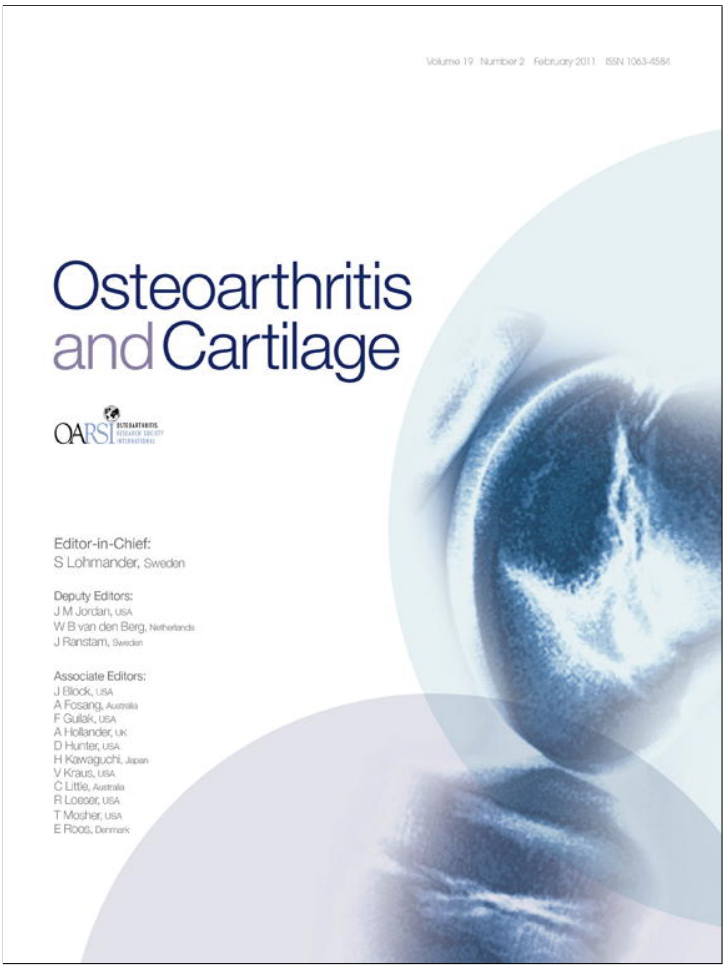

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PTHrP overexpression partially inhibits a mechanical strain-induced arthritic phenotype in chondrocytes

D. Wang †‡, J.M. Taboas †§, R.S. Tuan †§*

† Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis, and Musculoskeletal and Skin Diseases, National Institutes of Health, Department of Health and Human Services Bethesda, MD 20892, USA
‡ Howard Hughes Medical Institute-National Institutes of Health Research Scholars Program Bethesda, MD 20814, USA
§ Center for Cellular and Molecular Engineering, Department of Orthopaedic Surgery University of Pittsburgh School of Medicine, Pittsburgh, PA 15219, USA

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SUMMARY

Objective: Cell-based tissue engineering strategies are currently in clinical use and continue to be developed at a rapid pace for the repair of cartilage defects. Regardless of the repair methodology, chondrocytes within newly regenerated cartilage remain susceptible to the abnormal inflammatory and mechanical environments that underlie osteoarthritic disease, likely compromising the implant's integration, function, and longevity. The present study investigates the use of parathyroid hormone-related peptide (PTHrP) overexpression for chondroprotection.

Design: Bovine articular chondrocytes were transfected with human PTHrP (hPTHrP) constructs (1-141 or 1-173) and subjected to injurious cyclic tensile strain (CTS; 0.5 Hz and 16% elongation) for 48 h. mRNA expression of matrix remodeling, inflammatory signaling, hypertrophic, and apoptotic genes were examined with real-time reverse transcription polymerase chain reaction. Nitric oxide (NO) and prostaglandin E_2 (PGE_2) production were measured using the Griess assay and enzyme immunoassay (EIA), respectively.

Results: CTS-induced an arthritic phenotype in articular chondrocytes as indicated by increased gene expression of collagenases and aggrecanases and increased production of NO and PGE_2. Additionally, CTS increased collagen type X (Col10a1) mRNA expression, whereas overexpression of either hPTHrP isoform inhibited CTS-induced Col10a1 gene expression. However, hPTHrP 1-141 augmented CTS-induced NO and PGE_2 production, and neither hPTHrP isoform had any significant effect on apoptotic genes.

Conclusions: Our results suggest that chondrocytes overexpressing PTHrP resist mechanical strain-induced hypertrophic-like changes. Therapeutic PTHrP gene transfer may be considered for chondroprotection applications in newly regenerated cartilage.

Introduction

A number of surgical and cell-based regenerative strategies have been evaluated for the repair of cartilage defects. Current surgical techniques include mechanical penetration of the subchondral bone (i.e., abrasion arthroplasty, Pridie drilling, or microfracture) and autologous transplantation of periosteum, perichondrium, or osteochondral grafts[1]. Autologous chondrocyte implantation (ACI) is in current clinical application to mitigate progression to disease[2], and tissue engineering approaches that deliver a matrix seeded with chondrogenic cells and chondrogenic factors have also been evaluated experimentally. Regardless of the methodology, newly implanted chondrocytes within the defect remain susceptible to the abnormal inflammatory and mechanical environments that underlie osteoarthritic disease[3,4]. Subsequent arthritis-associated changes and aberrant functioning of these chondrocytes are likely to eventually compromise the integration, function, and longevity of regenerated cartilage[5]. Thus, chondroprotection of regenerated cartilage from the underlying disease would result in a more effective and durable therapy.

