Osteoarthritis and Cartilage (2008) **16,** 70–82

doi:10.1016/j.joca.2007.05.014

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Interaction between zonal populations of articular chondrocytes suppresses chondrocyte mineralization and this process is mediated by PTHrP

J. Jiang M.S.†, N. L. Leong B.S.†, J. C. Mung M.S.†, C. Hidaka M.D.§ and H. H. Lu Ph.D.†‡*

† *Biomaterials and Interface Tissue Engineering Laboratory, Department of Biomedical Engineering, Columbia University, New York, NY 10027, United States*

‡ *College of Dental Medicine, Columbia University, New York, NY 10032, United States*

§ *Soft Tissue Research Laboratory, Hospital for Special Surgery, New York, NY 10021, United States*

Summary

Objective: Articular cartilage is separated from subchondral bone by the tidemark and a calcified cartilage zone. Advancement of the calcified region and tidemark duplication are both hallmarks of osteoarthritis (OA). Currently the mechanisms controlling post-natal articular cartilage mineralization are poorly understood. The objective of this study is to test the hypothesis that cellular communication between different cartilage layers regulates articular chondrocyte mineralization.

Design: Co-culture models were established to evaluate the interaction of chondrocytes derived from the surface, middle and deep zones of articular cartilage. The cultures were stimulated with triiodothyronine (T3) to promote chondrocyte hypertrophy. The effects of zonal chondrocyte interactions on chondrocyte mineralization were examined over time.

Results: Co-culture of deep zone chondrocytes (DZCs) with surface zone chondrocytes (SZCs) suppressed the T3-induced increase in alkaline phosphatase (ALP) activity and related mineralization. Moreover, SZC–DZC co-culture was associated with a significantly higher parathyroid hormone-related peptide (PTHrP) expression when compared to controls. When PTHrP(1–40) was added to the DZC-only culture, it suppressed DZC ALP activity similar to the inhibition observed in co-culture with SZC. In addition, treatment with PTHrP reversed the effect of T3 stimulation on the expression of hypertrophic markers (Indian hedgehog, ALP, matrix metalloproteinases-13, Type X collagen) in the DZC cultures. Moreover, blocking the action of PTHrP significantly increased ALP activity in SZC + DZC co-culture.

Conclusion: Our findings demonstrate the role of zonal chondrocyte interactions in regulating cell mineralization and provide a plausible mechanism for the post-natal regulation of articular cartilage matrix organization. These findings also have significant implications in understanding the pathology of articular cartilage as well as devising strategies for functional cartilage repair.

Key words: Co-culture, Alkaline phosphatase activity, Indian hedgehog, Calcification, Articular cartilage, Parathyroid hormone-related peptide, Chondrocytes.

Introduction

Arthritis is the leading cause of disability among Americans[1]. The most common form of arthritis is osteoarthritis (OA), with 21 million Americans suffering from this degenerative condition[1]. Articular cartilage provides frictionless surfaces for joint articulation and its functional properties are derived from its structural organization and composition. Articular cartilage can be divided into three regions: the tangential (surface) zone, the transitional (middle) zone and the radial (deep) zone, with each region exhibiting characteristic cellular phenotype and matrix properties[2–8]. Below the radial zone lies the tidemark, separating articular cartilage from the calcified cartilage region[9–14]. The tidemark and the calcified cartilage collectively constitute the osteochondral interface, which functions as a physical barrier for vascularization and facilitates the pressurization and physiological loading of articular cartilage[15–17]. Advancement of the calcified region and tidemark duplication are observed with age[13,18–21], and has been associated with OA[22–28]. Elucidation of the biochemical processes responsible for the regulation of articular chondrocyte mineralization may be critical in the treatment and eventual prevention of OA.

Current understanding of cartilage mineralization derives largely from our knowledge of endochondral ossification during embryonic limb development[29–33], fracture healing[34–36], and from studies of pathological mineralization associated with crystal deposition arthropathies[27,37]. Systemic factors such as thyroid hormone promote hypertrophy of growth plate chondrocytes and the formation of mineralized cartilage, which is later remodeled into bone during endochondral ossification[20,32,38–41]. Articular cartilage in immature animals provides the matrix source for the growth of the epiphyseal nucleus[42]. Chondrocytes near the articular surface proliferate and form new cartilage, the matrix compartment then calcifies and the mineralization front

*Address correspondence and reprint requests to: Helen H. Lu, Ph.D., Department of Biomedical Engineering, Columbia University, 351 Engineering Terrace Building, MC 8904, 1210 Amsterdam Avenue, New York, NY 10027, United States. Tel: 1-212-854-4071; Fax: 1-212-854-8725; E-mail: hl2052@columbia.edu

Received 18 January 2007; revision accepted 26 May 2007.

advances toward the articular surface[20,21,32,38,39,42]. This process, however, slows down following puberty and becomes dormant upon reaching skeletal maturity[20,21,38].

Multiple theories have been proposed regarding the mechanisms underlying the observed age-[13,18–21] and disease-related[20,22–28] advancement of the calcification front in articular cartilage. Carter and Wong[43] postulated that upward advancement is modulated by intermittent hydrostatic pressure generated in the deep layers of cartilage during physiological loading. Another hypothesis suggests that cells residing near the tidemark exert an active suppression of the mineralization processes in this area[44]. It has also been postulated that cellular activity and mineralization are quiescent at or near the transition region, and that only after trauma or during the early phases of OA, the chondrocytes near the tidemark become active and begin to mineralize[44,45]. To date, direct experimental validation of these hypotheses has been limited and the mechanisms controlling chondrocyte mineralization remain elusive.

It is well established that molecular transport from the articular surface to the cartilage proper is mediated through dynamic loading[46,47]. Physiological loading is also believed to prevent tidemark advancement[48]; O'Connor[49] reported that unloading of the rat hind leg led to thickening of the calcified cartilage layer. Based on these observations and taking into consideration the stratified organization of articular cartilage, we propose here an original hypothesis that cellular communication within cartilage layers plays a regulatory role in cartilage mineralization. Specifically, we hypothesize that chondrocytes from the surface layer regulate the mineralization potential of chondrocytes residing in the deep zone. To test this hypothesis, the first objective of this study is to evaluate the interaction of chondrocytes isolated from the three different zones of articular cartilage using a direct co-culture model. The effects of co-culture on chondrocyte mineralization will be determined, and conditioned media studies will be conducted to evaluate the existence of paracrine effects. Thyroid hormone has previously been shown to induce hypertrophy in both growth plate and aging articular chondrocytes[32,38–41,50]. In this study, triiodothyronine (T3), a form of thyroid hormone, will be used to stimulate articular chondrocyte hypertrophy and mineralization[50]. Since the growth plate remains open before reaching skeletal maturity, exposure to systemic factors such as T3 in post-natal articular cartilage is not unexpected. Moreover, addition of T3 will stimulate the condition following injury to the osteochondral interface, after which the deep zone cartilage may be exposed to systemic factors via invasion of the subchondral bone vasculature[51].

The second objective of this study is to investigate the mechanism underlying any potential interactions between zonal sub-populations of articular chondrocytes. During development, the rate at which the growth plate cartilage mineralizes is highly regulated. It has been shown to be controlled via the parathyroid hormone-related peptide (PTHrP) and Indian hedgehog (*Ihh*) negative feedback loop[52–56]. During endochondral ossification, the hypertrophic and pre-hypertrophic chondrocytes secrete *Ihh* which promotes hypertrophy in adjacent chondrocytes[57–59] and induces PTHrP production in immature peri-articular chondrocytes[52,55,60,61]. Whether the PTHrP–*Ihh* control loop is significant in the regulation of articular chondrocyte mineralization during post-natal development is not known. *In situ* hybridization of rodent articular cartilage revealed that PTHrP expression is present in chondrocytes residing near the surface of articular cartilage at 20 weeks of age[62]. Based on these observations, we hypothesize that similar to growth plate chondrocytes, mineralization by deep zone chondrocytes (DZCs) is regulated by PTHrP, and chondrocytes at the surface and deep zones of articular cartilage may also communicate via the PTHrP–*Ihh* negative feedback loop. To test this hypothesis, experiments have been designed to determine the role of PTHrP in regulating *Ihh* expression and mineralization potential of DZCs. Specifically, exogenous PTHrP will be added to DZCs stimulated by T3, and the resultant effects on cell alkaline phosphatase (ALP) activity and gene expression will be investigated. It is expected that while stimulation of DZCs with T3 will increase *Ihh* expression, this effect will be countered by the addition of PTHrP. Findings from this study are anticipated to provide insight into the mechanisms governing articular cartilage mineralization, and can have a significant impact on the formulation of future treatment strategies for OA as well as functional cartilage repair.

