

Three-Dimensional Cartilage Tissue Engineering Using Adult Stem Cells From Osteoarthritis Patients

Wael Kafienah, Sanjay Mistry, Sally C. Dickinson, Trevor J. Sims, Ian Learmonth, and Anthony P. Hollander

Objective. To determine whether it is possible to engineer 3-dimensional hyaline cartilage using mesenchymal stem cells derived from the bone marrow (BMSCs) of patients with osteoarthritis (OA).

Methods. Expanded BMSCs derived from patients with hip OA were seeded onto polyglycolic acid scaffolds and differentiated using transforming growth factor $\beta 3$ in the presence or absence of parathyroid hormone-related protein (PTHrP) to regulate hypertrophy. Micromass pellet cultures were established using the same cells for comparison. At the end of culture, the constructs or pellets were processed for messenger RNA (mRNA) analysis by quantitative real-time reverse transcription-polymerase chain reaction. Matrix proteins were analyzed using specific assays.

Results. Cartilage constructs engineered from BMSCs were at least 5 times the weight of equivalent pellet cultures. Histologic, mRNA, and biochemical analyses of the constructs showed extensive synthesis of proteoglycan and type II collagen but only low levels of type I collagen. The protein content was almost identical to that of cartilage engineered from bovine nasal chondrocytes. Analysis of type X collagen mRNA revealed a high level of mRNA in chondrogenic constructs compared with that in undifferentiated BMSCs, indicating an increased risk of hypertrophy in the tissue-

engineered cells. However, the inclusion of PTHrP at a dose of 1 μM or 10 μM during the culture period resulted in significant suppression of type X collagen mRNA expression and a significant decrease in alkaline phosphatase activity, without any loss of the cartilage-specific matrix proteins.

Conclusion. Three-dimensional hyaline cartilage can be engineered using BMSCs from patients with OA. This method could thus be used for the repair of cartilage lesions.

An intact articular cartilage surface is essential for normal joint function (1). Loss of this tissue through degradation of type II collagen and proteoglycan components in the extracellular matrix is a well-described feature of osteoarthritis (OA) (1–4). In adults there is little or no capacity for self-repair of eroded articular cartilage, presumably because this tissue is avascular (5,6). Despite intensive research into the use of proteinase inhibitors to prevent cartilage loss in OA (7), no effective pharmaceutical therapies have emerged (8,9).

In recent years, various methods for the repair of articular cartilage lesions have been developed (5,10). These include osteochondral transplantation (11), microfracture (6), and autologous chondrocyte implantation (12,13) with or without the assistance of a scaffold matrix to deliver the cells (14). A feature of all of these techniques is that their use is limited to the repair of focal lesions. In patients with OA, cartilage lesions are generally large and unconfined (15) and thus do not provide an appropriate environment for the retention of chondrocytes or stem cells over a sufficient length of time to elaborate an extracellular matrix. Thus, the majority of patients with OA are excluded from these types of treatment. Successful repair of cartilage lesions in OA is therefore likely to be achieved only when 3-dimensional cartilage implants that have enough ex-

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Wael Kafienah, PhD, Sanjay Mistry, BSc, Sally C. Dickinson, PhD, Trevor J. Sims, Ian Learmonth, MD, Anthony P. Hollander, PhD: Southmead Hospital, and University of Bristol, Bristol, UK.

Dr. Kafienah, Mr. Mistry, Dr. Dickinson, Mr. Sims, and Drs. Learmonth and Hollander have a patent pending for a method of engineering cartilage tissue implants using adult stem cells.

Address correspondence and reprint requests to Anthony P. Hollander, PhD, Department of Clinical Science at North Bristol, AMBI Research Laboratories, Avon Orthopaedic Centre, Southmead Hospital, Bristol BS10 5NB, UK. E-mail: A.Hollander@Bristol.ac.uk.

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tracellular matrix for fixation within the joint can be generated.

Cartilage tissue engineering provides a potential method for the production of 3-dimensional implants (16,17). Effective engineering protocols have already been developed in which chondrocytes, usually from young animals, are seeded onto biodegradable scaffolds and cultured in a bioreactor (18,19). Generating 3-dimensional cartilage using adult human chondrocytes is far more challenging and, in the case of older OA patients, is probably impossible in the clinic setting, because of the lack of autologous donor tissue. One study demonstrated that OA chondrocytes can generate a cartilage-like matrix when grown on hyaluronic acid scaffolds (20), indicating the potential utility of these cells in OA. However, the cells synthesized significantly less collagen than was generated by chondrocytes from individuals without arthritis, suggesting that there may be a need for additional up-regulation of chondrogenesis if OA chondrocytes are ever to be used in a clinical setting.

This has led several groups of investigators to explore the use of mesenchymal stem cells (MSCs) for the generation of autologous chondrocytes (21). MSCs are multipotent cells with a self-renewing capacity (22,23). Many studies have utilized adherent bone marrow MSCs (BMSCs) cultured as small micromass pellets and stimulated with transforming growth factor β (TGF β) to drive chondrogenesis (24,25). From these studies there is strong histologic evidence that under these conditions, the BMSCs become chondrocytes and synthesize both type II collagen and proteoglycan. However, micromass pellets were designed for use in experimental models, and the amount of extracellular matrix produced by the pellets is too small to be of practical value for implantation (26). Furthermore, there is clear evidence that BMSCs stimulated with TGF β express type X collagen, an early marker of hypertrophy that is normally absent from hyaline cartilage (27).

It is not yet known whether BMSCs derived from OA patients have the capacity to become chondrocytes and generate hyaline cartilage. Most studies have utilized BMSCs from animals or healthy human donors (23–25,28). Nevertheless, one study investigated OA BMSCs cultured as pellets, and the results demonstrated a reduced chondrogenic capacity of these cells (29). The purpose of the present study was therefore to determine whether OA BMSCs can be used for tissue engineering of 3-dimensional hyaline cartilage.

PATIENTS AND METHODS

Patients. Bone marrow plugs were collected as trabecular bone biopsy specimens from the femoral heads of 23 patients with OA who were undergoing hip arthroplasty at Southmead Hospital (Bristol, UK), of whom 52% were men and 48% were women. The mean age of the 23 patients was 65.8 years (range 42–90 years). The study was carried out in full accordance with local ethics guidelines, and all of the patients gave their informed consent.

Isolation and characterization of BMSCs. Cells were isolated from the bone marrow plugs by washing in expansion medium consisting of low-glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 1% (volume/volume) Glutamax (1 \times ; Invitrogen, Carlsbad, CA), and 1% (v/v) penicillin (100 units/ml)–streptomycin (100 μ g/ml) (Invitrogen). The serum batch was selected to promote the growth and differentiation of MSCs (30).

The cell suspension was separated from any bone in the sample using a 19-gauge needle. The cells were centrifuged at 1,500 revolutions per minute for 5 minutes and the supernatant/fat was removed. The resulting cell pellet was resuspended in medium and then plated at a seeding density of $1.5\text{--}2.0 \times 10^5$ nucleated cells per cm^2 . The expansion medium was supplemented with 1 ng/ml fibroblast growth factor 2 (FGF-2) (PeproTech, London, UK) to enhance BMSC proliferation and differentiation (31,32). These flasks were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The first medium change was after 4 days, and the medium was then changed every other day until adherent cells reached 90% confluence and were ready for passaging. The cells were characterized for stem cell surface markers and multilineage potential, as described previously (30).

