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Harnessing the purinergic receptor pathway to develop functional engineered cartilage constructs

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SUMMARY

Objective: Mechanical stimulation is a widely used method to enhance the formation and properties of tissue-engineered cartilage. While this approach can be highly successful, it may be more efficient and effective to harness the known underlying mechanotransduction pathways responsible. With this aim, the purpose of this study was to assess the effect of directly stimulating the purinergic receptor pathway through exogenous adenosine 5′-triphosphate (ATP) in absence of externally applied forces.

Methods: Isolated bovine articular chondrocytes were seeded in high density, 3D culture and supplemented with varying doses of ATP for up to 4 weeks. The effects on biosynthesis, extracellular matrix accumulation and mechanical properties were then evaluated. Experiments were also conducted to assess whether exogenous ATP elicited any undesirable effects, such as: inflammatory mediator release, matrix turn-over and mineralization.

Results: Supplementation with ATP had a profound effect on the growth and maturation of the developed tissue. Exogenous ATP (62.5–250 μM) increased biosynthesis by 80–120%, and when stimulated for a period of 4 weeks resulted in increased matrix accumulation (80% increase in collagen and 60% increase in proteoglycans) and improved mechanical properties (6.5-fold increase in indentation modulus). While exogenous ATP did not stimulate the release of inflammatory mediators or induce mineralization, high doses of ATP (250 μM) elicited a 2-fold increase in matrix metalloproteinase-13 expression suggesting the emergence of a catabolic response.

Conclusions: Harnessing the ATP-purinergic receptor pathway is a highly effective approach to improve tissue formation and impart functional mechanical properties. However, the dose of ATP needs to be controlled as not to elicit a catabolic response.

Introduction

The formation of cartilaginous tissue *in vitro* is a promising alternative approach for the repair of damaged articular cartilage. However, it has been challenging to engineer articular cartilage that possesses similar properties to native tissue[1–4]. While engineered cartilaginous tissue constructs can accumulate substantial amounts of proteoglycans, the engineered tissues are typically deficient in collagen[2–4] and display inferior mechanical performance[1–5]. As the mechanical environment is involved in the development and maintenance of articular cartilage *in vivo*[6], much attention has focused on the use of mechanical stimuli as a means to upregulate matrix synthesis and to improve tissue properties[5,7–11]. Although this method has been highly successful in bench-scale investigations, there may be potential limitations to translate this approach to stimulate anatomically shaped constructs[12–14]. Direct mechanical stimulation of tissue constructs with irregular geometry and/or high radii of curvature may be problematic and limit the types of forces that can be effectively applied. However, by harnessing the known molecular pathways involved in the mechanotransduction cascade, it may be possible to elicit the same response in the absence of externally applied forces and overcome such limitations.

Recently, it has been demonstrated that chondrocytes release adenosine 5′-triphosphate (ATP) in response to mechanical loading which is then utilized as an autocrine and/or paracrine signal[15–17]. This pathway, termed the purinergic receptor pathway, is not limited to chondrocytes as other cells of the mesenchymal lineage[18,19] also utilize extracellular ATP as a signalling molecule

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during mechanotransduction. These cells express purinergic membrane receptors (P2 receptors) which are stimulated by extracellular nucleotides (e.g., ATP, uridine diphosphate – UDP)[15–19]. In response to mechanical loading, ATP is believed to be released from matrix vesicles[16] and/or connexin hemi-channels (e.g., connexion 43)[20] into the extracellular space where it can bind to the P2 receptor. While several different P2 membrane receptors have been identified on chondrocytes (e.g., $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$)[16] the predominant receptor implicated in ATP binding is the $P2Y_2$ receptor[15–17]. Following binding, there is an associated increase in intracellular calcium ($[Ca^{2+}]_i$) leading to the stimulation of extracellular matrix (ECM) gene expression and protein synthesis[16,17]. This pathway appears to be tightly regulated as extracellular ATP is not exclusively utilized as a mechano-transduction signalling molecule. High doses of ATP can also elicit undesirable effects, including stimulating the release of inflammatory mediators (e.g., nitric oxide (NO) and prostaglandin E_2 (PGE_2))[21,22] as well as initiating matrix turn-over[23,24] and mineralization[25]. Thus, in this study, we hypothesized that the direct stimulation of the purinergic receptor pathway by exogenous ATP would enhance cartilaginous matrix synthesis and improve tissue mechanical properties as a means to facilitate the development of functional engineered cartilage constructs.

Materials and methods

Cell isolation and high-density 3D culture

Tissue-engineered cartilage constructs were generated from isolated chondrocytes harvested from calf (12–18 months old) metacarpal–phalangeal articular cartilage by sequential enzymatic digestion, as described previously[4,26]. Tissue was obtained from several joints (up to 4 per experiment) and pooled together to collect a sufficient cell population. The cells were seeded on the surface of type II collagen-coated Millicell™ filters (Millipore, Billerica, MA, USA) in high-density 3D culture (2×10^6 cells/filter or 35,000 cells/mm^2)[26] and maintained in Ham's F12 media containing 10 mM glucose supplemented with 20% fetal bovine serum (FBS), 100 μg/mL ascorbate and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid) (Sigma–Aldrich Ltd., St. Louis, MI, USA). The cultures were grown in an incubator maintained at 37°C and 95% relative humidity supplemented with 5% CO_2: 95% atmospheric air. The culture medium (1 mL per filter) was changed every 2–3 days and fresh ascorbic acid was added with each change after day 7 of culture. All experiments were repeated three-times using separate cell isolations.

Exogenous ATP supplementation and assessment of ECM synthesis

Two days after seeding, cultures were supplemented with varying doses of freshly prepared ATP (Sigma–Aldrich Ltd.) (250, 62.5 or 0 μM) and incubated in the presence of both $[^{35}S]SO_4$ (5 μCi/culture) to label proteoglycans and $[^3H]$proline (5 μCi/culture) to label collagen for a period of 24 h. Although proline can be incorporated into different proteins, in chondrocyte cultures approximately 90% of proline becomes incorporated into collagen[27,28]. The unincorporated isotope from the tissue cultures was removed by gently washing the samples three-times in phosphate-buffered saline with 10 min incubations between each wash[28]. Using this method, the amount of incorporated isotope ($[^{35}S]SO_4$ and $[^3H]$proline) was between 5 and 10% of the original isotope dose (5 μCi) supplied to the cultures in these experiments. Cultures were then digested by papain (40 μg/ml in 20 mM ammonium acetate, 1 mM ethylenediaminetetraacetic acid – EDTA, and 2 mM dithiothreitol – DTT) for 48 h at 65°C. The accumulation of newly synthesized proteoglycan and collagen in the matrix was then estimated by quantifying radioisotope incorporation from aliquots of the papain digest using a β-liquid scintillation counter. The amounts of synthesized molecules were calculated relative to the DNA content of the tissue, determined from aliquots of the papain digest using the Hoechst dye 33258 assay[29] and expressed as a percentage of the unstimulated controls. Similar experiments were conducted with the addition of the P2 receptor antagonist Reactive Blue 2[30] (100 μM; Sigma–Aldrich Ltd.).

