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# Effects of Auricular Chondrocyte Expansion on Neocartilage Formation in Photocrosslinked Hyaluronic Acid Networks*

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## ABSTRACT

**The overall objective of this study was to examine the effects of in vitro expansion on neocartilage formation by auricular chondrocytes photoencapsulated in a hyaluronic acid (HA) hydrogel as a next step toward the clinical application of tissue engineering therapies for treatment of damaged cartilage. Swine auricular chondrocytes were encapsulated either directly after isolation ($p = 0$), or after further *in vitro* expansion ($p = 1$ and $p = 2$) in a 2 wt%, 50-kDa HA hydrogel and implanted subcutaneously in the dorsum of nude mice. After 12 weeks, constructs were explanted for mechanical testing and biochemical and immunohistochemical analysis and compared to controls of HA gels alone and native cartilage. The compressive equilibrium moduli of the $p = 0$ and $p = 1$ constructs ($51.2 \pm 8.0$ and $72.5 \pm 35.2$ kPa, respectively) were greater than the $p = 2$ constructs ($26.8 \pm 14.9$ kPa) and the control HA gel alone ($12.3 \pm 1.3$ kPa) and comparable to auricular cartilage ($35.1 \pm 12.2$ kPa). Biochemical analysis showed a general decrease in glycosaminoglycan (GAG), collagen, and elastin content with chondrocyte passage, though no significant differences were found between the $p = 0$ and $p = 1$ constructs for any of the analyses. Histological staining showed intense and uniform staining for aggrecan, as well as greater type II collagen versus type I collagen staining in all constructs. Overall, this study illustrates that constructs with the $p = 0$ and $p = 1$ auricular chondrocytes produced neocartilage tissue that resembled native auricular cartilage after 12 weeks *in vivo*. However, these results indicate that further expansion of the chondrocytes ($p = 2$) can lead to compromised tissue properties.**

## INTRODUCTION

As the need for cartilage repair becomes a growing problem in today's society, a wide range of materials and techniques for cartilage repair and regeneration are being developed.[1,2] Tissue engineering, in which cells are combined with a biocompatible, biodegradable scaffold to provide a suitable environment for tissue regeneration, may prove to be an ideal technique to repair damaged cartilage. One obstacle that cartilage tissue engineering faces is the development of a suitable environment for cell encapsulation that promotes the retention of the chondrogenic phenotype, the production of extracellular matrix (ECM), and the integration of scaffold and native tissue.

In previous studies, Elisseeff *et al.*[3] established photopolymerization of a hydrogel system as an efficient method to encapsulate chondrocytes, demonstrating chondrocyte survival, uniform cell distribution, and ECM protein production within poly(ethylene oxide)-based hydrogels. Recently, Nettles *et al.*[4] demonstrated that HA hydrogels provided a suitable environment for articular chondrocytes, where retention of a chondrogenic phenotype and the

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*Portions of this work were performed at the University of Pennsylvania (polymer synthesis, sample analysis) and portions were performed at the Massachusetts General Hospital (implantation in mice).

production of ECM were observed. Photopolymerization may be advantageous for clinical applications because defects can be filled directly with the liquid prepolymer solution containing cells and polymerized *in vivo*, providing filling of irregular defects and good contact between the hydrogel and surrounding cartilage tissue.

However, before reaching clinical feasibility, other challenges such as finding an efficient cell source and developing techniques to expand the cells while maintaining a chondrogenic phenotype need to be addressed.[5] To this effect, auricular cartilage proves to be a promising cell source for cartilage regeneration for applications in plastic surgery and potentially for articular surface repair. Auricular chondrocytes are easily harvested with little donor-site morbidity and can be obtained at yields twice as high as articular chondrocytes[6] and proliferate approximately 4 times faster than articular chondrocytes when grown in monolayer culture.[7] In addition, when implanted *in vivo* on 3-dimensional scaffolds, primary auricular chondrocytes have been shown to express high levels of type II collagen and glycosaminoglycans (GAGs).[6] Furthermore, auricular chondrocytes have been successfully encapsulated in a variety of materials such as poly(glycolic acid),[8,9] alginate,[10] chitosan,[11] and Pluronic F127,[12] and have been shown to produce extracellular matrix and form neocartilage. In a study by Xu *et al.,*[13] auricular chondrocytes encapsulated in fibrin polymer exhibited the highest equilibrium modulus compared to those encapsulated with articular and costal chondrocytes. In addition, we showed, in a previous study, that auricular chondrocytes that were photoencapsulated in hyaluronic acid (HA) hydrogels could produce neocartilage *in vivo* after the optimization of hydrogel properties.[14]

