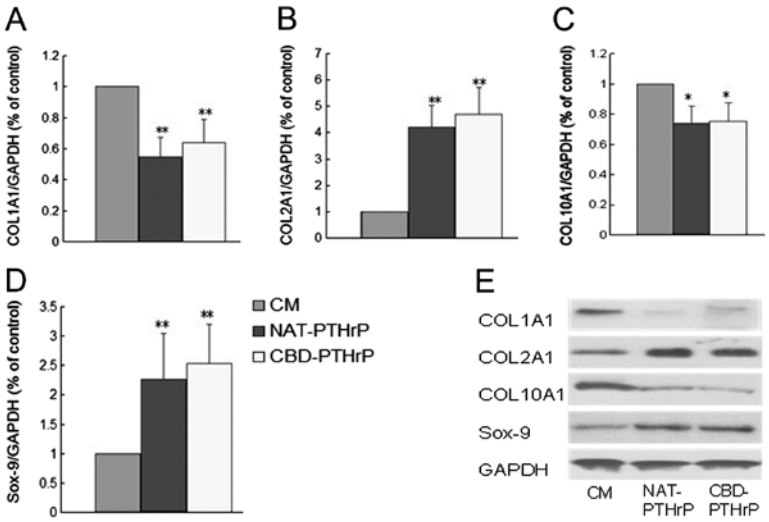

Figure 3. Reverse transcription and real-time PCR of the cells after 4 weeks of culture. The mRNA expression of (A) COL1A1, (B) COL2A1, (C) COL10A1 and (D) Sox-9 were measured and normalized vs. GAPDH. Values are expressed as fold change relative to the control. (E) The protein expression of COL1A1, COL2A1, COL10A1 and Sox-9 was measured by western blotting. Equal amount of protein was loaded on each gel lane. (CM, BM-MSCs treated with their respective chondrogenic medium). The bars represent means ± SEM (n=3: *P<0.05, **P<0.01).

Based on the binding curve, the dissociation constant Kd values for the 2 types of PTHrP binding to collagen (1 mg) were calculated by Scatchard analysis (Fig 2B). At each concentration, the ratio of bound counts to unbound counts was plotted against the amount of bound protein. The slope of the resulting straight line was -1/Kd. The Kd value for the binding of NAT-PTHrP and CBD-PTHrP to 1 mg collagen was 0.725 and 0.291 μM, respectively. The lower Kd value indicated that the protein had a higher binding ability to collagen. Thus, the results clearly demonstrated that CBD-PTHrP possessed stronger collagen-binding capacity vs. NAT-NGF.

Expression of COL1A1, COL2A1, COL10A1 and Sox-9 as determined by qRT-PCR and western blotting. In BM-MSCs, the expression levels of COL1A1, COL2A1, COL10A1 and Sox-9 were determined by qRT-PCR and western blotting at the mRNA and protein levels, respectively. The expression of COL1A1 decreased by 45.38% (P<0.01) and 40.81% (P<0.01) after treatment with 100 ng/ml NAT-PTHrP and CBD-PTHrP, respectively (Fig. 3A). Meantime, Sox-9 mRNA (Fig. 3D), the master gene of chondrogenesis, increased from 1 to 2.3- (P<0.01) and 2.5-fold (P<0.01) when compared to the untreated control following treatment with 100 ng/ml NAT-PTHrP and CBD-PTHrP. The expression of COL2A1 dramatically increased to 4.2-fold (P<0.01) following 100 ng/ml of NAT-PTHrP and 4.7-fold (P<0.01) following 100 ng/ml of CBD-PTHrP (Fig. 2B). COL10A1, the marker of hypertrophic chondrocytes, decreased 25.86% (P<0.05) following 100 ng/ml of NAT-PTHrP and 24.79% (P<0.05) following 100 ng/ml of CBD-PTHrP (Fig. 3C). Western blotting provided similar results as the qRT-PCR. As shown in Fig. 3E, the protein levels of COL2A1 and Sox-9 were significantly increased after treatment with 100 ng/ml NAT-PTHrP and CBD-PTHrP compared with the untreated group. Meanwhile, the protein expression of COL1A1 and COL10A1 was dramatically inhibited by NAT-PTHrP and CBD-PTHrP. There were no significant differences between NAT-PTHrP and CBD-PTHrP at either the mRNA or the protein level.