Materials and methods

CELLS AND CELL CULTURE

Primary bovine articular chondrocytes were used in this study. The cells were isolated from the knee joints of neonatal calves (~1 week old) obtained from an abattoir (Fresh Farm's Beef, Inc., VT). Due to differences in cartilage thickness between animals and isolation sites, a standardized protocol was developed based on published reports[3,5,6]. Specifically, the 10% (by height of the full thickness articular cartilage) closest to the articular surface was considered to be the surface zone, the middle 60% by height was deemed the middle zone, and the 30% closest to the subchondral bone was designated as the deep zone. Zonal populations of chondrocytes were then obtained by enzymatic digestions of tissue derived from each region following the methods of Hidaka *et al.*[8]. Briefly, the tissue was minced and digested overnight in 0.1% w/v collagenase (Sigma, St. Louis, MO) in Dulbecco's modified Eagle's medium (DMEM, Cellgro-Mediatech, Herndon, VA) supplemented with 1% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA) and 2% antibiotics (10,000 U/mL penicillin, 10 mg/mL streptomycin, Cellgro-Mediatech). The cells were seeded at high density (4×10^5 cells/cm^2) and maintained in fully-supplemented DMEM (10% FBS, 1% non-essential amino acids, 1% antibiotics). Three sub-populations of chondrocytes were isolated from the tibiofemoral joint: the surface zone chondrocytes (SZCs), middle zone chondrocyte (MZC), and DZCs. A full thickness chondrocyte (FC) culture was established from digestion of a mixture of all isolated cartilage tissue pieces, without distinguishing any one cartilage zone from another.

T3 STIMULATION OF CHONDROCYTES

Primary chondrocytes were seeded at high density (4×10^5 cells/cm^2), and the cultures were maintained in fully-supplemented media for 2–3 days after seeding. Cultures were then stimulated with T3 (Sigma, St. Louis, MO) following methods described by Rosenthal and Henry[50]. Briefly, all groups were pre-treated for 24 h with serum-free DMEM supplemented with 1% antibiotic and 0.35 mg/mL bovine serum albumin (Sigma). The cultures were then stimulated with T3 (0, 10 and 100 nM) for 4 days, after which all cultures were maintained for up to 2 weeks in mineralization media without T3, i.e., fully-supplemented DMEM plus 50 μg/mL ascorbic acid (Sigma) and 3 mM β-glycerophosphate (β-GP, Sigma). Chondrocyte ALP activity and mineral deposition were

assessed at the onset (day 0) as well as post T3 stimulation on days 2, 4, 7, 10, and 14.

CO-CULTURE MODELS OF ZONAL POPULATIONS OF CHONDROCYTES

To evaluate cellular interactions between chondrocyte populations, direct two-dimensional (2-D) co-cultures of DZC with either SZC and/or MZC were established. Specifically, co-cultures of SZC + DZC and MZC + DZC were initiated by seeding at high density (4×10^5 cells/cm^2) with a 1:1 mixture of each cell type. The co-culture ratio of SZC and DZC was chosen to approximate that of the *in vivo* cell ratio as previous reports have shown that the cell density in the surface zone cartilage is about two to three times higher than that of deep zone cartilage[7,63]. Based on our isolation technique, it is expected the total cell number of SZCs and DZCs are approximately equal. Individual cultures of SZC, MZC, and DZC, as well as FCs served as controls. All chondrocyte cultures were then stimulated with T3 as described above. The effects of co-culture on cell proliferation, ALP activity and mineral deposition were assessed at 2, 4, 7, 10, and 14 days.

MECHANISM OF CELLULAR INTERACTIONS: PARACRINE EFFECTS

To determine whether zonal chondrocyte interactions were mediated through paracrine effects, a conditioned media study was performed. Cultures of SZC, DZC, and SZC + DZC were first established in serum-free DMEM as described above, and conditioned media from these groups were collected at day 2 and again at day 4 of culture. The collected media were first centrifuged at 1500*g* for 10 min, and subsequently mixed with fresh media (1:1 volume). This media mixture was further supplemented with T3 to achieve a final T3 concentration of 0 or 10 nM. Parallel monolayer cultures of DZC were established, and these cultures were treated with the day 2-conditioned media at the start of the experiment, and then again with the day 4-conditioned media at media change 48 h later. Cell response to conditioned media, in particular ALP activity and cell proliferation, was evaluated at 96 h post treatment.

MECHANISM OF CELLULAR INTERACTIONS: ROLE OF PTHrP

To evaluate the role of PTHrP in mediating the interaction between zonal chondrocyte populations, PTHrP expression was measured for all groups. In addition, to directly assess its effect on DZC mineralization, exogenous PTHrP was added to DZC cultures stimulated with T3. Specifically, 50 nM of PTHrP (1−40, Sigma) was added along with T3 (0, 10, and 100 nM) to confluent DZC cultures. Cultures of DZCs stimulated by T3 but untreated with PTHrP served as controls. The effects of PTHrP on the mineralization potential of DZCs were analyzed at 0, 2 and 4 days.

The role of PTHrP-mediated interaction between zonal chondrocytes' population was further evaluated by blocking the actions of PTHrP using a PTHrP antagonist, parathyroid hormone (PTH) (7−34, Bachem, San Carlos, CA)[64]. Exogenous PTH (7−34) at 0, 0.1, 10, and 1000 nM was added to SZC + DZC cultures along with T3 (0 and 50 nM) under serum-free conditions. The effects of the antagonist on ALP activity of SZC + DZC cultures were analyzed at day 4.

THREE-DIMENSIONAL (3-D) CO-CULTURE MODEL

To ascertain that the observed responses were not limited to the 2-D model, a 3-D co-culture model was used to verify DZC and SZC interactions. Specifically, DZCs were first encapsulated in 2% agarose hydrogel at a concentration of 2×10^7 cells/mL and then co-cultured with a monolayer of SZCs in a culture well [Fig. 9(A)]. An acellular hydrogel disc separated the DZC-laden disc from the monolayer of SZCs. The effect of co-culture on ALP activity of DZC was evaluated at 1, 3, 7 and 14 days.

END-POINT ANALYSIS: CELL PROLIFERATION

Cell proliferation ($n = 6$) was determined by measuring total DNA per sample using the PicoGreen® assay (Molecular Probes, Eugene, OR) according to the manufacturer's suggested protocol. Briefly, the samples were first rinsed with phosphate buffered saline (PBS, Sigma) and the cells were lysed in 300 μL of 0.1% Triton X solution (Sigma). An aliquot of the sample (20 μL) was then added to 180 μL of the PicoGreen® working solution. Fluorescence was measured with a microplate reader (Tecan, Research Triangle Park, NC) with excitation and emission wavelengths of 485 and 535 nm, respectively. Total cell number in the sample was determined by converting the amount of DNA per sample to cell number using the conversion factor of 7.7 pg DNA/cell[65].

END-POINT ANALYSIS: GENE EXPRESSION

Gene expression was measured using reverse transcription followed by real-time polymerase chain reaction (PCR). The oligonucleotides were custom designed and the sequences are summarized in Table I. Total RNA was isolated using the TRIzol® reagent (Invitrogen, Carlsbad, CA) extraction method. The isolated RNA was reverse-transcribed into cDNA via the SuperScript™ III First-Strand Synthesis System (Invitrogen) following the manufacturer's suggested protocol. The cDNA product was amplified and quantified through real-time PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The expression levels of relevant genes indicative of chondrocyte maturation ($n = 6$) such as ALP, type X collagen, matrix metalloproteinases-13 (MMP13), as well as the expression of *Ihh* and PTHrP ($n = 3$) were measured and normalized to the expression of the housekeeping gene β-actin. All reactions were run for 40 cycles using

Table I
List of primer sequences utilized in real-time PCR analysis

Gene	Sense	Antisense
ALP	TGCGACTGACCCTTCACTCTC	CACCAGCAGGAAGAAGCCTTT
β-Actin	CTGCGGCATTCACGAAACTA	ACCGTGTTGGCGTAGAGGTC
Col X	TGGATCCAAAGGCGATGTG	GCCCAGTAGGTCCATTAAGGC
Ihh	CCTTCGTAATGCAGCGACT	ATCTCGGTGATGAACCAGTG
MMP13	ACATCCCAAAACGCCAGACAA	GATGCAGCCGCCAGAAGAAT
PTHrP	ACCTCGGAGGTGTCCCCTAA	GCCCTCATCATCAGACCCAA

the iCycler iQ Real-Time PCR Detection System (BioRad). Normalized expression levels reported were calculated based on difference between threshold cycles, namely, the difference in threshold cycle values between the gene of interest and the housekeeping gene β-actin.

END-POINT ANALYSIS: MINERALIZATION POTENTIAL

Cell mineralization potential was determined by examining ALP activity and mineral deposition. Quantitative ALP activity ($n = 6$) was measured using an enzymatic assay based on the hydrolysis of *p*-nitrophenyl phosphate (*p*NP-PO_4) to *p*-nitrophenol (*p*NP)[66]. The samples were lysed in 0.1% Triton X solution, then added to *p*NP-PO_4 solution (Sigma) and allowed to react for 30 min at 37°C. The reaction was terminated with 0.1 N NaOH (Sigma).

A quantitative Alizarin Red-S (Sigma) assay was used to measure mineralization, according to the method described by Puchtler *et al.*[67]. The samples ($n = 5$) were first rinsed with PBS and then fixed in 70% ethanol for 60 min at 4°C to preserve the mineral. The samples were incubated with the dye solution (40 mM) for 10 min, before rinsing with deionized water and PBS. A 10% (w/v) cetylpyridinium chloride solution (CPC, Sigma) was added in order to reconstitute the Alizarin Red-S dye. The amount of Alizarin Red-S, which reflects calcium deposition, was determined at the absorbance wavelength of 570 nm.

END-POINT ANALYSIS: HISTOLOGICAL ANALYSIS

Histological analysis was performed to visualize extracellular matrix deposition and ALP activity. Two samples ($n = 2$) from each group were used for each type of histological stain. All samples were washed and fixed for 10 min in neutral formalin. Distribution of ALP activity was visualized using Fast Blue RR Salt and AS-MX Phosphate (Sigma). After fixation, the samples were incubated with 300 μL of dye solution at room temperature for 30 min, and then rinsed with deionized water prior to viewing under light microscopy. For mineral distribution, the samples were stained with 2% Alizarin Red-S for 1 h and then rinsed with deionized water.