However, because of the large number of chondrocytes that would be needed to repair a clinically relevant cartilage defect, expansion of isolated chondrocytes may be necessary. Unfortunately, for rapid expansion in monolayer culture, chondrocytes isolated from both articular and auricular cartilage have been shown to dedifferentiate, losing their chondrogenic phenotype.[15,16] Originally rounded in shape, chondrocytes flatten and take on a more fibroblastic phenotype with *in vitro* expansion.[16,17] In addition, when chondrocytes are removed from their ECM environment, a decrease in type II collagen and an increase in type I collagen are seen,[18] leading to a mechanically inferior fibrocartilage tissue. Although dedifferentiation seems inevitable in monolayer culture, some studies have shown slower dedifferentiation, stabilization of the differentiated phenotype, or even redifferentiation (i.e., return to a chondrocytic phenotype after dedifferentiation) when chondrocytes are cultured under conditions such as in liquid suspension,[19] agarose,[15] alginate,[20] or methacrylated HA hydrogels.[4] In our previous work, we also showed retention of the chondrogenic phenotype by auricular chondrocytes when photoencapsulated in 2 wt%, 50-kDa HA hydrogels, which exhibited continued GAG and type II collagen production.[14]

The overall objective of this study was to examine the effects of *in vitro* expansion of auricular chondrocytes on neocartilage formation in a previously optimized HA hydrogel. This work will also allow more insight into the potential use of auricular chondrocytes as a cell source for cartilage regeneration. To accomplish this, initially isolated ($p = 0$) and expanded ($p = 1$ and $p = 2$) swine auricular chondrocytes were photoencapsulated in a HA hydrogel; implanted subcutaneously in nude mice for 12 weeks; and explanted for mechanical, biochemical, and immunohistological analysis with comparisons to controls of the HA gel alone and native cartilage tissue.

## MATERIALS AND METHODS

### *Macromer synthesis and polymerization*

Methacrylated HA (MeHA) was synthesized by the addition of methacrylic anhydride (Sigma) to a solution of 1 wt% HA (Lifecore, molecular mass = 50 kDa) in deionized water, adjusted to pH 8 with 5 N NaOH, and reacted on ice for 24 h, as previously reported.[21,22] The macromer solution was purified via dialysis (molecular mass cutoff, 5–8 kDa) against deionized water for a minimum of 48 h with repeated changes of water. The final product was obtained by lyophilization and stored at –20°C in powder form before use. The macromer was sterilized via a germicidal lamp in a laminar flow hood for 30 min and dissolved in a sterile solution of phosphate-buffered saline (PBS) containing 0.05 wt% 2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959, I2959) for cell encapsulation.

### *Chondrocyte isolation, expansion, and photoencapsulation*

Cartilage tissue was harvested in a sterile fashion from the ears (auricular) and the knees (articular) of 3- to 6-month-old swine that were euthanized with an overdose of pentobarbital (100 mg/kg IV). The harvested auricular cartilage was cut into $\sim$1 $mm^3$ pieces, washed in PBS, and digested overnight at 37°C in Ham's F-12 medium containing 0.1% collagenase (Worthington). Digested tissue was passed through a 100-μm filter and centrifuged to obtain a chondrocyte pellet. Chondrocytes were washed twice with PBS, counted via a hemacytometer, and determined viable using the trypan blue exclusion dye test before encapsulation and plating. Chondrocytes ($40\times10^6$ cells/mL) were photoencapsulated in hydrogel networks by suspension in a 2 wt% macromer (MeHA) solution containing 0.05 wt% I2959. The solution was pipetted into sterile molds (50 μL volume) and polymerized with $\sim$4 mW/$cm^2$ ultraviolet light for 10 min using a long-wave ultraviolet lamp (Model 100AP, Blak-Ray). Remaining chondrocytes were plated in T-150 flasks at a seeding density of $1\times10^6$ cells/150 $cm^2$ ($\sim$6700 chondrocytes/$cm^2$) for expansion in Ham's F-12 culture medium containing 10% fetal bovine

serum (FBS), 1% penicillin–streptomycin, and 1% non-essential amino acids. After reaching ~90% confluency, chondrocytes were trypsinized and photoencapsulated as stated earlier ($p = 1$) or replated at $1 \times 10^6$ cells/150 cm$^2$, trypsinized, and photoencapsulated after reaching ~90% confluency again ($p = 2$). Constructs were placed in culture media and implanted within 2 h of gelation.

### *Implantation in nude mice*

Nude mice were anesthetized with ketamine (80 mg/kg) and xylazine (12 mg/kg). A 2-cm midline incision was made on the dorsum of each mouse and 5 subcutaneous pockets were made using blunt dissection. One chondrocyte/hydrogel construct was placed in each of these pockets and the wound was closed with sterile stainless steel skin clips. After 12 weeks, mice were euthanized and constructs were harvested for analysis. NIH guidelines for the care and use of laboratory animals (NIH Publication 85-23 Rev. 1985) were observed.

