View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

OsteoArthritis and Cartilage (2006) **14,** 179–189
Published by Elsevier Ltd on behalf of OsteoArthritis Research Society International.
doi:10.1016/j.joca.2005.09.002

Osteoarthritis and Cartilage

International Cartilage Repair Society

Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture

Dr R. L. Mauck Ph.D., X. Yuan and Dr R. S. Tuan Ph.D., Branch Chief*
Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, MD, USA

Summary

Background: The developmental history of the chondrocyte results in a cell whose biosynthetic activities are optimized to maintain the concentration and organization of a mechanically functional cartilaginous extracellular matrix. While useful for cartilage tissue engineering studies, the limited supply of healthy autologous chondrocytes may preclude their clinical use. Consequently, multipotential mesenchymal stem cells (MSCs) have been proposed as an alternative cell source.

Objective: While MSCs undergo chondrogenesis, few studies have assessed the mechanical integrity of their forming matrix. Furthermore, efficiency of matrix formation must be determined in comparison to healthy chondrocytes from the same donor. Given the scarcity of healthy human tissue, this study determined the feasibility of isolating bovine chondrocytes and MSCs, and examined their long-term maturation in three-dimensional agarose culture.

Experimental design: Bovine MSCs were seeded in agarose and induced to undergo chondrogenesis. Mechanical and biochemical properties of MSC-laden constructs were monitored over a 10-week period and compared to those of chondrocytes derived from the same group of animals maintained similarly.

Results: Our results show that while chondrogenesis does occur in MSC-laden hydrogels, the amount of the forming matrix and measures of its mechanical properties are lower than that produced by chondrocytes under the same conditions. Furthermore, some important properties, particularly glycosaminoglycan content and equilibrium modulus, plateau with time in MSC-laden constructs, suggesting that diminished capacity is not the result of delayed differentiation.

Conclusions: These findings suggest that while MSCs do generate constructs with substantial cartilaginous properties, further optimization must be done to achieve levels similar to those produced by chondrocytes.
Published by Elsevier Ltd on behalf of OsteoArthritis Research Society International.

Key words: Mesenchymal stem cells, Agarose, Mechanical properties, Chondrogenesis, Chondrocyte, Cartilage.

Introduction

The high prevalence of osteoarthritis[1,2] and the poor intrinsic healing capacity of articular cartilage engender a demand for cell-based strategies for cartilage repair. Consequently, numerous studies have explored the production of cartilage equivalents via the combination of scaffolds or hydrogels with differentiated chondrocytes[3–10]. While the use of chondrocytes has been important in delineating the path by which tissue engineering may produce replacement tissues, it is likely that the use of only fully differentiated cells to populate engineered constructs is an untenable goal. This is due to the limited supply of such cells, their de-differentiation with extensive monolayer expansion[11,12]. Tissue harvest, even from non-weight bearing sites, may further complicate a damaged joint, and harvested cells may themselves be inferior in terms of matrix production due to age and/or disease processes[13,14].

One opportunity for overcoming such concerns is the use of multipotent, adult mesenchymal stem cells (MSCs)[15,16]. These cells are easily obtained from bone marrow aspirates and retain a multi-lineage differentiation potential; that is, they may differentiate along a number of different mesenchymal lineages, including fat, muscle, bone and cartilage[17–19] when provided the proper soluble and environmental cues. These cells are expandable in culture[20], and therefore may be grown in sufficient numbers to populate engineered scaffolds, serving as an alternative source for fully differentiated chondrocytes.

In general, chondrogenic differentiation of MSCs has been effected by the application of growth factors from the transforming growth factor-β (TGF-β) superfamily[21–25], in the presence of ascorbate and dexamethasone. Original studies of MSC chondrogenesis were carried out to mimic cartilage development in the embryonic limb bud, i.e., in high cell density micromass or pellet culture[21,26], to promote cellular condensation. Translating these studies to the realm of tissue engineering, a number of recent studies have shown that MSCs can differentiate in scaffolds such as fibrin[27], agarose[28,29], hyaluranon/gelatin[28], alginate hydrogels[25,28,30,31], photopolymerizing hydrogels[32], as well

*Address correspondence and reprint requests to: Dr Rocky S. Tuan, Ph.D., Branch Chief, Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, 50 South Drive, MSC 8022, Building 50, Room 1503, Bethesda, MD 20892-8022, USA. Tel: 1-301-451-6854; Fax: 1-301-480-4315; E-mail: tuanr@mail.nih.gov
Received 21 April 2005; revision accepted 5 September 2005.

as in polyester foams[33] and nanofibrous meshes[34] and in gel–fiber amalgams[35].