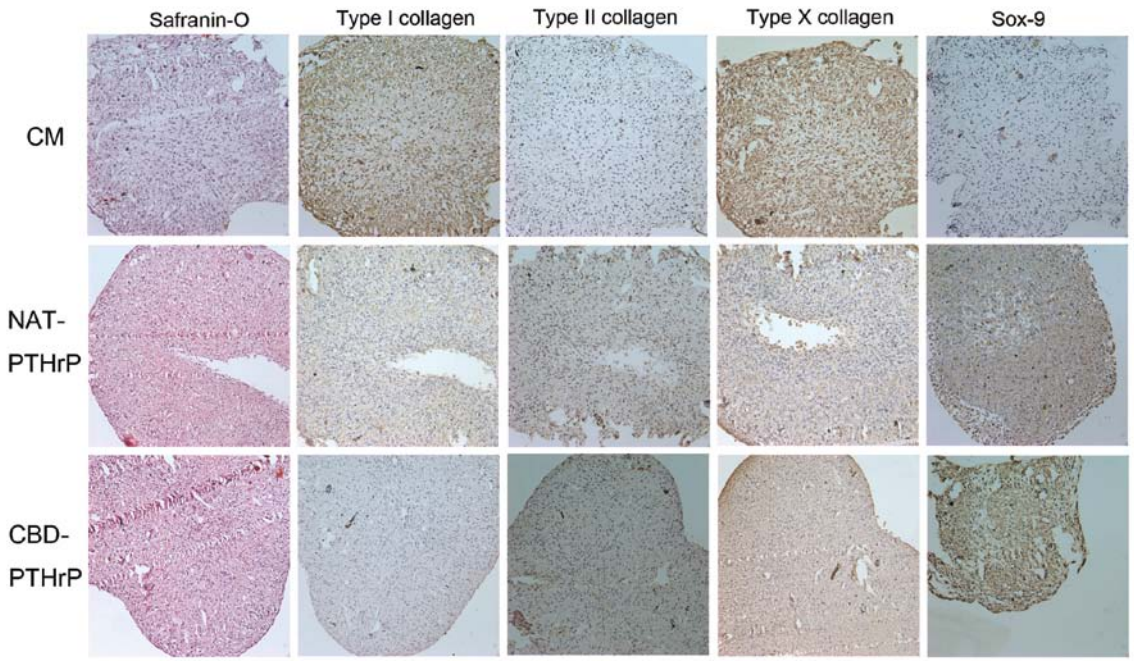

Figure 4. Histological findings of the pellets after 4 weeks of culture. Safranin-O staining (x200), immunohistochemistry (x200) for type I, II and X collagen and Sox-9 of cells cultured in pellets of BM-MSCs. CM, MSCs treated with their respective chondrogenic medium.

Histological findings. Histological findings of Safranin-O and immunohistochemistry for type I, II and X collagens and Sox-9 generally mirrored changes detected by qRT-PCR and western blotting with minor variations. Safranin-O staining showed an increase in the metachromatic staining after NAT-PTHrP and CBD-PTHrP treatment. Type II collagen expression markedly increased in both NAT-PTHrP and CBD-PTHrP treatment groups while type I collagen staining decreased in both treatment groups. Type X collagen expression decreased after NAT-PTHrP and CBD-PTHrP treatment. Sox-9 protein expression increased dramatically after NAT-PTHrP and CBD-PTHrP treatment in BM-MSCs (Fig. 4).

Discussion

MSCs are an attractive option for cartilage regeneration because of their abilities to proliferate and their easy accessibility (8-10). However, chondrocyte proliferation and chondrocyte hypertrophy are the main challenges for cartilage regeneration from MSCs (13,14). Previous studies suggest that PTHrP may circumvent these problems by promoting chondrogenesis and suppressing hypertrophy in the growth plate through the PTHrP-Indian hedgehog (IHH) axis (17-20). During chondrocyte induction from MSCs, the cells secrete PTHrP during the early phase of differentiation, until days 14-21, when the mRNA levels for PTHrP declined, whereas those for IHH were upregulated for the remaining weeks of culture (30). PTHrP has been shown to severely reduce type X collagen expression, AP activity, and cell enlargement of lower sternal chondrocytes from immature chicken, and these molecules are soluble factors produced by articular chondrocytes (31). These results make PTHrP an attractive candidate for chondrocyte induction.