Long-term culture and assessment of tissue properties

Tissue constructs were supplemented with ATP (250, 62.5 or 0 μM; as described previously) at each media change for a period of 4 weeks to assess the effect of exogenous ATP on tissue formation and resulting properties. After long-term culture, tissues were removed from the filter units and weighed (wet weight). The tissues were lyophilized overnight, and weighed again (dry weight). Tissues were then digested by papain (as described earlier) and stored at −20°C until analysis. Aliquots of the digest were assayed separately for proteoglycan, collagen and DNA contents. The proteoglycan content was estimated by quantifying the amount of sulphated glycosaminoglycans using the dimethylmethylene blue dye binding assay[31,32]. Collagen content was estimated from the determination of the hydroxyproline content. Aliquots of the papain digest were hydrolyzed in 6 N HCl at 110°C for 18 h and the hydroxyproline content of the hydrolyzate was then determined using chloramine-T/Ehrlich's reagent assay[33]. Collagen content was estimated assuming hydroxyproline accounts for 10% of the total collagen mass in cartilage[34]. The DNA content was also determined from aliquots of the papain digest (as described earlier).

Separate cultures were established for the determination of tissue mechanical properties. Immediately following long-term culture, tissue thickness was measured using the needle probe method reported by Hoch *et al.*[35]. Briefly, a 25 Ga. needle (Becton–Dickinson, Franklin Lakes, NJ, USA) was attached to a 1 kg load cell of a Mach-1 mechanical tester (Biosyntech Canada Inc., Laval, PQ, Canada) which was then displaced into the tissue at a rate of 5 μm/s. Abrupt changes in force were interpreted as needle contact with the tissue and the underlying support surface to determine the tissue thickness. Thickness measures were taken at two random locations and the average value was recorded. Compressive stiffness of the cartilaginous tissues was then determined by uniaxial, unconfined indentation tests[36] with a plane-ended indenter (2 mm diameter) in conjunction with a Mach-1 mechanical tester (Biosyntech Canada Inc.). The diameter of the indentation probe was selected such that ratio of the indentor radius-to-tissue thickness were within acceptable limits of this technique (0.2–8[36]). Tissues were pre-loaded to 5 mN which was then defined as the zero-strain state. Compression was then applied in incremental steps of 2% strain to a maximum of 20% strain. Each compressive step was held until equilibrium was reached, which was defined as a change in force of less than 2 mN/min. Equilibrium modulus was determined at 20% strain using the expression derived by Hayes *et al.* for indentation testing of cartilage[36]. All thickness and mechanical measurements were conducted in culture media at 37°C.

Histological and immunohistochemical assessment

Selected cultures (from each experimental group and not subjected to mechanical testing) were also collected for histological and immunohistochemical evaluation. Upon harvest, tissue constructs were fixed overnight in 4% paraformaldehyde (Sigma–Aldrich Ltd.) and embedded in paraffin. Undecalcified, thin sections

(5 μm thick) were cut and stained with hemotoxylin–eosin (H&E), toluidine blue (TB) and von Kossa (VK). All sections were examined by light microscopy. Immunohistochemistry with antibodies against type I and II collagen was performed according to a standard ABC protocol (Vector Laboratories Inc., Burlingame, CA, USA) with diaminobenzidine for colour development[37]. Prior to incubation with the primary antibody, sections were pre-digested with 0.25% trypsin (Sigma–Aldrich Ltd.) and 2.5% hyaluronidase (Sigma–Aldrich Ltd.) to facilitate immunostaining[38]. The following primary antibodies and dilutions were used: rabbit polyclonal type I collagen antibody (T40113R: Biodesign International, Saco, ME, USA), 1:200 dilution; mouse monoclonal type II collagen antibody (II-II6B3: Developmental Studies Hybridoma Bank, University of Iowa, IA, USA), 1:10 dilution. Non-specific staining was assessed (for each section) by omission of the primary antibody.

Release of inflammatory mediators

During long-term culture, conditioned culture media was collected weekly to determine the potential effect of exogenous ATP on the subsequent release of inflammatory mediators (NO and PGE_2). The concentration of nitrate, a stable end-product of NO, in the conditioned culture media was determined using the Griess assay[39]. Concentration of PGE_2 in the conditioned media was determined using a commercially available enzyme immunoassay (EIA) (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's instructions (minimum detection limit of 15 pg/mL).

Expression of catabolic matrix turn-over genes

The expression of catabolic matrix turn-over genes and their associated inhibitors as a result of exogenous ATP was determined by semi-quantitative end-point polymerase chain reaction (PCR). After 4 weeks of culture, tissue constructs were snap-frozen in liquid N_2 and total RNA was extracted using the TRI reagent (Sigma–Aldrich Ltd.). The concentration and purity of extracted RNA were measured using a NanoDrop Spectrophotometer (ND1000; Nanodrop Products, Wilmington, DE, USA) and the 260/280 ratio generally ranged between 1.9 and 2.0. First strand cDNA was synthesized using the Multiscribe™ Reverse Transcriptase Kit (Applied Biosystems Inc., Foster City, CA, USA) from 1 μg of total RNA using oligo $(dT)_{15}$ primers (Integrated DNA Technologies Inc., Coralville, IA, USA). PCR primer sets specific for catabolic genes associated with cartilage turn-over and 18S ribosomal RNA (housekeeping gene) (Table I) were constructed using published sequences (basic local alignment search tool – BLAST) and Primer3 software[40] (Integrated DNA Technologies Inc). The primer sets were engineered to span intron–exon boundaries in order to detect genomic contamination. Studies were undertaken for all primers to determine the appropriate conditions (cycle number, annealing temperatures) for amplification (Table I). PCR was performed using 2 μL of diluted cDNA (~2 ng of input RNA) in a 20 μL reaction volume using Taq Polymerase (UBI Life Sciences Inc., Saskatoon, SK, Canada) on a Bio-Rad C1000 thermal cycler (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Minus-reverse transcription (RT) and no template controls were included in every run. Semi-quantitative gene expression levels[41] were determined by densitometry of PCR-product agarose gels (2.5%), visualized using ethidium bromide staining detected under ultraviolet light (G:Box Chemi HR16, Syngene, Cambridge, UK). All expression values were normalized to the expression of 18S ribosomal RNA (housekeeping gene).