STATISTICAL ANALYSIS

Results are presented in the form of mean ± standard deviation, with *n* equal to the number of samples analyzed. A two-way analysis of variance (ANOVA) was performed to determine the effects of T3 concentration and co-culturing conditions on total cell number, ALP activity and mineral deposition. Similarly, a two-way ANOVA was used to determine effects of combined T3 and PTHrP treatment on cell response. The Tukey–Kramer *post hoc* test was used for all pair-wise comparisons, and significance was attained at $P < 0.05$. All statistical analyses were performed using the JMP software (SAS, Cary, NC).

Results

EFFECT OF T3 ON ZONAL POPULATIONS OF CHONDROCYTES

As the cultures were seeded at high density and maintained under serum-free conditions, total cell number remained relatively constant for the first 4 days of culture. Cell number increased after mineralization media were added at day 4 and peaked after 1 week for all groups examined, however, no significant difference was found between groups. The ALP activity of single cultures of SZC and MZC did not respond to T3 stimulation and remained at basal levels. In contrast, ALP activity of DZC increased significantly following T3 stimulation [Fig. 1(A), $P < 0.05$]. Histological staining [Fig. 2(B)] confirmed these results, with the T3-stimulated DZC cultures exhibiting stronger staining intensity when compared to the DZC control.

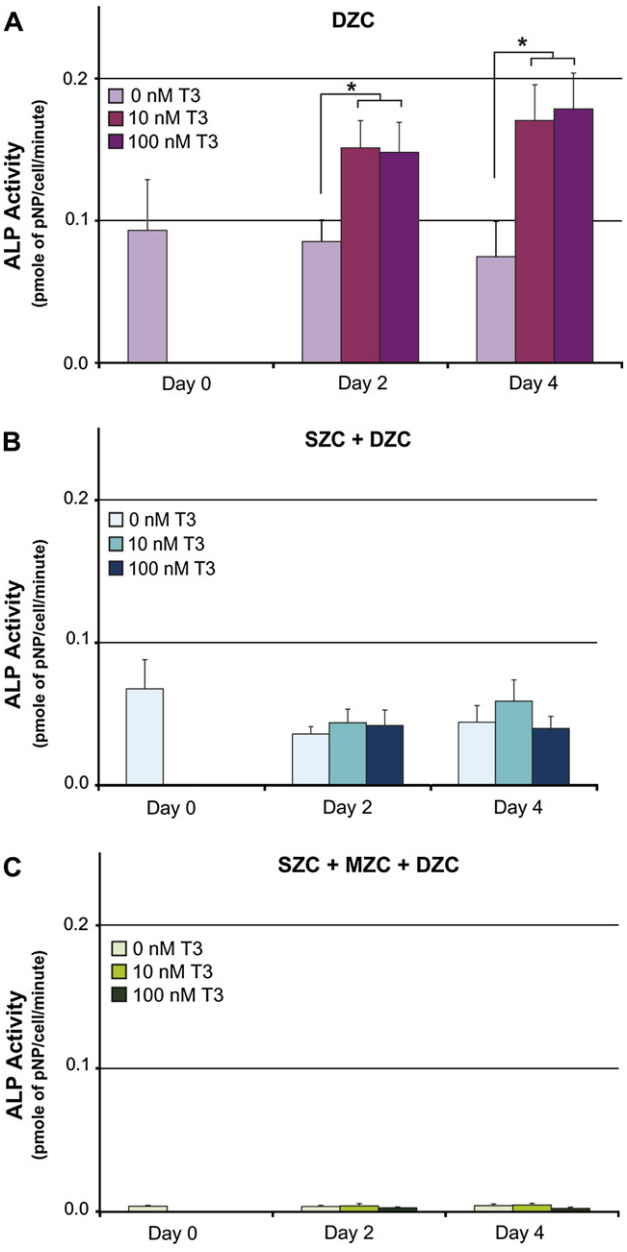

Fig. 1. Effects of T3 treatment on the ALP activities of single- and co-cultured chondrocytes derived from different cartilage zones ($n = 6$, *:$P < 0.05$). Minimal ALP activity was measured in the SZC and MZC groups (not shown), while ALP activity for DZC (A) exhibited a significant increase when stimulated with T3. In contrast, co-culture of DZC with SZC (B) suppressed this increase in ALP activity, and a similar response was found in the FC which consisted of chondrocytes derived from full thickness articular cartilage.

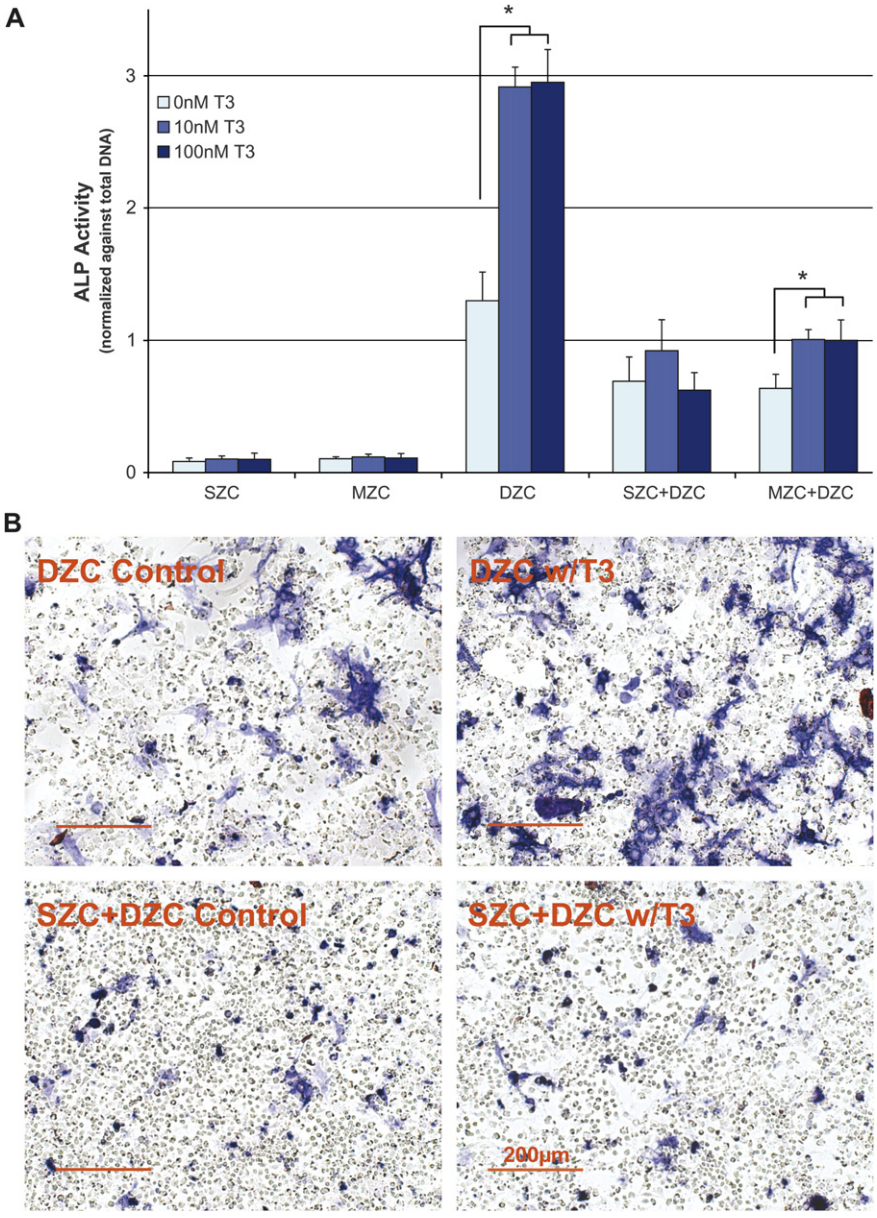

Fig. 2. Effects of cellular interactions on the ALP activity of T3-stimulated chondrocyte cultures (day 4, $n = 6$, *: $P < 0.05$). (A) T3 stimulation of DZC led to a significant increase in ALP activity over the DZC control. The co-culture of DZC with MZC also measured a significant increase in ALP activity after stimulation with T3. In contrast, co-culture of DZC with SZC under T3 stimulation did not result in a significant increase in ALP activity when compared to the un-stimulated control. (B) Histological staining corroborated the quantitative findings, as DZC stimulated with T3 (10 nM) exhibited higher staining intensity for ALP when compared to DZC control, while no difference was observed between the stimulated and un-stimulated co-cultured groups. (Fast Blue stain, day 4).

EFFECT OF CO-CULTURE ON CHONDROCYTE MINERALIZATION POTENTIAL

When DZCs were co-cultured with SZCs, the stimulatory effect of T3 on ALP activity was inhibited; no significant increase in ALP was measured in the SZC + DZC group over time [Fig. 1(B)]. A similar response was found in the FCs [Fig. 1(C)]. As shown in Fig. 2, in the two co-cultured groups (MZC + DZC and SZC + DZC), T3 stimulation of MZC + DZC resulted in significant increases in ALP activity as compared to the MZC + DZC control. On the other hand, co-culture of DZC with SZC led to no significant increase in ALP activity when compared to the un-stimulated control at day 4. Histological staining [Fig. 2(B)] confirmed these quantitative findings.