In the majority of these studies, chondrogenic differentiation has been characterized by the emergence of cartilage-specific markers, such as the expression and deposition of collagen type II, aggrecan, and sulfated proteoglycans. While many studies show that MSCs undergo chondrogenesis, few have assayed the efficiency of their chondrogenic activity or their ability to produce functional material properties within the scaffold. For tissue engineering of cartilage, MSCs must not only differentiate in the three-dimensional (3D) context but also produce constructs with mechanical properties similar to those achieved with differentiated chondrocytes[36–38]. Preliminary reports on the mechanical properties of adipose derived MSC-laden hydrogels suggest that while undergoing differentiation, the constructs do not acquire significant material properties over a 4-week culture period[28]. This failure to achieve significant stiffness over this time course raises a serious practical concern for growing functional tissue replacements.

To address these issues, the goals of this study were to first determine if the bovine model system, using animals derived from a slaughterhouse, would yield a sufficient supply of marrow derived multipotential MSCs. While numerous studies have been carried out with bovine chondrocytes, few have reported on MSCs from this animal source[39,40]. After identifying a multipotential population, the second goal of this study was to compare the development of mechanical properties in constructs seeded with chondrocytes and MSCs from the same animals seeded in an agarose hydrogel culture system. Given the well-documented sensitivity of mature chondrocytes to modulators of chondrogenesis (particularly those from the TGF-β superfamily)[41–47], we examined the response of both MSC- and chondrocyte-laden agarose hydrogels in a basal (BM) and TGF-β-containing chondrogenic medium (CM) over 10 weeks of free swelling culture. We hypothesized that after an initial delay, during which MSCs commit to the chondrogenic lineage, the efficiency of cartilage matrix production and accumulation of mechanical properties would be similar for both cell types exposed to the same stimuli.

Materials and methods

CHONDROCYTE AND MARROW STROMAL CELL ISOLATION AND CULTURE

Bone marrow derived MSCs were harvested from either the carpal bones or the tibia and femur of freshly slaughtered (<36 h) 3–6 month old calves (Fresh Farms Beef, Rutland, VT). Typically, six separate carpal bone marrow isolations (minimum of three animals) or four separate femur/tibia marrow isolations (minimum of four animals) were carried out simultaneously. The marrow was then removed and deposited into an equal volume of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1× penicillin/streptomycin/fungizone (PSF) with an additional 300 U/mL heparin. After vortexing for 5 min, marrow samples were separated from fat (by centrifugation for 5 min at 300 × *g*) and the resulting solution was plated onto a 15 cm tissue culture plate (Falcon Becton Dickinson Labware, Franklin Lakes, NJ). After an initial period of 48 h to allow for cell attachment, cultures were washed with phosphate buffered saline (PBS) and then maintained in Expansion Medium (EM) (described in *Assays for Multipotentiality*), with medium changed twice weekly, until cultures reached confluence. Subsequent sub-culturing was carried out at a 1:3 expansion ratio, with cultures up to passage four utilized for these studies. For initial differentiation studies, individual donor samples were maintained separately; for long-term agarose culture studies, MSCs from all donors were combined after culture expansion but prior to encapsulation.