Statistical analyses

All results were expressed as the mean ± 95% confidence interval for the mean (95% CI). Collected data was analyzed statistically using a one-way analysis of variance (ANOVA) and the Fisher's least square differences (LSD) post-hoc test (SPSS version 16, SPSS Inc., Chicago, IL, USA) to determine the effect of ATP dose. The data was checked prior to performing statistical tests for both normality and equal-variance. Significance was associated with *P*-values less than 0.05.

Results

Effect of exogenous ATP on ECM synthesis

To determine the effect of ATP stimulation on the synthesis of cartilaginous ECM macromolecules, isolated articular chondrocytes were seeded in high-density 3D culture supplemented with varying doses of ATP 2 days after seeding. As determined by radiolabel incorporation, stimulation by ATP significantly

Table I
Oligonucleotide primers used for semi-quantitative end-point PCR

Gene	Accession number	Primer sequence	Size	Number of cycles	Annealing temperature
MMP-1	NM_174112	Fwd: 5′ – TCCCTTGGACTTGCTCATTC – 3′ Rev: 5′ – ACTGGCTGAGTGGGATTTTG – 3′	143 bps	40	55°C
MMP-3	XM_586521	Fwd: 5′ – CTTGTCCTTCGATGCAGTCA – 3′ Rev: 5′ – CTGATGGCCCAGAACTGATT – 3′	213 bps	40	56.5°C
MMP-13	NM_1744389	Fwd: 5′ – CCCAGGAGCACTCATGTTTC – 3′ Rev: 5′ – GGCGTTTTGGGATGTTTAGA – 3′	138 bps	40	55°C
ADAMTS-1	NM_001101080	Fwd: 5′ – AGCCCTGGTCTCCCTGTAGT – 3′ Rev: 5′ – AGATCGGAGGGGAGCTGTAT – 3′	109 bps	40	55°C
ADAMTS-4	NM_181667	Fwd: 5′ – AGCAGTCTGGCTCCTTCAAA – 3′ Rev: 5′ – ATTCACCGTTGAGGGCATAG – 3′	165 bps	38	56°C
ADAMTS-5	XM_589193	Fwd: 5′ – GACGTGTGCAAACTGACCTG – 3′ Rev: 5′ – TGTATGGCCTGCACTCTGTC – 3′	94 bps	38	55°C
TIMP-1	NM_174471	Fwd: 5′ – GATGTTCAAAGGGTTCAGTGC – 3′ Rev: 5′ – GCAGAACTCATGCTGTTCCA – 3′	201 bps	35	55°C
TIMP-2	NM_174472	Fwd: 5′ – TACCAGATGGGCTGTGAGTG – 3′ Rev: 5′ – GTCCAGAAACTCCTGCTTGG – 3′	207 bps	35	55°C
TIMP-3	NM_174473	Fwd: 5′ – ATCGATATCACCTGGGCTGT – 3′ Rev: 5′ – TGCAAGCGTAGTGTTTGGAC – 3′	144 bps	33	55°C
TIMP-4	NM_001045871	Fwd: 5′ – GCCTTTTGATTCCTCCCTCT – 3′ Rev: 5′ – TTCTCCCAGGGCTCAATGTA – 3′	132 bps	38	55°C
18S	DQ222453	Fwd: 5′ – ATGGCCGTTCTTAGTTGGTG – 3′ Rev: 5′ – ATGGCCGTTCTTAGTTGGTG – 3′	136 bps	21	55°C

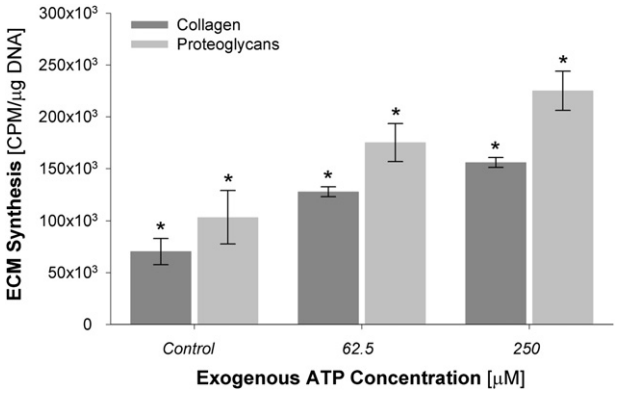

Fig. 1. Effect of exogenous ATP on collagen and proteoglycan synthesis determined 24 h after exposure to the nucleotide. Data expressed as the mean ± 95% CI for the mean ($n = 6–8$ for all groups). * Denotes a significance difference between all other groups ($P < 0.05$).

affected ECM synthesis ($P < 0.05$, Fig. 1). The synthesis of collagen and proteoglycans appeared to be similarly affected by ATP supplementation with an increase in synthesis between 70–81% (relative to control) observed under the low dose (62.5 μM) and 118–122% (relative to control) under the high dose (250 μM). ATP supplementation also had no apparent effect on tissue cellularity under the dose range investigated (data not shown). To confirm whether these changes were due to directly stimulating the purinergic receptor pathway, similar experiments were conducted in the presence of the P2 receptor antagonist Reactive Blue 2. In the presence of the inhibitor (100 μM), there was no significant effect of exogenous ATP on ECM synthesis ($P < 0.05$) and the level of inhibition ranged from 45 to 55% for both collagen and proteoglycan synthesis (Fig. 2).

Effect of ATP supplementation on tissue formation and properties

To determine the long-term effect of ATP supplementation on the growth and properties of cartilaginous tissue formed *in vitro*, tissue-engineered cartilage constructs were cultured in the presence of exogenous ATP for a period of 4 weeks. In response to long-term ATP supplementation, there was an approximate 40% decline in the wet

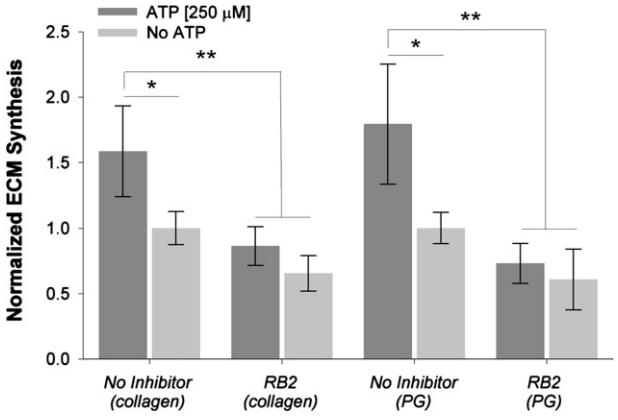

Fig. 2. Effect of exogenous ATP (250 μM) on collagen and proteoglycan (PG) synthesis in presence, or absence, of the P2 receptor inhibitor Reactive Blue 2 (RB2; 100 μM) determined 24 h after exposure. Synthesis data was normalized to the no-ATP controls and expressed as the mean ± 95% CI for the mean ($n = 6$ for all groups). * Denotes a significance difference between the ATP stimulated and corresponding no-ATP control group ($P < 0.05$); ** denotes a significant difference between inhibitor groups (ATP stimulated and no-ATP control) and corresponding ATP-stimulated group (no inhibitor) ($P < 0.05$).