Calcium deposition increased in the chondrocyte cultures after the addition of mineralization media at day 4 in all groups over time (Fig. 3). Stimulation of DZC with 100 nM T3 resulted in a significant increase in calcium deposition at day 7 compared to the control DZC that did not receive T3 ($P < 0.05$). This increase in calcium deposition was not present after day 7, likely due to the termination of T3 stimulation at day 4. When DZCs were co-cultured with SZCs, no significant increase was found in calcium deposition regardless of T3 stimulation at any time. Similarly, the

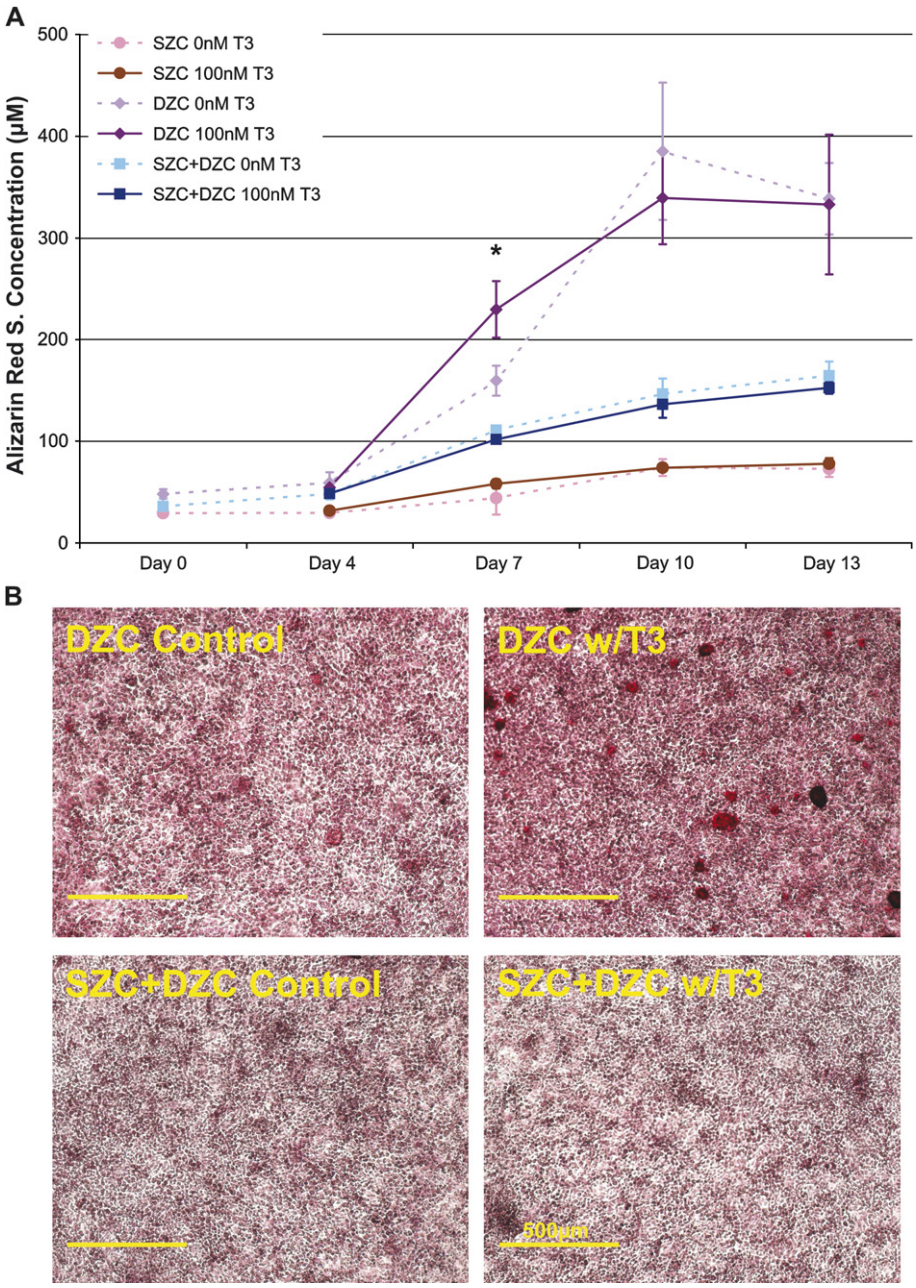

Fig. 3. Effects of cellular interactions on mineralization ($n = 5$, *:$P < 0.05$). (A) Calcium deposition in the DZC cultures stimulated with T3 increased significantly at day 7 compared to DZC control, while no such increase was found in co-culture regardless of T3 stimulation. (B) Histological staining for mineralization corroborated the quantitative findings (Alizarin Red-S, day 8).

SZC-only, MZC-only and FC groups accumulated minimal mineral and T3 did not promote calcium deposition in these groups over time. These quantitative results were confirmed by Alizarin Red-S staining [Fig. 3(B)], where DZC stimulated with 100 nM T3 exhibited higher staining intensity compared to DZC control at day 7.

Expression of marker genes characteristic of hypertrophic chondrocytes, including ALP, type X collagen and MMP13, was determined following T3 stimulation. As expected, both the DZC-only and DZC + MZC co-cultured group showed an increase in gene expression for these hypertrophic markers with T3 stimulation (data not shown). The expression for ALP at day 4 increased significantly for the DZC-only and DZC + MZC groups, while no increase in ALP was detected in the SZC + DZC group.

MECHANISM OF CELLULAR INTERACTIONS: PARACRINE EFFECTS

As shown in Fig. 4, when conditioned media from the DZC group were added to DZC cultures (DZC → DZC), an increase in ALP activity was measured, and this increase was significantly higher at 10 nM T3 when compared to the untreated DZC control ($P < 0.05$). In contrast, the

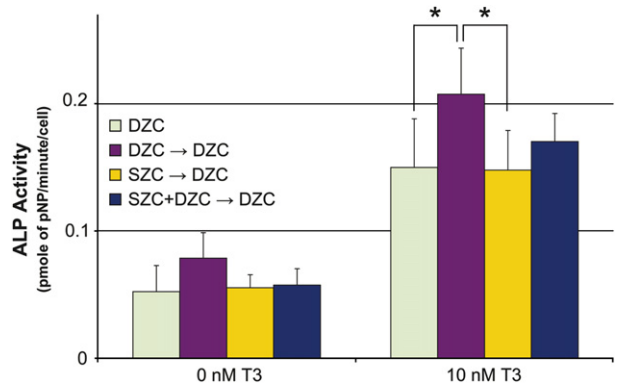

Fig. 4. Effects of conditioned media treatment on the ALP activity of DZC cultures (day 4, $n = 6$, *:$P < 0.05$). Addition of the DZC-conditioned media resulted in a significant increase in DZC ALP activity, while no such increase was found in DZC cultures treated with conditioned media from either the co-cultured (SZC + DZC) or SZC-only groups. (→ indicate addition of conditioned media).

level of ALP activity in DZC cultures treated with SZC-conditioned media (SZC → DZC) was comparable to the un-stimulated controls. A similar response was observed when the DZC group was stimulated with conditioned media from the co-cultured group (SZC + DZC → DZC).

MECHANISM OF CELLULAR INTERACTIONS: ROLE OF PTHrP

Since chondrocyte mineralization potential was suppressed in the SZC + DZC co-cultured group, gene expression for PTHrP, a known regulator of chondrocyte hypertrophy, was determined. As shown in Fig. 5, PTHrP gene expression increased significantly when DZCs were co-cultured with SZCs. In contrast, the co-culture of DZC with MZC did not lead to increased PTHrP gene expression at day 4.

To further assess the role of PTHrP in mediating SZC and DZC interactions, DZC cultures stimulated with T3 were treated with exogenous PTHrP. As shown in Fig. 6, when the DZC culture was treated with PTHrP (50 nM), the previously observed increase in ALP activity following T3 stimulation was inhibited [Fig. 6(A)]. These findings were corroborated by ALP staining results [Fig. 6(B)]. Treatment with PTHrP also modulated the effect of T3 stimulation on the expression of markers of chondrocyte hypertrophy (*Ihh*, ALP, Type X collagen) in DZC cultures at day 4 (Fig. 7, $P < 0.05$). Addition of PTHrP also inhibited the increase in ALP expression level for DZC cultures stimulated with 10 and 100 nM T3. Moreover, type X collagen expression was significantly lower after treatment with PTHrP at 10 nM T3, while PTHrP had no significant effect on MMP13 expression level. Since PTHrP regulates chondrocyte maturation through the PTHrP−*Ihh* negative feedback loop[52,55,68], gene expression for *Ihh* was also determined in DZC cultures stimulated with T3. A dose-dependent increase in *Ihh* expression was measured for DZC cultures stimulated by 10 and 100 nM of T3. This increase was, however, inhibited when the DZC cultures were treated with 50 nM of PTHrP ($P < 0.05$, Fig. 7).

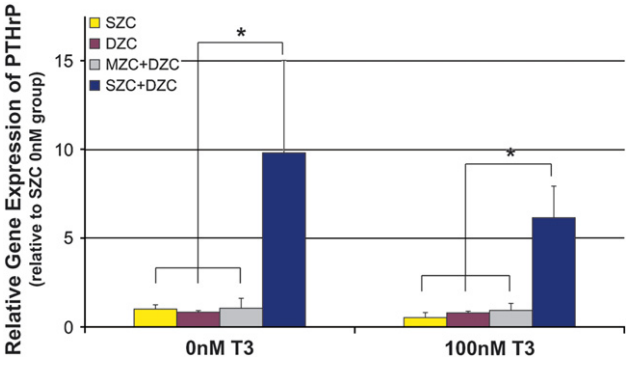

Fig. 5. Effects of cellular interactions on the relative gene expression of PTHrP (day 4, $n = 3$, *:$P < 0.05$). Gene expression for PTHrP increased significantly when DZC was co-cultured with SZC. The co-culture of DZC with MZC did not increase PTHrP gene expression.