### *Mechanical testing*

Samples ($n = 5$) were cored with a 3/16 inch diameter punch and weighed (wet wt). Cored samples were mechanically tested in confined compression in a PBS bath. For complete confinement, samples were initially loaded in creep to a tare load of 5 g until reaching equilibrium (defined as less than 10 μm of change in 10 min) before undergoing stress relaxation. Stress relaxation was carried out by applying a ramped displacement to 10% strain, and then the sample was allowed to relax to equilibrium (defined as less than 0.5 g of change in 10 min). The equilibrium confined compression modulus ($H_A$) for each sample was calculated by dividing the equilibrium load by the area loaded and the applied strain.

### *Biochemical analysis*

For biochemical analysis ($n = 5$), mechanically tested samples were lyophilized, weighed (dry weight), and digested in a proteinase K solution (200 μg/mL of proteinase K (Roche), 100 mM ammonium acetate, pH 7.0) overnight at 60°C. Proteinase K was then inactivated at 100°C for 5 min. Total DNA, GAG, and collagen contents were determined using the PicoGreen dsDNA Assay,[23] the dimethylmethylene blue dye method[24] with chondroitin sulfate as a standard, and the hydroxyproline assay[25] using a collagen to hydroxyproline ratio of 7.25,[26,27] respectively. Values reported for DNA, GAG, and collagen content were normalized to the sample wet weight. Elastin content was measured using the Fastin Elastin Assay (Accurate Chemical & Scientific Corp)[28] with an α-elastin solution as a standard. Briefly stated, 100 μL of the sample digest solution was combined with 200 μL of 90% ammonium sulfate and 1 mL of Fastin dye reagent to form the elastin–dye complex. Contents were reacted for 1 h and centrifuged to pellet the complex. The pellet was solubilized with the Fastin dissociation reagent and the absorbance was measured at a wavelength of 513 nm. The proteinase K digestion solution was used as a negative control for the hydroxyproline and elastin assays.

### *Histological analysis*

For histological analysis, constructs were fixed in 10% formalin for 24 h, embedded in paraffin, and processed using standard histological procedures. The histological sections (7 μm thick) were stained for chondroitin sulfate and collagen distributions using the Vectastain ABC kit (Vector Labs) and the DAB Substrate kit for peroxidase (Vector Labs). Sections were predigested in 0.5 mg/mL of hyaluronidase for 30 min at 37°C and incubated in 0.5 N acetic acid for 4 h at 4°C to swell the samples prior to overnight incubation with primary antibodies at dilutions of 1:100, 1:200, and 1:3 for chondroitin sulfate (mouse monoclonal anti-chondroitin sulfate, Sigma-Aldrich), and type I (mouse monoclonal anti-collagen type 1, Sigma-Aldrich) and type II collagen antibodies (mouse monoclonal anti-collagen type II, Developmental Studies Hybridoma Bank), respectively. The same procedure was used for nonimmune controls, but without primary antibody incubation.

### *Statistical analysis*

ANOVA with Tukey's *post hoc* test was used to determine significant differences among groups, with $p < 0.05$. All values are reported as the mean ± the standard deviation.

## RESULTS

To determine the effects of auricular chondrocyte expansion on neocartilage formation in photopolymerizable HA hydrogels, swine auricular chondrocytes isolated directly from cartilage or expanded *in vitro* were encapsulated and implanted in the dorsum of nude mice for 12 weeks. The specific hydrogel composition used (2 wt%, 50 kDa MeHA) was previously optimized for chondrocyte encapsulation and neocartilage formation.[14] Constructs were explanted, mechanically tested, analyzed, and compared to controls of the HA gel alone and native auricular and articular cartilage. A schematic of this process is shown in Fig. 1. Macroscopically, the explants were white and opaque and resembled native cartilage tissue. The $p = 0$ and $p = 1$ constructs are noticeably larger (0.5 cm diameter when implanted) and more opaque than the $p = 2$ constructs (Fig. 2), which are slightly translucent.

### *Mechanical behavior*

Samples were tested in confined compression in a PBS bath to simulate a cartilage defect environment. The compressive equilibrium moduli were calculated by dividing the equilibrium load by the area loaded and the

