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Influence of 3D Hyaluronic Acid Microenvironments on Mesenchymal Stem Cell Chondrogenesis

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Abstract

Mesenchymal stem cells (MSCs) are multipotent progenitor cells whose plasticity and self-renewal capacity have generated significant interest for applications in tissue engineering. The objective of this study was to investigate MSC chondrogenesis in photocrosslinked hyaluronic acid (HA) hydrogels. Since HA is a native component of cartilage and MSCs may interact with HA via surface receptors, these hydrogels could influence stem cell differentiation. Both *in vitro* and *in vivo* cultures of MSC-laden HA hydrogels permitted chondrogenesis, measured by the early gene expression and production of cartilage specific matrix proteins. For *in vivo* culture, MSCs were encapsulated with and without TGF-β3, or pre-cultured for 2 weeks in chondrogenic media prior to implantation. All groups exhibited up-regulation of type II collagen, aggrecan, and sox 9 compared to MSCs at the time of encapsulation, and the addition of TGF-β3 enhanced expression of these genes. To assess the influence of scaffold chemistry on chondrogenesis, HA hydrogels were compared to relatively inert poly(ethylene glycol) (PEG) hydrogels, and showed enhanced expression of cartilage specific markers. Differences between HA and PEG hydrogels *in vivo* were most noticeable for MSCs and polymer alone, indicating that hydrogel chemistry influences the commitment of MSCs to undergo chondrogenesis (e.g., ~43-fold up-regulation of type II collagen of MSCs in HA over PEG hydrogels). Although this study only investigated early markers towards tissue regeneration, these results emphasize the importance of material cues in MSC differentiation microenvironments, potentially through material/cell receptor interactions.

Keywords

mesenchymal stem cells; hyaluronic acid; chondrogenesis; hydrogel

Introduction

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that have the ability to self-replicate and differentiate down multiple cell lineages when given the appropriate environmental cues[1]. Although they were first identified in bone marrow by Friedenstein and colleagues[2] in the 1970s, MSCs have since been isolated from various adult tissues and differentiated into several cell types, including osteoblasts, chondrocytes, and adipocytes[1,

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[3–5]. With their plasticity and self-renewal capacity, these cells have generated significant interest for applications in cell replacement therapies and tissue regeneration.

Particularly, MSCs have garnered interest as an alternative cell source for cartilage tissue engineering, since they can be isolated from adults via a bone marrow biopsy. To induce chondrogenic differentiation, MSCs are typically grown in pellet culture in the presence of transforming growth factor-βs (TGF- βs)[6–10], and differentiation is monitored by the production of cartilaginous matrix proteins such as sulfated glycosaminoglycans (GAGs) and type II collagen. In recent years, both natural and synthetic biomaterials have been used to create niches or microenvironments to control stem cell behavior and differentiation towards cartilage formation[11, 12]. These biomaterials serve as 3D scaffolds capable of enhancing and templating tissue formation through cell morphology and organization, intercellular interactions, mechanical forces, and the delivery of bioactive molecules[11, 13].

One molecule of particular interest is hyaluronic acid (HA), which is found natively in cartilage tissue. HA, a linear polysaccharide of alternating D-glucuronic acid and N-acetyl-D-glucosamine, functions as a core molecule for the binding of keratin sulfate and chondroitin sulfate in forming aggrecan[14]. It degrades enzymatically by hyaluronidases found in the body and through oxidative mechanisms to yield oligosaccharides and glucuronic acid. This natural polymer plays a role in cartilage homeostasis, is involved in cellular processes like cell proliferation, morphogenesis, inflammation, and wound repair[15–17], and can interact with cell surface receptors for HA[18] (e.g., CD44, CD54, and CD168). HA can be modified through its carboxyl and hydroxyl groups and subsequently crosslinked into hydrogels or made hydrophobic and processed into macroporous scaffolds. These modification strategies include: esterfication[19, 20], methacrylation[21, 22], and crosslinking with divinyl sulfone[23, 24] or dialdehyde[25].

To date, researchers have developed HA-based scaffolds in the form of hydrogels[26–30], sponges[31], and meshes[32]. These scaffolds are biocompatible and can serve as delivery vehicles for cells and bioactive molecules. Chondrocytes and MSCs have been successfully encapsulated in HA and HA composite hydrogels[22, 26, 33]. Liu *et al*, showed that MSC-laden HA-gelatin hydrogels were able to produce elastic, firm, translucent cartilage with zonal architecture in rabbit osteochondral defects[26]. Sponges and non-woven meshes made of hydrophobic HA ester derivatives (Hyaff®-7 and -11) seeded with MSCs and chondrocytes have been shown to support a chondrocyte phenotype and the production of cartilaginous extracellular matrix (ECM) proteins[31, 32, 34, 35]. In the clinical setting, Hyalograft®-C, a tissue-engineered graft composed of Hyaff®-11 scaffolds seeded with autologous chondrocytes, has been used to treat cartilage lesions in patients. Results from 2 to 5 year follow ups showed improved repair of cartilage lesions in 91.5% of patients compared to pre-operative assessments, and the repaired cartilage was hyaline-like in appearance[20]. Recently, a thiolated HA derivative has been successfully electrospun into a nanofibrous mesh with the potential to more closely mimic the size-scale of native ECM[36].

For this study, we utilized photocrosslinked HA hydrogels to investigate the chondrogenesis of MSCs in HA microenvironments. Previously, we optimized a methacrylated HA (MeHA) hydrogel system for the encapsulation of chondrocytes and characterized cytocompatibility, phenotype retention, and neocartilage formation within these hydrogels using both auricular and articular chondrocytes[30, 33, 37, 38]. However, inherent limitations to the use of chondrocytes (e.g., low cell yields and a tendency to dedifferentiate when expanded *in vitro*) have motivated the use of MSCs as an alternative cell source. MSCs are easily expanded *in vitro* without loss of differentiation potential and express CD44[39], one of the primary receptors for HA. Thus, we hypothesize that photocrosslinked MeHA hydrogels can provide a favorable niche for MSC chondrogenesis.