Resident chondrocytes provide the essential function of maintaining cartilage matrix homeostasis. This activity is impaired after traumatic mechanical loads damage articular cartilage during post-traumatic arthritis and osteoarthritis (OA)[6,7]. Derangement of the extracellular matrix (ECM) significantly alters chondrocyte

* Address correspondence and reprint requests to: Rocky S. Tuan, Center for Cellular and Molecular Engineering, Department of Orthopaedic Surgery, University of Pittsburgh School of Medicine, 450 Technology Drive, Room 221, Pittsburgh, PA 15219, USA. Tel: 412-648-2603; Fax: 412-624-5544.
E-mail address: rst13@pitt.edu (R.S. Tuan).

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behavior and phenotype, and the cells play a direct role in the degradation process by up-regulation of matrix-degrading proteases, such as matrix metalloproteinases (MMPs) and a disintegrin and MMP with thrombospondin motifs (ADAMTSs), as well as inflammatory intermediaries, including nitric oxide (NO) and prostaglandins (PGs)[8]. These increased proteolytic activities are not sufficiently counterbalanced by an increase in chondrocyte anabolic activities, resulting in matrix erosion. Another interesting aspect in the arthritis disease process is the recapitulation of molecular mechanisms that occur during fetal skeletogenesis. In addition to changes in anabolic and catabolic events, there are changes in cellular phenotype similar to those found in endochondral ossification, including hypertrophic differentiation, matrix calcification, and apoptosis[9]. Previous studies have found increased expression of hypertrophic markers in OA cartilage, including type X collagen, Indian hedgehog (Ihh), MMP-13, and alkaline phosphatase (ALP)[9–12]. Expression of these markers, which are typically low to absent in normal healthy cartilage, suggests that the progression of the degenerative cascade may involve the loss of inhibitory control over terminal differentiation genes in osteoarthritic chondrocytes.

Parathyroid hormone-related protein (PTHrP) maintains the function of proliferating chondrocytes and inhibits chondrocyte differentiation toward hypertrophy in the growth plate[13]. The anti-hypertrophy activity has been shown to result from binding of the N-terminus of PTHrP to its cell surface receptor (PTH1R), activating sex determining region Y box 9 (Sox9)[14,15]. PTHrP also stimulates proliferation of endochondral chondrocytes and inhibits apoptosis, partly *via* induction of B-cell lymphoma 2 (Bcl-2)[16,17]. Therefore, PTHrP may be a therapeutic option for the protection of articular chondrocytes. Additionally, previous studies have used gene transfer methods to provide sustained transgene expression for the purposes of cartilage repair and chondroprotection[18]. The efficacy of sustained PTHrP transgene expression by articular chondrocytes in preserving phenotypic homeostasis remains unknown.

The present study explored the use of human PTHrP (hPTHrP) overexpression by articular chondrocytes to suppress arthritic-like changes induced by mechanical trauma. Two different PTHrP isoforms, 1-141 and 1-173, were tested. A cyclic tensile strain (CTS) model was used to promote arthritic changes in articular chondrocytes *in vitro*.

Materials and methods

Chondrocyte isolation and culture

Chondrocytes were isolated from the knees of adult steers (2–3 years old) using previously described methods[19]. Briefly, cartilage slices were diced finely and digested in a stirring flask for 8–10 h with 0.2% collagenase type II (Sigma) in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with antibiotics at 37°C under 5% CO_2. After filtration through a 40 μm nylon mesh cell strainer, the cells were re-suspended at a density of 1.5×10^5 cells/cm^2 in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics for 2 days, and frozen for later use in liquid nitrogen.

Transfection procedure and plasmid DNA

Cells were thawed in DMEM with 10% FBS for 2 days before transfection. First, chondrocytes were treated with 40 U/ml bovine testicular hyaluronidase (Sigma) for 3 h. Next, medium was replaced with a transfection solution containing Opti-MEM-I + GlutaMAX-I (Invitrogen), 4 U/ml hyaluronidase, FuGENE 6 (Roche), and plasmid DNA. Optimal transfection of bovine articular chondrocytes used a lipid/DNA ratio of 3:1[20]. After 6 h, the transfection solution was replaced with DMEM with 10% FBS. Transfection efficiency using this protocol was observed to be approximately 35% with GFP control plasmids.

hPTHrP 1-141 and 1-173 plasmids were generous gifts from Dr. Leonard J. Deftos, University of California, San Diego[21]. Both constructs were inserted downstream of a cytomegalovirus (CMV) promoter/enhancer in the pCI-neo expression plasmid vector (Promega). Control cells were simultaneously transfected with the blank pCI-neo vector. All plasmids were amplified and purified by MTR Scientific (Ijamsville, MD).

Application of CTS

Chondrocytes were trypsinized and cultured onto fibronectin-coated Bioflex plates (Flexcell) at a density of 40,000 cells/cm^2 in DMEM with 1% FBS for 1 day. Bioflex plates were coated with 5 μg/ml bovine plasma fibronectin (Sigma) in 0.1 M bicarbonate solution for 3 h at 4°C, then replaced with DMEM with 10% FBS and incubated overnight to remove non-adherent fibronectin. Wells were rinsed with Hanks Balanced Salt Solution (HBSS) immediately before seeding. Cells were subjected to equibiaxial tensile strain using a custom manufactured vacuum-operated loading device. A pulsed waveform 16% CTS and 0.5 Hz frequency was applied. Seeding of cells onto the Bioflex plates and application of CTS were completed at 37°C under 5% CO_2 and 5% O_2. A 5.0% ambient oxygen tension environment was maintained with a dual gas incubator (Forma II 3130, Thermo Scientific) and used to simulate normal physiologic conditions and reduce the production of reactive oxygen species relative to standard normoxic culture conditions. Cell viability at the end of experimentation was observed using LIVE/DEAD staining (Invitrogen) to detect live (green fluorescence) and dead (red fluorescence) cells.