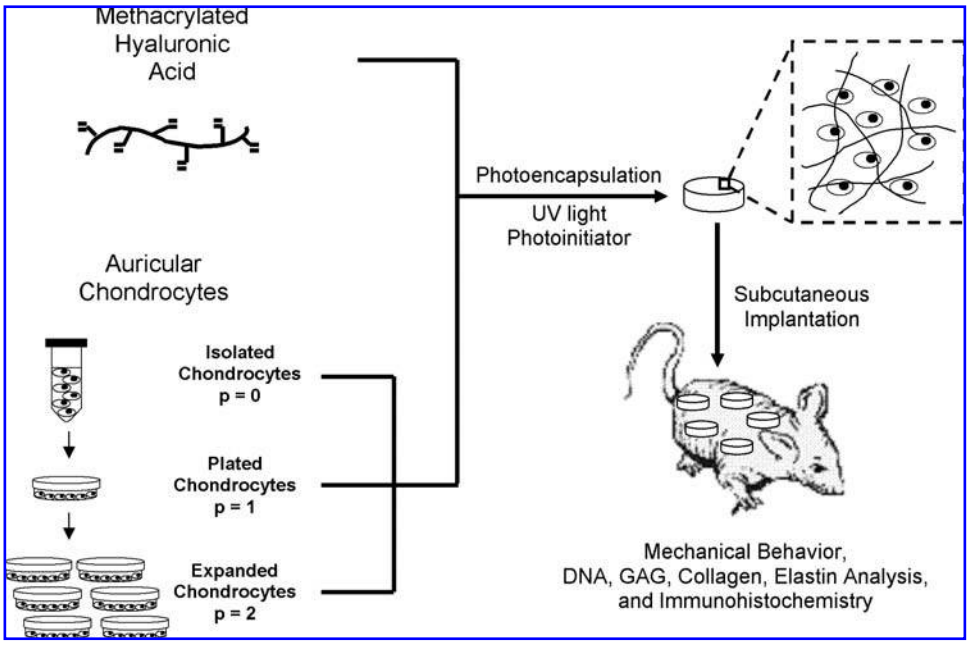


**FIG. 1.** General schematic of chondrocyte expansion, photoencapsulation, and subsequent analysis.

applied strain and are reported in Fig. 3. The $p=0$ and $p=1$ engineered constructs ($51.2 \pm 8.0$ kPa and $72.5 \pm 35.2$ kPa, respectively) exhibited a significant increase in compressive equilibrium moduli from the HA gel ($12.3 \pm 1.3$ kPa), while no significant difference was detected for the $p=2$ constructs ($26.8 \pm 14.9$ kPa). Though the moduli of the engineered constructs were all significantly lower than the articular cartilage ($259.2 \pm 20.0$ kPa), they were all either higher than or not statistically different than that of native auricular cartilage ($35.1 \pm 12.2$ kPa).

### *Biochemical analysis*

The water content of the tissue engineered constructs and control samples were determined from wet and dry weights (Fig. 4). In general, constructs exhibited an increase in water content with passage number. All constructs exhibited a significantly lower water content when compared to the HA gel ($97.0 \pm 0.3\%$ water) and showed a significantly higher water content when compared to native auricular ($66.5 \pm 2.7\%$ water) and articular ($72.7 \pm 4.0\%$ water) cartilage. The $p=0$ constructs ($83.1 \pm 1.3\%$ water) were most comparable to native articular cartilage.

Total DNA content was determined using the dsPicoGreen assay and normalized to sample wet weight in Fig. 5. Minimal background fluorescence was detected for the HA gel and was determined to be insignificant when normalized to wet weight. In general, no significant differences were detected among sample groups, with DNA content ranging from $\sim 0.3$ to 0.5 pg DNA/μg wet weight. However, the $p=1$ constructs exhibited the lowest amount of DNA/wet weight among the engineered constructs.