Materials and Methods

CD44 Staining and Flow Cytometry

To determine the presence of CD44 receptors, human MSCs (Lonza Walkersville, Inc.) were cultured in 2D on glass coverslips and fixed in accustain (Sigma) for immunofluorescent staining. Briefly, the cells were blocked with 5% fetal bovine serum (FBS), stained with primary antibody anti-CD44 clone A3D8 (4μg/ml, Sigma) for 2 hours, incubated with secondary antibody anti-mouse IgG (whole molecule) $F(ab')_2$ fragment-FITC (1:50 dilution) for 15 minutes, and counterstained with DAPI (2μg/ml) for nuclei visualization. In addition, MSCs were labeled with phycoerythrin (PE)-conjugated CD44 (0.25 μg/ml, eBioscience) monoclonal antibody for 1 hr on ice and analyzed by flow cytometry (Guava EasyCyte 3.10).

Macromer Syntheses

Methacrylated HA (MeHA) was synthesized as previously reported[21]. Briefly, methacrylic anhydride (Sigma) was added to a solution of 1 wt% HA (Lifecore, MW = 64 kDa) in deionized water, adjusted to a pH of 8 with 5 N NaOH, and reacted on ice for 24 hours. The macromer solution was purified via dialysis (MW cutoff 6–8k) against deionized water for a minimum of 48 hours with repeated changes of water. The final product was obtained by lyophilization and stored at −20°C in powder form prior to use. Poly(ethylene glycol)-diacrylate (PEGDA) was synthesized as previously reported[40]. Briefly, triethylamine (1.5 molar excess) was added to PEG-4600 (Sigma) dissolved in methylene chloride. Acryloyl chloride (1.5 molar excess) was added dropwise under nitrogen and reacted on ice for 6 hrs, followed by reaction at room temperature for 30 hours. The product was precipitated in ethyl ether, filtered, dried in a vacuum oven, redissolved in deionized water, dialyzed for 3 days, lyophilized, and stored at −20°C in powder form prior to use. The macromers were characterized by ^{1}H NMR (Bruker Advance 360 MHz, Bruker). Macromers were sterilized using a germicidal lamp in a laminar flow hood for 30 minutes 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 polymerization. Hydrogels were polymerized by injecting the macromer solution into a mold or between glass slides and exposing to ultraviolet light (Eiko, ~1.9 mW/cm^2) for 10 minutes.

Mechanical Characterization

Acellular hydrogels (n=5) with ~7mm diameter and ~1mm thickness were tested in unconfined compression with a Dynamic Mechanical Analyzer Q800 (DMAQ800, TA Instruments) in a PBS bath. Hydrogels were compressed at a rate of 10%/min until failure or until 70% of the initial thickness. The modulus was determined as the slope of the stress versus strain culture at low strains (<20%). The elastic modulus of PEG hydrogels was matched to 2wt% MeHA hydrogels by varying the macromer concentration (5–10 wt%).

MSC Photoencapsulation and Culture

Human MSCs were expanded to passage 4 in growth media consisting of α-MEM with 16.7% FBS and 1% penicillin/streptomycin. MSCs (20×10^6 cells/ml) were photoencapsulated in hydrogels by suspension in 2 wt% MeHA or 5.5 wt% PEG solutions with or without 200ng/ml TGF-β3 (R&D Systems). The cell/macromer solutions were pipetted into sterile molds (50 μl volume) and polymerized with ultraviolet light (Eiko, ~1.9 mW/cm^2) for 10 minutes.

To evaluate chondrogenesis, MSC-laden MeHA hydrogels were cultured *in vitro* in either growth media or DMEM supplemented with 1% penicillin/streptomycin, 1% ITS+, 1mM sodium pyruvate, 40mg/ml L-proline, 100nM dexamethasone, 50μg/ml ascorbic acid 2-phosphate, and 10ng/mL TGF-β3 (chondrogenic media). For *in vivo* culture, MSCs were

encapsulated in MeHA hydrogels with (HA+T3) and without (HA-MSCs) TGF-β3 and implanted in nude mice, or cultured *in vitro* in chondrogenic media for 2 weeks prior to implantation (HA-C). Nude mice were anesthetized with isoflurane, a 2 cm midline incision was made on the dorsum of each mouse, and 5 subcutaneous pockets were made using blunt dissection. One construct was placed in each pocket and the wound was closed with sterile stainless steel skin clips. Constructs were cultured *in vivo* for 2 weeks. NIH guidelines for the care and use of laboratory animals (NIH Publication #85–23 Rev. 1985) were observed. For scaffold comparison, MSCs were encapsulated in PEG hydrogels and cultured *in vitro* and *in vivo* in the same manner as the MeHA hydrogels.

Viability

The viability of MSCs in the MeHA and PEG hydrogels was assessed using a live/dead cytotoxicity kit (Molecular Probes) and an MTT assay (ATCC). Live/dead images were taken 1 and 24 hrs after encapsulation. Mitochondrial activity (n=3) was assessed after 7 and 14 days of *in vitro* culture. Briefly, 100μl of MTT reagent was added to 1ml of media and incubated for 4 hours. Samples were then removed from the media, homogenized in the detergent solution with a tissue grinder, incubated for 4 hrs, and read at an absorbance of 570nm in a Synergy HT™ (Bio-Tek Instruments) spectrophotometer.

