
An improved collagen scaffold for skeletal regeneration

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Abstract: Bone repair and regeneration is one of the most extensively studied areas in the field of tissue engineering. All of the current tissue engineering approaches to create bone focus on intramembranous ossification, ignoring the other mechanism of bone formation, endochondral ossification. We propose to create a transient cartilage template *in vitro*, which could serve as an intermediate for bone formation by the endochondral mechanism once implanted *in vivo*. The goals of the study are (1) to prepare and characterize type I collagen sponges as a scaffold for the cartilage template, and (2) to establish a method of culturing chondrocytes in type I collagen sponges and induce cell maturation. Collagen sponges were generated from a 1% solution of type I collagen using a freeze/dry technique followed by UV light crosslinking. Chondrocytes isolated from two locations in chick embryo sterna were cultured in these sponges and treated with retinoic acid to induce chondrocyte maturation

and extracellular matrix deposition. Material strength testing as well as microscopic and biochemical analyzes were conducted to evaluate the properties of sponges and cell behavior during the culture period. We found that our collagen sponges presented improved stiffness and supported chondrocyte attachment and proliferation. Cells underwent maturation, depositing an abundant extracellular matrix throughout the scaffold, expressing high levels of type X collagen, type I collagen and alkaline phosphatase. These results demonstrate that we have created a transient cartilage template with potential to direct endochondral bone formation after implantation. © 2010 Wiley Periodicals, Inc. *J Biomed Mater Res* 94A: 371–379, 2010

Key words: chondrocytes; type I collagen; type X collagen; alkaline phosphatase; tissue engineering; endochondral ossification

INTRODUCTION

Bone and cartilage regeneration are two important areas of intense research effort worldwide.¹ Primarily, those studies focus on regenerative approaches for bone and cartilage defects^{2–5} and secondarily they try to understand the molecular mechanisms responsible for degenerative processes of the skeletal system such as osteoarthritis.^{6,7}

Cartilage regeneration approaches initially attempted to use the patients own chondrocytes.^{1,8} These chondrocytes were harvest, allowed to proliferate

in vitro and finally implanted into the defects. This strategy not only involved two surgeries, but allowed only a small number of cells to be harvested, requiring *in vitro* expansion/proliferation for long periods of time before implantation.² To overcome these difficulties, many studies using stem cells have been conducted over the last years.^{1,9–11} The bone regeneration field faces similar difficulties, having explored the use of osteoblasts like cells, stem cells and growth factors or their combination, to create osteoinductible scaffolds.^{12–14}

Both bone or cartilage regeneration approaches require a biocompatible material as a scaffold to support cells proliferation as well as to withstand mechanical solicitations. Ceramic materials were for several years the main scaffolds used in bone regeneration. However their low biodegradability, high hardness and high modulus lead researchers to study other alternatives such as polymers.³ Natural polymers, such as chitosan, alginate and collagen present great solutions and their use has been growing

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exponentially.^{15–17} Since bone is mainly a combination of organic and inorganic compounds, studies attempted to recreate its structure by using scaffolds prepared from a combination of polymers and ceramics.^{17,18}

Among natural polymers, collagen is the most abundant protein in mammals. It provides structural and mechanical support to tissues and organs,¹⁹ and fulfill biomechanical functions in bone, cartilage, skin, tendon, and ligament. Collagen scaffolds have been used in numerous medical applications: drug delivery, hemostatic pads, skin substitutes, soft tissue augmentation, suturing and as tissue engineering substrate.^{20–23} Collagen scaffolds are processed in a variety of forms.²⁴ Thin sheets and gels are substrates for smooth muscle,^{25–27} renal,²⁸ hepatic,²⁹ endothelial,²⁷ and epithelial cells,³⁰ while sponges are often used to engineer skeletal tissues such as cartilage,^{31,32} tendon^{33,34} and bone.^{35,36} Collagen is biodegradable and has low or negligible antigenicity.^{37,38}

Forms of collagen type I, commonly extracted from bovine tendon, are biocompatible and adequate scaffolds for tissue engineering in terms of mechanical properties, pore structure, permeability, hydrophilicity and *in vivo* stability.³⁹ Several immunological studies (animal models) of injectable collagen gels and implanted collagen sponges, confirm little or no antibodies to collagen type I are detected.^{40,41} Collagen type I has been shown to support osteoblast, osteoclast, and chondrocyte attachment, proliferation, and differentiation *in vitro* as well as *in vivo*.^{31,35,36,42,43}

To the best of our knowledge, collagen scaffolds were never used as support for chondrocytes maturation to obtain a transient cartilage structure for endochondral bone formation. As opposed to intramembranous ossification, where bone is formed by differentiation of mesenchymal cells into osteoblasts, endochondral ossification requires an intermediate stage, where a transient cartilage model of the future bone is gradually converted into the final bone structure. This pathway is responsible for formation of most of the bones in our bodies.