Micromass pellet cultures using OA BMSCs. Expanded BMSCs were trypsinized and cultured in micromass pellets as described previously (24), with slight modification. Briefly, 500,000 cells were placed in a 15-ml conical polypropylene tube and resuspended in 1.0 ml chondrogenic differentiation medium consisting of DMEM containing 4.5 gm/liter glucose supplemented with 10 ng/ml of TGF β 3 (R&D Systems, Abingdon, UK), 1 mM sodium pyruvate (Sigma), 50 μ g/ml ascorbic acid-2-phosphate (Sigma), 1×10^{-7} M dexamethasone (Sigma), 1% insulin–transferrin–selenium (Invitrogen), and 1% (v/v) penicillin (100 units/ml)–streptomycin (100 μ g/ml) (Invitrogen). Cells were centrifuged at 1,500 rpm for 5 minutes at 20°C. The pellets were maintained in culture with 1 pellet/tube and 1.0 ml chondrogenic medium/tube. Medium was changed every 2–3 days. After the first week the medium was further supplemented with 50 μ g/ml insulin (Sigma) until the end of culture. Chondrogenic pellets were harvested at 21 days for messenger RNA (mRNA), matrix proteins, and histologic analyses.

Tissue engineering using OA BMSCs. Polyglycolic acid (PGA) scaffolds (a kind gift from Dr. James Huckle, Smith & Nephew, York, UK) were produced as 5-mm (diameter) \times 2-mm (thick) discs according to an established method (33). The scaffolds were presoaked in 100 μ g/ml human fibronectin (Sigma) in phosphate buffered saline (PBS) to support BMSC adherence to PGA fibers. BMSCs from passage 2 or passage 3 were trypsinized and suspended in 30 μ l of expansion medium.

The suspension was loaded drop-wise onto the scaffold in tissue-culture wells precoated with 1% (weight/volume) agarose (Sigma) to prevent cell adherence to plastic. After incubation for 4 hours, the scaffolds were turned over and incubated for a further 4 hours to allow for even distribution of cells across the scaffold.

The constructs were maintained in chondrogenic differentiation medium, as described above for micromass pellet cultures. After the first week, the medium was further supplemented with 50 $\mu\text{g/ml}$ insulin (PeproTech) until the end of culture. Human recombinant parathyroid hormone-related protein (PTHrP) (Sigma) was included in the differentiation medium at 1 μM or 10 μM , as appropriate. The medium was changed 3 times a week. The constructs were incubated at 37°C, in 5% CO₂ on a rotating platform at 50 rpm for 35 days. Harvested samples were digested with collagenase to release the cells, which were stored at -70°C for subsequent RNA extraction or alkaline phosphatase activity assay. Other samples were stored at -20°C prior to quantitative biochemical analysis (see below). In some experiments, cartilage was engineered using bovine nasal chondrocytes (BNCs) that were isolated as described previously (34).

Histologic and immunohistochemical analyses of micromass pellets and engineered cartilage. Micromass pellets and mature cartilage engineered from stem cells were frozen in OCT embedding matrix (BDH Chemicals, Poole, UK). Full-depth sections (thickness 7 μm) were cut with a cryostat and fixed in 4% (w/v) paraformaldehyde (Sigma) in PBS, pH 7.6. Some sections were stained with hematoxylin and eosin or 0.1% (w/v) Safranin O (both from Sigma) to evaluate the distribution of matrix and proteoglycan, respectively. Other sections were immunostained with monoclonal antibodies against types I and II collagen (Southern Biotechnology, Birmingham, AL) and type X collagen (a kind gift from Dr. Alvin Kwan, Cardiff University, UK), as previously described (34,35). Biotinylated secondary antibodies were detected with a peroxidase-labeled biotin-streptavidin complex (Vectastain Elite kit; Vector Laboratories, Peterborough, UK) with diaminobenzidine substrate (Vector Laboratories). Natural cartilage and tendon were used as positive controls for detection of type II collagen and type I collagen, respectively. Normal goat serum was used as a negative control, and all sections were counterstained with hematoxylin (Vector Laboratories).

Quantitative biochemical analyses of engineered cartilage. Dry weights of the constructs were determined after freeze-drying. The samples were then solubilized with trypsin and processed for complete biochemical analysis as recently described (36). Briefly, samples were digested with 2 mg/ml TPCK-treated bovine pancreatic trypsin containing 1 mM iodoacetamide, 1 mM EDTA, and 10 $\mu\text{g/ml}$ pepstatin A (all from Sigma). An initial incubation for 15 hours at 37°C with 250 μl trypsin was followed by a further 2-hour incubation at 65°C after the addition of a further 250 μl of the freshly prepared proteinase. All samples were boiled for 15 minutes at the end of incubation to destroy any remaining enzyme activity. The extracts were assayed by inhibition enzyme-linked immunosorbent assay (ELISA) using a mouse IgG monoclonal antibody to denatured type II collagen, COL2-3/4m, as previously described (2). Peptide CB11B (CGKVGPSGAP[OH]GEDGRP[OH]GPP[OH]GPQY) was synthesized using 9-fluorenylmethoxycarbonyl chemistry (a gift from Dr. A. Moir, Krieb's

Institute, Sheffield University, UK) and was used as a standard in all of the immunoassays. The extracts were also assayed by inhibition ELISA using a rabbit anti-peptide antibody to type I collagen, as previously described (36). Peptide SFLPQPPQ was synthesized using 9-fluorenylmethoxycarbonyl chemistry (also from Dr. A. Moir) and was used as a standard in all of the immunoassays. Proteoglycan in the digests was measured by the detection of sulfated glycosaminoglycan using colorimetric assay with dimethylmethylene blue (Aldrich, Gillingham, UK) as previously described (37).

Alkaline phosphatase activity. Cells in engineered cartilage constructs were assayed for alkaline phosphatase activity after collagenase digestion of the extracellular matrix, as described previously (38). Although it is theoretically possible that the collagenase digestion could itself influence alkaline phosphatase activity, this is unlikely to affect the interpretation of our results, since all samples were extracted under identical conditions and assayed as soon as possible after completion of the extraction process. Briefly, the cells were lysed with 0.1 ml of 25 mM sodium carbonate (pH 10.3), 0.1% (v/v) Triton X-100. After 2 minutes, each sample was treated with 0.2 ml of 15 mM *p*-nitrophenyl phosphate (di-tri salt; Sigma) in 250 mM sodium carbonate (pH 10.3), 1.5 mM MgCl₂. Lysates were then incubated at 37°C for 2 hours. After the incubation period, 0.1-ml aliquots were transferred to a 96-well microtiter plate and the absorbance was read at 405 nm. An ascending series of *p*-nitrophenol (25–500 μM) prepared in the incubation buffer enabled quantification of product formation.