RNA extraction and quantitative real-time PCR

Chondrocytes were homogenized in TRIzol reagent (Invitrogen) at 0, 12, 24, and 48 h of CTS to extract total cellular RNA, which was further treated with Turbo DNase (Ambion) for 25 min at 37°C to remove genomic DNA. RNA concentration and purity were estimated spectrophotometrically on the basis of absorbance values at 260 nm and 280 nm. Reverse transcription of equal quantities of RNA (1 μg) from each sample was performed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen).

Real-time PCR was performed using the Stratagene Mx3000P instrument and Power SYBR Green PCR Master Mix (Applied Biosystems). Select PCR primers were designed using Primer3 v0.4.0. (Whitehead Institute for Biomedical Research) on the basis of bovine mRNA sequences (primer sequences available on request). The amplification program was as follows: denaturation, 95°C, 10 min; 40 amplification cycles, 95°C, 30 s; and annealing, 60°C, 1 min. After amplification, melt analysis was performed by heating the reaction mixture from 60°C to 95°C at a rate of 0.2°C/s. Relative fold changes relative to the time zero control samples (24 h post seeding but pre-CTS) were calculated using the comparative threshold cycle (C_T) method ($\Delta\Delta C_T$) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the control gene. Four housekeeping genes were tested (GAPDH, HPRT, β2 M, RPL13a), and GAPDH was found to be the most stable with a max fold change of 0.26 from baseline after 48 h. Neither CTS nor PTHrP significantly affected expression of the housekeeping genes.

Enzyme immunoassay (EIA) and biochemical analysis

Culture medium aliquots were stored at −80°C prior to biochemical analyses. Relative concentrations of NO were measured in the culture media using the Griess assay (Promega).

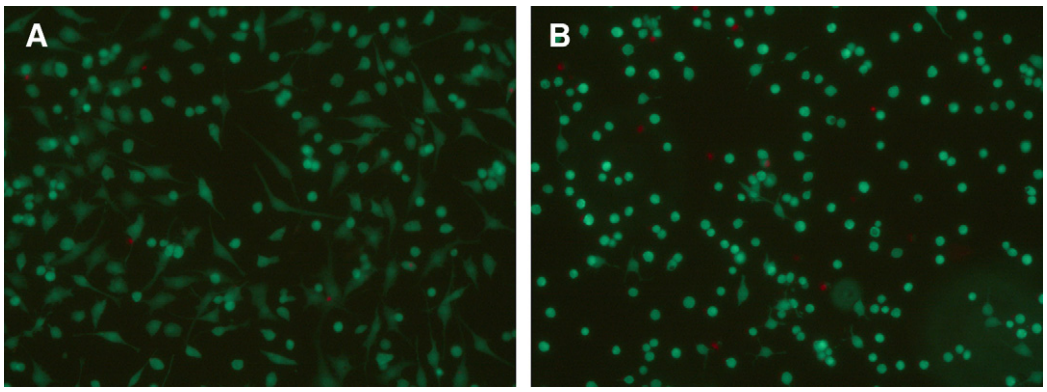

Fig. 1. Cell viability at the end of experimentation was observed using LIVE/DEAD staining to detect live (green fluorescence) and dead (red fluorescence) cells. The majority of chondrocytes (>90%) remained viable after 48 h of (A) No CTS and (B) CTS.

The Griess reaction is based on the chemical diazotization reaction that uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system measures nitrite, which is one of two primary, stable and nonvolatile breakdown products of NO. PGE_2 production was measured in the culture media using a high-sensitivity EIA kit (Assay Designs). Total PTHrP production was measured in the culture media using an EIA kit (Bachem), which is designed to measure the 1–34 peptide sequence and detects both human and bovine PTHrP, but does not cross-react with PTH. All biochemical assays were normalized to number of viable cells, which was determined quantitatively using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega).

Statistical analysis

Each experiment was performed three times with different chondrocyte sources. Proportional fold changes are presented using the means [±95%confidence interval (CI)] from a single replicate experiment, and statistical analyses were carried out by GraphPad Prism five using three replicate experiments. Two-tailed *t*-tests were used to compare gene expression differences between CTS and no CTS conditions. Two-way analysis of variance (ANOVA) with *post hoc* Bonferroni-corrected *t*-tests were used to compare differences between the three different PTHrP treatment conditions (control, 1-141, 1-173) for both CTS and no CTS conditions. Statistical significance was based on $P < 0.05$.

Results

Effects of 16% CTS on articular chondrocytes

When observed under light microscopy, unstrained cells continued to adhere and spread in monolayer during the experimental period. CTS caused most adherent cells to retain their round shape, while others took on a more stellate-shaped appearance. The majority of chondrocytes (>90%), regardless of their shape, remained viable after undergoing 48 h of CTS (Fig. 1). Furthermore, the number of viable cells between CTS and No CTS conditions, as measured by proliferation assays, was similar at all time points. The effects of CTS were ascertained by measuring the mRNA levels of multiple genes (Table I). All MMP, ADAMTS, and tissue inhibitors of metalloproteinases (TIMP) gene levels were up-regulated by CTS. MMP-3 (stromelysin 1) and MMP-13 (collagenase 3) mRNA levels in strained chondrocytes were increased 3.31 and 3.73 times, respectively, over levels in unstrained chondrocytes after 48 h [Fig. 2(A)]. ADAMTS-4 (aggrecanase 1) and ADAMTS-5 (aggrecanase 2) mRNA levels were increased in strained chondrocytes 10.63 and 1.14 times, respectively, over levels in unstrained cells after 48 h [Fig. 2(B)]. In comparison, mRNA levels of these genes in unstrained cells did not change by more than 0.81-fold from time zero levels; changes in MMP-9 and MMP-13 mRNA levels were the only exceptions (−2.39 and −2.36, respectively). CTS decreased gene expression of collagen type IIα1 (Col2a1) by 0.37-fold from no CTS controls but did not alter expression of aggrecan over 48 h [Fig. 2(C)]. The hypertrophy-associated genes, Col10a1 and runt-related transcription factor 2 (Runx2), were significantly up-regulated 3.00-fold and 0.43-fold, respectively, by CTS over no CTS conditions after 48 h [Fig. 2(D)]. Col10a1 gene expression was immediately increased with application of CTS, with significant increases at all measured time points. In comparison, Col10a1 mRNA levels increased by an average of only 0.93-fold over the 48-h experimental period under no CTS conditions. Gene expression of