Exploring the endochondral pathway for bone formation presents significant advantages such as of the high rate of chondrocyte proliferation, these cells resistance to low oxygen^{44,45} and the ability of chondrocyte to induce angiogenesis and osteogenesis.^{46,47} In our previous studies we used chitosan scaffolds and calcium phosphate materials^{48,49} to prepare transient cartilage templates for bone formation. Indeed, endochondral ossification was induced as early as one month after scaffold implantation *in vivo*.⁵⁰

While collagen presents better biocompatibility and biodegradability than other polymers, in terms of mechanical properties, polymers such as chitosan provide stronger and stiffer scaffolds⁴⁸ even after crosslinking of collagen.⁵¹ In this project we decided

to use collagen type I as a scaffold for a transient cartilage template for bone regeneration because of its ability to support both chondrocytes and osteoblasts proliferation and differentiation. We reasoned that since transient cartilage templates mineralize the extracellular matrix, our approach will combine collagen excellent biocompatibility with improved osteoinductive and mechanical properties, provided by chondrocyte's signals and the abundant mineralized extracellular matrix.

MATERIALS AND METHODS

Bovine collagen gel (2%), prepared as described in Yost et al.⁵² was diluted to a 1% solution with 0.012 N HCl, centrifuged to remove air, transferred to a 24 well plate again centrifuged for an even surface, kept overnight -80°C , and freeze dried (Virtis Freezemobile-12EL, Gardiner, NY) to obtain a porous structure. These sponges were crosslinked under UV light ($120\ \mu\text{W}/\text{cm}^2$), and neutralized with distilled water four times (30 min each) then Dulbecco's Modified Eagle Medium (DMEM) (Cellgro, Mediatech, Fairlawn, NJ) twice (20 min each).

Uniaxial load at a constant strain rate of 0.1 mm/s was applied with a 30 mm diameter cylindrical probe on six fully hydrated sponges (diameter 15.0 ± 0.5 mm, thickness 2.9 ± 0.2 mm), (Texture Analyser (TA-XT2i, Stable Micro Systems, Surrey, UK). Force and displacement and "stress versus strain" curves plotted. Both compression strength and elastic modulus were determined on linear area of curves (Fig. 1) for 30% of deformation, as per Mow et al.⁵³ Compression strength was obtained directly from curves "stress versus strain" [Fig. 1(A)] while elastic modulus was computed using the linear equation of trend-lines at 30% of deformation [Fig. 1(B)].

Chondrocytes were isolated from two locations in the sternum of 14 day chick embryos using method of Iwamoto et al.⁵⁴ The cephalic (CP) sternal chondrocytes (upper region of sternum) behave like transient cartilage cells and can undergo hypertrophy and induce bone formation, while caudal (CD) sternal chondrocytes (lower region of sternum) behave as permanent cartilage cells, do not undergo hypertrophy, and therefore were used as control.⁴⁸ Freshly isolated chondrocytes were plated in 100 mm tissue culture dishes for 7 days in 10 mL complete medium [DMEM containing 10% Nu serum (Fisher scientific, Fairlawn, NJ), 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin (Sigma, St Louis, MO)]. After one week, 200,000 chondrocytes in 10 μL media were seeded on each collagen type I sponge in a 96 well plate, and incubated 2 h, before adding 200 μL of complete medium carefully not to disturb the sponge and cells. Cultures were maintained at 37°C , 5% CO_2 atmosphere. Media changed every day for 5 days before treatment with 100 nM all trans-retinoic acid and 50 $\mu\text{g}/\text{mL}$ of ascorbic acid (every day for another 5 days), to induce chondrocyte maturation/hypertrophy and matrix synthesis. These specimens were then used for testing and imaging.