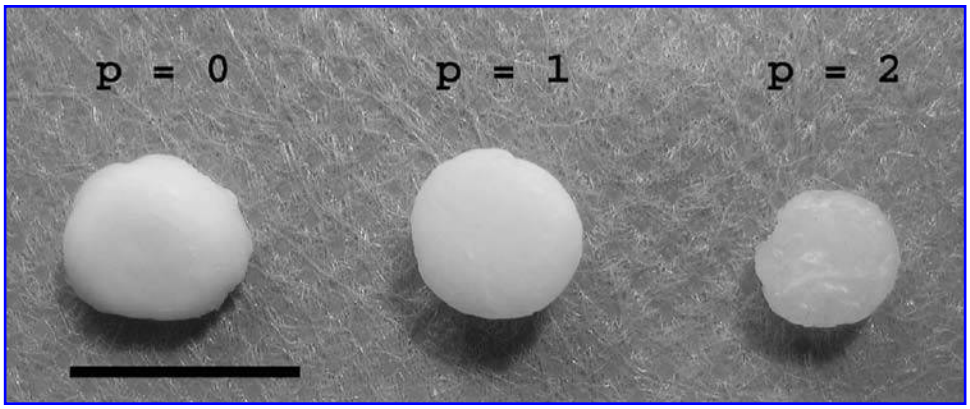


**FIG. 2.** Explanted HA constructs 12 weeks after subcutaneous implantation in nude mice. Scale bar = 1 cm.

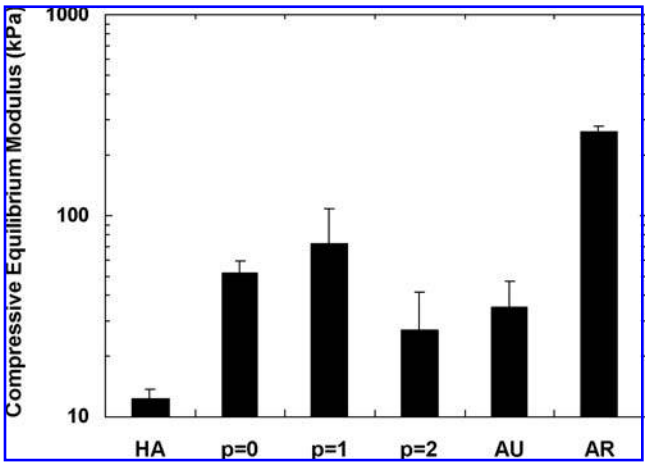


**FIG. 3.** Compressive equilibrium modulus of constructs after 12 weeks of subcutaneous implantation in nude mice compared to controls of the HA gel alone and native auricular and articular cartilage. The moduli of the $p=0$ and $p=1$ constructs are significantly greater than the HA gel and the average modulus of the $p=1$ constructs is significantly greater than both the $p=2$ constructs and auricular cartilage. In addition, the average modulus of the articular cartilage is significantly greater than in all other groups.

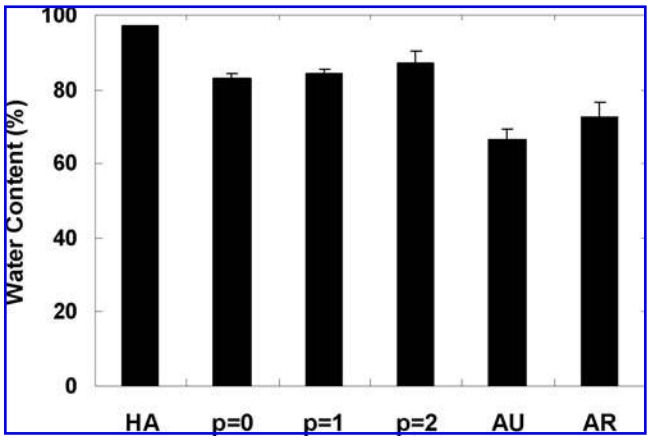


**FIG. 4.** Water content of constructs after 12 weeks of subcutaneous implantation in nude mice compared to controls of the HA gel alone and auricular and articular cartilage. The HA gel exhibits a significantly greater water content than all other groups. The water content also showed slight increases with auricular chondrocyte passage number, although no significant differences were measured. The water content of auricular and articular cartilage is significantly lower than in both the HA gel and the tissue engineered constructs.

In Fig. 6, GAG content is reported as the quantity of chondroitin sulfate normalized to sample wet weight. In general, constructs exhibited a decrease in GAG content with passage number, where $p=0$ and $p=1$ constructs ($0.049 \pm 0.009$ and $0.046 \pm 0.004$ μg CS/μg wet weight, respectively) are significantly greater than the $p=2$ constructs ($0.029 \pm 0.005$ μg CS/μg wet wt). When compared to native cartilage, the $p=0$ and $p=1$ constructs are ~75–80% and ~53–57% of the GAG content measured for auricular and articular cartilage, respectively. Articular cartilage was significantly greater than all engineered constructs, but there was no significant difference detected in GAG content between auricular cartilage and the $p=0$ constructs. Minimal GAG content was detected for the HA gels.

Total collagen content, normalized to wet weight and reported in Fig. 7, exhibited similar trends to those observed for GAG content, with a general decrease in collagen observed with passage number. The collagen content of the $p=0$ constructs ($0.051 \pm 0.017$ μg collagen/μg wet wt) was most comparable to native cartilage (i.e., ~57% and ~50% of measured collagen content for auricular and articular cartilage, respectively). Again, the control HA gels showed minimal levels with this assay.

As a final measure of construct biochemical levels, elastin was quantified to determine if the implanted auricular chondrocytes still produced elastin after isolation and expansion. In general, elastin content decreased with passage number, with a significant decrease from $p=1$ to $p=2$ (Fig. 8). However, no significant difference for $p=0$ ($2.7 \pm 0.4$ pg elastin/μg wet weight) or $p=1$ constructs ($2.5 \pm 0.5$ pg elastin/μg wet weight) was observed when compared to auricular cartilage ($2.9 \pm 0.5$ pg elastin/μg wet weight). Lower levels of elastin were detected in articular cartilage and $p=2$ constructs ($0.3 \pm 0.4$ and $1.1 \pm 0.7$ pg elastin/μg wet weight, respectively), which were significantly lower than elastin found in the $p=0$ and $p=1$ constructs and auricular cartilage. No elastin was detected in the HA gels.