Articular cartilage was harvested from the carpometacarpal joints of the same group of animals. Cartilage was removed from the joint, rinsed in DMEM containing 2× PSF, and incubated overnight at 37 °C in a humidified 5% CO_2 incubator. Cartilage pieces were combined and digested sequentially with pronase and collagenase as described previously[10]. The chondrocyte suspension was filtered (40 μm cell strainer, BD Falcon, Bedford, MA), pelleted (5 min, 300 × *g*), resuspended in DMEM, and viable cells counted. In order to match chondrocytes from the same isolation with their accompanying MSC population, chondrocytes were slowly culture expanded through passage two. Typically, chondrocytes were cultured at ~70,000 cells/cm^2 to achieve high initial confluency so as to limit cell division and maintain the chondrocyte phenotype[48], with chondrocyte sub-culturing occurring once every 2 weeks. Chondrocytes were maintained in the same EM as used for the MSCs.

ASSAYS FOR MULTI-LINEAGE DIFFERENTIATION POTENTIAL

Bovine marrow derived MSCs were tested for three different differentiation lineages: adipogenesis, osteogenesis, and chondrogenesis as described previously[49]. All cell populations were expanded individually in EM, consisting of high glucose (hg) DMEM containing 1× PSF and 10% fetal bovine serum (FBS).

To test for osteogenesis and adipogenesis, MSCs were plated in six-well plates (Corning Costar, Inc., Corning, NY) at 200,000 cells/well and exposed to osteogenic medium (OS), adipogenic medium (AS), or EM for 21 days. OS consisted of hgDMEM supplemented with 10% FBS, 1× PSF, 10 nM dexamethasone, 10 mM β-glycerophosphate, 50 μg/mL ascorbate 2-phosphate and 10 nM 1,25-dihydroxyvitamin D3. AS consisted of hgDMEM supplemented with 10% FBS, 1× PSF, 1 μM dexamethasone, 1 μg/mL insulin and 0.5 mM 3-isobutyl-1-methylxanthine. Media were changed twice weekly. Osteogenesis was determined by fixing monolayers in isopropanol and staining with Alizarin Red (2%, Rowley Biochemicals, Danvers, MA) for mineralized matrix. Adipogenesis was assessed by fixing with paraformaldehyde and staining with freshly prepared Oil Red O (Sigma, 0.18% w/v in 60% isopropanol) to visualize lipid droplets.

Chondrogenic potential of the bovine MSC populations was evaluated using the pellet culture system[18,19,21]. Cells were released with 0.25% trypsin, counted, and total RNA from 1 × 10^6 cells from each animal was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). Baseline expression of specific genes (see below) were assayed by reverse-transcriptase polymerase chain reaction (RT-PCR). To assay for chondrogenic potential of the MSC populations, pellets containing 250,000 bovine MSCs were formed in 96-well polypropylene plates (Nalge Nunc International, Rochester, NY) via centrifugation for 5 min at 300 × *g*. Cultures were maintained in CM consisting of hgDMEM supplemented with 1× PSF, 0.1 μM dexamethasone, 50 μg/mL ascorbate 2-phosphate, 40 μg/mL L-proline, 100 μg/mL sodium pyruvate, 1× insulins–transferrin–selenous acid+ with (CM+) and without (CM−) TGF-β3 (R&D Systems, Minneapolis, MN, 10 ng/mL), with medium changed twice

weekly. Chondrogenic pellets were harvested on Days 3, 7 and 11, and fixed for histology or frozen in 1 mL Trizol reagent. After paraffin embedding and sectioning, pellets were stained with Safranin O (for proteoglycans) and Picrosirius Red (for collagens). For RT-PCR, total RNA from four to six pellets per donor was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). Reverse transcription of 500 ng RNA was performed using the First Strand cDNA Synthesis kit (Invitrogen Life Technologies) with oligo(dT) primers. PCR amplification was carried out with primers specific for aggrecan, cartilage oligomeric matrix protein (COMP), collagen types I, II, and IX, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase, as described previously[34]. PCR products were resolved on a 2% agarose gel, visualized with ethidium bromide.