RNA isolation and reverse transcription. RNA was extracted from cell cultures using the GenElute Mammalian Total RNA kit (Sigma), according to the manufacturer's instructions. Reverse transcription (RT) was carried out using the Superscript II system (Invitrogen). Total RNA (2 μg) was reverse transcribed in a 20- μl reaction volume containing Superscript II (200 units), random primers (25 μM), and dNTP (0.5 mM each) at 42°C for 50 minutes.

Primer design. The coding sequences for human type X, type II, and type I collagens (accession no. NM_000493, NM_001844, and NM_000088, respectively) were used to design primers using the online software, Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA). The primers span intronic junctions to avoid the amplification of genomic sequences. They were also checked for the amplification of potential pseudogenes. A BLAST search against all known sequences confirmed specificity. Published primers for the housekeeping gene β -actin (39) were used as a reference for normalization in all RT-polymerase chain reactions (RT-PCRs). In preliminary studies (results not shown) we found that this gene was expressed more stably than GAPDH or ribosomal RNA for a range of differentiated progeny of stem cells. All of our primers were specifically designed not to coamplify processed pseudogenes in contaminating genomic DNA and they all generated the correct sizes of the PCR fragments with no nonspecific products, thus confirming the specificity of the real-time RT-PCR (results not shown). Details of the primers used in the study are as follows: for type X collagen α , forward GACACAGTTCTTCATCCCTACAC and reverse GCAACCCTGGCTCTCCTT; for type II collagen α 1 (A+B), forward CAACACTGCCAACGTCCAGAT and reverse CTGCTTCGTCCAGATAGGCAAT; for type I colla-

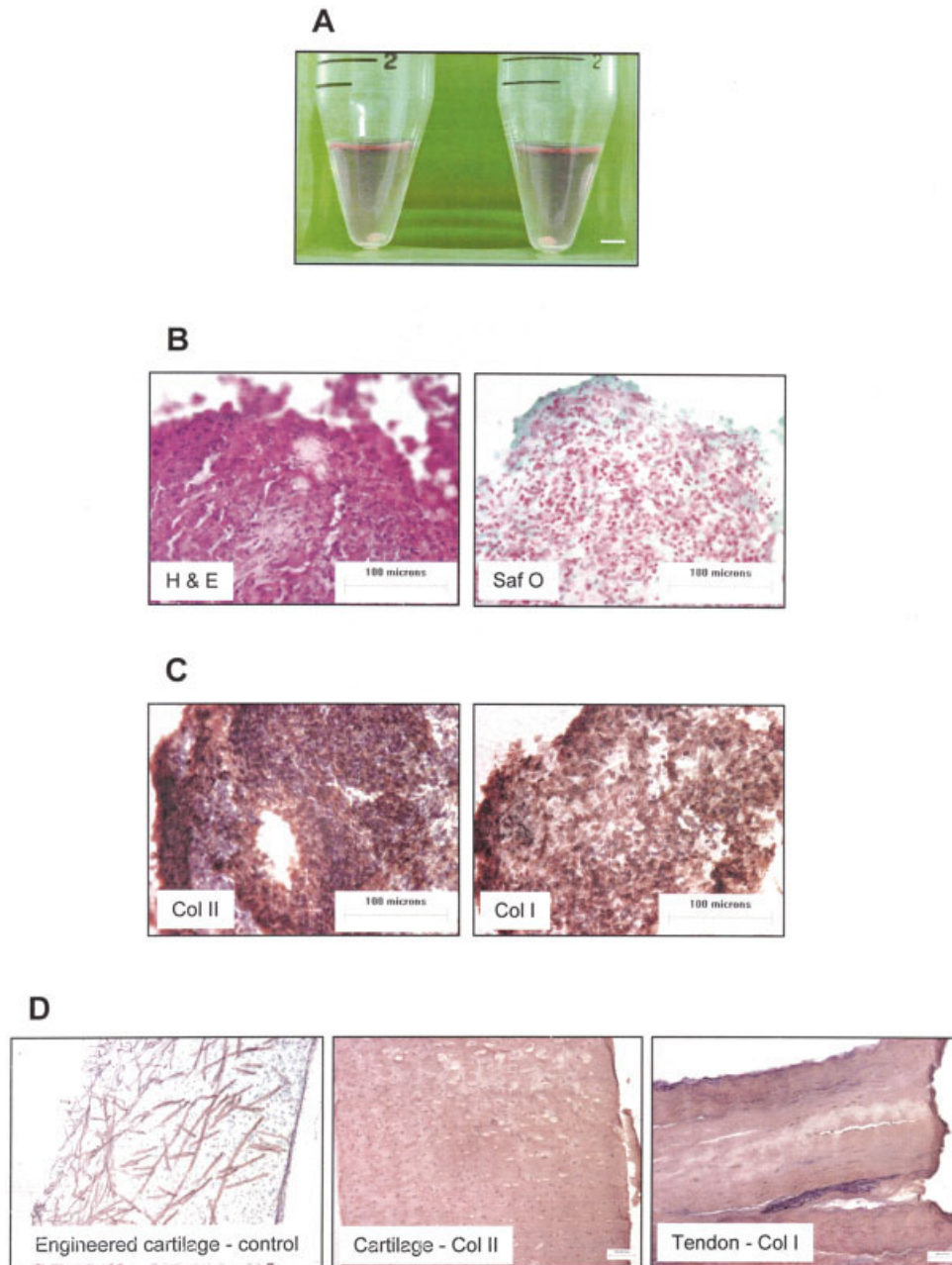


Figure 1. Chondrogenesis in pellet cultures of bone marrow mesenchymal stem cells (BMSCs) from patients with hip osteoarthritis (OA). Expanded OA BMSCs from passages 2 or 3 were cultured as 3-dimensional pellets. Representative results are shown for 1 of 5 different patients. **A**, Macroscopic appearance of pellets. Bar = 3 mm. **B**, Histologic appearance of pellets at the end of culture. Sections were stained with hematoxylin and eosin (H&E) or Safranin O (Saf O) for sulfated proteoglycans. **C**, Immunohistochemical detection of type I collagen (Col I) and type II collagen (Col II) at the end of culture, using specific antibodies for immunostaining of sections. **D**, Controls for immunostaining, comprising the negative control (tissue-engineered cartilage with normal goat serum) (left) in which staining of the remaining polyglycolic acid scaffold but not the extracellular matrix is evident, and positive controls for Col II in hyaline cartilage (middle) and Col I in tendon (right). Bar = 100 μ m. (Original magnification $\times 40$ in **B** and **C**; $\times 10$ in **D**.)

gen $\alpha 1$, forward AGGGCCAAGACGAAGACATC and reverse CAACACTGCCAACGTCCAGAT; and for β -actin, forward GACAGGATGCAGAAGGAGATTACT and reverse TGATCCACATCTGCTGGAAGGT.

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR was performed in a 25- μ l reaction volume containing 12.5 μ l of the SYBR Green PCR master mix (Sigma), 5 μ l of the RT reaction mixture, and 300 nM each primer using the Smart Cycler II System (Cepheid, Sunnyvale, CA). For the β -actin gene, the RT reaction mixture was diluted 100 times. The amplification program consisted of initial denaturation at 95°C for 2 minutes followed by 40 cycles of 95°C for 15 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 15 seconds. After amplification, melt analysis was performed by heating the reaction mixture from 60°C to 95°C at a rate of 0.2°C/second. The threshold cycle (C_t) value for each gene of interest was measured for each RT sample. The C_t value for β -actin was used as an endogenous reference for normalization. Real-time RT-PCR assays were done in duplicate or triplicate, with each set of assays repeated 2–4 times.