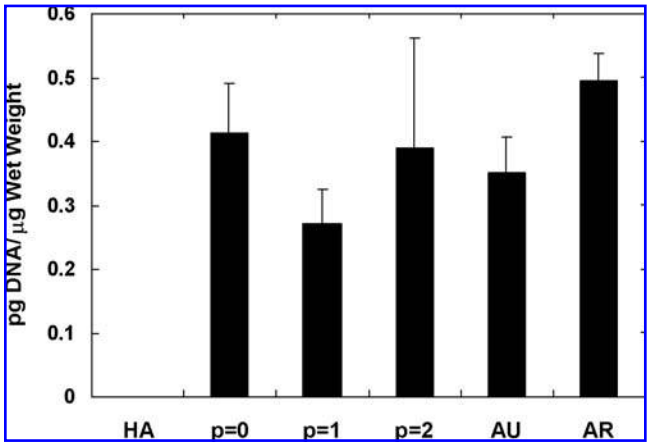


**FIG. 5.** DNA content normalized to wet weight for constructs after 12 weeks of subcutaneous implantation in nude mice compared to controls of the HA gel alone and native auricular and articular cartilage. No significant differences were detected among groups with the exception of $p=1$ constructs vs. articular cartilage. The HA gel showed insignificant DNA measurements and thus, exhibited no interference with the fluorescent assay.

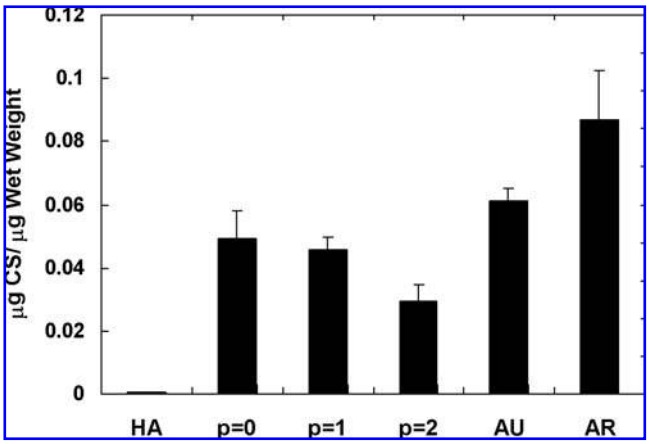


**FIG. 6.** Glycosaminoglycan content of samples normalized to construct wet weight after 12 weeks of subcutaneous implantation in nude mice compared to controls of the HA gel alone and native auricular and articular cartilage. Articular cartilage has significantly greater GAG content than all other groups while no significant difference was detected between auricular cartilage and the $p=0$ constructs. In the engineered constructs, the GAG content generally decreased with chondrocyte passage, with the GAG content of the $p=0$ and $p=1$ constructs significantly greater than that of the $p=2$ constructs. The GAG content detected in the HA gels was minimal and statistically lower than all constructs and native cartilage.

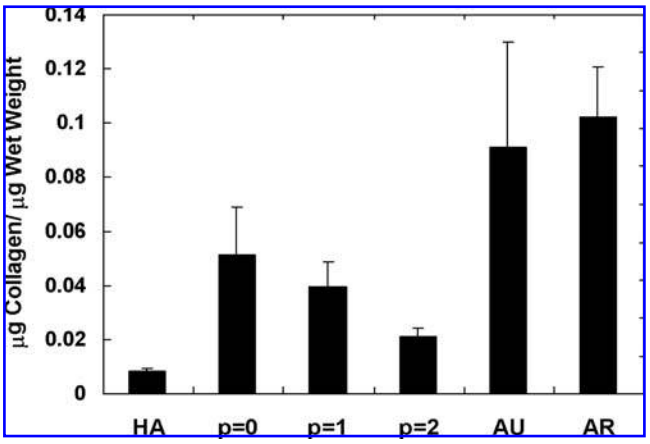


**FIG. 7.** Collagen content of constructs normalized to construct wet weight after 12 weeks of subcutaneous implantation in nude mice compared to controls of the HA gel alone and native auricular and articular cartilage. Collagen content in articular cartilage is significantly greater than in the HA gel and all engineered constructs. No significant difference was found between auricular cartilage and the $p=0$ constructs, but there is significantly more collagen in auricular cartilage than the HA gel and the $p=1$ and $p=2$ constructs. In general, the collagen content decreased with chondrocyte passage.

### *Immunohistochemical analysis*

Representative stains for chondroitin sulfate, type I collagen, and type II collagen are shown in Fig. 9 with nonimmune controls for comparison. In general, histological sections illustrate the morphology and distribution of the auricular chondrocytes and the distribution of extracellular components. Chondroitin sulfate is evenly distributed throughout all constructs with similar intensity. Though little to no type I collagen staining was detected, type II collagen staining was detected in all constructs, where it was most intense and most widely distributed for the $p=0$ constructs. Overall, aggrecan is more evenly distributed throughout the constructs than collagen. The nonimmune controls exhibited no background staining of the constructs.