To further test the role of PTHrP in the interaction between SZC and DZC, a known PTHrP antagonist, PTH (7−34) was added to SZC + DZC co-culture concomitant with T3 stimulation. The PTHrP antagonist had no effect on cell number and no cell toxicity was observed in the cultures at all concentrations of PTH (0−1000 nM). PTHrP antagonist was able to block the inhibitory effect of SZC + DZC co-culture on DZC mineralization potential (Fig. 8). At 1000 nM PTH (7−34), a significant increase in ALP activity was detected in SZC + DZC co-culture stimulated with T3. These findings were corroborated by ALP staining (data not shown).

3-D CO-CULTURE MODEL

As shown in Fig. 9, when SZCs and DZCs were co-cultured in the 3-D model, a significant decrease in DZC ALP activity was measured when compared with the DZC only control at day 7. Measured ALP activity decreased thereafter for all groups. Since ALP is an early indicator for cartilage calcification and acts as an enzyme to facilitate the hydrolyzation of organic phosphate, its activity is expected decrease after the onset of mineralization[69−71].

Discussion

The objectives of this study were to determine the role of interactions between zonal populations of articular chondrocytes in post-natal regulation of mineralization and to elucidate the mechanisms governing these cellular interactions. To this end, we established direct co-culture models of cells derived from the three zones of articular cartilage (surface, middle, and deep), and found that cellular interactions, especially those between chondrocytes derived from the surface and deep zones, are critical for the inhibition of deep zone mineralization. Moreover, the regulation of chondrocyte mineralization is mediated by local paracrine factors such as PTHrP, most likely through the PTHrP−*Ihh* negative feedback loop reminiscent of endochondral ossification.

Stimulation with thyroid hormone significantly increased ALP activity in the DZCs and also during their co-culture with MZCs. In contrast, little change in ALP activity was found when DZCs co-cultured with SZCs were stimulated with T3. Gene expression for PTHrP was significantly higher in the co-culture of SZCs and DZCs, suggesting that PTHrP may be a key modulator of the observed interactions between these two cell populations. Moreover, exogenous PTHrP inhibited increases in DZC ALP activity, while decreasing both type X collagen and *Ihh* expression

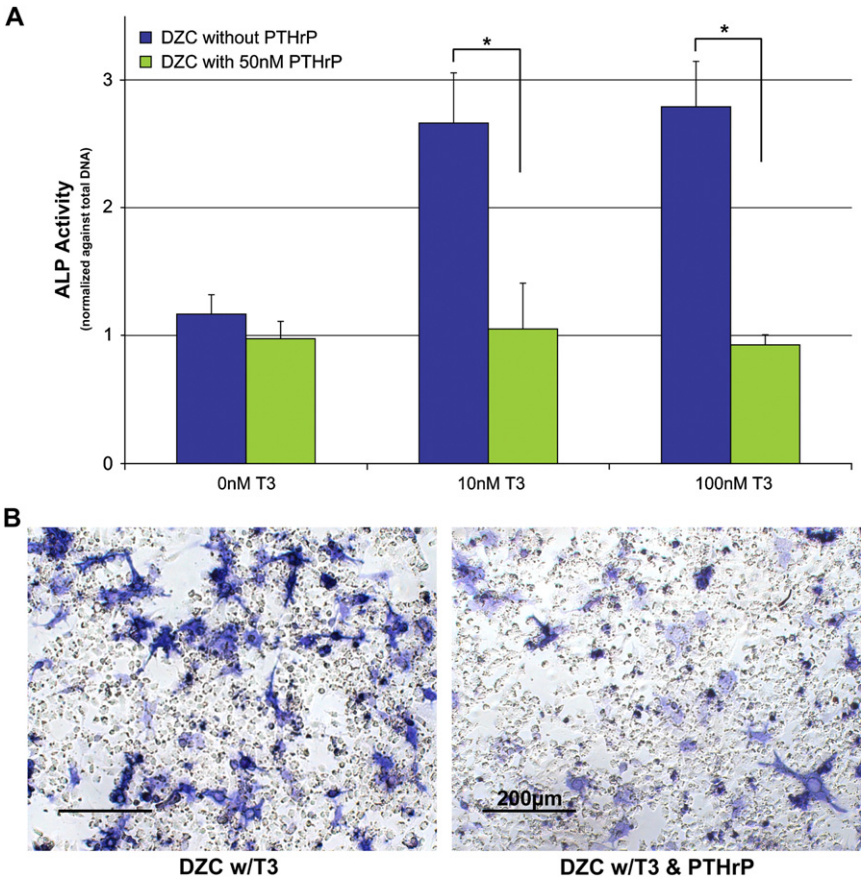

Fig. 6. Effects of PTHrP on the ALP activity of T3-stimulated DZC cultures (day 4, $n = 4$, *:$P < 0.05$). (A) All samples treated with PTHrP measured a similar ALP activity level as the non-stimulated DZC control. (B) Fast Blue staining confirmed that treating DZC cultures with PTHrP (50 nM) prevented the increase in ALP activity normally associated with T3 stimulation (10 nM, day 4). This regulatory effect of PTHrP is similar to that found when DZC is co-cultured with SZC.

in these cells. Further evidence supporting the role of PTHrP in mediating SZC and DZC interactions was observed in the blocking of PTHrP in the SZC + DZC co-culture group, which effectively reduced the inhibitory effect of SZC on DZC mineralization potential under T3 stimulation. The potential of PTHrP to regulate growth plate chondrocyte hypertrophy via the PTHrP–*Ihh* negative feedback loop is well established[52–56], as the presence of PTHrP promotes chondrocyte proliferation and suppresses the maturation of pre-hypertrophic chondrocytes, thereby preventing further mineralization. The findings of this study suggest that a similar mechanism may be implicated in the regulation of articular chondrocyte mineralization.

One limitation of the 2-D model is the dilution effect present in co-culture, as only half of the cell type is present when compared to single cultures. Due to this dilution effect the results should only be compared within the co-culture groups, and not between single cultured and co-cultured groups. For example, DZC stimulated with T3 measured over 100% increase in ALP activity when compared to the DZC control [Fig. 2(A)], while T3 stimulation of MZC + DZC resulted in only a 50% increase over the MZC + DZC control [Fig. 2(A)]. The less pronounced change seen in ALP activity for the MZC + DZC group is likely due to a dilution effect. By the same token, a similar dilution effect is expected when SZC is co-cultured with DZC. Surprisingly, no significant increase was found in SZC + DZC group stimulated with T3, suggesting that the observed suppression of ALP activity is likely due to SZC + DZC interactions. In addition, when comparing the two co-culture groups (SZC + DZC and MZC + DZC), SZC + DZC group measured a significantly lower ALP activity when compared to the MZC + DZC group stimulated with 100 nM T3 [Fig. 2(A)]. This conclusion is further strengthened by the results of our 3-D model (Fig. 8), in which any dilution effect is eliminated by directly evaluating the ALP activity of DZC in co-culture. A similar suppression of the ALP activity was found in 2-D and 3-D co-cultures, thus confirming that these zonal chondrocyte interactions are physiologically relevant and not artifacts of the 2-D culturing system. The inhibitory effect in 2-D co-culture however, occurred sooner and at higher magnitudes, as direct co-culture circumvents any potential diffusion limitations associated with the static 3-D system. Future studies will focus on utilizing the 3-D model to elucidate the specific roles of cell sub-populations in the regulation of articular chondrocyte mineralization.

During development, PTHrP is secreted by peri-articular chondrocytes of the epiphyseal growth plate[52,55,58,72]; recent studies have shown that post-natal PTHrP expression is localized toward the articular surface[62,73]. It is thus likely that in our co-culture model, the SZCs are responsible for secreting the PTHrP that led to the suppression of deep zone mineralization. However, the PTHrP expression and conditioned media results point to a more complex