Gene Expression Analysis

Samples (n=4) were homogenized in Trizol Reagent (Invitrogen) with a tissue grinder and RNA was extracted according to the manufacturer's instructions. RNA concentration was determined using an ND-1000 spectrophotometer (Nanodrop Technologies). One microgram of RNA from each sample was reverse transcribed into cDNA using reverse transcriptase (Superscript II, Invitrogen) and oligoDT (Invitrogen). Polymerase chain reaction (PCR) was performed on an Applied Biosystems 7300 Real-Time PCR system using a 25μl reaction volume for Taqman (5′-nuclease) reactions. Primers and probes specific for glyceraldehydes 3-phosphate dehydrogenase (GAPDH), type I and type II collagen, aggrecan, sox 9 and hyaluronidases (Hyal) 1, 2 and 3 are listed in Table 1. GAPDH was used as the housekeeping gene. Relative gene expression was calculated using the $\Delta\Delta C_T$ method, where fold difference was calculated using the expression $2^{-\Delta\Delta Ct}$.

Histological Analysis

For histological analysis, constructs were fixed in 10% formalin for 24 hours, embedded in paraffin, and processed using standard histological procedures. The histological sections (7 μm thick) were stained for aggrecan 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 hyaluronidase for 30 min at 37°C and incubated in 0.5 N acetic acid for 4 hours 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), and type I (mouse monoclonal anti-collagen type 1, Sigma) and type II collagen antibodies (mouse monoclonal anti-collagen type II, Developmental Studies Hybridoma Bank), respectively. Non-immune controls underwent the same procedure without primary antibody incubation.

TGF-β3 Release

TGF-β3 (10 ng/50 μl gel) was encapsulated in acellular MeHA hydrogels, and release of the growth factor was monitored via diffusion in PBS alone or in the presence of hyaluronidase (500U/mL) at 37°C on an orbital shaker. PBS and hyaluronidase solutions were changed every other day and aliquots were stored at −20°C until analysis with a TGF-β3 enzyme-linked immuno-adsorbent assay (ELISA) (R&D systems).

Statistical Analysis

All values are reported as the mean ± SEM. Student's t-test and Wilcoxon Sum-Rank test were used to determine significant differences among groups, with $p < 0.05$.

Results

For this study, we investigated the chondrogenesis of MSCs in photocrosslinked HA hydrogels. The chondrogenic differentiation of MSCs in HA hydrogels was monitored both *in vitro* and *in vivo*, where increases in the gene expression and production of cartilaginous matrix proteins were used as markers for chondrogenesis, as well as limited expression and production of type II collagen. In addition, benefits of potential cell/HA scaffold interactions were explored using short-term culture comparisons to a relatively inert PEG hydrogel system that would provide minimal direct interactions with encapsulated cells.

MSC interactions with HA

The potential for MSC/HA interactions was first assessed using immunofluorescent staining and flow cytometry (Figure 1A and 1B) for CD44. This HA receptor was present on 99.6% of the cell population and stained uniformly on MSCs cultured in 2D. Additionally, MSCs cultured in 2D expressed multiple isoforms of hyaluronidases (e.g., Hyal 1, Hyal 2, Hyal 3) (results not shown). When photoencapsulated in 3D HA hydrogels with the MeHA macromer, nearly all of the MSCs remained viable (>98%) as indicated by live/dead staining 6 hours after encapsulation (Figure 1C).

MSC Chondrogenesis

Chondrogenic differentiation was induced *in vitro* with the addition of TGF-β3 to cultures of MSCs in HA hydrogels. Comparisons between MSC-laden HA hydrogels cultured in growth and chondrogenic media (+TGF-β3) showed significant differences in gene expression at 3, 7, and 14 days of culture (Figure 2). Specifically, up-regulation of sox 9, type II collagen, and aggrecan was observed for constructs cultured in chondrogenic media over cultures in growth media at all time points. Importantly, except for aggrecan at day 3, an up-regulation of the chondrogenic genes (type II collagen, aggrecan, sox 9) was observed compared to initially encapsulated cells. Significant differences in hyaluronidase expression were also observed based on culture media for hyal 2 and 3 (Figure 3) at several time points. In addition, type I collagen was down regulated in both growth and chondrogenic media when compared to expression at the time of encapsulation. Histologically, increased deposition of type II collagen and chondroitin sulfate (CS) was observed for MSC-laden HA hydrogels cultured in chondrogenic media (Figure 4), where intense pericellular staining was observed after 14 days of culture. The cells remained rounded in all gels and no obvious spatial variations were observed between the perimeter of the gels and the central areas. Light staining for CS in hydrogels cultured in growth media and type I collagen in hydrogels cultured in chondrogenic media was also observed.

When cultured *in vivo*, MSCs in all groups (HA-MSC, HA+T3, HA-C) exhibited increased expression for all genes of interest (Figure 5) compared to cells at the time of encapsulation after 14 days of implantation. Without growth factors present, there were ~3000-, ~18-, and ~13-fold increases in type II collagen, aggrecan, and sox 9 gene expression, respectively. With the addition of TGF-β3 (HA+T3 group) and with 2 weeks of pre-culture in chondrogenic media (HA-C group), type II collagen increased ~17.5- and ~2370-fold and aggrecan increased ~3.7 and ~4.6-fold, respectively, compared to the HA-MSC group. In addition, *in vitro* assessment of TGF-β3 release in the HA+T3 groups indicated that there was TGF-3 remaining in the hydrogels after 2 weeks, and that release could be triggered by the addition of exogenous hyaluronidase (data not shown).