Table I
Gene expression changes in articular chondrocytes subjected to 48 h of CTS

Gene	CTS*	No CTS*	*P*-value†
Col2a1	−0.11	0.25	0.050
Aggrecan	0.15	0.17	0.148
MMP-1	1.28	0.34	0.010
MMP-3	2.49	−0.81	0.080
MMP-9	3.29	−2.39	0.010
MMP-13	1.37	−2.36	0.049
ADAMTS-4	9.92	−0.72	0.087
ADAMTS-5	0.34	−0.81	0.074
TIMP-1	0.23	−0.20	0.067
TIMP-2	0.36	−0.10	0.073
TIMP-3	2.21	−0.31	0.005
Col10a1	3.93	0.93	0.007
ALP	−0.19	0.27	0.159
Runx2	0.57	0.14	0.047
bPTHrP	3.72	−0.56	0.000
PTHR1	−1.04	0.13	0.016
CTGF	−0.62	0.01	0.010
Sox9	0.51	0.18	0.044
iNOS	4.66	−0.80	0.010
COX-2	0.85	−0.21	0.006
Bcl-2	1.17	−0.04	0.006
Bcl-xL	1.08	−0.07	0.018
Bax	0.24	−0.03	0.067

ADAMTS – a disintegrin and metalloproteinase with thrombospondin motifs; Col10a1 – collagen type Xα1; PTHR1 – PTHrP receptor; CTGF – connective tissue growth factor.

* Proportional fold changes of mRNA levels over 48 h, compared to time zero, of CTS and no CTS conditions presented as mean of $N = 3$.

† *P*-Values were calculated from two-tailed *t*-tests.

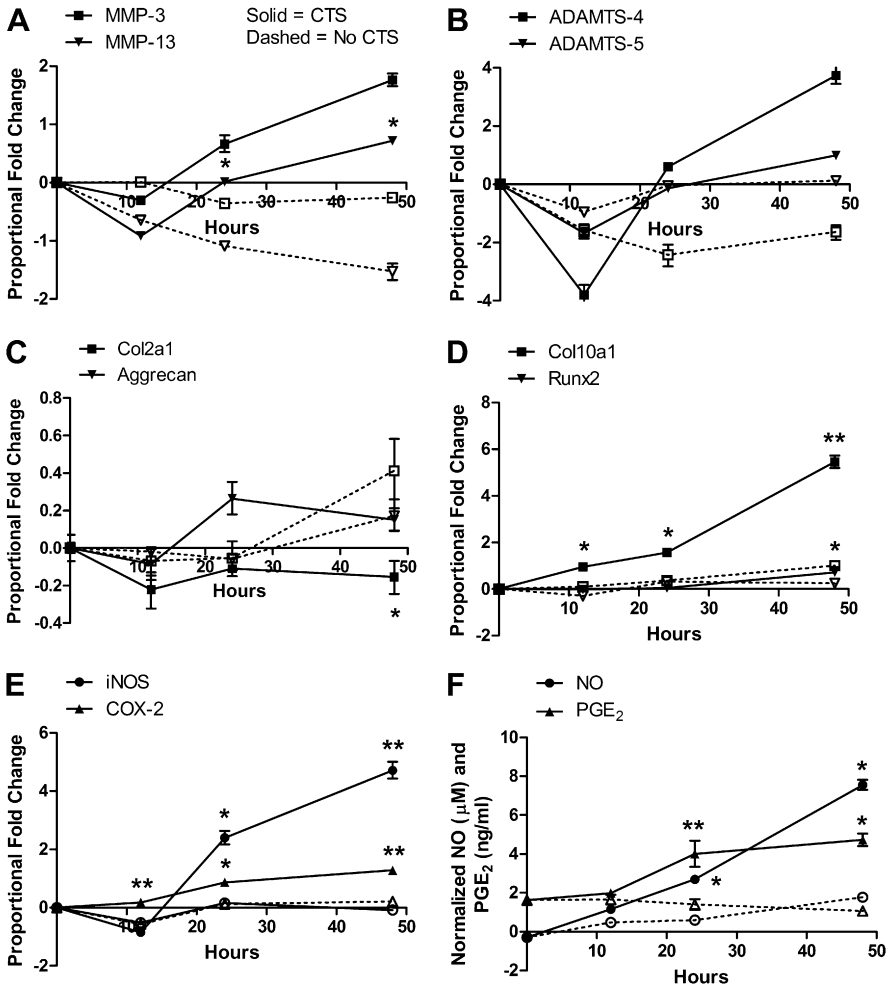

Fig. 2. Effects of 16% CTS on normal bovine articular chondrocytes. CTS increased gene expression of (A) MMPs, (B) ADAMTSs, (D) hypertrophic genes, and (F) iNOS and COX-2 and decreased gene expression of (C) Col2a1. CTS increased secretion of (D) NO and PGE_2, which correlates with (E). Error bars represent ±95% CIs ($N = 3$). Solid lines = CTS; Dotted lines = No CTS. $^{*} = P < 0.05$ and $^{**} = P < 0.01$ vs control. Specifically, MMP-13 48 h $P = 0.0493$, 24 h $P = 0.0465$; Col2a1 48 h $P = 0.0498$; Col10al $P = 0.0072$, 24 h $P = 0.0124$, 12 h $P = 0.0122$; Runx2 48 h $P = 0.0471$; iNOS 48 h $P = 0.0097$, 24 h $P = 0.0139$; COX-2 48 h $P = 0.0059$, 24 h $P = 0.0130$, 12 h $P = 0.0075$; NO 48 h $P = 0.0397$, 24 h $P = 0.0301$; PGE_2 48 h $P = 0.0147$, 24 h $P = 0.0066$.