For scanning electron microscopy (SEM) collagen scaffolds, alone or with chondrocytes, were washed with phos-

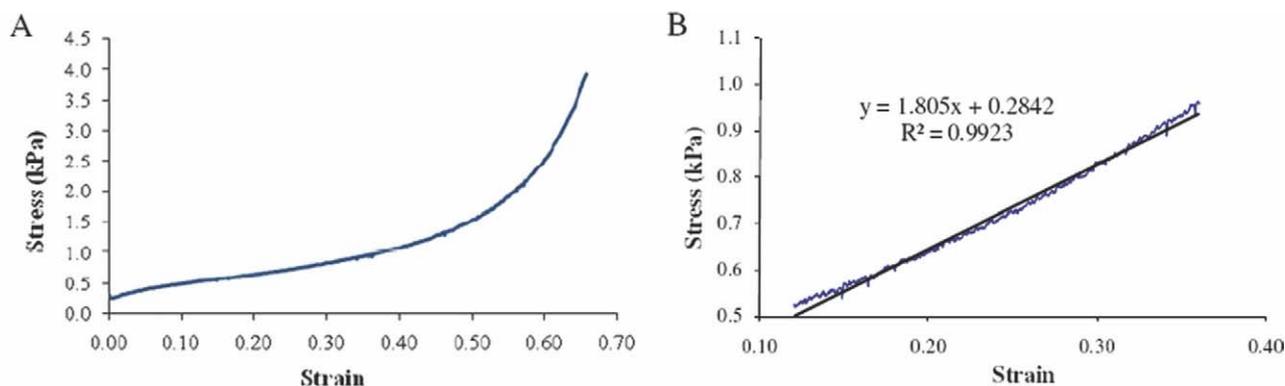


Figure 1. Stress versus strain curves of hydrated collagen type I sponges. Sponges deformation was evaluated in compression tests. Stress and strain was calculated from the recorded values of force and displacement. Image A shows whole compression curve while image B shows the linear equation and the trend-line of a restrict area close to 30% of deformation.

phate buffered saline, fixed (2% glutaraldehyde in 0.1 M sodium cacodylate HCl buffer with 0.1 M sucrose, pH 7.2) overnight at 4°C, dehydrated in ethanol series at room temperature, and critical point dried (Denton Vacuum, Cherry hill, NJ). Samples were mounted, sputter coated with gold palladium (Emitech K-650), and viewed on SEM (Hitachi S-3500 N, Schaumburg, IL) using secondary electron emission at 10 kv.

For immunohistochemistry, samples were embedded in paraffin and 5 μ m sections cut. After deparaffinization and rehydration, immunostaining was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and antibodies against chick alkaline phosphatase and collagen type X. Sections were counterstained with Alcian blue (1% alcian blue in 3% acetic acid), and light green, mounted, and scanned on Scan Scope GL optical microscope (Aperio, Bristol, UK) at 10X.

Cell supernatant for DNA and protein content, and alkaline phosphatase activity was extracted from four sets of two sponges with CP and CD chondrocytes by collection in 150 μ L 0.1% Triton-X (Fisher Scientific, Fairlawn, NJ), homogenized in eppendorf tubes (on ice, 1 hour), and scaffolds crushed to extract cells and proteins. Cell extract centrifuged at 3000 rpm, 2 minutes.

DNA measurements were performed as in Teixeira et al.⁵⁵ using a bisBenzidazole dye (Hoechst 33,258 dye, Polyscience, Northampton, UK). Fluorescence was measured by spectrofluorometer (Beckman Coulter DU-640) at wavelengths of 365 and 460 nm. A standard curve using calf thymus DNA (Sigma-Aldrich, St Louis, MO) and regression analysis was used to calculate DNA amount.

Protein measurements were performed using DC Protein Assay (BioRad, Hercules, CA). A spectrophotometer (Beckman Coulter DU-640, Fullerton, CA) was used to measure at an absorbance of 750 nm. A standard curve of bovine serum albumin and regression analysis were used to calculate protein content in the samples.

Alkaline phosphatase (AP) activity was measured as in Leboy et al.⁵⁶ Quantification of AP activity used a colorimetric assay in which *p*-Nitrophenyl phosphate is hydrolyzed into *p*-Nitrophenol and inorganic phosphate, forming a yellow complex measured at 400–420 nm. AP activity was expressed as nmol of product/minute/mg protein; 1 absorbance unit change = 64 nmol product.