Table II
Effect of the exogenous ATP on cartilaginous tissue formation and properties

	Control ($n = 8$)	62.5 μM ATP ($n = 8$)	250 μM ATP ($n = 8$)
Wet weight [mg]	11 ± 2†	9.4 ± 0.6	7.9 ± 0.4
Dry weight [mg]	1.0 ± 0.4	2.0 ± 0.4†	1.4 ± 0.4
Water content [%]	86 ± 2†	70 ± 2	72 ± 2
Thickness [μm]	230 ± 9†	354 ± 69	346 ± 15
DNA [μg]	3.7 ± 0.6†	6 ± 2	4.5 ± 0.8
GAG/dry weight [μg/mg]	96 ± 4†	128 ± 15	138 ± 4
GAG/DNA [μg/μg]	28 ± 8	34 ± 12	28 ± 4
Collagen/dry weight [μg/mg]	154 ± 16†	262 ± 12	264 ± 15
Collagen/DNA [μg/μg]	40 ± 8†	58 ± 4†	72 ± 11†
Collagen-to-GAG ratio	1.3 ± 0.5†	1.7 ± 0.6	1.8 ± 0.4
Indentation modulus* [kPa]	54 ± 25†	352 ± 152	277 ± 122

Data presented as mean ± 95% CI for the mean.
* Indentation modulus determined at 20% compressive strain.
† Significantly different from all other experimental groups ($P < 0.05$).

weight of the tissue (independent of dose) without a corresponding decline in tissue dry weight (Table II). This resulted in an apparent decrease in tissue water content to a level of approximately 70% (Table II). ATP-stimulated constructs were also significantly thicker (by 50–55%; $P < 0.05$) than the unstimulated controls (independent of dose) (Table II). Further analysis of the accumulated ECM revealed that ATP stimulation resulted in a significant increase in collagen (by 50–80%; $P < 0.05$) and proteoglycans (by 30–60%; $P < 0.05$) compared to the unstimulated controls (independent of dose) (Fig. 3). The substantial effect on collagen accumulation (with respect to proteoglycan accumulation) also resulted in an associated increase in the collagen-to-glycosaminoglycan ratio (by 30–40%; $P < 0.05$) (Table II). In contrast to the short-term studies, long-term stimulation with exogenous ATP appeared to elicit a proliferative response with the stimulated cultures having an approximately 20–60% higher DNA content ($P < 0.05$) compared to the unstimulated controls (independent of dose) (Table II). Histological assessment of the developed tissues indicated that all of the cultures stained positive for sulphated proteoglycans and displayed the same trends in tissue thickness as a result of long-term ATP stimulation (Fig. 4). Similarly, long-term supplementation with ATP elicited no adverse effects on tissue formation or induced evidence of matrix mineralization (Fig. 4). Immunohistochemical evaluation confirmed that the increased collagen accumulation as a result of ATP stimulation was primarily type II with no detectable presence of type I collagen synthesized by the cells (Fig. 5). Stimulation by exogenous ATP resulted in substantially improved mechanical properties of the

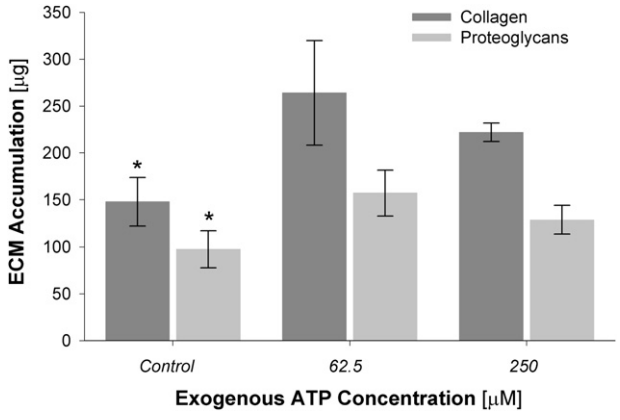

Fig. 3. Effect of long-term ATP stimulation on collagen and proteoglycan accumulation determined after exposure to the nucleotide for 4 weeks. Data expressed as the mean ± 95% CI for the mean ($n = 8$ for all groups). * Denotes a significance difference between all other groups ($P < 0.05$).

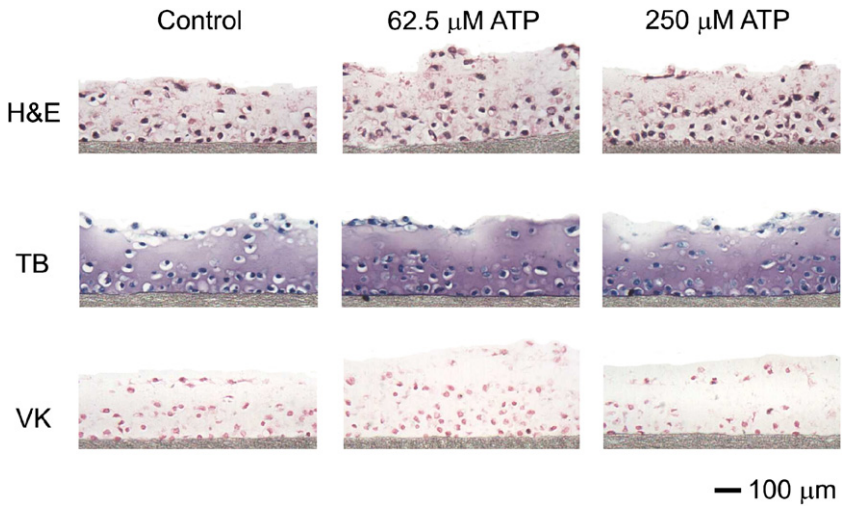

Fig. 4. Histological assessment of cartilaginous tissue constructs after 4 weeks of ATP stimulation. Tissue sections were stained with H&E, TB and VK. Original magnification of 100× and the scale bar represents 100 μm.

developed tissues. Constructs grown in the presence of ATP displayed a 5- to 6.5-fold increase ($P < 0.01$) in indentation modulus (independent of dose) (Table II).

Release of inflammatory mediators

As extracellular ATP has been shown to stimulate the release of inflammatory mediators, the conditioned culture media was collected and assayed for the presence of NO and PGE_2. While both nitrate (the stable end-product of NO) and PGE_2 were detected in the conditioned culture media at all time points investigated, there was no apparent effect of ATP stimulation on their release (Fig. 6). Both nitrate and PGE_2 media concentrations were at high levels early in the culture period and appeared to stabilize to lower levels after 1–2 weeks of culture (Fig. 6).