LONG-TERM AGAROSE CULTURE

For long-term agarose culture, three separate preparations of MSCs and chondrocytes from at least three animals were used. MSCs were expanded through passage three, while chondrocytes from the same donors were expanded through passage two. To produce constructs, a sterile agarose solution (42°C, 4% w/v in PBS, type VII, Sigma) was combined 1:1 with MSCs or chondrocytes in DMEM (40 million cells/mL), and cast between parallel glass plates, as described previously[10,50]. After 20 min gelation at room temperature, cylindrical samples were cored using a dermal punch (∅ 5 mm, Miltex, York, PA). Constructs (25–30/dish) laden with MSCs and chondrocytes were cultured in 30 mL medium (CM+, CM−, or BM) that was changed twice weekly over a 10-week period, with ascorbate, dexamethasone, and TGF-β3 added fresh with each change. BM consisted of hgDMEM supplemented with 10% FBS, amino acids (0.5× essential amino acids, 1× non-essential amino acids), buffering agents (10 mM 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid, 10 mM $NaHCO_3$, 10 mM N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid, 10 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid), and 1× PSF with fresh 50 μg/mL ascorbate. At 2-week intervals, three to four samples from each preparation were removed for mechanical, biochemical, and histological analyses.

MECHANICAL TESTING

Mechanical testing was performed using a custom apparatus[50,51]. Sample thickness and diameter were measured with a digital micrometer. Constructs were tested in unconfined compression between two smooth impermeable surfaces in PBS at room temperature. Samples were first tested in creep under a tare load of 0.02 N applied until equilibrium was achieved (~300 s). Subsequently, stress relaxation tests were carried out with a compressive deformation of 1 μm/s to 10%, after which samples were allowed to relax to equilibrium (1200 s). The equilibrium compressive Young's modulus (E_{Y-}) was determined from the equilibrium force normalized to the original cross-sectional area divided by the equilibrium compressive strain. After equilibrium was achieved, dynamic testing was carried out by applying 1% oscillatory deformation at 0.1, 0.5, and 1.0 Hz. The dynamic modulus (E_D) for each frequency was calculated from the slope of the compressive stress as a function of the compressive strain, as in Ref.[52]. After testing, samples were frozen at −20°C for subsequent biochemical analysis.

BIOCHEMICAL ANALYSIS

To determine the biochemical composition of samples at various time points, assays were performed to determine the proteoglycan, DNA, and bulk collagen (*ortho*-hydroxyproline (OHP)) content. Samples were first weighed wet and digested for 16 h in papain at 60°C. Aliquots were analyzed for sulfated glycosaminoglycan (sGAG) content using the 1,9-dimethylmethylene blue dye-binding assay[53], for DNA content using the PicoGreen dsDNA Quantification kit (Molecular Probes, Eugene, OR), and for OHP content (after acid hydrolysis) by reaction with chloramine T and dimethylaminobenzaldehyde[54]. OHP was converted to collagen content using a 1:10 ratio of OHP:collagen[55]. Each constituent (DNA, sGAG, OHP) was normalized to the wet weight (ww). On separate Day 70 samples, collagen type II content was assessed using a sandwich ELISA kit (Chondrex, Inc., Redmond, WA) according the manufacturer's recommendations.

HISTOLOGY

Samples for histology were fixed overnight at 4°C in paraformaldehyde, dehydrated in a graded series of ethanol, embedded in paraffin (Tissue Prep, Fisher Scientific), and sectioned at 8 μm thickness. Sections were stained with hematoxylin and eosin (H&E, Sigma, St. Louis, MO) for general histology, with Safranin O (1% aqueous solution, pH 6.7) or Alcian Blue (pH 1.0) to detect sulfated proteoglycans, and with Picrosirius Red (0.1% w/v in saturated picric acid) to view collagen deposition. Stained specimens were imaged with a color charge coupled device camera and an inverted microscope. To assess cell viability on Day 77, chondrocyte- and MSC-laden constructs were stained using the LIVE/DEAD cell viability kit (Molecular Probes, Eugene, OR) according to the manufacturer's protocol, and imaged in cross-section using a fluorescent microscope.

STATISTICAL ANALYSIS

All data are reported as the mean and standard deviation of three to nine samples (depending on the group, measure, and time point). Analysis of variance was performed using the STATISTICA software package (Statsoft, Tulsa, OK) with cell source (chondrocyte or MSC), media (BM, CM−, or CM+) and time in culture (0, 14, 28, 42, 56, and 70 days) as independent variables and thickness, diameter, wet weight, Young's and dynamic moduli, sGAG/ww, DNA/ww, and Collagen/ww as dependent variables. *Post hoc* comparisons were made using the Tukey's honest significant difference test for unequal sample sizes, with $\alpha = 0.05$.