RNA was isolated from scaffolds at day 5 and 10 (5 days of RA treatment) for PCR. Total RNA was extracted using Trizol[®] reagent (Life Technologies, Gaithersburg, MD) per manufacturer's instructions with modifications. Briefly, samples were liquid nitrogen frozen and crushed, Trizol[®] reagent added, vortexed 30 sec, then kept at 4°C for 2 h. 0.2 volume chloroform added for 15 min, then solution centrifuged at 12,000 g, 30 min, at 4°C for phase separation. The aqueous phase containing the RNA was collected, mixed with high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl and isopropanol) and centrifuged at maximum speed for 30 min at 4°C. RNA was purified using RNA micro kit (Qiagen, Chatsworth, CA) according to RNeasy cleanup protocol. Real-time RT-PCR was performed using QuantiTect SYBR Green RT-PCR kit (Qiagen), a DNA Engine Optican2 system (Roche Molecular Systems, Pleasanton, CA), and primers specific for chick genes: type X collagen (forward: AGTGCTGTCATTGATCTCATTGGA, reverse: TCAGAGGAATAGAGACCATGGATT), alkaline phosphatase (forward: CCTGACATCGAGGTGATCCT, reverse: GAGACCCAGCAGGAAGTCCA), type I collagen (forward: GCCGTGACCTCAGACTTAGC, reverse: TTTTGTCCTGGGGTCTTG). Acidic ribosomal protein (RP) mRNA was used as a reference for quantification (forward: AACATGTTGAACATCTCCCC, reverse: ATCTGCAGACAGACGCTGGC). Primers were purchased from Qiagen (Valencia, CA). Results were presented as "fold change" in gene expression and calculated using the threshold cycle (Ct) and the formula below, where "CD" refers to CD chondrocytes, "CP" refers to CP chondrocytes, and "RP" refers to the acidic ribosomal protein: $x = 2^{\Delta\Delta Ct}$, in which $\Delta\Delta Ct = \Delta E - \Delta C$, and $\Delta E = Ct_{CP} - Ct_{RP}$, and $\Delta C = Ct_{CD} - Ct_{RP}$. A $\Delta\Delta Ct < 0$ was considered a decrease while a $\Delta\Delta Ct > 0$ was considered an increase in gene expression.

For statistical analysis, all experiments were repeated 3–4 times and the mean and standard deviation were determined. Significant differences between test groups and controls were assessed by ANOVA. Significance was set at $p < 0.05$.

RESULTS

Scanning electron microscopy was performed in different cross sections to evaluate the porosity.

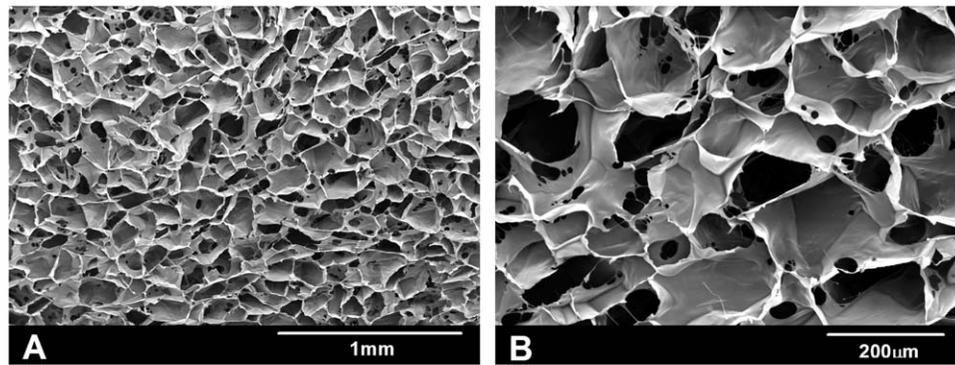


Figure 2. Scanning electron microscopy of collagen type I sponges. Photomicrographs of cross sections of collagen sponges are shown. Image A shows homogenous size and distribution of pores and image B shows the good interconnectivity between the pores.