Statistical analysis. Comparison of differences between individual groups was performed using the 2-tailed Mann-Whitney U test. For multiple comparisons, the groups were compared by analysis of variance using the nonparametric Kruskal-Wallis test. When significant variance was demonstrated, differences between individual groups were then determined using the 2-tailed Mann-Whitney U test with Dunn's post hoc correction. In all analyses, P values less than 0.05 were considered significant.

RESULTS

Phenotype of isolated OA BMSCs. A well-characterized population of stem cells was used for the 3-dimensional engineering of cartilage tissue. We have previously described this cell population as being positive for CD105, CD106, CD49a, CD117, STRO-1, and bone morphogenetic protein receptor type 1A and negative for CD34 (30). We have also shown the population to be multipotential, in that it consistently is able to differentiate into adipogenic, chondrogenic, and osteogenic lineages (30). In the present study there was no significant variation in the extent of stem cell differentiation among the patient samples ($n = 23$), between male and female patients, or between different age groups (results not shown). Therefore, we were able to confirm that the BMSC population used was consistently multipotent, as expected for stem cells that have been expanded with the use of FGF-2 (30–32).

Chondrocyte formation from OA BMSCs. In our initial experiments, we cultured OA BMSCs with TGF β 3 in high-density pellets. Under these conditions, the pellets grew into cartilage-like nodules that were visible to the naked eye (Figure 1A). At the histologic

level, these nodules contained a large number of cells as well as an extracellular matrix that stained consistently for proteoglycan, although this was largely in a pericellular location (Figure 1B). Similarly, there was staining for types I and II collagen throughout the pellets and this was most intense around the cells (Figure 1C). The extent of immunostaining for type I collagen was relatively similar to that for type II collagen, suggesting that the pellet tissue was likely to be predominantly fibrocartilage rather than hyaline cartilage. Negative and positive controls for the immunostaining are shown in Figure 1D.

Cartilage tissue engineering using OA BMSCs.

We were able to successfully engineer 3-dimensional cartilage using a carefully ordered sequence of signals, as described above. First, the OA BMSCs were expanded in 10% FBS and 1 ng/ml FGF-2. Second, the expanded cells were seeded onto PGA scaffolds that had been precoated with fibronectin. Third, the cells were cultured on a gently rotating platform for 1 week with 10 ng/ml TGF β 3 in differentiation medium. Fourth, the cells were cultured for a further 4 weeks on the rotating platform in differentiation medium with 50 μ g/ml insulin as well as 10 ng/ml TGF β 3.

Under these carefully defined conditions, we were able to generate a white, shiny tissue that resembled hyaline cartilage at a macroscopic level (Figure 2A). On histologic analysis, these cartilage constructs were found to contain an extracellular matrix that stained extensively for proteoglycan, moderately for type II collagen, and weakly for type I collagen (Figure 2B). At higher magnification, it was possible to observe rounded cells, some pericellular staining for type X collagen, and staining for type II collagen (strongly) and type I collagen (weakly) in the interterritorial matrix.

Biochemical findings in engineered cartilage. We undertook an extensive quantitative analysis of the engineered cartilage using a series of well-validated and specific assays (36). For comparison, micromass pellet cultures were also analyzed. Despite the sensitive nature of our assays (36), we had to use a minimum of 500,000 cells per micromass pellet, and at the end of culture we had to combine 3 of these pellets in order to generate enough extracellular matrix for quantitative analysis. For tissue engineering, we were able to use as few as 300,000 cells per scaffold and to analyze one sample at a time. Cultures were maintained in TGF β 3 for up to 35 days prior to analysis. However, in cultures with the micromass pellets, there was evidence of some loss of matrix beyond 21 days, and therefore the pellet cultures were

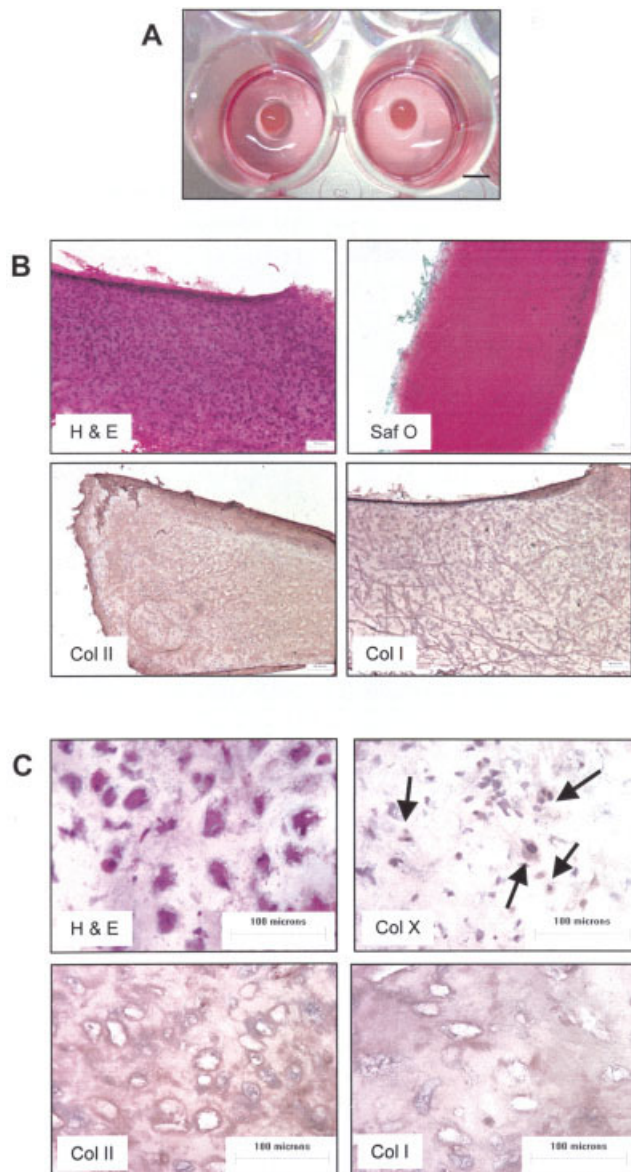


Figure 2. Cartilage tissue engineering from BMSCs. Expanded OA BMSCs from passages 2 or 3 were used to engineer cartilage on polyglycolic acid scaffolds. Representative results are shown for 1 of 8 different patients. **A**, Macroscopic appearance of engineered cartilage. Bar = 3 mm. **B**, Histologic appearance of engineered cartilage at the end of culture, in sections stained with H&E or Saf O for sulfated proteoglycans and immunostained for Col II and Col I using specific antibodies. Bar = 100 μ m. **C**, Histologic appearance of engineered cartilage at the end of culture, in sections stained with H&E and immunostained for types X, II, and I collagens using specific antibodies; arrows indicate staining for Col X limited to the pericellular region. See Figure 1 for other definitions. (Original magnification \times 10 in **B**; \times 40 in **C**.)

stopped at this optimal time point. After 35 days in culture, the mean dry weight was 0.08 mg, the type II

collagen content was 0.4%, and proteoglycan content was 0.75%, whereas the type I collagen content was undetectable; the equivalent findings after 21 days in pellet cultures were a mean 0.23 mg dry weight, 0.57% type II collagen, 1.14% proteoglycan, and 0.26% type I collagen. The dry weights of pellets and engineered tissue were calculated after freeze-drying. In the case of engineered tissue, the weight of any remaining PGA scaffold was determined after enzymatic digestion of the extracellular matrix, and this was subtracted from the total dry weight.