Expression of catabolic matrix turn-over genes

The expression of catabolic matrix turn-over genes and their associated inhibitors was determined after long-term ATP supplementation as extracellular ATP has also been implicated to induce cartilage resorption. Of the 10 genes investigated, the only gene that was differentially expressed as a result of long-term ATP stimulation was matrix metalloproteinase-13 (MMP-13) ($P < 0.05$, Fig. 7). In response to the high dose of ATP (250 μM), MMP-13 expression was over 2-fold compared to both the low dose (62.5 μM) and the unstimulated controls (Fig. 7). Although not statistically significant, there were slight increases in the expression of MMP-1 (1.4-fold over control) and TIMP-2 (1.4- to 1.6-fold over control) with ATP stimulation.

Discussion

This study demonstrates that by harnessing the molecular pathways involved in the mechanotransduction cascade, the anabolic effects of mechanical stimuli can be achieved in the absence of externally applied forces. While several different mechanotransduction pathways have been identified in chondrocytes[42,43], here we investigated the effect of directly stimulating the ATP-purinergic receptor pathway. Previous studies have shown that chondrocytes release ATP in response to mechanical loading[15–17] where it can then bind to the P2 receptor leading to the stimulation of ECM gene expression and matrix synthesis[16,17]. Although the amount of released ATP appears to be dependent on the magnitude of the applied loads[15], the concentration of ATP in the vicinity of the cells as a result of mechanical loading has been suggested to be in the order of 10^{-5} M[15]. In contrast, higher doses of ATP, of the order of 10^{-4} to 10^{-3} M, have previously been shown to elicit the release of inflammatory mediators[21,22] or induce matrix mineralization[25]. For this reason in the present study, the upper bound ATP dose (250 μM)

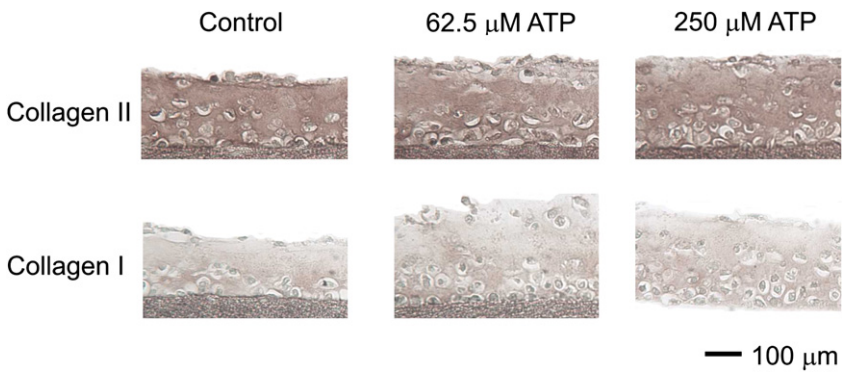

Fig. 5. Immunolocalization of collagen types II and I in the cartilaginous tissue constructs after 4 weeks of ATP stimulation. Original magnification of 100× and the scale bar represents 100 μm.

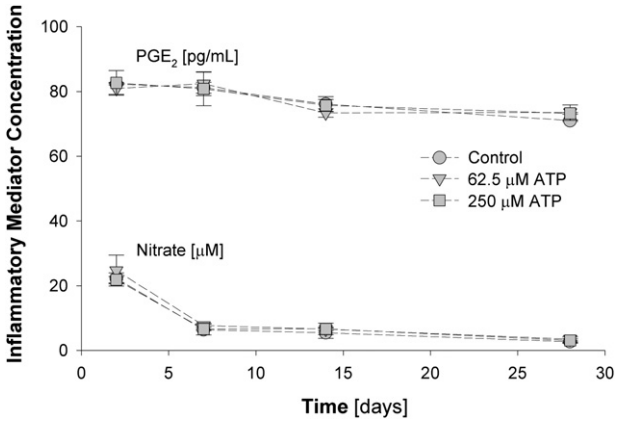

Fig. 6. Inflammatory mediator concentration (NO and PGE_2) in the conditioned media as a function of culture time during long-term ATP stimulation over a 4 week period. Data expressed as the mean ± 95% CI for the mean ($n = 6$ for all groups).

was selected to be well below this limit and the lower bound dose (62.5 μM) was then arbitrarily selected as 25% of the upper bound dose. In response to exogenous ATP, cartilaginous matrix synthesis (collagen and proteoglycans) was increased within the dose range investigated (62.5–250 μM). While there was no differential effect on synthesis of collagen as opposed to proteoglycans, ATP supplementation resulted in an increase in matrix synthesis of approximately 80–120% (relative to control). This effect appeared to be stimulated through the purinergic receptor pathway, as the response was abolished in the presence of a P2 receptor antagonist (Reactive Blue 2). A previous study also investigated the effect of extracellular nucleotides (ATP, uridine 5′-triphosphate – UTP) on the synthesis of ECM macromolecules in a bovine chondrocyte pellet culture system[44]. Although the effect of dose was not investigated, supplementation with 500 μM of UTP resulted in similar, but slightly lower changes in matrix synthesis (50–70% increase).

Long-term stimulation with exogenous ATP for a period of 4 weeks had a profound affect on both the growth and maturation of the developed tissue. Not only were the ATP-stimulated tissues significantly thicker, they also displayed a decreased water content (relative to control) that was similar to the values reported for native articular cartilage (~70% overall)[4,45]. Similarly, supplementation with ATP further stimulated the accumulation of ECM with a far greater influence on collagen compared to proteoglycans. Proteoglycan levels in the stimulated tissues appeared to approach that reported for native articular cartilage (~200 μg/mg dry weight[4]) whereas the levels of collagen were still substantially lower (~700 μg/mg dry weight[4]). Interestingly, these changes in ECM accumulation as a result of ATP stimulation also resulted in an associated increase in the collagen-to-glycosaminoglycan ratio that also appeared to approach that of native cartilage (ranging from 2:1 to 3:1 overall)[4,45]. In the previous study involving bovine chondrocyte pellet cultures, a single dose of extracellular nucleotides (500 μM ATP or UTP) also resulted in the greater accumulation of collagen as opposed to proteoglycans determined 21 days after exposure[44]. Paralleling the changes in ECM accumulation, ATP-stimulated tissues displayed substantial increases in indentation modulus (by 5- to 6.5-fold over control) which again appeared to approach the level of native bovine cartilage (ranging from 490 to 630 kPa)[46–48]. The improvements in physical properties are most likely attributable to the observed increase in collagen content which has been previously shown to correlate with decreased hydration[49] and improved mechanical properties[10]. Histological and immunohistochemical assessment of the tissue constructs revealed no detectable changes in phenotype as a result of ATP stimulation. The ECM of the developed tissues stained positive for the presence of sulphated proteoglycans and type II collagen without the detectable presence of type I collagen expression.