There is a homogeneous distribution of pores throughout the sponges (Fig. 2). The average pore size is $150 \pm 25 \mu\text{m}$. Compression tests (Fig. 1) showed that strength of sponges reached $0.79 \pm 0.04 \text{ kPa}$ and the elastic modulus was $1.80 \pm 0.13 \text{ kPa}$.

Scanning electron microscopy analyses of collagen sponges cultured with either CP or CD sternal chondrocytes (Fig. 3) reveal that cells attached and proliferated deep into the scaffold, filling most of the pores with extracellular matrix after only 5 days in culture. At the end of 10 days in culture (day 5 of maturation treatment), the pores were completely packed with cells encased in a compact extracellular matrix. No difference in matrix amount for CP or CD chondrocytes was seen. Measurement of DNA content in sponges cultured for 5 and 10 days, con-

firmed extensive cell proliferation in the scaffolds (Fig. 4).

To evaluate chondrocyte maturation in response to ascorbic and retinoic acid, we performed immunohistochemical staining for alkaline phosphatase and collagen type X, important markers of hypertrophic phenotype.^{57,58} The CP chondrocytes produced higher levels of AP [Fig. 5(B)] and collagen type X [Fig. 5(D)], visualized by dark brown staining. Furthermore, measured AP activity increased significantly in CP chondrocytes in response to retinoic acid and ascorbic acid treatment (Fig. 6). In contrast, CD chondrocytes did not respond to maturation agents, and AP enzymatic activity remained low. The 10-fold difference in AP activity between CP and CD chondrocytes at day 10 ($6.9 \pm 1 \text{ nmol/min/mg}$

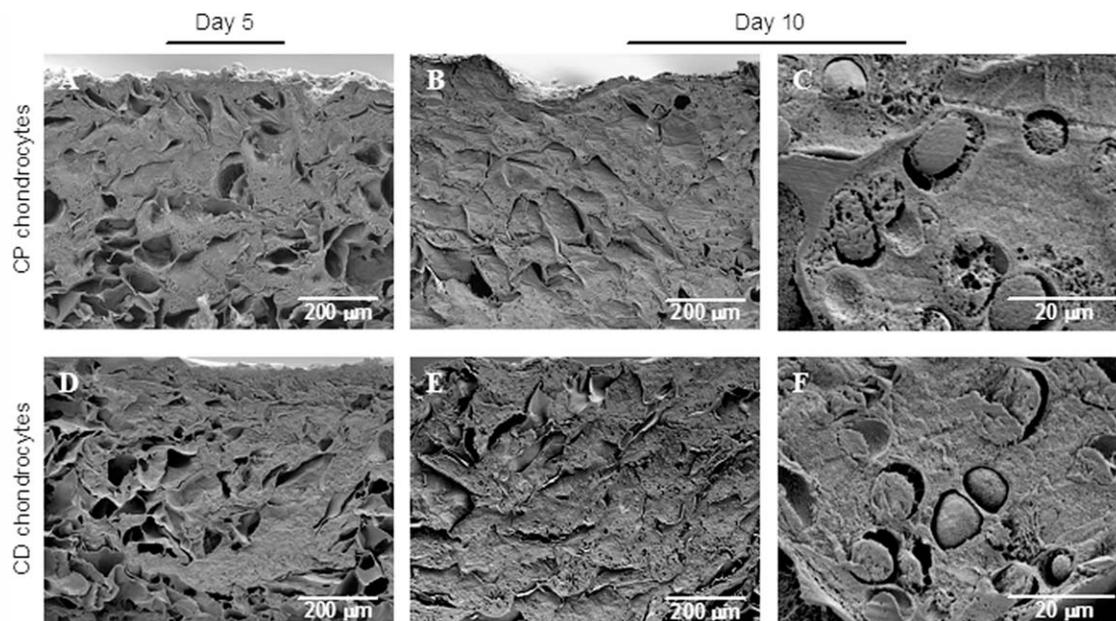


Figure 3. Scanning electron microscopy of collagen sponges cultured with chondrocytes. CP and CD chondrocytes were grown on collagen sponges for 5 days and then treated with retinoic acid for the next 5 days to induce maturation. CP chondrocyte cultures, A (day 5), B and C (day 10). CD chondrocyte cultures, D (day 5), E and F (day 10).