inducible enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were up-regulated 5.46-fold and 1.06-fold, respectively, over no CTS controls after 48 h of CTS. Secretion of their respective mediators, NO and PGE_2, were significantly increased 5.17 and 4.50 times over controls after 48 h of CTS [Figs. 2 (E and F)]. NO and PGE_2 levels in unstrained chondrocytes remained low and changed minimally over the course of the experimental period.

Total PTHrP secretion after transfection of articular chondrocytes

Chondrocytes transfected with either hPTHrP construct secreted more total PTHrP under both CTS and no CTS conditions [Fig. 3(A), CTS data shown]. Under CTS conditions, chondrocytes expressing 1-141 and 1-173 secreted an average 1.59 and 1.39 times more total PTHrP than control cells, respectively, at 48 h; under no CTS conditions, chondrocytes expressing 1-141 and 1-173 secreted an average of 1.27 and 1.12 times more total PTHrP than control cells, respectively, at 48 h. Additionally, chondrocytes expressing hPTHrP 1-141 secreted more total PTHrP than those expressing hPTHrP 1-173 at all measured times, although the differences were not significant.

Several trends in PTHrP secretion by control cells were observed. When comparing CTS and no CTS conditions, CTS significantly increased PTHrP secretion by 1.31 times over that in no CTS cells at 24 h [Fig. 3(A)]. Additionally, while total PTHrP secretion generally trended down during experimental period with CTS, endogenous PTHrP gene expression was significantly up-regulated by CTS as measured by real-time PCR [Fig. 3(B)]. After 48 h of CTS, bovine parathyroid hormone-related peptide (bPTHrP) gene expression increased 4.28-fold over no CTS controls. However, chondrocytes expressing hPTHrP 1-141 and 1-173 demonstrated a 1.68- and 1.21-fold feedback inhibition, respectively, of endogenous bPTHrP mRNA levels.

hPTHrP overexpression inhibits CTS-induced Col10a1 gene expression

PTHrP overexpression suppressed both basal and CTS-induced Col10a1 gene expression. hPTHrP 1-141 and 1-173 expression suppressed CTS-induced Col10a1 mRNA levels during all measured time points [Fig. 4(A)]. Increases in Col10a1 gene levels by CTS were inhibited significantly by 2.39- and 1.86-fold, respectively, at 48 h and by 0.85- and 0.92-fold, respectively, at 24 h. In comparison,

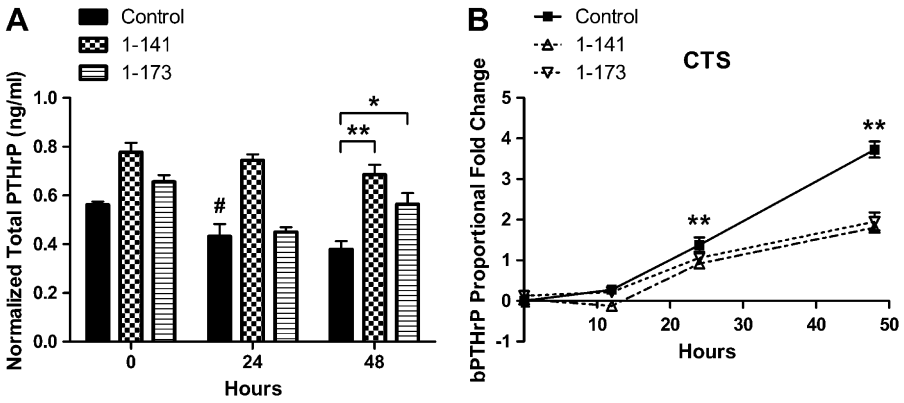

Fig. 3. Total PTHrP secretion by articular chondrocytes transfected with hPTHrP expression constructs. (A) Chondrocytes expressing hPTHrP constructs secrete more total PTHrP (bovine and human) under CTS and No CTS conditions (only CTS data shown). Error bars represent ±95% CIs ($N = 3$). * = $P < 0.05$ and ** = $P < 0.01$ vs control. # Denotes $P = 0.0102$ vs No CTS control. (B) Endogenous PTHrP (bPTHrP) gene expression, as measured by RT-PCR using a primer specific for the bovine gene, was up-regulated by CTS. Chondrocytes expressing hPTHrP 1-141 and 1-173 demonstrated feedback inhibition of endogenous bPTHrP mRNA levels. Error bars represent ±95% CIs ($N = 3$). ** = $P < 0.01$ control vs hPTHrP 1-141 and 1-173.

hPTHrP 1-141 and 1-173 suppressed basal Col10a1 mRNA levels significantly by 0.84- and 0.62-fold, respectively, at 48 h in no CTS cells [Fig. 4(B)]. hPTHrP did not significantly affect expression of matrix remodeling genes.