Comparison between HA and PEG hydrogels

To explore potential cell/HA scaffold interactions, HA hydrogel cultures were compared to a relatively inert PEG hydrogel in short-term *in vitro* and *in vivo* cultures. First, the elastic modulus of PEG hydrogels was matched to 2 wt% MeHA hydrogels by altering the PEGDA macromer concentration. A 5.5 wt% PEG formulation with a modulus of 13.3±1.0 kPa was found to be comparable (i.e., no statistical differences between moduli) to the 2 wt% MeHA hydrogels (13.0±1.4 kPa) and was used for all comparison studies to minimize mechanical influences on cellular differentiation (Figure 6A). In addition, live/dead staining and an MTT assay (Figure 6B, 6C) demonstrated that viable MSCs were successfully encapsulated in both hydrogel systems and there were no statistical differences in cell viability between hydrogel types.

With both *in vitro* and *in vivo* cultures, the gene expression of encapsulated MSCs differed depending on the hydrogel chemistry. With *in vitro* culture, type II collagen expression by MSCs in MeHA hydrogels was up-regulated ~7.3- and ~6.6-fold over PEG counterparts after 7 and 14 days, respectively (Figure 7). Aggrecan was also up-regulated in MeHA hydrogels (~1.5- and ~1.2-fold after 7 and 14 days, respectively), but to a lesser extent. These differences were also observed in immunohistochemical staining, where more intense type II collagen and CS staining is observed in MeHA over PEG hydrogels (Figure 8).

For *in vivo* culture, differences between MeHA and PEG hydrogels were most noticeable for MSCs plus scaffold alone, where type II collagen and aggrecan were up-regulated by ~43-and ~6-fold, respectively, for MSCs in MeHA hydrogels compared to PEG hydrogels (Figure 9). These differences between MeHA and PEG decreased to ~11.5- and ~4.1-fold with the addition of TGF-β3 directly to the hydrogel and became negligible (~0.7- and ~1.5-fold) when chondro-induced MSCs are encapsulated in both hydrogel systems. Hyaluronidase expression also differed, where the expression of enzymes was down-regulated *in vitro* but up-regulated *in vivo* for HA+T3 and HA-C groups when compared to their PEG counterparts.

Discussion

Recently, MSCs have been explored as an alternative cell source for cartilage regeneration and repair due to their chondrogenic potential and their ease of isolation from sources such as bone marrow without damage to native cartilage tissue. To this end, 3D scaffolds have been developed to create microenvironments for stem cells, where numerous factors including material chemistry, functionalization with biological cues, interactions with surrounding cells, and mechanical properties[11] play a role in directing stem cell differentiation, in addition to soluble cues. In our laboratory, we investigated the use of a photocrosslinked HA hydrogel to provide a favorable niche for MSC chondrogenesis both *in vitro* and *in vivo* by providing cell interactive cues with a naturally found polysaccharide.

One of the advantages of using an HA-based scaffold is the potential for cell/scaffold interactions via cell surface receptors, which could direct cell behaviors and assist in stem cell differentiation. CD44 is a cell surface receptor that binds to HA, providing a means to retain and anchor proteoglycan aggregates to the plasma membrane of a cell. In addition, intimate association with the underlying cytoskeleton permits CD44 to initiate intracellular signaling[41, 42], allowing it to sense changes in the ECM environment and signal a cellular response. Furthermore, this receptor is of particular interest because it is essential for the maintenance of cartilage homeostasis[42] and plays a role in the catabolism of HA via phagocytosis[43]. To demonstrate the potential of MSCs to interact with our HA scaffold, CD44 expression was verified with immunofluorescent staining and flow cytometry. In addition, the expression of hyaluronidases was observed in MSCs indicating the potential to remodel the MeHA hydrogel. Hyaluronidases are enzymes that cleave the β-1,4-glycosidic bonds between

glucuronic acid and N-acetylglucosamine[44], which can affect cell differentiation and matrix catabolism. Each enzyme isoform plays a specific role in cleaving HA into discrete fragment sizes that regulate different cellular processes[45–47].

The high viability of MSCs after photoencapsulation in MeHA hydrogels demonstrated that these hydrogels could be successfully used as cell delivery vehicles. In addition, photopolymerization, with its numerous advantages for a clinical setting, served as a facile means to encapsulate cells uniformly in a 3D hydrogel matrix. MSC chondrogenesis in MeHA hydrogels was induced *in vitro* by culture in chondrogenic media containing TGF-β3, which has been shown to induce chondrogenesis in a variety of other scaffolds[48, 49]. Accordingly, the culture of MSC-laden MeHA hydrogels in chondrogenic media resulted in the up-regulation of type II collagen and aggrecan, which are typical markers for chondrogenic differentiation, and sox 9, a transcription factor that is required for successive steps in chondrogenesis. This up-regulation of cartilaginous protein expression was also reflected in immunohistochemical staining, which showed intense pericellular staining of type II collagen and CS after only 2 weeks of culture. It is interesting to note that culture in growth media also resulted in the down-regulation of type I collagen and the slight up-regulation of type II collagen compared to the cells at the time of encapsulation, suggesting that the scaffold alone could promote chondrogenesis. Likewise, this was also observed through the immunohistochemical staining of CS. This could be due to both the morphology of the cells in the hydrogels (rounded in 3D, versus spread in 2D culture) and interactions with the hydrogel. Importantly, there were no spatial distributions in ECM elaboration, indicating that growth factor transport through the hydrogels is not inhibited.