Using these calculations, we determined that the extracellular matrix of engineered tissue was at least 5 times that of pellet cultures, and this difference was significant (Table 1). Furthermore, the engineered cartilage contained significantly more proteoglycan and type II collagen than was observed in micromass pellet cultures. There was also a slightly higher type I collagen content in engineered cartilage, although this was still $<10\%$ of the type II collagen content in engineered cartilage.

We previously demonstrated that the best results from cartilage tissue engineering could be achieved using BNCs (34). We therefore compared the results of cartilage engineering using human OA BMSCs with those using BNCs. There was no significant difference in the content of type II collagen, type I collagen, or proteoglycan between the 2 cell types (Figure 3), indicating that chondrocytes derived from OA BMSCs are as effective as BNCs.

Inhibition of hypertrophy by PTHrP. In preliminary experiments, we observed that OA BMSCs cultured with TGF β 3 in micromass pellets displayed increased expression of type X collagen, indicating that these cells

Table 1. Biochemical analysis of cartilage extracellular matrix

Parameter	Pellet culture (n = 10)	Tissue-engineered cartilage (n = 15)
Number of cells seeded*	500,000	300,000
Dry weight, mean \pm SEM mg	0.23 \pm 0.03	1.25 \pm 0.77 \dagger
Type II collagen, mean \pm SEM % of dry weight \ddagger	0.57 \pm 0.07	17.21 \pm 2.88 \dagger
Proteoglycan, mean \pm SEM % of dry weight \S	1.14 \pm 0.13	29.96 \pm 3.45 \dagger
Type I collagen, mean \pm SEM % of dry weight \ddagger	0.26 \pm 0.11	1.76 \pm 0.25 \dagger

* Minimum number of cells required for accurate quantification using biochemical assays.

\dagger $P < 0.0001$ versus pellet culture, by Mann-Whitney U test.

\ddagger Determined by specific immunoassay after selective extraction of peptide epitopes.

\S Determined by dimethylmethylene blue colorimetric assay of glycosaminoglycans.

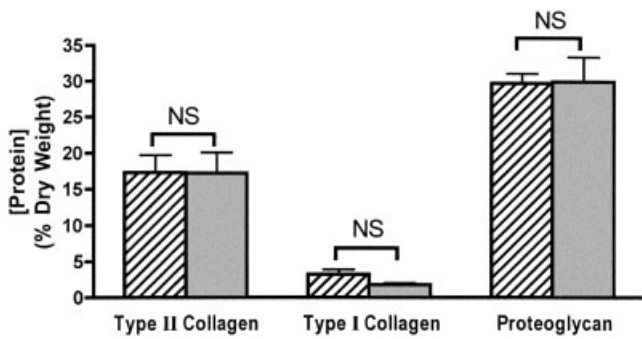


Figure 3. Quantitative comparison of cartilage engineered from bovine nasal chondrocytes (BNCs) (hatched bars; n = 18 animals) and cartilage engineered from human OA BMSCs (shaded bars; n = 19 patients). Cartilage was engineered from BNCs or from expanded OA BMSCs at passages 2 or 3 and then digested with trypsin. Digests were assayed for types I and II collagen using specific immunoassays. Proteoglycan was measured as sulfated glycosaminoglycans using the dimethylmethylene blue colorimetric assay. Bars show the mean and SEM content of each protein expressed as a percentage of dry weight. NS = *P* not significant, by 2-tailed Mann-Whitney U test. See Figure 1 for other definitions.

were likely to generate hypertrophic cartilage rather than hyaline cartilage. This was confirmed by immunostaining of sections from tissue-engineered cartilage (see Figure 2C). We therefore investigated the potential of inhibiting this hypertrophy using PTHrP, which has been shown to prevent maturation of prehypertrophic chondrocytes in the growth plate.

BMSCs cultured in monolayer without TGFβ3 expressed very little type X collagen; however, expression of type X collagen was significantly up-regulated when the same cells were used to engineer cartilage in a TGFβ3-driven system (Figure 4A). PTHrP suppressed this up-regulation of type X collagen mRNA in a significant and dose-dependent manner (Figure 4A). Similarly, PTHrP at 10 μM significantly reduced the alkaline phosphatase content of the cells from our engineered cartilage (Figure 4B).

Having demonstrated that PTHrP can suppress early markers of hypertrophy, we considered it important to establish that there was no reduction in the quality of engineered cartilage in the PTHrP cultures. PTHrP had no effect on cartilage-specific type II collagen mRNA or protein (Figure 5A). Type I collagen, which is normally absent from hyaline cartilage, was further reduced from its already low level in these cartilage constructs, at both the mRNA and protein levels (Figure 5B). This led to a 4-fold, significant improvement in the ratio of type II collagen to type I

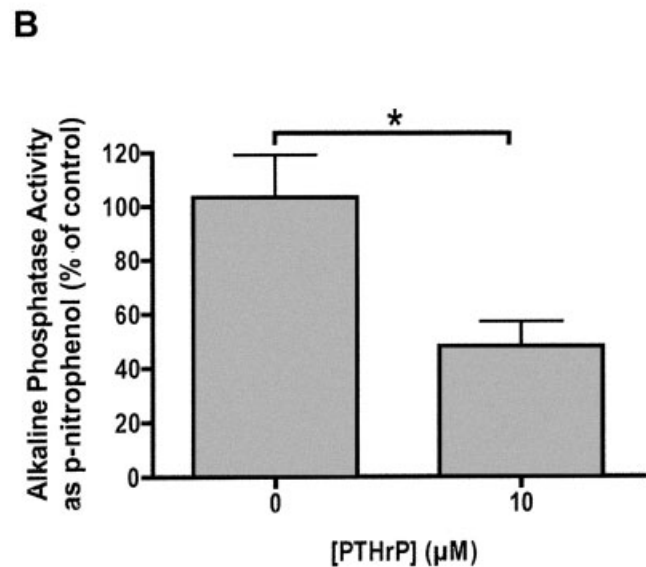
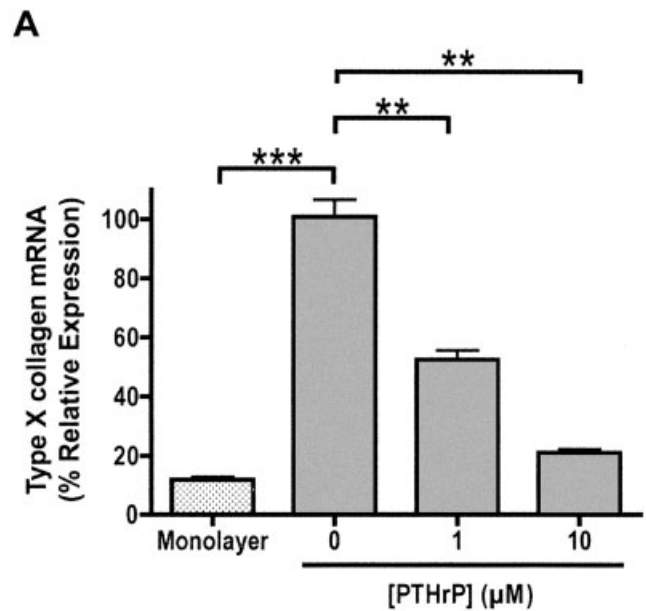