As extracellular ATP can induce a variety of undesirable responses[21–25] (in terms of engineering cartilaginous tissue), the effect of long-term exposure to exogenous ATP on inflammatory mediator release and matrix mineralization was also assessed. While both NO and PGE_2 were present in the conditioned culture media, there was no observable influence of exogenous ATP under the dose range investigated. Similarly, no evidence of matrix mineralization was observed upon long-term stimulation by ATP. This suggests that the dose range investigated in the present study (up to 250 μM) was well below that which has been previously shown to elicit the release of inflammatory mediators (~500 μM)[21,22] or induce matrix mineralization (~1 mM)[25].

In the long-term ATP stimulation study, there was no effect of nucleotide dose on ECM accumulation where a significant dose effect was observed on ECM synthesis. As previous work has shown that exogenous ATP can also initiate matrix turn-over[23,24], this led to an investigation of the expression of matrix turn-over genes in response to long-term exposure to ATP. Of the 10 genes investigated (MMP-1, -3, -13, ADAMTS-1, -4, -5, TIMP-1, -2, -3, -4), only MMP-13 was differentially expressed as a result of ATP stimulation. Under the high dose of ATP (250 μM), MMP-13 expression was over 2-fold that of the low dose (62.5 μM) or control cultures. The change in MMP-13 expression could account for the lack of a dose effect observed after long-term stimulation with ATP; however, additional experiments on MMP-13 protein expression and activity are required to confirm this assertion. MMP-13 (also known as collagenase-3) is highly expressed in osteoarthritic cartilage and is well known for its role in cartilage matrix turn-over[50]. Recent work has shown that MMP-13 can also be expressed in response to mechanical loading as part of the remodelling process[51]. This peptidase cleaves fibrillar collagen and is more effective at cleaving collagen type II than either type I or III[52]. In addition, as with most MMPs, MMP-13 can also cleave the Asn^{341}–Phe^{342} bond of aggrecan (the MMP site)[53]. This result could also account for the lack of ECM accumulation observed upon repeated ATP doses (500 μM) in bovine chondrocyte pellet culture observed previously[44], assuming that the trend of MMP-13 expression continues with increasing doses of exogenous ATP. While it is not exactly known why only MMP-13 was expressed in response to elevated concentrations of ATP, previous studies have suggested that this response may be due to an excess of inorganic pyrophosphate (PP_i)[54]. ATP can be catabolized by both soluble (e.g., tissue non-specific alkaline phosphatase, tissue transglutaminase) and membrane-bound (e.g., nucleotide pyrophosphatase/phosphodiesterases, ecto-5′ nucleotidase) nucleotide degrading enzymes leading to the generation of PP_i. Excess PP_i (as little as 100 nM) has been shown to directly elicit the expression of MMP-13 in chondrocyte cultures observed within 2 h after exposure[55]. Thus, these results taken together suggest that extracellular ATP most likely serves multiple roles depending on concentration. At low concentrations the nucleotide acts as an anabolic mediator whereas at higher concentrations the nucleotide can initiate a catabolic response.

The results of this study demonstrate that it can be possible to influence the growth and maturation of tissue-engineered cartilage through the direct stimulation of the ATP-purinergic receptor pathway in absence of externally applied forces. Recently, the purinergic receptor pathway has been implicated in the mechanotransduction cascade of chondrocytes and other cells of the mesenchymal lineage[15–19]. ATP is released in response to mechanical forces and is utilized as an autocrine and/or paracrine signal[15–19]. While exogenous extracellular ATP had a profound effect, the dose of the nucleotide needs to be controlled as the