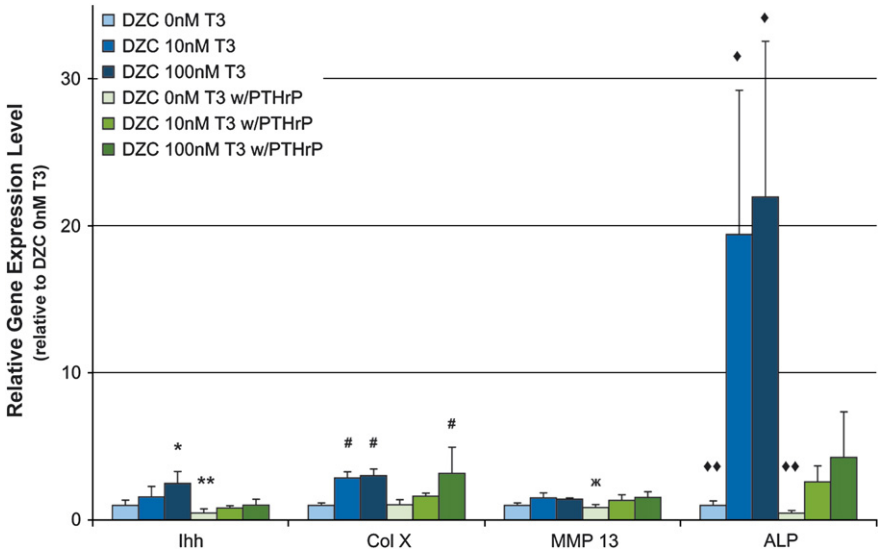

Fig. 7. Effects of PTHrP treatment on the relative gene expression of chondrocyte hypertrophy markers (day 4, $P < 0.05$, $n = 6$). Treatment with PTHrP suppressed the gene expression of *Ihh*, type X collagen, MMP13 and ALP. All gene expressions were normalized to expression of the housekeeping gene β-actin. *: Significantly higher than 0 nM T3, 0 nM T3 w/PTHrP, 10 nM T3 w/PTHrP, and 100 nM T3 w/PTHrP. **: Significantly lower than 0 nM T3, 10 nM T3, 100 nM T3, and 100 nM T3 w/PTHrP. #: Significantly higher than 0 nM T3, 0 nM T3 w/PTHrP and 10 nM T3 w/PTHrP. ж: Significantly lower than 10 nM T3, 100 nM T3, 10 nM T3 w/PTHrP, and 100 nM T3 w/PTHrP. ◆: Significantly higher than 0 nM T3, 0 nM T3 w/PTHrP, 10 nM w/PTHrP, and 100 nM w/PTHrP. ◆◆: Significantly lower than 10 nM T3, 100 nM T3, 10 nM T3 w/PTHrP, and 100 nM T3 w/PTHrP.

interaction. SZC-only cultures expressed relatively low levels of PTHrP and accordingly, conditioned media from these cells had no effect on DZC ALP activity. The up-regulation of PTHrP was only measured in co-culture, which suggests that cellular interaction is a pre-requisite for PTHrP secretion; this is consistent with the active PTHrP−*Ihh* feedback loop present in endochondral bone formation.

The DZC autocrine interactions, which led to an increase in DZC ALP activity after treatment with DZC-conditioned media, may be directed by a similar mechanism as reported for pre-hypertrophic chondrocytes, which secrete *Ihh* to promote hypertrophy in adjacent chondrocytes independent of PTHrP during endochondral ossification[57–59]. Based on our results, the conditioned media from SZC and DZC co-culture were expected to inhibit the ALP activity of DZCs. However, no significant decrease in ALP activity was measured following treatment with the co-cultured media. There are several possible explanations. The 2-D co-culture model permits autocrine and paracrine interactions, as well as physical contact between chondrocyte sub-populations. Preliminary results from our 3-D co-culture model suggest that the co-culture interactions are independent of cell-to-cell contact, as a similar suppressive effect on ALP activity was found in the segregated co-culture of SZCs and DZCs. Therefore, considering the dilution effect of

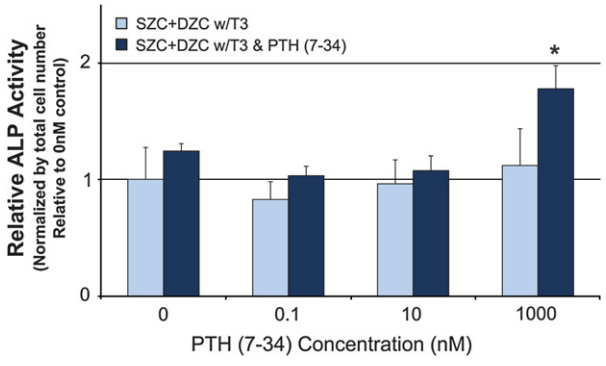

Fig. 8. Effects of PTHrP antagonist on the ALP activity of T3-stimulated SZC + DZC co-culture (day 4, $n = 6$, *: $P < 0.05$). In the presence of T3 (50 nM), blocking of PTHrP with PTH(7-34) in SZC + DZC co-culture inhibited the suppressive effect of SZC on DZC mineralization potential. Samples treated with 1000 nM of PTHrP antagonist and 50 nM T3 measured significantly higher ALP activity compared to all other groups.

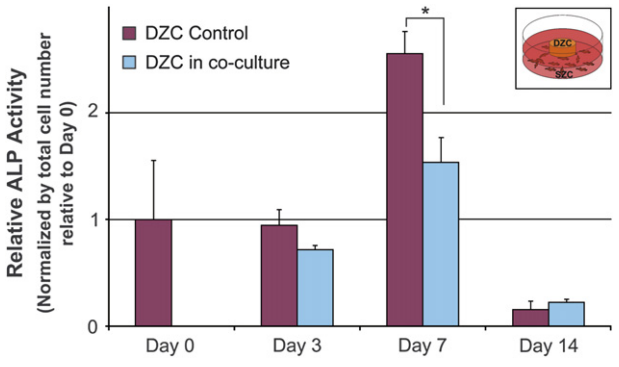

Fig. 9. Effects of cellular interactions on DZC ALP activity in a 3-D co-culture model ($n = 6$,*: $P < 0.05$). In the 3-D model, DZCs were first encapsulated in a hydrogel and then co-cultured with a monolayer of SZCs. An acellular hydrogel disc separated the DZC disc from the SZC monolayer. As in the 2-D co-culture models, SZC−DZC co-culture decreased the ALP activity of DZC embedded in the hydrogel. A significant decrease in DZC ALP activity was found in co-culture when compared to the DZC-only group at day 7.

co-culture (e.g., lower seeding density per cell type) coupled with limitations associated with conditioned media studies (e.g., nutrient depletion vs dilution effects), our results suggest that the amount of PTHrP present in the conditioned media is not sufficient to suppress the ALP activity of the treated cultures. Yoshida *et al.*[54] reported that a threshold PTHrP concentration is required for the suppression of growth plate chondrocyte hypertrophy. The conditioned media used here originated from day 4 co-cultures which are presently associated with a significant increase in PTHrP expression. Thus media from a later time point would likely be more potent. Furthermore, as an *active* negative PTHrP−*Ihh* feedback loop is necessary for regulating chondrocyte mineralization, conditioned media study is inherently limited in its inability to fully mimic the *in situ* cellular communication occurring in co-culture.

Articular chondrocytes isolated from the deep, middle and surface zones exhibit distinct morphology and phenotypes[5,6,8,21,27]. It is thus not surprising that communication exists between cells derived from surface and deep zones of cartilage, since cellular interactions are essential for structural organization and maintenance of the controlled matrix heterogeneity inherent in complex tissues and organs. The results of this study provide a functional rationale for the structural organization of articular cartilage into these three distinct zones and suggest an important role for PTHrP in SZC and DZC interactions. Molecular transport increases from the articular surface to cartilage proper under dynamic loading[46,47], and unloaded joints exhibit an accelerated tidemark advancement[49]. Thus regulatory molecules such as PTHrP secreted by the SZCs are most likely transported to DZCs during joint articulation and physiological loading.

Our results suggest that in immature animals, the increased mineralization potential of DZCs induced by systemic factors such as thyroid hormone is regulated by surface zone through the PTHrP−*Ihh* feedback loop. A major difference between adult and immature articular cartilage resides in the characteristics of the calcified cartilage region. Published studies have reported that the calcified cartilage region is avascular, relatively impermeable, and acts as a barrier against subchondral bone in adult animals[16,74–77], while substantial vasculature that facilitates molecular transport has been measured across the calcified cartilage region in immature animals[78]. The mechanism regulating the maintenance of the osteochondral interface in adult cartilage is not known. Based on the findings of this study, we propose that with age, the continued depletion of chondrocytes at the surface zone during articulation attenuates their regulation of DZCs. This decrease in overall secretion of regulatory molecules (e.g., PTHrP and others) permits DZC mineralization and advancement of the calcification front into the non-mineralized cartilage region. A resident population of progenitor cells have been identified at the articular surface, periosteum as well as in the synovium[79,80], thus these cells may replenish the population of SZCs. The regulation of DZC mineralization is reactivated once a sufficient number of SZCs has been regenerated. This newly formed calcified matrix can then serve as the neo-osteochondral interface, separating the remaining articular cartilage from the underlying calcified cartilage and bone. Given the expected differences in physiology between articular cartilage in mature and immature animals, future studies are needed to confirm this hypothesis.

Interpretation of our cellular interaction findings may also be relevant for understanding the pathology of OA. It is possible that the loss of SZCs following microtrauma associated with OA may lead to tidemark duplication and the apparent advancement of the calcification zone through DZC-mediated mineralization. Moreover, damage to the subchondral plate in OA could re-expose the DZCs to systemic factors such as T3. In this case, if coupled with wear of the cartilage surface or depletion of SZCs, unchecked mineralization by DZCs can occur. High concentration of thyroid hormone has been shown to induce ectopic mineralization in adult chondrocytes isolated from full thickness articular cartilage[50]. However, it has also been reported that PTHrP level is elevated in both osteoarthritic and rheumatic joints[81,82]. It is likely that in OA, the PTHrP−*Ihh* negative feedback look may be disrupted or compensatory mechanisms are responsible for the maintenance of structural organization in articular cartilage. Recent studies exploring osteoblasts and chondrocytes' co-culture[83,84] have suggested that heterotypic cellular communications may also be relevant for regulating articular chondrocyte mineralization. The mechanisms of these cellular interactions will be explored in future studies.