Previous studies have used the PTHrP protein for the purpose of cartilage chondrocyte induction (15). However, simple absorption of PTHrP to the collagen scaffold would allow the diffusion of PTHrP into extracellular fluids, and would rapidly lose its activity. In addition, overexpression of PTHrP by a gene transfer method was also used and induced an arthritic phenotype in articular chondrocytes (32). In the present study, we first engineered PTHrP to construct a collagen-targeting system. Safranin-O staining showed that the recombined protein CBD-PTHrP increased metachromatic staining after treatment (Fig. 4A). At the same time, CBD-PTHrP treatment increased the expression of COL2A1 and Sox-9 and decreased the expression of COL1A1 and COL10A1 at the mRNA and protein levels (Figs. 3 and 4). These results were slightly different than those of Kafienah *et al* (33) and Kim *et al* (15). Kafienah *et al* (33) found that type II collagen expression was unchanged and expression of type I and X collagen was suppressed while Kim *et al* (15) found that type X collagen expression was gradually decreased, type I collagen expression was suppressed and type II collagen expression was increased after treating chondrogenic cultures of human MSCs with PTHrP.

Tissue engineering of skin, bone, vascular and nerve offers a promising means of producing 3-dimensional neocartilages for clinical treatment (34), and various factors for tissue engineering have been developed, such as bFGF, NGF and PDGF (35-37). However, in clinical practice, factors simply delivered in solution are difficult to be retained at the injured site due to their rapid diffusion in extracellular fluids. Many attempts have been made to overcome the difficulties in using factors as a therapeutic agent. In order to maintain adequate factor concentration, multiple injections are needed. However, this would increase the cost and surgical risks, and may even have adverse effects. Immobilization of bFGF

on heparin-Sepharose beads prolonged the storage and release (38). Recently, the most abundant component of extracellular matrices, collagen, has been widely used in drug delivery (39,24). Meanwhile, in tissue engineering, many types of collagen-based scaffolds have been fabricated, and they have shown good characteristics in wound repair (40,41). During the process, collagen was found to play an important role in providing a cell anchorage site, mechanical stability and structural guidance. They also provided the interface to respond to physiological and biological changes, and to remodel the extracellular matrix to integrate with the surrounding native tissue (42,43). Moreover, collagen is commonly used as an attractive targeted site for exogenous peptide growth factors. Targeted growth factors on collagen may not only retain its activity and control its diffusion but may also repair injured tissues locally. Nerve growth factor-β, platelet-derived growth factor and basic fibroblast growth factor have been added with a collagen-binding peptide, and the results have shown that CBD specifically binds to native collagen, and the modified growth factors achieve better repair compared to the native growth factors at the same concentration (28,44,45). In the present study, CBD which is a peptide of 7 amino acids was first used to engineer PTHrP to specially target the PTHrP to collagen. Our results showed that CBD-PTHrP has a higher binding ability to collagen than NAT-PTHrP (Fig. 2). The ability of CBD-PTHrP to induce chondrogenesis in MSCs was also measured by an *in vitro* pellet assay. As shown in Figs. 3 and 4, CBD-PTHrP induced chondrogenesis and inhibited chondrocyte differentiation toward hypertrophy as well as NAT-PTHrP. In future studies, the collagen-based scaffolds of bone will be used to determine the ability of CBD-PTHrP to produce MSC-derived tissue-engineered cartilage *in vivo*.

In conclusion, our study suggests that CBD-PTHrP is an attractive recombined protein for use in cartilage tissue engineering from MSCs. It demonstrated that CBD-PTHrP has a higher binding ability to collagen than NAT-PTHrP and significantly enhances chondrogenesis and suppresses hypertrophy in BM-MSCs. Further investigations are warranted to confirm the ability of CBD-PTHrP to produce MSC-derived tissue-engineered cartilage *in vivo* based on collagen-based scaffolds of bone.

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