Effects of hPTHrP overexpression on CTS-induced iNOS and COX-2 gene expression and NO and PGE_2 production

Expression of hPTHrP 1-141 augmented CTS-induced iNOS gene expression at 24 h by 1.99-fold, although the differences were not significant [Fig. 5(A)]. However, hPTHrP 1-141 augmented CTS-induced NO production significantly at all measured time points, with an average 1.81 times increased production vs control at 48 h [Fig. 5(B)]. In addition, hPTHrP 1-141 augmented both COX-2 gene levels and PGE_2 production by 1.20- and 1.78-fold over control levels at 48 h [Fig. 5(C and D)]. Chondrocytes overexpressing hPTHrP 1-173 exhibited similar iNOS and COX-2 mRNA levels as control, with a maximum 0.61-fold change during the experimental period. NO and PGE_2 production between 1-173 and control groups were almost identical as well, with a maximum 0.10-fold change during the experimental period. Under no CTS conditions, neither hPTHrP isoform affected iNOS and COX-2 mRNA levels or NO and PGE_2 production, all of which remained low and showed minimal change during the experimental period.

Effect of hPTHrP overexpression on CTS-induced apoptotic gene expression

CTS increased Bcl-2 and Bcl-2-associated X protein (Bax) gene expression by 1.21- and 0.27-fold, respectively, over no CTS controls after 48 h. Neither hPTHrP isoform had any significant effect on Bcl-2 or Bax mRNA levels [Fig. 6(A and B), CTS data shown]. The Bcl-2/Bax mRNA ratio was calculated, which also did not change significantly upon hPTHrP overexpression when compared to control [Fig. 6(C)]. Finally, basal Bcl-2 and Bax expression levels in unstrained chondrocytes remained low and changed minimally during the experimental period and were not altered by hPTHrP overexpression (data not shown).

Discussion

Cell-based tissue engineering strategies are currently in clinical use and continue to be developed at a rapid pace for the repair of cartilage defects. Regardless of the repair methodology, newly created joint cartilage will still be susceptible to the abnormal inflammatory and mechanical environments that underlie osteoarthritic disease, potentially compromising its integration, function, and longevity. Chondroprotection measures must therefore be designed for the chondrocytes of regenerated cartilage, which are in close proximity to matrix proteases, bioactive ECM fragments, and inflammatory mediators responsible for degenerative OA. Previous

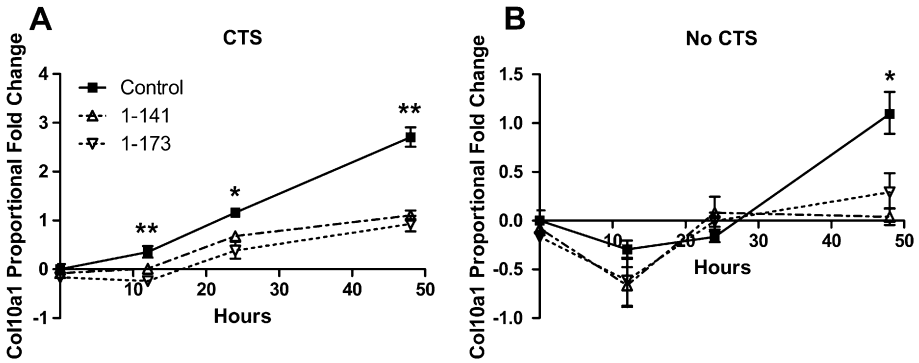

Fig. 4. Inhibition of Col10a1 gene expression in articular chondrocytes by PTHrP overexpression. Expression of exogenous hPTHrP in chondrocytes suppressed (A) CTS-induced Col10a1 gene expression and (B) basal Col10a1 gene expression. hPTHrP overexpression had no significant effect on matrix remodeling genes (data not shown). Error bars represent ±95% CIs ($N = 3$). * = $P < 0.05$ and ** = $P < 0.01$ vs hPTHrP 1-141 and 1-173.

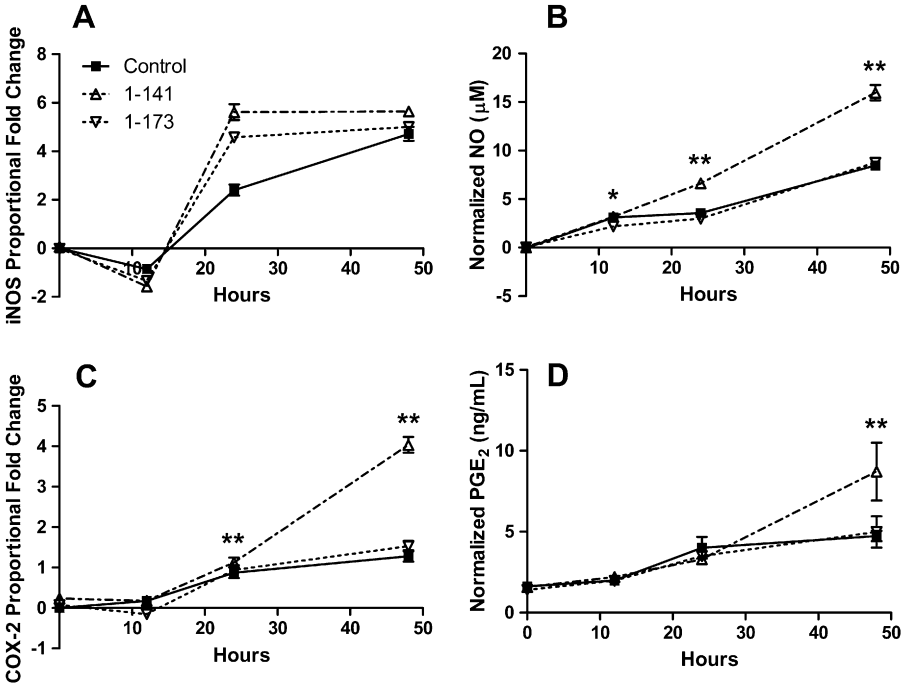

Fig. 5. Effects of hPTHrP overexpression on CTS-induced iNOS and COX-2 gene expression and NO and PGE_2 production in articular chondrocytes. Overexpression of hPTHrP 1-141 augmented gene expression of inducible enzyme isoforms, (A) iNOS and (C) COX-2, and production of their respective inflammatory mediators, (B) NO and (D) PGE_2. Chondrocytes overexpressing hPTHrP 1-173 did not show significant changes in iNOS and COX-2 gene expression or production of NO and PGE_2 when compared to control. Error bars represent ±95% CIs ($N = 3$). * = $P < 0.05$ and ** = $P < 0.01$ vs control.