The findings of this study are also significant in the context of current efforts in functional cartilage tissue regeneration. Engineered cartilage constructs incorporating a biomimetic zonal distribution of articular chondrocytes have been reported[85–87] and our findings provide additional rationale for such sophisticated mimicry. Furthermore, regeneration of the osteochondral interface on cartilage grafts could be critical for long-term graft stability and functional matrix organization post-implantation[88,89]. Our results provide initial evidence that zonal chondrocyte cellular communication plays an important role in the regulation and maintenance of the calcified cartilage zone in articular cartilage, and these insights can be utilized for the regeneration of a stable and functional osteochondral interface on tissue-engineered cartilage grafts.

Conclusions

Cellular communication between zonal sub-populations of articular chondrocytes regulates chondrocyte mineralization potential. Specifically, chondrocytes residing at the articular surface actively suppress mineralization by chondrocytes in the deep layer of articular cartilage. Our results suggest that this suppression is regulated, at least in part, through PTHrP. The findings of this study represent the first reported investigation of the role of zonal cellular interactions in articular chondrocyte mineralization and provide a novel rationale for the zonal organization of articular cartilage. These findings also present a probable mechanism for the post-natal regulation of articular cartilage matrix organization and have implications for OA as well as functional cartilage repair.

Acknowledgments

The authors would like to thank Christina Cheng for technical assistance with real-time PCR analysis. This study was funded by start-up funds from Columbia University (HHL) and National Institutes of Health (HHL). Dr Chisa Hidaka was supported by grants from the National Institutes of Health and the Arthritis Foundation.

References

1. Lethbridge-Cejku M, Schiller JS, Bernadel L. Summary health statistics for U.S. adults: National Health

Interview Survey, 2002. Vital Health Stat 10 2004;222: 1–151.
2. Minns RJ, Steven FS. The collagen fibril organization in human articular cartilage. J Anat 1977;123:437–57.
3. Freeman MAR. Adult Articular Cartilage. Tunbridge Wells (England): Pitman Medical 1979.
4. Aydelotte MB, Kuettner KE. Differences between subpopulations of cultured bovine articular chondrocytes. I. Morphology and cartilage matrix production. Connect Tissue Res 1988;18:205–22.
5. Wong M, Wuethrich P, Eggli P, Hunziker E. Zone-specific cell biosynthetic activity in mature bovine articular cartilage: a new method using confocal microscopic stereology and quantitative autoradiography. J Orthop Res 1996;14:424–32.
6. Sun Y, Kandel R. Deep zone articular chondrocytes *in vitro* express genes that show specific changes with mineralization. J Bone Miner Res 1999;14:1916–25.
7. Jadin KD, Wong BL, Bae WC, Li KW, Williamson AK, Schumacher BL, *et al*. Depth-varying density and organization of chondrocytes in immature and mature bovine articular cartilage assessed by 3d imaging and analysis. J Histochem Cytochem 2005;53: 1109–19.
8. Hidaka C, Cheng C, Alexandre D, Bhargava M, Torzilli PA. Maturational differences in superficial and deep zone articular chondrocytes. Cell Tissue Res 2006;323:127–35.
9. Fawns HT, Landells JW. Histochemical studies of rheumatic conditions. I. Observations on the fine structures of the matrix of normal bone and cartilage. Ann Rheum Dis 1953;12:105–13.
10. Bullough PG, Jagannath A. The morphology of the calcification front in articular cartilage. Its significance in joint function. J Bone Joint Surg Br 1983;65:72–8.
11. Havelka S, Horn V, Spohrova D, Valouch P. The calcified-non-calcified cartilage interface: the tidemark. Acta Biol Hung 1984;35:271–9.
12. Oettmeier R, Abendroth K, Oettmeier S. Analyses of the tidemark on human femoral heads. I. Histochemical, ultrastructural and microanalytic characterization of the normal structure of the intercartilaginous junction. Acta Morphol Hung 1989;37:155–68.
13. Oegema TR Jr, Thompson RC Jr. Cartilage–bone interface (tidemark). In: Brandt K, Ed. Cartilage Changes in Osteoarthritis. Indianapolis (IN): Indiana School of Medicine Publ. 1990:43–52.
14. Lyons TJ, Stoddart RW, McClure SF, McClure J. The tidemark of the chondro-osseous junction of the normal human knee joint. J Mol Histol 2005;36:207–15.
15. Collins DH. The Pathology of Articular and Spinal Diseases. Baltimore (MD): William & Wilkins 1950.
16. Redler I, Mow VC, Zimny ML, Mansell J. The ultrastructure and biomechanical significance of the tidemark of articular cartilage. Clin Orthop Relat Res 1975;112:357–62.
17. Mow VC, Proctor CS, Kelly MA. Biomechanics of articular cartilage. In: Nordin M, Victor HF, Forssen K, Eds. Basic Biomechanics of the Musculoskeletal System. Philadelphia (PA): Lea and Febiger 1989:31–58.
18. Lemperg R. The subchondral bone plate of the femoral head in adult rabbits. I. Spontaneous remodelling studied by microradiography and tetracycline labelling. Virchows Arch A Pathol Pathol Anat 1971;352:1–13.
19. Haynes DW. The mineralization front of articular cartilage. Metab Bone Dis Relat Res 1980;2(Suppl):55–9.
20. Lane LB, Bullough PG. Age-related changes in the thickness of the calcified zone and the number of tidemarks in adult human articular cartilage. J Bone Joint Surg Br 1980;62:372–5.
21. Oegema TR Jr, Carpenter RJ, Hofmeister F, Thompson RC Jr. The interaction of the zone of calcified cartilage and subchondral bone in osteoarthritis. Microsc Res Tech 1997;37:324–32.
22. Bullough PG. The geometry of diarthrodial joints, its physiologic maintenance, and the possible significance of age-related changes in geometry-to-load distribution and the development of osteoarthritis. Clin Orthop Relat Res 1981:61–6.
23. Oettmeier R, Abendroth K, Oettmeier S. Analyses of the tidemark on human femoral heads. II. Tidemark changes in osteoarthrosis—a histological and histomorphometric study in non-decalcified preparations. Acta Morphol Hung 1989;37:169–80.
24. Oegema TR Jr, Thompson RC Jr. The zone of calcified cartilage. Its role in osteoarthritis. In: Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC, Eds. Articular Cartilage and Osteoarthritis. New York (NY): Raven Press 1992:319–31.
25. Oegema TR Jr, Johnson SL, Meglitsch T, Carpenter RJ. Prostaglandins and the zone of calcified cartilage in osteoarthritis. Am J Ther 1996;3: 139–49.
26. Burr DB. Anatomy and physiology of the mineralized tissues: role in the pathogenesis of osteoarthrosis. Osteoarthritis Cartilage 2004;12(Suppl A):S20–30.
27. Bullough PG. The role of joint architecture in the etiology of arthritis. Osteoarthritis Cartilage 2004;12 (Suppl A):S2–9.
28. Radin EL, Burr DB, Caterson B, Fyhrie D, Brown TD, Boyd RD. Mechanical determinants of osteoarthrosis. Semin Arthritis Rheum 1991;21:12–21.
29. Hunziker EB. Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. Microsc Res Tech 1994;28:505–19.
30. Iannotti JP, Goldstein S, Kuhn J, Lipiello L, Kaplan FS, Zaleske DJ. The formation and growth of skeletal tissue. In: Buckwalter JA, Einhorn TA, Simon SR, Eds. Orthopaedic Basic Science: Biology and Biomechanics of the Musculoskeletal System. Rosemont (IL): American Academy of Orthopaedic Surgeons 2000: 77–110.
31. Kronenberg HM. Developmental regulation of the growth plate. Nature 2003;423:332–6.
32. Pacifici M, Koyama E, Iwamoto M, Gentili C. Development of articular cartilage: what do we know about it and how may it occur? Connect Tissue Res 2000; 41:175–84.
33. Tuan RS. Biology of developmental and regenerative skeletogenesis. Clin Orthop Relat Res 2004: S105–17.
34. Grant WT, Wang GJ, Balian G. Type X collagen synthesis during endochondral ossification in fracture repair. J Biol Chem 1987;262:9844–9.
35. Einhorn TA. The cell and molecular biology of fracture healing. Clin Orthop Relat Res 1998:S7–S21.
36. Barnes GL, Kostenuik PJ, Gerstenfeld LC, Einhorn TA. Growth factor regulation of fracture repair. J Bone Miner Res 1999;14:1805–15.
37. Boskey AL, Bullough PG. Cartilage calcification: normal and aberrant. Scan Electron Microsc 1984; 943–52.
38. Clark JM, Norman A, Notzli H. Postnatal development of the collagen matrix in rabbit tibial plateau articular cartilage. J Anat 1997;191(Pt 2):215–21.