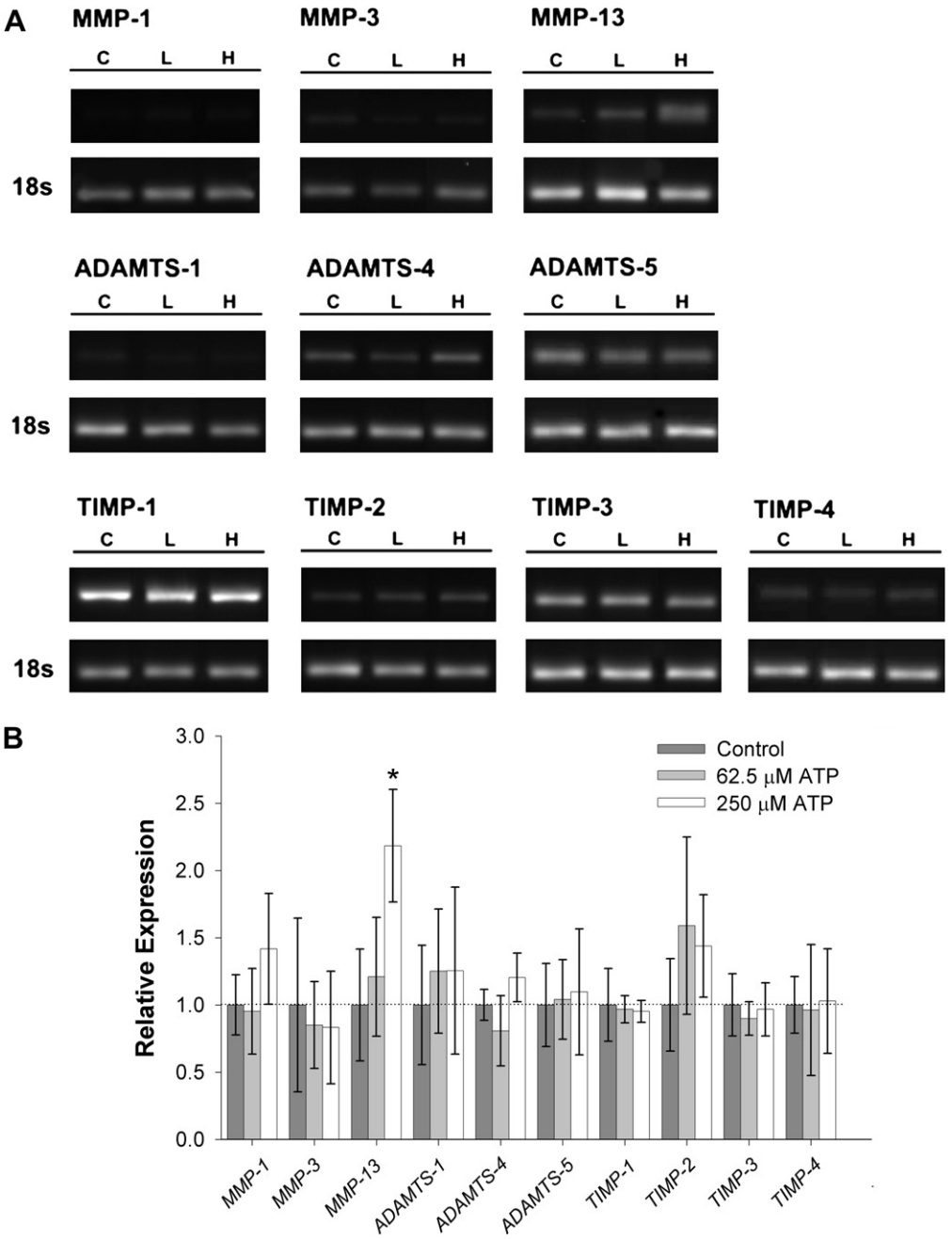

Fig. 7. Semi-quantitative end-point PCR results for the expression of catabolic matrix turn-over genes after 4 weeks of ATP stimulation. (A) Representative expression of PCR products analyzed by electrophoresis on 2.5% agarose gels, (B) relative expression of PCR products normalized to 18S ribosomal RNA (housekeeping gene) by densitometry. Data expressed as the mean ± 95% CI for the mean ($n = 4$ for all groups). * Denotes a significance difference between all other groups ($P < 0.05$).

emergence of a catabolic response (increased MMP-13 expression) was observed with higher doses (250 μM). Future studies will be undertaken to assess MMP protein expression and activity as well as to determine the threshold catabolic response in order to maximize cartilaginous tissue growth and maturation.

Conflict of interest

None.

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References

1. Buschmann MD, Gluzband YA, Grodzinsky AJ, Kimura JH, Hunziker EB. Chondrocytes in agarose culture synthesize a mechanically functional extracellular matrix. J Orthop Res 1992;10:745–58.

2. Vunjak-Novakovic G, Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R, *et al*. Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. J Orthop Res 1999;17:130–8.
3. Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R, Freed LE, *et al*. Modulation of the mechanical properties of tissue engineered cartilage. Biorheology 2000;37:141–7.
4. Waldman SD, Grynpas MD, Pilliar RM, Kandel RA. Characterization of cartilagenous tissue formed on calcium polyphosphate substrates in vitro. J Biomed Mater Res 2002;62:323–30.
5. Jin M, Frank EH, Quinn TM, Hunziker EB, Grodzinsky AJ. Tissue shear deformation stimulates proteoglycan and protein biosynthesis in bovine cartilage explants. Arch Biochem Biophys 2001;395:41–8.
6. Grodzinsky AJ, Levenston ML, Jin M, Frank EH. Cartilage tissue remodeling in response to mechanical forces. Annu Rev Biomed Eng 2000;2:691–713.
7. Quinn TM, Grodzinsky AJ, Buschmann MD, Kim YJ, Hunziker EB. Mechanical compression alters proteoglycan deposition and matrix deformation around individual cells in cartilage explants. J Cell Sci 1998;111:573–83.
8. Lee DA, Noguchi T, Frean SP, Lees P, Bader DL. The influence of mechanical loading on isolated chondrocytes seeded in agarose constructs. Biorheology 2000;37:149–61.
9. Bonassar LJ, Grodzinsky AJ, Frank EH, Davila SG, Bhaktav NR, Trippel SB. The effect of dynamic compression on the response of articular cartilage to insulin-like growth factor-I. J Orthop Res 2001;19:11–7.
10. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Kandel RA. Long-term intermittent shear deformation improves the quality of cartilaginous tissue formed in vitro. J Orthop Res 2003;21:590–6.
11. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Kandel RA. Long-term intermittent compressive stimulation improves the composition and mechanical properties of tissue-engineered cartilage. Tissue Eng 2004;10:1323–31.
12. Hung CT, Lima EG, Mauck RL, Takai E, LeRoux MA, Lu HH, *et al*. Anatomically shaped osteochondral constructs for articular cartilage repair. J Biomech 2003;36:1853–64.
13. Han E, Bae WC, Hsieh-Bonassera ND, Wong VW, Schumacher BL, Görtz S, *et al*. Shaped, stratified, scaffold-free grafts for articular cartilage defects. Clin Orthop Relat Res 2008;466:1912–20.
14. Ballyns JJ, Gleghorn JP, Niebrzydowski V, Rawlinson JJ, Potter HG, Maher SA, *et al*. Image-guided tissue engineering of anatomically shaped implants via MRI and micro-CT using injection molding. Tissue Eng Part A 2008;14:1195–202.
15. Graff RD, Lazarowski ER, Banes AJ, Lee GM. ATP release by mechanically loaded porcine chondrons in pellet culture. Arthritis Rheum 2000;43:1571–9.
16. Graff RD, Picher M, Lee GM. Extracellular nucleotides, cartilage stress, and calcium crystal formation. Curr Opin Rheumatol 2003;15:315–20.
17. Pingguan-Murphy B, El-Azzeh M, Bader DL, Knight MM. Cyclic compression of chondrocytes modulates a purinergic calcium signalling pathway in a strain rate- and frequency-dependent manner. J Cell Physiol 2006;209:389–97.
18. Tsuzaki M, Bynum D, Almekinders L, Faber J, Banes AJ. Mechanical loading stimulates ecto-ATPase activity in human tendon cells. J Cell Biochem 2005;96:117–25.
19. Yamazaki S, Weinhold PS, Graff RD, Tsuzaki M, Kawakami M, Minchew JT, *et al*. Annulus cells release ATP in response to vibratory loading in vitro. J Cell Biochem 2003;90:812–8.
20. Knight MM, McGlashan SR, Garcia M, Jensen CG, Poole CA. Articular chondrocytes express connexin 43 hemichannels and P2 receptors – a putative mechanoreceptor complex involving the primary cilium? J Anat 2009;214:275–83.
21. Caswell AM, Leong WS, Russell RG. Evidence for the presence of P2-purinoceptors at the surface of human articular chondrocytes in monolayer culture. Biochim Biophys Acta 1991;1074:151–8.
22. Varani K, De Mattei M, Vincenzi F, Tosi A, Gessi S, Merighi S, *et al*. Pharmacological characterization of P2X1 and P2X3 purinergic receptors in bovine chondrocytes. Osteoarthritis Cartilage 2008;16:1421–9.
23. Leong WS, Russell RG, Caswell AM. Stimulation of cartilage resorption by extracellular ATP acting at P2-purinoceptors. Biochim Biophys Acta 1994;1201:298–304.
24. Brown CJ, Caswell AM, Rahman S, Russell RG, Buttle DJ. Proteoglycan breakdown from bovine nasal cartilage is increased, and from articular cartilage is decreased, by extracellular ATP. Biochim Biophys Acta 1997;1362:208–20.
25. Kandel R, Hurtig M, Grynpas M. Characterization of the mineral in calcified articular cartilagenous tissue formed in vitro. Tissue Eng 1999;5:25–34.
26. Boyle J, Luan B, Cruz TF, Kandel RA. Characterization of proteoglycan accumulation during formation of cartilagenous tissue in vitro. Osteoarthritis Cartilage 1995;3:117–25.
27. Peterkofsky B, Diegelmann R. Use of a mixture of protease-free collagenases for specific assay of radioactive collagen in the presence of other proteins. Biochemistry 1971;10:988–94.
28. Sun YL, Kandel RA. Deep zone articular chondrocytes in vitro express genes that show specific changes with mineralization. J Bone Miner Res 1999;14:1916–25.
29. Kim YJ, Sah RL, Doong JY, Grodzinsky AJ. Fluorometric assay of DNA in cartilage explants using Hoechst 33258. Anal Biochem 1988;174:168–76.
30. Inoue K, Nakazawa K, Ohara-Imaizumi M, Obama T, Fujimori K, Takanaka A. Antagonism by reactive blue 2 but not by brilliant blue G of extracellular ATP-evoked responses in PC12 phaeochromocytoma cells. Br J Pharmacol 1991;102:851–4.
31. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883:173–7.
32. Goldberg RL, Kolibas LM. An improved method for determining proteoglycans synthesized by chondrocytes in culture. Connect Tissue Res 1990;24:265–75.
33. Woessner JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. Arch Biochem Biophys 1961;93:440–7.
34. Heinegard D, Bayliss M, Lorenzo P. Biochemistry and metabolism of normal and osteoarthritic cartilage. In: Brandt KD, Doherty M, Lohmander LS, Eds. Osteoarthritis. New York: Oxford University Press; 1998:74–84.
35. Hoch DH, Grodzinsky AJ, Koob TJ, Albert ML, Eyre DR. Early changes in material properties of rabbit articular cartilage after meniscectomy. J Orthop Res 1983;1:4–12.
36. Hayes WC, Keer LM, Herrmann G, Mockros LF. A mathematical analysis for indentation tests of articular cartilage. J Biomech 1972;5:541–51.
37. Poole CA, Glant TT, Schofield JR. Chondrons from articular cartilage. (IV). Immunolocalization of proteoglycan epitopes in isolated canine tibial chondrons. J Histochem Cytochem 1991;39:1175–87.
38. Chevrier A, Rossomacha E, Buschmann MD, Hoemann CD. Optimization of histoprocessing methods to detect