Results

CHARACTERIZATION OF THE MULTIPOTENTIALITY OF BOVINE MESENCHYMAL CELLS

MSCs and chondrocytes isolated from bovine bone marrow and articular cartilage exhibited distinct cellular morphologies in monolayer culture. Analysis of gene expression showed a consistent expression of cartilage marker genes (aggrecan and COMP) in chondrocytes, indicating a retention of phenotype during expansion, whereas MSCs from six donors failed to express these genes (data not shown). When these MSCs were cultured in the presence of either adipogenic or OS for 21 days, each population deposited fat droplets [Fig. 1(B)] or mineralized matrix

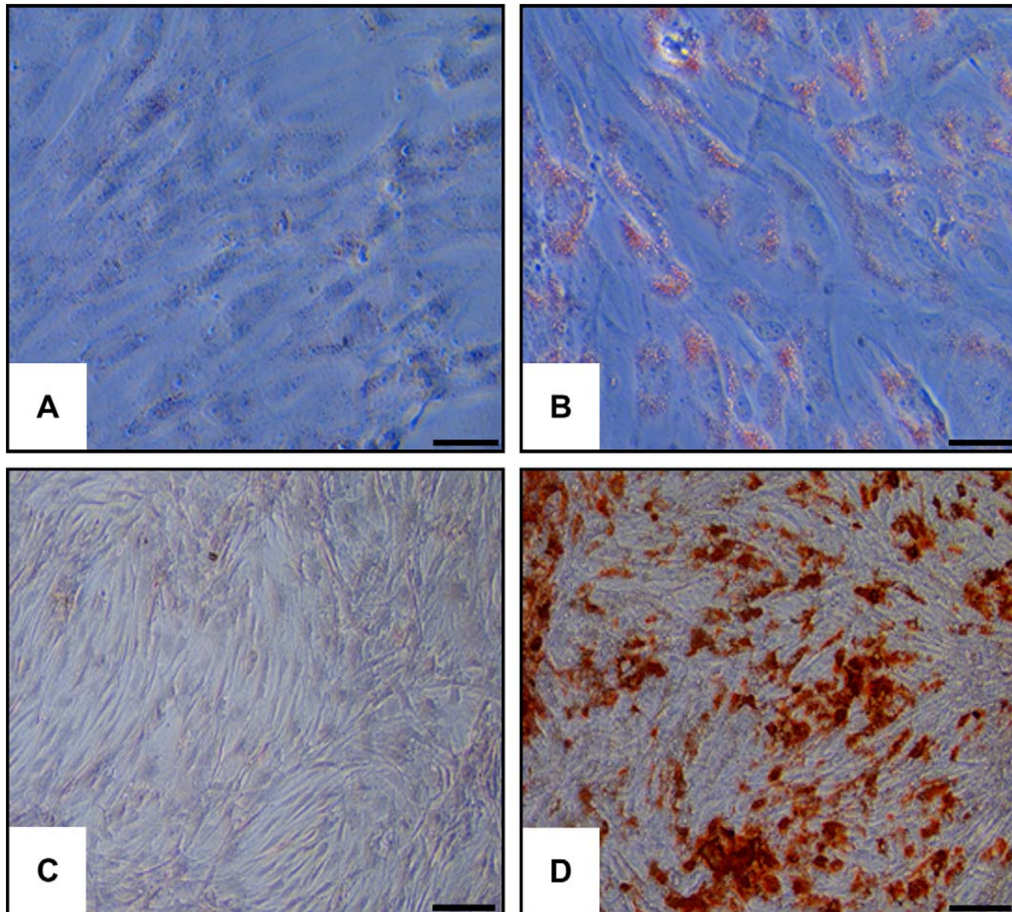

Fig. 1. Bovine MSCs undergo both adipogenesis and osteogenesis in confluent monolayer cultures after 21 days of exposure to adipogenic (B) or osteogenic (D) medium compared to those cultured in BM (A and C). (A and B) Oil Red O staining; (C and D) Alizarin Red staining. Scale bar: 50 μm (A and B) or 200 μm (C and D).