## DISCUSSION

One of the major obstacles for cartilage tissue engineering is finding a cell source and adequate cell numbers for delivery to a defect. For this study, auricular chondrocytes were chosen for their ease of harvest, high yield, and rapid proliferation.[6] Our previous work screened various HA macromer concentrations and molecular weights for their ability to support neocartilage formation and we found that hydrogels fabricated from 2 wt% of a 50-kDa HA macromer most resembled the properties of native cartilage and showed the greatest promise for cartilage regeneration. In this study, we examined the effects of auricular chondrocyte expansion on the final properties of engineered cartilage in photopolymerizable HA hydrogels. Directly isolated ($p=0$) and expanded ($p=1$ and $p=2$) auricular chondrocytes were photoencapsulated in HA gels, cultured

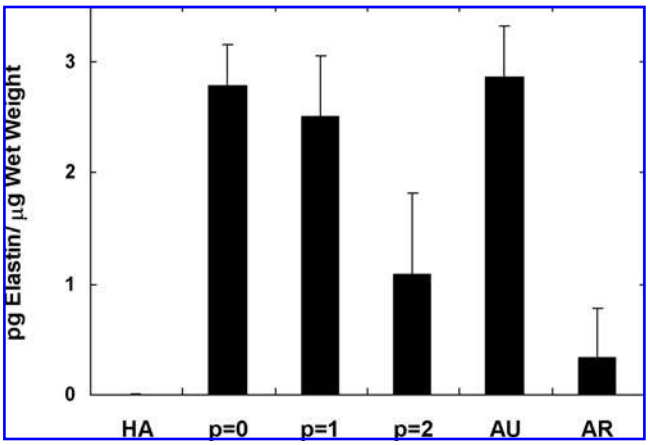


**FIG. 8.** Elastin content of constructs normalized to construct wet weight after 12 weeks of subcutaneous implantation in nude mice compared to controls of the HA gel alone and native auricular and articular cartilage. There was no significant difference between auricular cartilage when compared to either the $p=0$ or $p=1$ constructs. Minimal elastin was found in articular cartilage and the elastin content in the $p=2$ constructs was significantly lower than that in the $p=0$ and $p=1$ constructs and auricular cartilage. No elastin was detected in the HA gels.

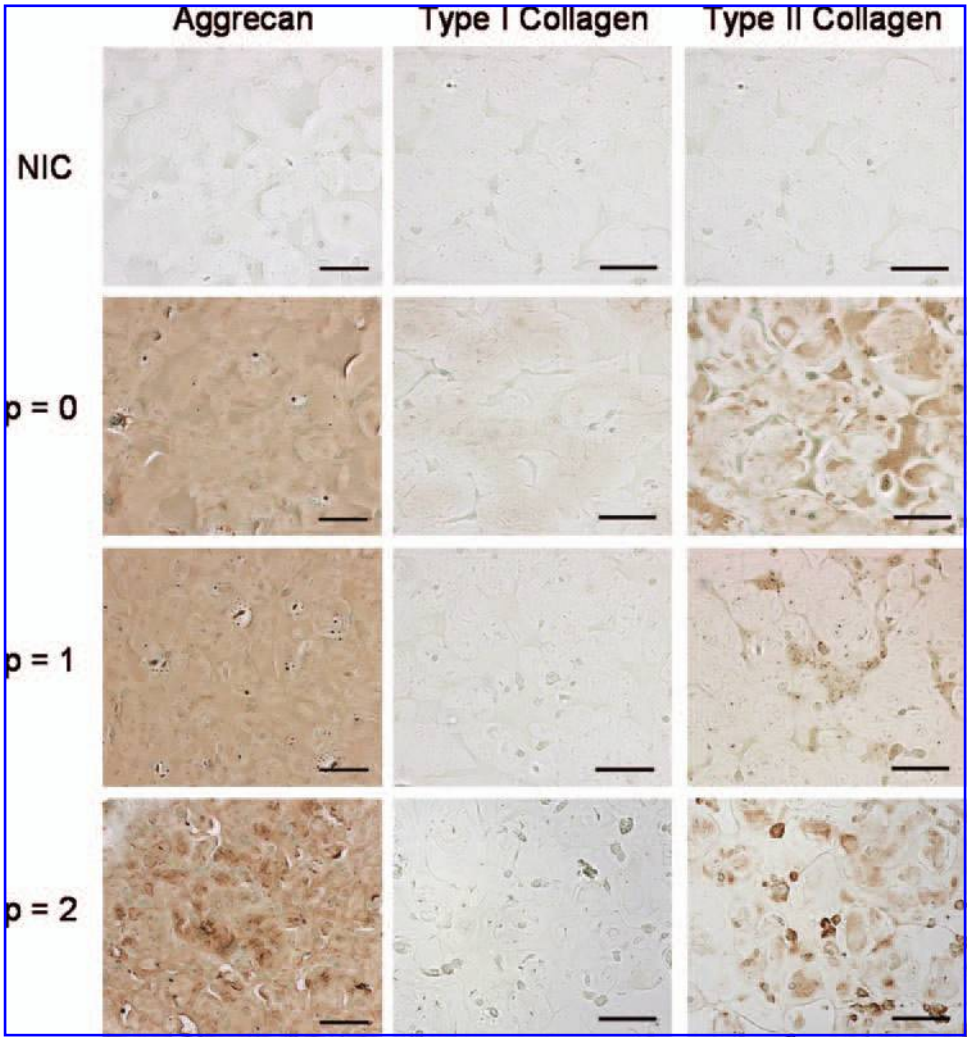


**FIG. 9.** Histological sections of constructs stained for chondroitin sulfate and type I and type II collagen compared to nonimmune controls (NIC), with no primary antibody, after 12 weeks of subcutaneous implantation in nude mice. Chondroitin sulfate is evenly distributed throughout all constructs with similar intensity, regardless of passage number. All constructs exhibited greater type II collagen staining vs. type I collagen, where the $p = 0$ constructs exhibit the greatest intensity and distribution of type II collagen. Scale bar = 100 μm. Color images available online at www.liebertpub.com/ten.