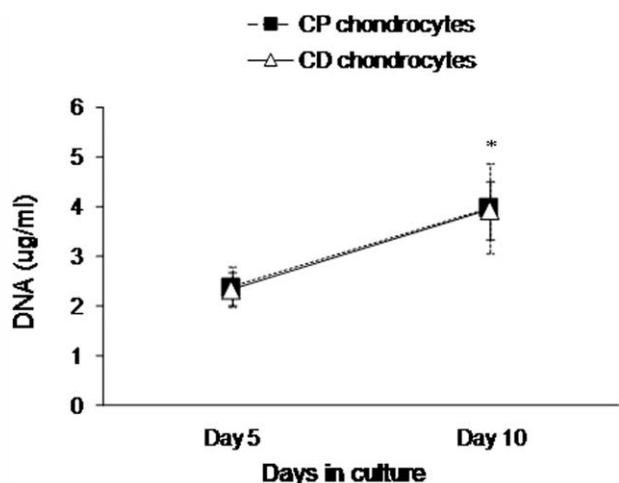


Figure 4. Rapid proliferation of chondrocytes on collagen sponges. CP and CD chondrocytes were grown on collagen sponge for 5 days and then treated with retinoic acid for the next 5 days to induce chondrocyte maturation. DNA was measured after 5 and 10 days in culture. *Significant difference from day 5 for both CP and CD chondrocytes.

vs. 60 ± 12 nmol/min/mg) highlights these different chondrocyte phenotypes.

To further investigate the differences in phenotype, we studied the gene expression profile at the

end of the culture period by RT-PCR. A value higher than 1, corresponds to a higher gene expression level in CP chondrocytes when compared to CD cells. Except for collagen type II (no change) all genes studied were expressed at higher levels in CP chondrocytes than in CD chondrocytes. As seen in Figure 7, collagen type X (early hypertrophy marker), and collagen type I (late hypertrophy marker) expression was significantly higher in CP cells. While AP gene expression was higher for CP chondrocytes, it was not statistically significant.

DISCUSSION

The collagen sponges we developed are ~20 times stiffer than those reported in the literature (≈ 0.1 kPa), comparable only to collagen sponges incorporating 40% of poly(glycolic acid) fibers (≈ 2.5 kPa).⁵¹ While we did not measure pore connectivity, our results show that proliferating chondrocytes reached the interior of the scaffold and initiated extensive intracellular matrix deposition in only 5 days. Our sponges maintained their shape/stability, while providing adequate porosity for cells to fully migrate, and supporting cell phenotype and correct differentiation. Therefore, these scaffolds successfully meet