In vivo, MSC chondrogenesis was explored with and without TGF-β3, which was delivered without a carrier via direct encapsulation within the hydrogel. The assessment of TGF-β3 release from MeHA hydrogels *in vitro* indicated that there was growth factor remaining in the hydrogels after 14 days, unless release was triggered by the addition of exogenous hyaluronidase. Thus, we hypothesized that TGF-β3 could be retained within the hydrogel to induce chondrogenic differentiation when implanted. Gene expression analysis after 2 weeks of *in vivo* culture reflected increases in type II collagen, aggrecan, and sox 9, as was found *in vitro*, for all groups. These results indicate that the MeHA hydrogel as a cell delivery vehicle alone supports some MSC chondrogenesis, which is then further enhanced with the addition of TGF-β3. It is important to note that the single dose of encapsulated TGF-β3 was capable of altering gene expression during short-term *in vivo* culture. In addition, data showed that the pre-programming of MSCs toward chondrogenesis with 2 weeks of pre-culture *in vitro* was also sufficient to maintain chondrogenesis in short-term *in vivo* culture. Furthermore, increases in hyaluronidase expression *in vivo* may reflect potential cell-dictated remodeling of the MeHA hydrogel.

To better evaluate the effect of scaffold material on MSC chondrogenesis, MeHA hydrogels were compared to a relatively inert PEG hydrogel system. PEG hydrogels were used as comparative controls due to their resistance to protein adsorption and minimal interaction with cells. To eliminate the influence of other parameters on MSC differentiation, the mechanical properties of the hydrogels were correlated. Although the macromers have different structures (diacrylate for PEG and an acrylated chain for HA) and molecular weights, the modulus is proportional to the hydrogel crosslinking density and should reflect the mechanics that the cells observe and also the diffusion of growth factors to the cells. It should also be noted that the 2wt% MeHA hydrogel has not been optimized for MSC differentiation and was chosen based on our previous work with chondrocytes. Thus, there is ample opportunity for further iteration on the HA hydrogel properties (e.g., mechanics and degradation) to influence chondrogenesis.

In vitro cultures reflected significant differences in gene expression for type II collagen and hyaluronidases between the hydrogels (i.e., greater expression in HA over PEG hydrogels). Accordingly, the up-regulation in gene expression translated to increased type II collagen and CS deposition within the HA hydrogels. For *in vivo* culture, differences between HA and PEG hydrogels were most noticeable for MSCs and polymer alone, indicating that hydrogel chemistry alone can greatly influence the commitment of MSCs to undergo chondrogenesis. However, these differences decreased with the addition of TGF-β3, suggesting that the hydrogel chemistry may play a less prominent role when chondrogenic growth factors are present. Furthermore, once chondro-induced, MSC gene expression for chondrogenic markers between polymers was comparable *in vivo*. With pericellular deposition after 2 weeks of *in vitro* culture, MSCs may begin interacting more with newly deposited matrix rather than the surrounding scaffold material; thus, differences as a result of polymer choice may be minimized when compared in longer *in vivo* cultures.

It is important to note that others have successfully used PEG hydrogels for cartilage tissue engineering with both chondrocytes[50] and stem cells[51,52] and that the inertness of the hydrogels may be advantageous for many applications since they can be modified to specifically control interactions. Additionally, PEG hydrogels have been further designed to incorporate degradable moieties, bioactive molecules, and adhesive functionality to control overall matrix distribution and cell interactions[12, 53–56]. Also, HA has been used to direct embryonic stem cell differentiation in hydrogels either as the major matrix component[57] or intermixed with PEG hydrogels[58]. This work indicates that the cell type is important in the cellular response and that the method of exposure (e.g., bound versus soluble) is also important.

In conclusion, we have shown that MSCs undergo chondrogenesis in photocrosslinked HA hydrogels *in vitro* and *in vivo* in short-term culture. Gene expression also showed that scaffold choice affects the expression of cartilaginous matrix proteins, where favorable cell/scaffold interactions can assist in chondrogenic differentiation. Additionally, TGF-β3 can be delivered within HA hydrogels and alter gene expression of encapsulated MSCs. The next step in assessing the use of MSCs as a cell source for cartilage regeneration in HA hydrogels is the completion of long term studies to assess the quality and function of the matrix formed by MSCs with a range of methods for the delivery of TGF-β3.

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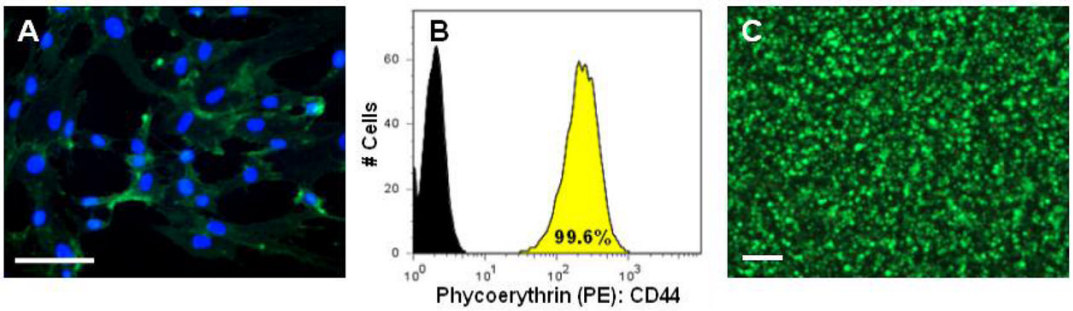

Figure 1. MSC interactions with HA

Immunofluorescence staining of CD44 (green) with nuclear counterstain (blue) of MSCs cultured in 2D on glass coverslips (scale bar = 100μm) (A), flow cytometry staining for CD44 (yellow) compared to unstained (black) population of MSCs prior to encapsulation (B), and live (green)/dead (red) image of MSCs encapsulated in MeHA hydrogel 6 hours after photopolymerization (scale bar = 200μm) (C).