Figure 4. Inhibition of hypertrophy by parathyroid hormone-related protein (PTHrP). Expanded osteoarthritis bone marrow mesenchymal stem cells from passages 2 or 3 were cultured in monolayer (stippled bar) or used to engineer cartilage on polyglycolic acid scaffolds with or without PTHrP (shaded bars). **A**, Analysis of type X collagen mRNA by quantitative real-time polymerase chain reaction at the end of culture. Results were normalized to values in the control culture with transforming growth factor β alone, and are shown as the mean and SEM in 7 patients. **B**, Alkaline phosphatase content determined by reaction with *p*-nitrophenyl phosphate; the enzyme activity was normalized to the values in the control culture without PTHrP. Results are the mean and SEM in 6 patients. * = *P* < 0.05; ** = *P* < 0.01; *** = *P* < 0.0001, by 2-tailed Mann-Whitney U test with Dunn's post hoc correction.

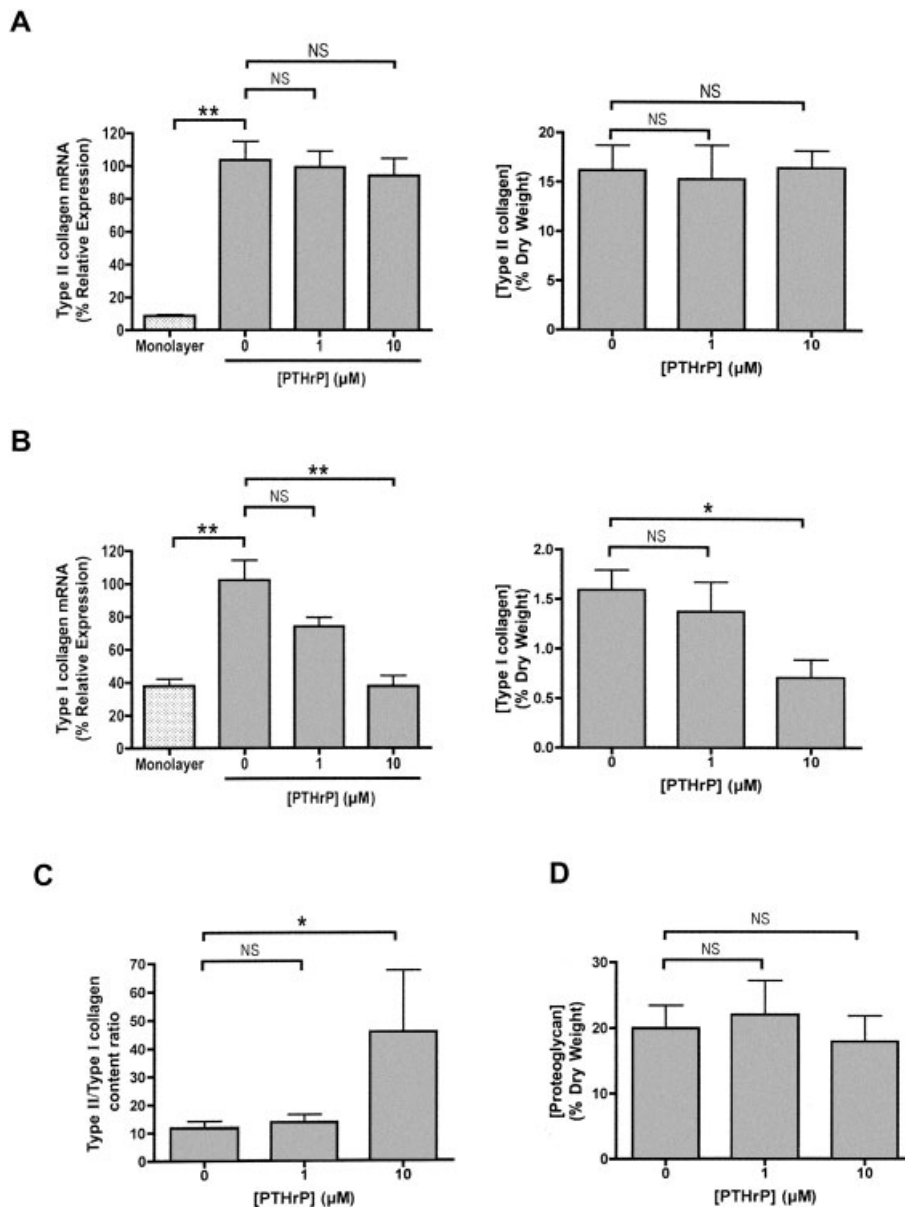


Figure 5. Effect of parathyroid hormone-related protein (PTHrP) on the extracellular matrix of engineered cartilage. **A** and **B**, Expanded osteoarthritis bone marrow mesenchymal stem cells from passages 2 or 3 were cultured in monolayer (stippled bar) or used to engineer cartilage on polyglycolic acid scaffolds with or without PTHrP (shaded bars). Type II collagen (**A**) and type I collagen (**B**) were analyzed by quantitative real-time polymerase chain reaction for mRNA expression (left) ($n = 6$ patients in each) and by specific immunoassay of trypsin digests for protein expression (right) ($n = 7$ patients in each). The mRNA results were normalized to the values in control culture with transforming growth factor β alone. **C**, Ratio of type II collagen to type I collagen measured as protein ($n = 7$ patients). **D**, Proteoglycan content measured as sulfated glycosaminoglycans using the dimethylmethylene blue colorimetric assay ($n = 7$ patients). All results are the mean and SEM. * = $P < 0.05$; ** = $P < 0.01$, by 2-tailed Mann-Whitney U test with Dunn's post hoc correction. NS = not significant.

collagen (Figure 5C), whereas no effect of PTHrP on the proteoglycan content was observed (Figure 5D).

DISCUSSION

The present study is the first to demonstrate the feasibility of tissue engineering of hyaline cartilage from OA BMSCs. Biochemically, the cartilage quality was comparable with that achieved using the best available cell source, namely BNCs (34). Furthermore, we were able to show that the tendency of BMSCs to become hypertrophic can be down-regulated using PTHrP. These findings suggest that it will be feasible to develop a method of cartilage repair in OA patients using their own BMSCs to generate 3-dimensional cartilage implants.

This study investigated the use of stem cells derived from patients with hip OA who were undergoing arthroplasty at a large orthopedic referral center in the UK. These patients are likely to be typical of individuals who might benefit from cartilage implantation. We demonstrated that despite the poor capacity of the cells to produce any extracellular matrix in micromass pellet cultures, the cells could be directed to produce cartilage through the use of a series of specific molecular signals, applied in appropriate order. We cannot be certain that this set of conditions is the best possible for cartilage formation, but our results demonstrate that the sequence of signals described herein can be used successfully to generate hyaline cartilage.