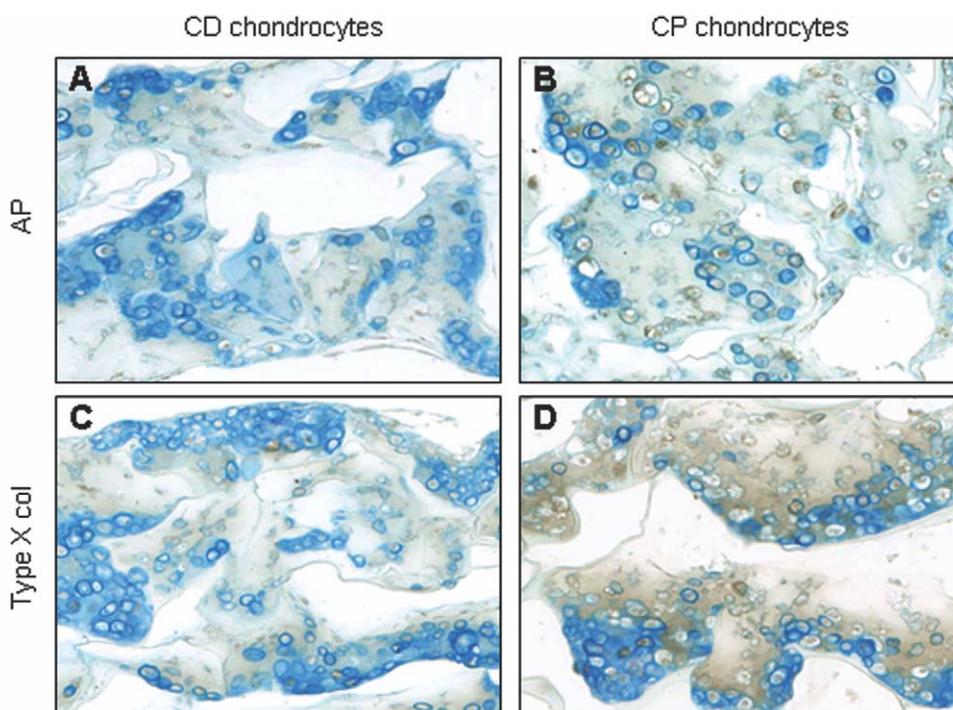


Figure 5. Immunohistochemical staining collagen sponges cultured with chondrocytes. Histological cross sections of sponges collected after 10 days in culture were immunostained with antibodies against alkaline phosphatase (A,B), and type X collagen (C,D). A and C are cross sectional views of the sponges with CD chondrocytes, while B and D are sponges with CP chondrocytes. AP and type X collagen are evidenced by the presence of brown color. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

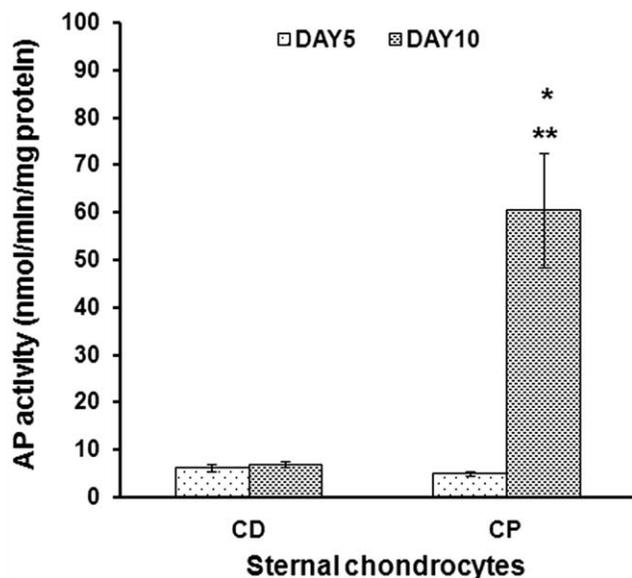


Figure 6. Retinoic acid treatment increases AP activity in CP chondrocytes. CP and CD chondrocytes were grown on collagen sponges for 5 days and then treated with retinoic acid for the next 5 days to induce chondrocyte maturation. Graph shows AP activity levels, measured spectrophotometrically, and normalized for total protein content in the samples. *Significant difference from CP chondrocytes at day 5. **Significant difference from CD chondrocytes day 10.

the major challenges in tissue engineering, unlike previous studies. Takahiro et al.³² cultured articular chondrocytes in bovine collagen type I sponges for 4 weeks, resulting in uncompleted cell penetration into the scaffold (as seen in histological sections) and loss of the cartilage phenotype (decreased collagen type II expression). A study by Chen et al.⁵⁹ using a polymer/collagen type I hybrid sponge to culture bovine articular chondrocytes, showed that while supporting chondrocyte proliferation and differentiation *in vitro*, the sponges collapsed and lost shape, *in vivo*.

But collagen type I has mostly been used as a scaffold for bone formation with bone marrow stromal cells or differentiated osteoblasts. Mizuno et al.⁴² cultured bone marrow stromal cells on different collagen gels for 20 days before subcutaneous implantation in nude mice for 4 weeks. These cells differentiated into osteoblasts only in collagen type I gel, suggesting that it alone offers a suitable environment for the induction of osteoblastic phenotype *in vitro* and osteogenesis *in vivo*.³⁸ In a study by Domaschke et al.³⁵ osteoblasts and osteoclasts were cocultured on a mineralized collagen Type I membrane. Both cell types attached and proliferated, maintained phenotype (evaluated by RT-PCR), and completely covered the membrane within 2 weeks. Rodrigues et al.³⁶ using human osteoblasts cultured in mineralized collagen Type I gel showed similar results. These studies used collagen membrane or gel that

had to be mineralized for improved mechanical properties. Collagen sponges can provide a better porous structure and 3D platform for bone deposition and blood vessel ingrowth. Indeed, our sponges proved stronger than others, and did not need mineralization for improved stability.