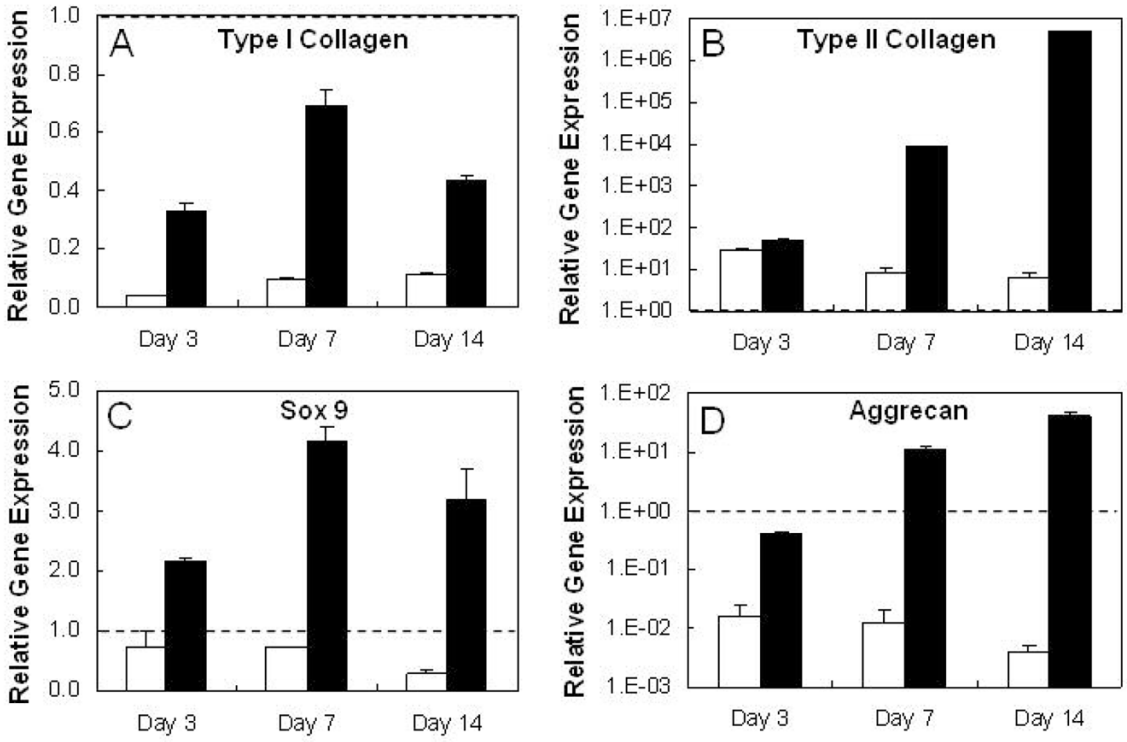

Figure 2. MSC chondrogenesis in MeHA hydrogels *in vitro*

Relative gene expression of type I (A) and type II (B) collagen, sox 9 (C) and aggrecan (D) for MSCs encapsulated in hydrogels cultured in growth (white) and chondrogenic (black) media. GAPDH is used as the housekeeping gene and expression is normalized to cells at the time of encapsulation (indicated by the dashed line). Gene expression of MSCs cultured in chondrogenic media is significantly different than MSCs cultured in growth media ($p<0.05$) for all genes at all time points.

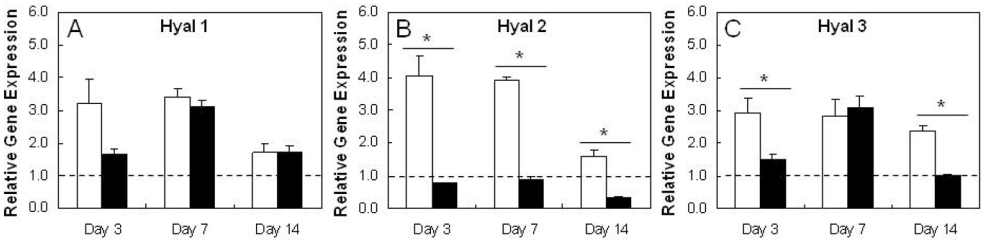

Figure 3. Hyaluronidase expression of MSC-laden MeHA hydrogels *in vitro*

Relative gene expression of hyaluronidases (Hyal) for MSCs encapsulated in hydrogels cultured in growth (white) and chondrogenic (black) media. GAPDH is used as the housekeeping gene and expression is normalized to cells at the time of encapsulation (indicated by the dashed line). Significant differences ($p<0.05$) between hydrogels cultured in growth and chondrogenic media are denoted by *.