[Fig. 1(D)], respectively. When cultured as pellets in CM supplemented with 10 ng/mL TGF-β3, MSCs showed increased pellet size and time-dependent induction of aggrecan, COMP, and collagen types II and IX expression (Fig. 2). These results indicate that bovine marrow derived mesenchymal cells possess a multi-lineage differentiation potential, including adipogenesis, osteogenesis, and chondrogenesis.

LONG-TERM CULTURE OF CELL-LADEN AGAROSE CONSTRUCTS

Cell morphology, viability, and physical parameters in 3D culture

To examine the long-term properties of MSCs and chondrocytes in 3D culture, each cell type was encapsulated in agarose at 20 million cells/mL. Both cell types survived the encapsulation process and took on a spherical morphology, with MSCs appearing larger than chondrocytes (Fig. 3). The physical parameters of constructs (thickness, diameter, and wet weight) were dependent on time in culture ($P < 0.001$), cell type (MSC or chondrocyte, $P < 0.001$), and medium conditions (BM, CM−, or CM+, $P < 0.001$). In particular, chondrocyte- and MSC-laden constructs showed significant increases in thickness (∼33% and ∼19%, $P < 0.01$ vs Day 0) and diameter (∼22%, $P < 0.01$ vs Day 0 and 4%, not significant) in CM+ (data not shown), with Day 70 chondrocyte seeded constructs in CM+ significantly larger and heavier than MSC-laden constructs similarly maintained ($P < 0.02$).

Extracellular matrix distribution and content in 3D culture

In addition to changes in physical parameters, there were clear changes in the amount and distribution of the forming extracellular matrix (ECM) depending on cell type and culture condition. Staining of chondrocyte-laden constructs with Alcian Blue for sulfated proteoglycan revealed a steady accretion of positive staining with all culture conditions (Fig. 4). For MSC-laden constructs, little to no staining was observed in BM medium, while some spontaneous differentiation was observed in CM−. When cultured in CM+, MSC-laden gels showed positive staining of a similar intensity to that achieved in chondrocyte-laden constructs similarly maintained.

Quantifying the biochemical constituents in these constructs also revealed differences that were dependent on time in culture ($P < 0.001$), cell type (MSC or chondrocyte, $P < 0.001$), and medium condition (BM, CM−, or CM+, $P < 0.001$).