39. Archer CW, Morrison H, Pitsillides AA. Cellular aspects of the development of diarthrodial joints and articular cartilage. J Anat 1994;184(Pt 3):447–56.
40. Williams GR, Robson H, Shalet SM. Thyroid hormone actions on cartilage and bone: interactions with other hormones at the epiphyseal plate and effects on linear growth. J Endocrinol 1998;157:391–403.
41. Alini M, Kofsky Y, Wu W, Pidoux I, Poole AR. In serum-free culture thyroid hormones can induce full expression of chondrocyte hypertrophy leading to matrix calcification. J Bone Miner Res 1996;11:105–13.
42. Hayes AJ, MacPherson S, Morrison H, Dowthwaite G, Archer CW. The development of articular cartilage: evidence for an appositional growth mechanism. Anat Embryol (Berl) 2001;203:469–79.
43. Carter DR, Wong M. Mechanical stresses in joint morphogenesis and maintenance. In: Mow VC, Ratcliffe A, Woo SL-Y, Eds. Biomechanics of Diarthrodial Joints. New York (NY): Springer-Verlag 1990: 155–74.
44. Kuettner KE, Schleyerbach R, Hascall VC. Articular Cartilage Biochemistry (Workshop Conference Hoechst-Werk Albert, 1985: Wiesbaden, Germany). New York (NY): Raven Press 1986.
45. Burr DB, Radin EL. Microfractures and microcracks in subchondral bone: are they relevant to osteoarthrosis? Rheum Dis Clin North Am 2003;29:675–85.
46. Maroudas A, Bullough P, Swanson SA, Freeman MA. The permeability of articular cartilage. J Bone Joint Surg Br 1968;50:166–77.
47. Maroudas A. Transport through articular cartilage and some physiological implications. In: Ali SY, Elves MW, Leaback DH, Eds. Normal and Osteoarthrotic Articular Cartilage. London (Britain): Institute of Orthopaedics 1974:492–500.
48. Palmoski MJ, Colyer RA, Brandt KD. Joint motion in the absence of normal loading does not maintain normal articular cartilage. Arthritis Rheum 1980;23: 325–34.
49. O'Connor KM. Unweighting accelerates tidemark advancement in articular cartilage at the knee joint of rats. J Bone Miner Res 1997;12:580–9.
50. Rosenthal AK, Henry LA. Thyroid hormones induce features of the hypertrophic phenotype and stimulate correlates of CPPD crystal formation in articular chondrocytes. J Rheumatol 1999;26:395–401.
51. Hunziker EB, Driesang IM. Functional barrier principle for growth-factor-based articular cartilage repair. Osteoarthritis Cartilage 2003;11:320–7.
52. Vortkamp A, Lee K, Lanske B, Segre GV, Kronenberg HM, Tabin CJ. Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 1996;273:613–22.
53. Karp SJ, Schipani E, St-Jacques B, Hunzelman J, Kronenberg H, McMahon AP. Indian hedgehog coordinates endochondral bone growth and morphogenesis via parathyroid hormone related-protein-dependent and -independent pathways. Development 2000;127: 543–8.
54. Yoshida E, Noshiro M, Kawamoto T, Tsutsumi S, Kuruta Y, Kato Y. Direct inhibition of Indian hedgehog expression by parathyroid hormone (PTH)/PTH-related peptide and up-regulation by retinoic acid in growth plate chondrocyte cultures. Exp Cell Res 2001;265:64–72.
55. Kobayashi T, Chung UI, Schipani E, Starbuck M, Karsenty G, Katagiri T, *et al*. PTHrP and Indian hedgehog control differentiation of growth plate chondrocytes at multiple steps. Development 2002;129: 2977–86.
56. Kronenberg HM. PTHrP and skeletal development. Ann N Y Acad Sci 2006;1068:1–13.
57. Stott NS, Chuong CM. Dual action of sonic hedgehog on chondrocyte hypertrophy: retrovirus mediated ectopic sonic hedgehog expression in limb bud micromass culture induces novel cartilage nodules that are positive for alkaline phosphatase and type X collagen. J Cell Sci 1997;110:2691–701.
58. St-Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev 1999;13:2072–86.
59. Karaplis AC. PTHrP: novel roles in skeletal biology. Curr Pharm Des 2001;7:655–70.
60. Kartsogiannis V, Moseley J, McKelvie B, Chou ST, Hards DK, Ng KW, *et al*. Temporal expression of PTHrP during endochondral bone formation in mouse and intra-membranous bone formation in an *in vivo* rabbit model. Bone 1997;21:385–92.
61. Lee K, Deeds JD, Segre GV. Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. Endocrinology 1995;136:453–63.
62. Tsukazaki T, Ohtsuru A, Enomoto H, Yano H, Motomura K, Ito M, *et al*. Expression of parathyroid hormone-related protein in rat articular cartilage. Calcif Tissue Int 1995;57:196–200.
63. Eggli PS, Hunziker EB, Schenk RK. Quantitation of structural features characterizing weight- and less-weight-bearing regions in articular cartilage: a stereological analysis of medial femoral condyles in young adult rabbits. Anat Rec 1988;222:217–27.
64. Horiuchi N, Holick MF, Potts JT Jr, Rosenblatt M. A parathyroid hormone inhibitor *in vivo*: design and biological evaluation of a hormone analog. Science 1983;220:1053–5.
65. Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem 1988;174:168–76.
66. Teixeira CC, Hatori M, Leboy PS, Pacifici M, Shapiro IM. A rapid and ultrasensitive method for measurement of DNA, calcium and protein content, and alkaline phosphatase activity of chondrocyte cultures. Calcif Tissue Int 1995;56:252–6.
67. Puchtler H, Meloan SN, Terry MS. On the history and mechanism of alizarin and alizarin red-S stains for calcium. J Histochem Cytochem 1969;17:110–24.
68. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, *et al*. PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. Science 1996;273:663–6.
69. Kato Y, Iwamoto M, Koike T, Suzuki F, Takano Y. Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: regulation by transforming growth factor beta and serum factors. Proc Natl Acad Sci U S A 1988;85:9552–6.
70. Tacchetti C, Quarto R, Campanile G, Cancedda R. Calcification of *in vitro* developed hypertrophic cartilage. Dev Biol 1989;132:442–7.
71. Hunter GK, Holmyard DP, Pritzker KP. Calcification of chick vertebral chondrocytes grown in agarose gels: a biochemical and ultrastructural study. J Cell Sci 1993;104(Pt 4):1031–8.
72. Kobayashi T, Soegiarto DW, Yang Y, Lanske B, Schipani E, McMahon AP, *et al*. Indian hedgehog

stimulates periarticular chondrocyte differentiation to regulate growth plate length independently of PTHrP. J Clin Invest 2005;115:1734–42.
73. Chen X, Macica CM, Dreyer BE, Hammond VE, Hens JR, Philbrick WM, *et al*. Initial characterization of PTH-related protein gene-driven lacZ expression in the mouse. J Bone Miner Res 2006;21:113–23.
74. Trueta J, Harrison MH. The normal vascular anatomy of the femoral head in adult man. J Bone Joint Surg Br 1953;35-B:442–61.
75. Haynes DW, Woods CG. Nutritional pathways for adult human articular cartilage. Orthopedics 1975;8:1.
76. Lane LB, Villacin A, Bullough PG. The vascularity and remodelling of subchondrial bone and calcified cartilage in adult human femoral and humeral heads. An age- and stress-related phenomenon. J Bone Joint Surg Br 1977;59:272–8.
77. Clark JM. The structure of vascular channels in the subchondral plate. J Anat 1990;171:105–15.
78. Holmdahl DE, Ingelmark BE. The contact between the articular cartilage and the medullary cavities of the bone. Acta Orthop Scand 1950;20:156–65.
79. Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, Evans DJ, *et al*. The surface of articular cartilage contains a progenitor cell population. J Cell Sci 2004;117:889–97.
80. Iwata H, Ono S, Sato K, Sato T, Kawamura M. Bone morphogenetic protein-induced muscle- and synovium-derived cartilage differentiation *in vitro*. Clin Orthop 1993;295–300.
81. Terkeltaub R, Lotz M, Johnson K, Deng D, Hashimoto S, Goldring MB, *et al*. Parathyroid hormone-related proteins is abundant in osteoarthritic cartilage, and the parathyroid hormone-related protein 1–173 isoform is selectively induced by transforming growth factor beta in articular chondrocytes and suppresses generation of extracellular inorganic pyrophosphate. Arthritis Rheum 1998; 41:2152–64.
82. Gomez-Barrena E, Sanchez-Pernaute O, Largo R, Calvo E, Esbrit P, Herrero-Beaumont G. Sequential changes of parathyroid hormone related protein (PTHrP) in articular cartilage during progression of inflammatory and degenerative arthritis. Ann Rheum Dis 2004;63:917–22.
83. Jiang J, Nicoll SB, Lu HH. Co-culture of osteoblasts and chondrocytes modulates cellular differentiation *in vitro*. Biochem Biophys Res Commun 2005;338: 762–70.
84. Sanchez C, Deberg MA, Piccardi N, Msika P, Reginster JY, Henrotin YE. Subchondral bone osteoblasts induce phenotypic changes in human osteoarthritic chondrocytes. Osteoarthritis Cartilage 2005;13: 988–97.
85. Klein TJ, Schumacher BL, Schmidt TA, Li KW, Voegtline MS, Masuda K, *et al*. Tissue engineering of stratified articular cartilage from chondrocyte subpopulations. Osteoarthritis Cartilage 2003;11:595–602.
86. Waldman SD, Grynpas MD, Pilliar RM, Kandel RA. The use of specific chondrocyte populations to modulate the properties of tissue-engineered cartilage. J Orthop Res 2003;21:132–8.
87. Sharma B, Elisseeff JH. Engineering structurally organized cartilage and bone tissues. Ann Biomed Eng 2004;32:148–59.
88. Lu HH, Jiang J. Interface tissue engineering and the formulation of multiple-tissue systems. Adv Biochem Eng Biotechnol 2006;102:91–111.
89. Lu HH, Jiang J, Tang A, Hung CT, Guo XE. Development of controlled heterogeneity on a polymer-ceramic hydrogel scaffold for osteochondral repair. Key Eng Mater 2005;17:607–10.