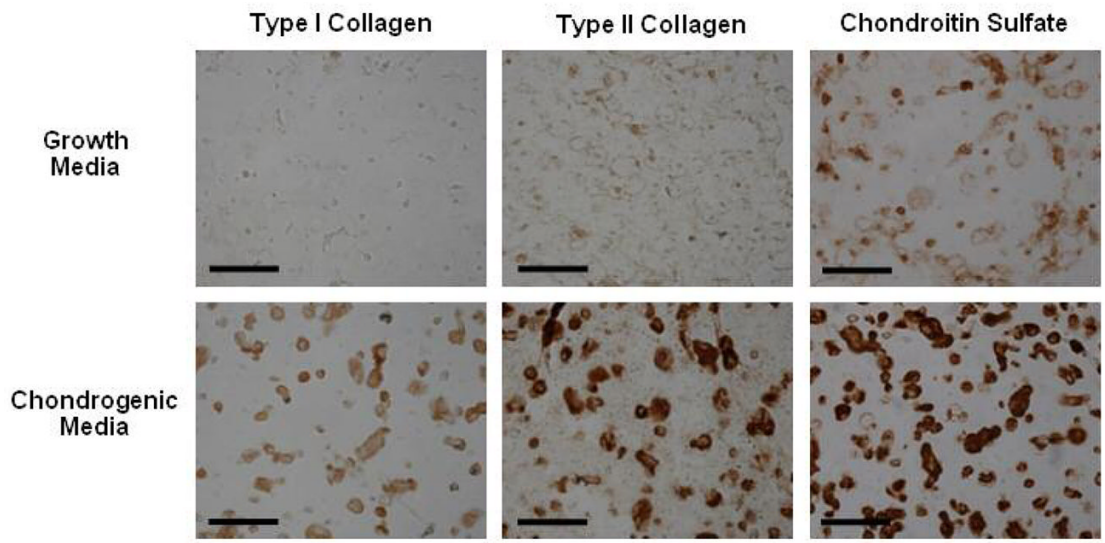

Figure 4. Immunohistochemistry of MSC-laden MeHA hydrogels *in vitro*

Representative stains for type I and II collagen and chondroitin sulfate for MSC-laden hydrogels cultured in growth and chondrogenic media for 14 days *in vitro*. Scale bar = 100μm.

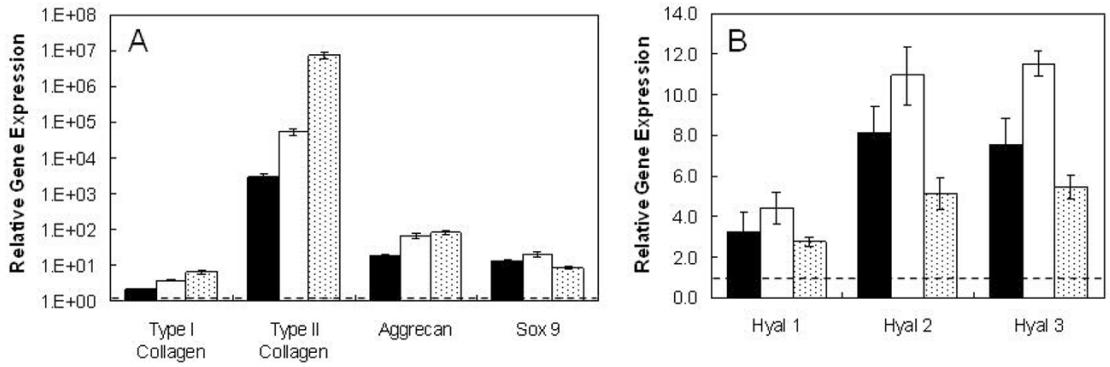

Figure 5. MSC-laden MeHA hydrogels *in vivo*

Relative gene expression for type I and type II collagen, aggrecan, sox 9 (A) and hyaluronidases (B) for MSCs encapsulated in hydrogels cultured 2 weeks *in vivo*. GAPDH is used as the housekeeping gene and expression is normalized to cells at the time of encapsulation (indicated by the dashed line). The groups included the hydrogel alone (HA-MSC, black), hydrogels with TGF-β3 co-encapsulated with cells (HA+T3, white), and hydrogels pre-cultured in chondrogenic media for 2 weeks (HA-C, shaded). All groups are significantly different ($p<0.05$) for type I and II collagen, while HA-MSC is significantly different from both HA+T3 and HA-C for aggrecan. In addition, HA+T3 is significantly different from HA-C for Hyal 2 and 3 and sox 9.

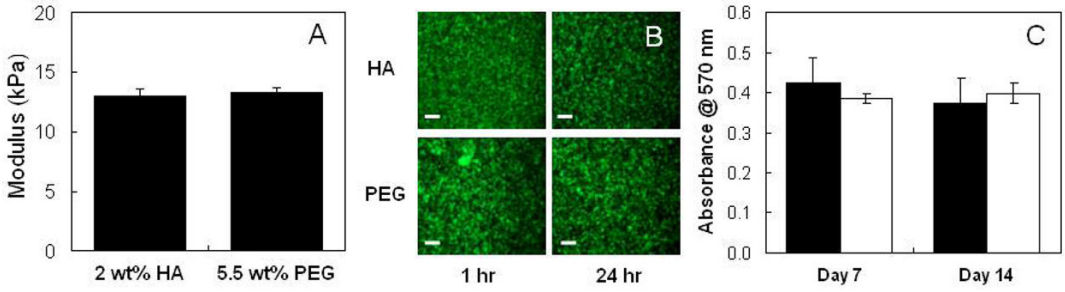

Figure 6. MeHA compared to PEG

Modulus of acellular HA and PEG hydrogels (A), live (green)/dead (dead) images of MSC-laden hydrogels at 1 and 24 hrs after polymerization; scale bar = 200 μm (B), relative mitochondrial activity for HA (black) and PEG (white) hydrogels after 7 and 14 days of *in vitro* culture (C). There were no statistical differences in hydrogel moduli and viability between HA and PEG groups.