glycosaminoglycan, collagen type II, and collagen type I in decalcified rabbit osteochondral sections. J Histotechnol 2005;28:165–75.
39. Van de Loo FA, Arntz OJ, Van den Berg WB. Effect of interleukin 1 and leukaemia inhibitory factor on chondrocyte metabolism in articular cartilage from normal and interleukin-6-deficient mice: role of nitric oxide and IL-6 in the suppression of proteoglycan synthesis. Cytokine 1997;9: 453–62.
40. Rozen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, Eds. Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa: Humana Press; 2000:365–86.
41. Rolland-Valognes G. Semiquantitative analysis of gene expression in cultured chondrocytes by RT-PCR (Chapter 6). In: Sabatini M, Pastoureau P, De Ceuninck F, Eds. Cartilage and Osteoarthritis. Cellular and Molecular Tools. Totowa: Humana Press Inc.; 2004;Volume 1:69–78.
42. Lammi MJ. Current perspectives on cartilage and chondrocyte mechanobiology. Biorheology 2004;41:593–6.
43. Buckwalter JA, Martin JA, Brown TD. Perspectives on chondrocyte mechanobiology and osteoarthritis. Biorheology 2006; 43:603–9.
44. Croucher LJ, Crawford A, Hatton PV, Russell RG, Buttle DJ. Extracellular ATP and UTP stimulate cartilage proteoglycan and collagen accumulation in bovine articular chondrocyte pellet cultures. Biochim Biophys Acta 2000;1502:297–306.
45. Mankin HJ, Mow VC, Buckwalter JA, Iannotti JP, Ratcliffe A. Form and function of articular cartilage (Chapter 1). In: Simon SR, Ed. Orthopaedic Basic Science. Columbus: American Academy of Orthopaedic Surgeons; 1994:1–44.
46. Korhonen RK, Laasanen MS, Töyräs J, Rieppo J, Hirvonen J, Helminen HJ, *et al*. Comparison of the equilibrium response of articular cartilage in unconfined compression, confined compression and indentation. J Biomech 2002;35:903–9.
47. Simha NK, Jin H, Hall ML, Chiravarambath S, Lewis JL. Effect of indenter size on elastic modulus of cartilage measured by indentation. J Biomech Eng 2007;129:767–75.
48. Hall ML, Krawczak DA, Simha NK, Lewis JL. Effect of dermatan sulfate on the indentation and tensile properties of articular cartilage. Osteoarthritis Cartilage 2009;17:655–61.
49. Reynolds JJ. The effect of ascorbic acid on the growth of chick bone rudiments in chemically defined medium. Exp Cell Res 1966;42:178–88.
50. Takaishi H, Kimura T, Dalal S, Okada Y, D'Armiento J. Joint diseases and matrix metalloproteinases: a role for MMP-13. Curr Pharm Biotechnol 2008;9:47–54.
51. De Croos JN, Dhaliwal SS, Grynpas MD, Pilliar RM, Kandel RA. Cyclic compressive mechanical stimulation induces sequential catabolic and anabolic gene changes in chondrocytes resulting in increased extracellular matrix accumulation. Matrix Biol 2006;25:323–31.
52. Knäuper V, López-Otin C, Smith B, Knight G, Murphy G. Biochemical characterization of human collagenase-3. J Biol Chem 1996;271:1544–50.
53. Fosang AJ, Neame PJ, Last K, Hardingham TE, Murphy G, Hamilton JA. The interglobular domain of cartilage aggrecan is cleaved by PUMP, gelatinases, and cathepsin B. J Biol Chem 1992;267:19470–4.
54. Terkeltaub R. Physiologic and pathologic functions of the NPP nucleotide pyrophosphatase/phosphodiesterase family focusing on NPP1 in calcification. Purinergic Signal 2006;2:371–7.
55. Johnson K, Terkeltaub R. Upregulated ank expression in osteoarthritis can promote both chondrocyte MMP-13 expression and calcification via chondrocyte extracellular PPi excess. Osteoarthritis Cartilage 2004;12:321–35.