reports have indicated that many of the biologic changes in articular chondrocytes during the progression of OA are similar to those in the cartilage growth plate during developmental endochondral ossification. Specifically, articular chondrocytes reinitiate a sequence of phenotypic changes that begin with hypertrophy and progress toward mineralization and apoptosis[9,10,22,23]. Increased production of catabolic proteases, including MMP-1, MMP-3, MMP-9, and MMP-13, has been observed both in the growth plate and OA cartilage, resulting in matrix degradation in both processes[12,24–28].

Because the progression of the degenerative cascade is thought to involve the loss of inhibitory control over terminal differentiation genes, we hypothesized that an anti-hypertrophic therapy may protect healthy chondrocytes from arthritic disease. PTHrP is an important signaling factor in the regulation of endochondral ossification in the epiphyseal growth plate of long bones. Within cells, PTHrP is processed by members of the family of prohormone convertases to at least three fragments with different biologic functions[29,30]. Several direct effects of these fragments make it an attractive option for chondroprotection therapy. Suppression of hypertrophy and terminal differentiation is mediated through parathyroid hormone receptor 1 (PTHR1). Activation of PTHR1 leads to subsequent activation of the PKA signaling pathway, increasing chondrocyte proliferation by decreasing p57 and Runx2 levels and increasing Sox9 phosphorylation[31]. Because the N-terminal regions of PTHrP and PTH are similar and share the same receptor (PTHR1), previous reports have shown the ability of these peptides to suppress hypertrophic changes and terminal differentiation of articular chondrocytes induced by T3 and 5-azacytidine[32,33]. Additionally, PTHrP has a well-documented anti-apoptotic effect through its translocation to the nucleus *via* a mid-region bipartite nuclear targeting sequence (NTS)[17,34–36]. Thus, PTHrP plays an important role in maintaining the survival and chondrogenesis of proliferating chondrocytes and suppressing terminal differentiation in the growth plate.

In the present study, the effects of PTHrP in suppressing early arthritic-like changes were ascertained in a mechanical CTS model.

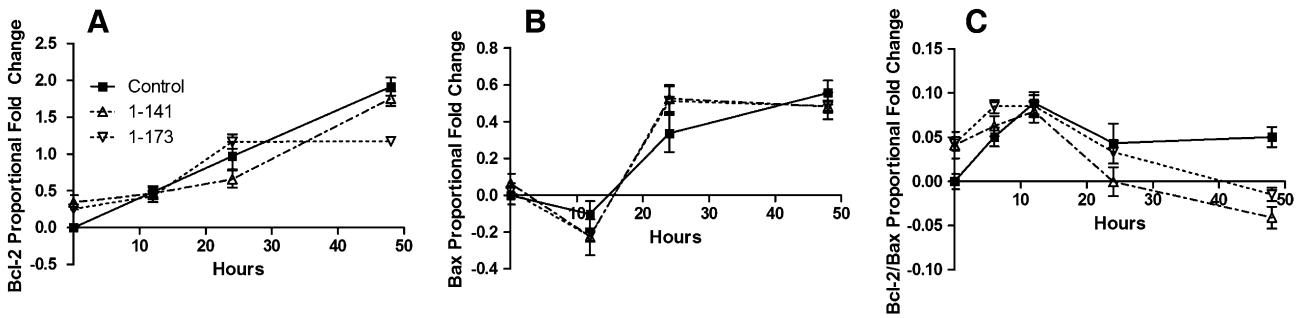

Fig. 6. Effects of hPTHrP overexpression on CTS-induced apoptotic gene expression in articular chondrocytes. Chondrocytes overexpressing either exogenous hPTHrP isoform did not show any significant change in CTS-induced (A) Bcl-2 and (B) Bax gene expression levels. (C) hPTHrP overexpression also did not significantly affect Bcl-2/Bax ratio. Error bars represent ±95% CIs ($N = 3$).

Because the half-life of the N-terminal fragment is only 6–8 min[37], sustained delivery of PTHrP will eventually be needed to inhibit the effects of a chronic disease like OA to achieve optimal therapeutic results. Such a mechanism of sustained PTHrP delivery was tested by non-viral gene transfer of hPTHrP cDNA into the articular chondrocytes themselves. This study contributes several novel approaches and results. First, high magnitude CTS model induced an arthritic phenotype with a similar pattern of changes as OA chondrocytes, including increased gene expression of the collagenases and aggrecanases, as well as increased production of NO and PGE_2. Additionally, high magnitude CTS was discovered to increase gene expression of Col10a1, a marker of chondrocyte hypertrophy, and this phenomenon may be mediated by Runx2 due to their concurrent increase. Endogenous bPTHrP gene expression increased with CTS, consistent with increased PTHrP secretion seen in human osteoarthritic cartilage[38,39] and thought to be a self-repair response to damaged cartilage[40]. Interestingly, gene expression of PTH1R, a marker of pre-hypertrophy, decreased initially for the first 12 h with CTS but steadily increased for the next 36 h (data not shown). This bimodal regulation may arise from CTS overriding the autocrine feedback down-regulation of receptor expression in cells producing PTHrP. However, cellular changes that occur during recovery from trypsinization may also modulate this response. Gene expression of ALP, a recognized marker for terminal differentiation, did not change significantly with 48 h of CTS. It may be possible that only early hypertrophic genes were up-regulated within the first 48 h of CTS, while late hypertrophic genes would have been up-regulated with a longer duration of CTS. Second, sustained PTHrP delivery to chondrocytes was achieved by means of FuGENE 6-mediated transfection of hPTHrP constructs. As expected, chondrocytes expressing exogenous hPTHrP showed higher secreted levels of total PTHrP (bovine and human) than controls. Chondrocytes expressing isoform 1-141 generally secreted more total PTHrP than those expressing isoform 1-173, which is consistent with previous data showing that serial truncation of the C-terminal end increases the amount of secreted PTHrP[21].