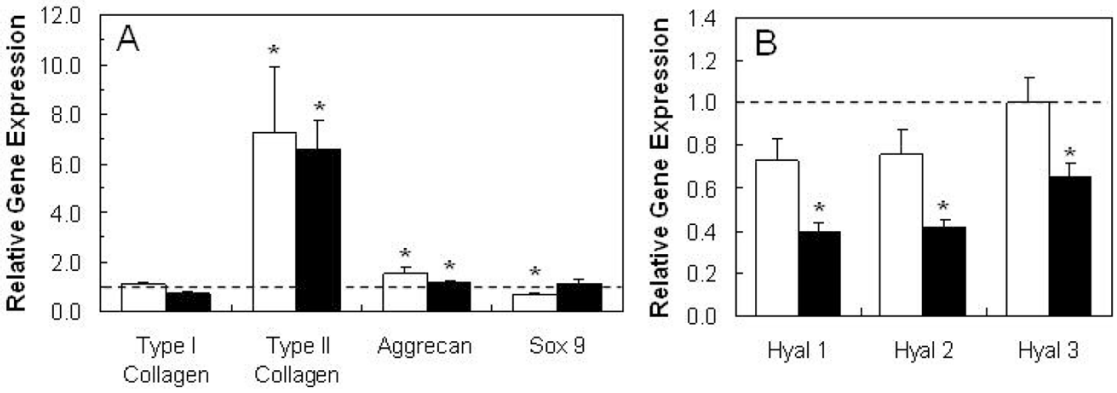

Figure 7. MeHA compared to PEG *in vitro*

Relative gene expression of type I and type II collagen, sox 9, and aggrecan (A) and hyaluronidases (B) for MSCs encapsulated in HA hydrogels cultured *in vitro* in chondrogenic media for 7 (white) and 14 days (black). GAPDH is used as the housekeeping gene and expression is normalized to PEG counterparts (indicated by the dashed line). Significant differences ($p<0.05$) between HA and PEG hydrogels are denoted by *.

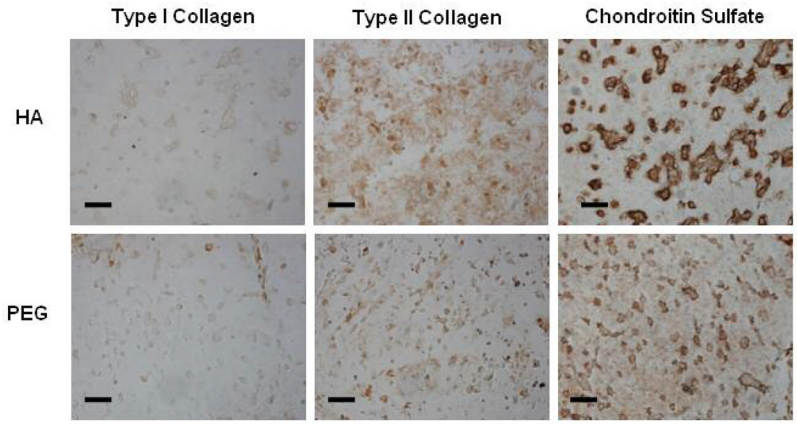

Figure 8. Immunohistochemistry of MSC-laden MeHA and PEG hydrogels *in vitro*
Representative stains for type I and II collagen and chondroitin sulfate for MSC-laden HA and PEG hydrogels cultured in chondrogenic media for 14 days *in vitro*. Scale bar = 200μm.

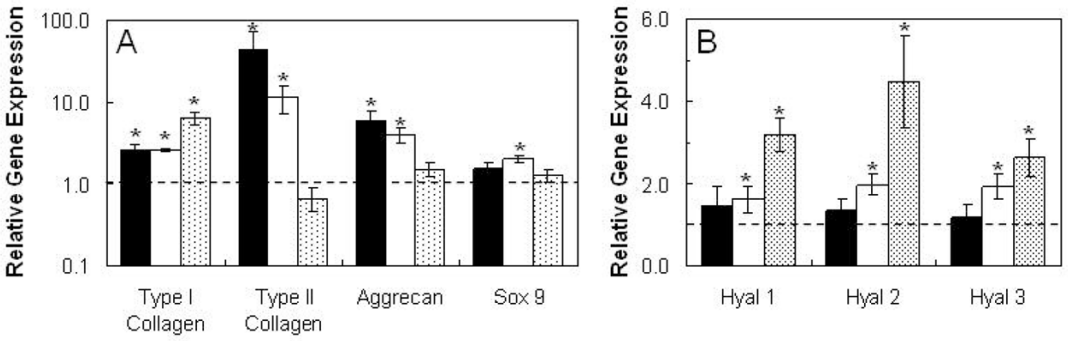

Figure 9. MeHA compared to PEG *in vivo*

Relative gene expression for type I and II collagen, aggrecan, sox 9 (A) and hyaluronidases (B) of MSCs in HA-MSC (black), HA+T3 (white), and HA-C (shaded) groups cultured *in vivo* for 2 weeks. GAPDH is used as the housekeeping gene and expression is normalized to PEG counterparts (indicated by the dashed line). Significant differences ($p<0.05$) between HA and PEG hydrogels are denoted by *.

Table 1

Human quantitative PCR primers and probes.

Gene	Forward Primer	Reverse Primer	Probe
GAPDH	AGGGCTGCTTTTAACTCTGGTAAA	GAATTTGCCATGGGTGGAAT	CCTCAACTACATGGTTTAC
Type I Collagen	AGGACAAGAGGCATGTCTGGTT	GGACATCAGGCGCAGGAA	TTCCAGTTCGAGTATGGC
Type II Collagen	GGCAATAGCAGGTTCACGTACA	CGATAACAGTCTTGCCCCACTT	CTGCACGAAACATAC
Aggrecan	TCGAGGACAGCGAGGCC	TCGAGGGTGTAGCGTGTAGAGA	ATGGAACACGATGCCTTTCACCACGA
Sox 9	AAGCTCTGGAGACTTCTGAACGA	GCCCGTTCTTCACCGACTT	
HYAL1	AAAATACAAGAACCAAGGAATCATGTC	CGGAGCACAGGGCTTGACT	
HYAL2	GGCGCAGCTGGTGTCATC	CCGTGTCAGGTAATCTTTGAGGTA	
HYAL3	GCCTCACACACCGGAGATCT	GCTGCACTCACACCAATGGA	