First, the adherent mesenchymal cells must be driven to proliferate so that their cell number can be expanded within a reasonable time frame. Murphy et al (29) found that the proliferation rate of OA BMSCs cultured in 10% serum was reduced compared with that of control cells. In the present study we used 1 ng/ml FGF-2 in addition to serum. This growth factor has been previously shown to enhance the proliferation of normal BMSCs (31,32). More recently, we found that proliferation of OA BMSCs is enhanced by FGF-2 and that the mechanism is dependent on the stem cell nucleolar protein nucleostemin (30). This suggests that the reduced proliferative capacity identified by Murphy et al can be overcome by using this growth factor.

The second molecular signal used was fibronectin, coated onto the PGA scaffolds in order to enhance adhesion of the OA BMSCs. Fibronectin has been previously shown to promote the adhesion of normal mesenchymal cells (40), and our experiments revealed the same effect on BMSCs derived from OA patients. The third molecular signal was TGF β 3. There is exten-

sive evidence indicating that growth factors of the TGF superfamily promote chondrogenesis in micromass pellet cultures of normal human or animal BMSCs (23–25,28), and our results showed that TGF β 3 is effective at driving chondrogenesis in BMSCs from OA patients. The fourth signal was 50 μ g/ml insulin, which was added to the tissue-engineering cultures 1 week after the start of differentiation by TGF β , to promote the formation of extracellular matrix by the differentiated cells (41).

Finally, as a fifth molecular signal, we investigated the use of PTHrP. Previous studies (23,27) have shown that the TGF β s promote the formation of hypertrophic chondrocytes, as shown by the up-regulation of type X collagen mRNA. It is also possible that dexamethasone, which was included in our culture medium, can contribute to hypertrophy. PTHrP is known to down-regulate the maturation of prehypertrophic chondrocytes in the growth plate (42), and we therefore considered it logical to investigate the effects of PTHrP in our tissue-engineering cultures. Not only did PTHrP down-regulate the early hypertrophic markers, it also enhanced the biochemical quality of our extracellular matrix, as shown by the down-regulation of type I collagen and the maintenance of both type II collagen and proteoglycan.

We were able to generate cartilage that was of the highest quality, comparable with that of cartilage generated using BNCs, which we have previously shown to be an excellent source of chondrocytes for cartilage engineering (34). However, this engineered cartilage had a lower collagen content than that found in natural tissue, a feature that is true of all cartilage engineered in vitro (18,19,34,41,43). Although there is growing evidence that even very immature cartilage constructs can mature into natural hyaline cartilage once implanted within the joint (44,45), it would nevertheless be preferable to engineer fully matured tissue in vitro prior to implantation. It is, at present, unclear whether such maturation can be achieved in vitro.

Our findings support and build on the work of other investigators who have shown that normal BMSCs can be used to generate chondrocytes (21,23–26,28). Murphy et al (29) described the poor capacity of OA BMSCs to proliferate and to form chondrocytes in micromass pellet cultures. We were able to overcome this reduced potential of the OA-derived cells by testing the range of molecular signals as described above and by the use of a PGA scaffold. Li et al (26) described the importance of using scaffolds to generate cartilage with a sufficient volume and mass to be implanted. However, in their studies, the histologic results suggested that the

cartilage quality was no better than that achieved using micromass pellet cultures. The reason that we were able to successfully generate constructs of enhanced quality is presumably because of the use of the specific molecular signals in conjunction with the use of a biomaterial scaffold.

Our conclusion that OA BMSCs can be used to generate relatively mature cartilage implants opens up the possibility of developing a cartilage therapy utilizing autologous stem cells. The use of autologous cells has several advantages. It avoids the risk of immune system rejection or the need for immunosuppression that would be required for donor cells. It also avoids the risk of disease transmission from donor to patient. There is currently intensive research into the use of embryonic stem cells (22,46) as well as other cells to generate chondrocytes. Albeit this is of scientific importance, it is currently unclear whether embryonic cell lines will ever be used in the clinical setting. Apart from the concerns of some patients regarding ethics, there is an inherent risk of teratoma formation as well as the potential for immune system rejection that must be managed (22).

Autologous stem cells provide an attractive option for patients and clinicians. However, it must also be recognized that autologous therapies are expensive, requiring growth of cells and tissue over several weeks in specialized ultraclean rooms. Therefore, it will be important to develop our tissue-engineering protocol so that it can be undertaken in the shortest possible time in order to reduce costs. We also need to develop methods of attaching the cartilage implants to the subchondral bone and of promoting integration of the implant with surrounding tissue. Despite these challenges, our findings represent a step forward in the development of an autologous cartilage replacement therapy.

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AUTHOR CONTRIBUTIONS

Dr. Hollander had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Dr. Kafienah, Mr. Mistry, and Dr. Hollander.

Acquisition of data. Dr. Kafienah, Mr. Mistry, Dr. Dickinson, Mr. Sims, and Drs. Learmonth and Hollander.

Analysis and interpretation of data. Dr. Kafienah, Mr. Mistry, Dr. Dickinson, Mr. Sims, and Drs. Learmonth and Hollander.

Manuscript preparation. Dr. Kafienah, Mr. Mistry, Dr. Dickinson, Mr. Sims, and Drs. Learmonth and Hollander.

Statistical analysis. Mr. Mistry and Dr. Hollander.