subcutaneously in the dorsum of nude mice for 12 weeks, and explanted for testing.

Macroscopic observations of the explants showed an increase in size and opaqueness of all constructs since initial implantation, regardless of expansion. The $p = 0$ and $p = 1$ constructs were larger and more opaque than the $p = 2$ constructs, potentially indicating more ECM production and neocartilage formation. The testing protocol used in this analysis is similar to previously used protocols for measuring the equilibrium modulus of hydrogel systems[29] and native cartilage.[30] Our mechanical testing results show an increase in the moduli of the $p = 0$ and $p = 1$ constructs over the HA hydrogel alone. This expected increase in compressive equilibrium modulus is indicative of neocartilage formation (i.e., chondrocyte proliferation and ECM deposition). However, the significant difference between all engineered constructs and articular cartilage may limit the application of these hydrogels to load bearing joints. It is important to note that the equilibrium moduli reported here for native cartilage is lower than the aggregate moduli reported previously for auricular[10] and articular[30] cartilage. These differences may be attributed to the specific conditions of the testing system (e.g., percent total strain) or testing method (e.g., indentation). Even though comparison among literature may be difficult, within our paradigm, relative comparisons can be made between our hydrogel constructs and native cartilage. Further, the equilibrium moduli we obtained for articular cartilage is comparable to that determined by Strauss *et al.*,[31] using a similar protocol.

As expected, the water content of all constructs was significantly lower than the control HA gel because of the

production of ECM molecules, which replaced water within the construct during the 12-week culture. Biochemical analysis of GAG and collagen content exhibited similar trends, with a general decrease observed with chondrocyte expansion. All engineered constructs exhibited a significantly higher GAG content than the control HA gels, indicating neocartilage formation, while GAG content significantly decreased from $p = 1$ to $p = 2$, indicating potential dedifferentiation. Further, no significant difference was found between $p = 0$ constructs and auricular cartilage, but constructs with expanded chondrocytes exhibited significantly lower collagen content than that of native cartilage, again showing a potential loss of phenotype. The trend for elastin content was similar to results from the GAG content analysis. This observed trend is similar to findings by van Osch *et al.*,[6] which showed that as the cells are expanded 2-dimensionally *in vitro*, their phenotype changes and a decrease in elastin, a component of auricular cartilage, is seen. No significant differences were found between the $p = 0$ and $p = 1$ constructs when compared to each other and to auricular cartilage, indicating a retention of the auricular chondrocyte phenotype.

Immunohistochemical analysis was used to detect the distribution of aggrecan and type I and type II collagen within the constructs. The histological sections of all constructs showed intense, uniform staining for chondroitin sulfate, which reflected the even distribution of chondrocytes within the hydrogel. Compared to type II collagen staining, the more even distribution of chondroitin sulfate, a major component of aggrecan, may have resulted from a difference in molecule size, in which smaller chondroitin sulfate molecules were able to fill the voids of the hydrogel with greater ease. Histological observations indicate ECM production by encapsulated chondrocytes and are consistent with the results from the biochemical analysis.

Overall, this analysis shows that $p = 0$ and $p = 1$ auricular chondrocytes retain a more chondrogenic phenotype when photoencapsulated in a HA hydrogel for 12 weeks while possible changes in phenotype may occur after $p = 1$ and may compromise neocartilage formation *in vivo*. Although constructs were all mechanically inferior to articular cartilage, the $p = 0$ and $p = 1$ constructs showed comparable if not greater compressive moduli than auricular cartilage and show an increase in modulus over the HA gel. Even though biochemical content generally decreased with passage, significant decreases were only found after $p = 1$. Histologically, all constructs exhibited aggrecan and type II collagen staining, characteristic of native cartilage. These results show that $p = 0$ and $p = 1$ auricular chondrocytes produce cartilaginous tissue in a 2 wt%, 50-kDa HA hydrogel that is comparable to auricular cartilage, but are lower than values for articular cartilage. However, it is possible that mechanical stimuli or the introduction of growth factors could lead to the production of more hyaline-like cartilage (i.e., articular cartilage). Auricular chondrocytes may be a viable source of cells for cartilage regeneration, but extensive expansion of the cells 2-dimensionally *in vitro* must be limited to prevent compromised properties.

## ACKNOWLEDGMENTS

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