Third, overexpression of both hPTHrP isoforms inhibited CTS-induced and basal Col10a1 expression in articular chondrocytes. Although CTS significantly increased Runx2 mRNA levels, this change was so slight that whether inhibition of Col10a1 expression by hPTHrP was *via* Runx2 could not be ascertained. hPTHrP had no significant inhibitory effect on CTS-induced MMP-13 gene expression. MMP-13 is expressed by hypertrophic chondrocytes to degrade the cartilage matrix, to facilitate vascular invasion and matrix mineralization by osteoblasts. Because MMP-13 is expressed in only the most terminally differentiated hypertrophic chondrocytes[41], the immediate MMP-13 up-regulation observed in this CTS model is most likely attributed to mechanotransduction rather than phenotype modulation. hPTHrP overexpression was not able to immediately inhibit the catabolic changes along with the hypertrophic-like changes, suggesting that the two pathways are regulated separately.

Lastly, CTS increased NO and PGE_2 production, which both act as strong catabolic signals in cartilage by altering chondrocyte function and enhancing chondrocyte apoptotic potential[42]. hPTHrP isoform 1-141 potentiated the production of these mediators with CTS by upregulating transcriptional levels of iNOS and COX-2. Because these effects were not observed with isoform 1-171, the underlying mechanism may be specific to the amount of secreted PTHrP or to the 140–141 region that differs between the two isoforms. Although PTHrP is known to stimulate NO release from endothelial cells for local regulation of vascular tone[43], a possible mechanism for PTHrP-mediated NO release in chondrocytes has not been elucidated. Additionally, studies have found that PGE_2 increases PTHrP production in chondrocytes[44,45], but the reverse has not been reported. Increased production of pro-inflammatory intermediaries is expected to promote apoptosis through regulation of Bcl-2 and Bax gene expression in chondrocytes. Bax forms heterodimers with Bcl-2, and when overexpressed, counters the anti-apoptotic effect of Bcl-2, causing accelerated cell death. Therefore, it is the ratio of Bcl-2 to Bax that determines the apoptotic fate of a cell. Our results show that Bcl-2 and Bax gene up-regulation by CTS follows a similar temporal pattern as hypertrophic chondrocytes in the growth plate[16,46]. Anti-apoptotic Bcl-2 gene expression was immediately increased, possibly in a rescue attempt for survival, while pro-apoptotic Bax gene expression was not increased until 12–24 h of CTS, when the ratio of the expression level of Bcl-2 and Bax gradually shifts in favor of Bax. Intracellular hPTHrP expression was expected to effect a pro-survival advantage to chondrocytes due to translocation of the NTS to the nucleus, as shown in previous work in the literature[17,34–36]. However, hPTHrP overexpression had no significant effect on Bcl-2 and Bax gene expression. This result may be attributed to the short testing period. The intact viability of the majority of cells after 48 h of CTS indicates that the only initial stages of the apoptotic cascade were activated. Longer follow-up times may be needed to appreciate any apoptosis and cell death cause by CTS and thus, any anti-apoptotic effects of hPTHrP overexpression. Interestingly, B-cell lymphoma – extra large (Bcl-xL) gene expression was up-regulated with CTS and augmented by expression of hPTHrP 1-141 at 48 h (data not shown).

Several studies have reported the use of inflammation-responsive promoters to regulate OA gene therapy[47–49]. Regulation of hPTHrP expression may be necessary to selectively activate targeted pathways because high concentrations of PTHrP can facilitate activation of other heterotrimeric G proteins, including the G_q family. The effect of G_q activation is opposite from that of G_s activation in that it mildly accelerates chondrocyte differentiation[50]. The purpose of these opposing actions may be to regulate the proliferation of chondrocytes based on their location, thus creating a functional gradient of PTHrP across the growth plate[31]. PTHrP overexpression may expose chondrocytes to concentrations that are too high, simultaneously activating G_s and G_q pathways. Therefore, we are currently testing the use of an inflammation-responsive, self-limiting promoter consisting of NF-κB repeat sequences to optimize PTHrP therapy.

This study demonstrates that PTHrP overexpression inhibits CTS-induced hypertrophic-like changes of articular chondrocytes. Several gene therapy approaches are currently being investigated for the repair of cartilage defects and the inhibition of OA progression. However, most of these approaches (i.e., MMP inhibitors) target only one of several players in the disease pathogenesis, and none have attempted to target the early hypertrophic-like changes seen in OA. Ultimately, a combination of these approaches may be needed for effective chondroprotection of regenerated cartilage. Because CTS only initiated early stage arthritic-like changes, the long-term protective effects of PTHrP may be even greater than indicated in this study. Future work will examine longer-term treatment regimens to better assess the effects of PTHrP overexpression on overall matrix metabolism and chondrocyte survival under mechanical loading conditions.

Author contributions

All authors contributed to the conception and design of the study, analysis and interpretation of data, and preparation and approval of the manuscript. In addition, DW and JMT contributed to data acquisition, and DW (wangd@ccf.org) drafted the manuscript and takes responsibility for the integrity of the work.

Conflict of interest

The authors had no competing interests.

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