REFERENCES

- Poole AR. Cartilage in health and disease. In: Koopman WJ, editor. *Arthritis and allied conditions: a textbook of rheumatology*. 13th ed. Baltimore: Williams & Wilkins; 1997. p. 255–308.
- Hollander AP, Heathfield TF, Webber C, Iwata Y, Bourne R, Rorabeck C, et al. Increased damage to type II collagen in osteoarthritic articular cartilage detected by a new immunoassay. *J Clin Invest* 1994;93:1722–32.
- Hollander AP, Pidoux I, Reiner A, Rorabeck C, Bourne R, Poole AR. Damage to type II collagen in aging and osteoarthritis starts at the articular surface, originates around chondrocytes, and extends into the cartilage with progressive degeneration. *J Clin Invest* 1995;96:2859–69.
- Caterson B, Flannery CR, Hughes CE, Little CB. Mechanisms of proteoglycan metabolism that lead to cartilage destruction in the pathogenesis of arthritis. *Drugs Today (Barc)* 1999;35:397–402.
- Hunziker EB. Articular cartilage repair: are the intrinsic biological constraints undermining this process insuperable? *Osteoarthritis Cartilage* 1999;7:15–28.
- Steadman JR, Rodkey WG, Rodrigo JJ. Microfracture: surgical technique and rehabilitation to treat chondral defects. *Clin Orthop Relat Res* 2001;391 Suppl:S362–9.
- Cawston TE. Blocking cartilage destruction with metalloproteinase inhibitors: a valid therapeutic target? *Ann Rheum Dis* 1993; 52:769–70.
- Greenwald RA. Thirty-six years in the clinic without an MMP inhibitor: what hath collagenase wrought? *Ann N Y Acad Sci* 1999;878:413–9.
- Close DR. Matrix metalloproteinase inhibitors in rheumatic diseases. *Ann Rheum Dis* 2001;60 Suppl 3:iii62–7.
- Smith GD, Knutsen G, Richardson JB. A clinical review of cartilage repair techniques. *J Bone Joint Surg Br* 2005;87:445–9.
- Hangody L, Feczko P, Bartha L, Bodo G, Kish G. Mosaicplasty for the treatment of articular defects of the knee and ankle. *Clin Orthop Relat Res* 2001;391 Suppl:S328–36.
- Peterson L, Minas T, Brittberg M, Nilsson A, Sjogren-Jansson E, Lindahl A. Two- to 9-year outcome after autologous chondrocyte transplantation of the knee. *Clin Orthop Relat Res* 2000;374: 212–34.
- Minas T, Peterson L. Advanced techniques in autologous chondrocyte transplantation. *Clin Sports Med* 1999;18:13–44.
- Trattng S, Ba-Salamah A, Pinker K, Plank C, Vecsei V, Marlovits S. Matrix-based autologous chondrocyte implantation for cartilage repair: noninvasive monitoring by high-resolution magnetic resonance imaging. *Magn Reson Imaging* 2005;23:779–87.
- Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, et al. Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis Cartilage* 2006;14:13–29.
- Langer R, Vacanti JP. Tissue engineering. *Science* 1993;260: 920–6.
- Langer R. 1994 Whitaker lecture: polymers for drug delivery and tissue engineering. *Ann Biomed Eng* 1995;23:101–11.
- Freed LE, Hollander AP, Martin I, Barry JR, Langer R, Vunjak-Novakovic G. Chondrogenesis in a cell-polymer bioreactor system. *Exp Cell Res* 1998;240:58–65.
- Riesle J, Hollander AP, Langer R, Freed LE, Vunjak-Novakovic G. Collagen in tissue engineered cartilage; types, structure and crosslinks. *J Cell Biochem* 1998;71:313–27.
- Tallheden T, Bengtsson C, Brantsing C, Sjogren-Jansson E, Carlsson L, Peterson L, et al. Proliferation and differentiation potential

- of chondrocytes from osteoarthritic patients. *Arthritis Res Ther* 2005;7:R560–8.
21. Raghunath J, Salacinski HJ, Sales KM, Butler PE, Seifalian AM. Advancing cartilage tissue engineering: the application of stem cell technology. *Curr Opin Biotechnol* 2005;16:503–9.
 22. Vats A, Bielby RC, Tolley NS, Nerem R, Polak JM. Stem cells. *Lancet* 2005;366:592–602.
 23. Martin I, Shastri VP, Padera RF, Yang J, Mackay AJ, Langer R, et al. Selective differentiation of mammalian bone marrow stromal cells cultured on three-dimensional polymer foams. *J Biomed Mater Res* 2001;55:229–35.
 24. Yoo JU, Barthel TS, Nishimura K, Solchaga L, Caplan AI, Goldberg VM, et al. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J Bone Joint Surg Am* 1998;80:1745–57.
 25. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998;238:265–72.
 26. Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, et al. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. *Biomaterials* 2005;26:599–609.
 27. Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L, et al. Osteogenic activity of the fourteen types of human bone morphogenetic proteins. *J Bone Joint Surg Am* 2003;85-A:1544–52.
 28. Majumdar MK, Banks V, Peluso DP, Morris EA. Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells. *J Cell Physiol* 2000;185:98–106.
 29. Murphy JM, Dixon K, Beck S, Fabian D, Feldman A, Barry F. Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. *Arthritis Rheum* 2002;46:704–13.
 30. Kafienah W, Mistry S, Williams C, Hollander AP. Nucleostemin is a marker of proliferating stromal stem cells in adult human bone marrow. *Stem Cells* 2006;24:1113–20.
 31. Martin I, Muraglia A, Campanile G, Cancedda R, Quarto R. Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic precursors from human bone marrow. *Endocrinology* 1997;138:4456–62.
 32. Solchaga LA, Penick K, Porter JD, Goldberg VM, Caplan AI, Welter JF. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. *J Cell Physiol* 2005;203:398–409.
 33. Freed LE, Vunjak-Novakovic G, Biron RJ, Eagles DB, Lesnoy DC, Barlow SK, et al. Biodegradable polymer scaffolds for tissue engineering. *Biotechnology (NY)* 1994;12:689–93.
 34. Kafienah W, Jakob M, Demarteau O, Frazer A, Barker MD, Martin I, et al. Three dimensional tissue engineering of hyaline cartilage: comparison of adult nasal and articular chondrocytes. *Tissue Eng* 2002;8:817–26.
 35. Stephens M, Kwan AP, Bayliss MT, Archer CW. Human articular surface chondrocytes initiate alkaline phosphatase and type X collagen synthesis in suspension culture. *J Cell Sci* 1992;103:1111–6.
 36. Dickinson SC, Sims TJ, Pittarello L, Soranzo C, Pavesio A, Hollander AP. Quantitative outcome measures of cartilage repair in patients treated by tissue engineering. *Tissue Eng* 2005;11:277–87.
 37. Handley CJ, Buttle DJ. Assay of proteoglycan degradation. *Methods Enzymol* 1995;248:47–58.
 38. Yarram SJ, Tasman C, Gidley J, Clare M, Sandy JR, Mansell JP. Epidermal growth factor and calcitriol synergistically induce osteoblast maturation. *Mol Cell Endocrinol* 2004;220:9–20.
 39. Raff T, van der Giet M, Endemann D, Wiederholt T, Paul M. Design and testing of β -actin primers for RT-PCR that do not co-amplify processed pseudogenes. *Biotechniques* 1997;23:456–60.
 40. Cool SM, Nurcombe V. Substrate induction of osteogenesis from marrow-derived mesenchymal precursors. *Stem Cells Dev* 2005;14:632–42.
 41. Blunk T, Sieminski AL, Gooch KJ, Courter DL, Hollander AP, Nahir AM, et al. Differential effects of growth factors on tissue-engineered cartilage. *Tissue Eng* 2002;8:73–84.
 42. 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(Pt 21):2691–701.
 43. Marijnissen WJ, van Osch GJ, Aigner J, Verwoerd-Verhoef HL, Verhaar JA. Tissue-engineered cartilage using serially passaged articular chondrocytes: chondrocytes in alginate, combined in vivo with a synthetic (E210) or biologic biodegradable carrier (DBM). *Biomaterials* 2000;21:571–80.
 44. Hollander AP, Dickinson SC, Sims TJ, Soranzo C, Pavesio A. Quantitative analysis of repair tissue biopsies following chondrocyte implantation. *Novartis Found Symp* 2003;249:218–29.
 45. Hollander AP, Dickinson SC, Sims TJ, Brun P, Cortivo R, Kon E, et al. Maturation of tissue engineered cartilage implanted in injured and osteoarthritic human knees. *Tissue Eng* 2006;12:1787–98.
 46. Vats A, Bielby RC, Tolley N, Dickinson SC, Boccacini AR, Hollander AP, et al. Chondrogenic differentiation of human embryonic stem cells: the effect of the micro-environment. *Tissue Eng* 2006;12:1687–97.