All these previous studies support the use of collagen Type I as a scaffold to create a transient cartilage template, sustaining both chondrocyte and osteoblast proliferation and differentiation. Our results confirm that chondrocytes attach, proliferate and mature on a collagen Type I scaffold, maintaining their characteristic ovoid shape in the matrix lacunae. We induced chondrocyte hypertrophy by treatment with retinoic and ascorbic acid, which resulted in an increase in mRNA levels of hypertrophy markers (AP and collagen Type X). We also observed an increase in the expression of Type I collagen by hypertrophic cells. The expression of genes characteristic of the osteoblast phenotype by growth plate chondrocytes has previously been reported,⁶⁰ typically including the expression of high levels of alka-

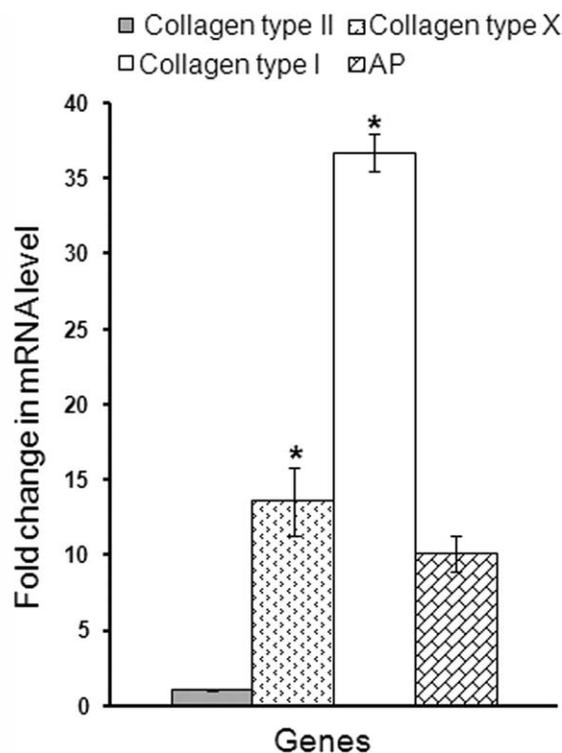


Figure 7. Gene expression profile of upper sternal chondrocytes when compared to lower sternal chondrocytes. CP and CD chondrocytes were grown to confluence for 5 days and then treated with retinoic acid for additional 5 days. mRNA was extracted at the end of the culture period (10 days) from CP and CD chondrocytes. RT-PCR was performed using primers specific for chick genes. Expression levels are presented as "fold change" in mRNA levels in CP chondrocytes in relation to CD chondrocytes. *Significantly different from CD chondrocytes.

line phosphatase, collagen Type I, and several non collagenous matrix proteins enriched in bone (osteonectin, osteopontin, bone sialoprotein and osteocalcin).^{60–66} Both *in vivo* and *in vitro* studies provide evidence that the hypertrophic chondrocyte may undergo further differentiation and express bone cell markers,^{61,63} suggesting a role for chondrocytes at the initial stages of endochondral bone deposition.

Previous bone engineering approaches focused on bone formation via intramembranous ossification (when using bone marrow stromal cells) or direct osteoblast activity, and most cartilage engineering approaches aim to regenerate permanent cartilage,^{31,32,59,67} however our objective is to create a transient cartilage as an improved osteoinductive template for endochondral ossification. Our study used hypertrophic chondrocytes that are well adapted to low oxygen tension, resisting hypoxic conditions like the ones most likely created by a large mass of cells.^{44,45} In addition, chondrocytes can induce osteogenic differentiation of mesenchymal stem cells.⁶⁸ Hypertrophic chondrocytes also secrete vascular endothelial growth factor, that has been shown to induce vascularization, osteoblast migration and differentiation, and osteoclast survival and resorption activity.^{46,47} All these mechanisms are required during the process of endochondral bone formation. Therefore, we believe that our approach to bone tissue engineering, takes advantage of this effective chondrocyte signaling mechanism to develop an osteoinductive scaffold. Our work using chitosan scaffolds shows that hypertrophic chondrocytes efficiently induce extensive bone formation once implanted *in vivo*.⁵⁰

In conclusion, while current research aims at clarifying some of the factors and signaling pathways controlling bone formation, approaches such as ours that provide a reservoir of differentiation factors in the form of a transient cartilage template have great potential for bone regeneration and tissue engineering.

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