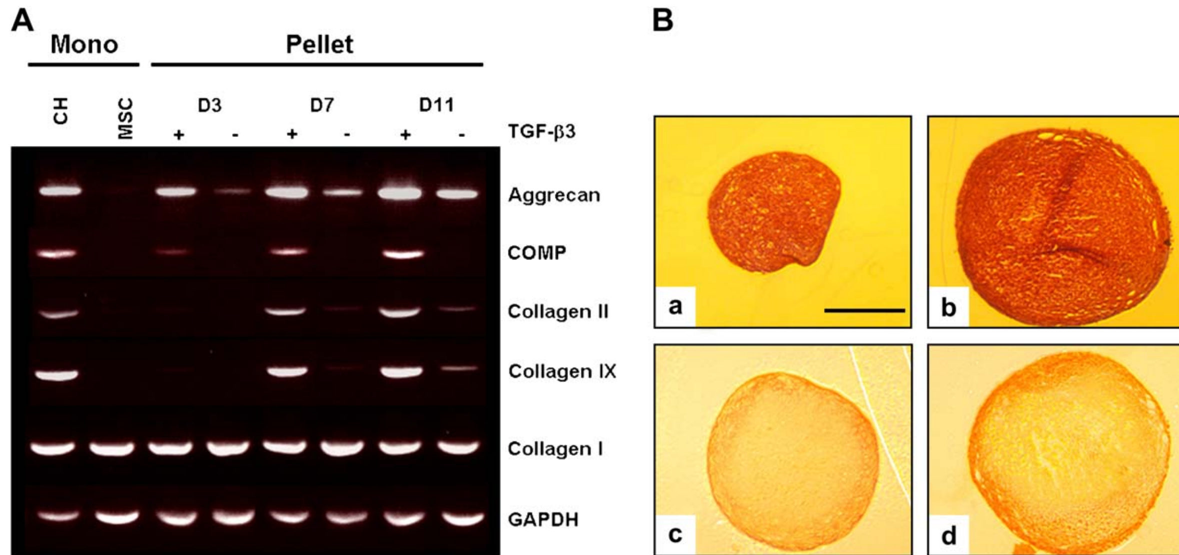

Fig. 2. Analysis of cartilage phenotype in bovine chondrocyte and MSC cultures. (A) Chondrogenic gene expression in bovine chondrocytes (CH) or MSCs in monolayer (mono) culture and MSCs in pellet culture in CM with (+) or without (−) 10 ng/mL TGF-β3. MSCs in pellet culture show a time-dependent increase in cartilage-specific matrix gene expression in the presence of TGF-β3. (B) Sections of Day 11 MSC pellets cultured in CM. a, b: Safranin O staining; c, d: Picrosirius Red staining. a, c: CM−; b, d: CM+; scale bar: 300 μm.

sGAG content (% wet weight) increased in chondrocyte-laden disks under each medium condition ($P < 0.01$ vs Day 0, Fig. 5). MSC-laden constructs increased in sGAG content only when cultured in CM+ ($P < 0.001$ vs Day 0). After 70 days, sGAG content of chondrocyte-laden constructs was higher than that of all other groups ($P < 0.0002$).

Collagen content (% wet weight) increased in chondrocyte-laden disks under each medium condition ($P < 0.01$ vs Day 0, Fig. 5). MSC-laden constructs increased in collagen content when cultured in both CM+ and CM− ($P < 0.001$ vs Day 0), but not in BM. After 70 days of culture, the collagen content of chondrocyte-laden constructs in CM+ was not different from MSC-laden constructs. The content of collagen type II of these CM+ constructs was not different from one another (CH: 107 ± 6 μg/construct vs MSC: 107 ± 21 μg/construct, $n = 3$), although concerns related to completeness of extraction precluded an exact comparison.

DNA content (normalized to wet weight) increased in chondrocyte-laden disks in CM+ by Day 42 ($P < 0.01$, Fig. 5) and then fell to levels not different than starting levels on Day 70. These fluctuations represent a marked change in DNA amount, which was partially countermanded by the concomitant increases in wet weight. MSC-laden constructs displayed only marginal increases in DNA content. After 70 days in CM+, DNA content (per wet weight) of chondrocyte-laden constructs was ~two-fold higher than that of MSC-laden constructs (~three-fold difference in total DNA content per construct). Viability staining revealed that chondrocytes survived in each media with proliferation in CM+, while MSCs survived only in CM− and CM+ (Fig. 4).

Mechanical properties of constructs in 3D culture

The equilibrium and dynamic mechanical properties of constructs were dependent on time in culture ($P < 0.001$), cell type (MSC or chondrocyte, $P < 0.001$), and medium condition (BM, CM−, or CM+, $P < 0.001$). The equilibrium Young's modulus of chondrocyte-laden constructs increased in CM+, reaching 140 ± 18 kPa by Day 70 ($P < 0.0001$ vs Day 0, Fig. 6). Chondrocyte-laden constructs in CM− and BM were significantly softer ($P < 0.0001$), reaching 23 ± 4 kPa and 16 ± 4 kPa by Day 70, respectively. MSC-laden constructs in CM+ also increased in stiffness, reaching 48 ± 12 kPa by Day 70 ($P < 0.01$ vs Day 0, Fig. 6). MSC-laden constructs in CM− and BM were significantly softer ($P < 0.0001$), reaching 6 ± 1 kPa and 4 ± 2 kPa by Day 70, respectively. After 70 days in CM+, chondrocyte-laden constructs were significantly stiffer than MSC-laden constructs maintained similarly ($P < 0.0001$). The dynamic modulus (measured at 1 Hz) of these constructs showed a similar pattern (Fig. 6). By Day 70, chondrocyte-laden constructs in CM+ achieved a dynamic modulus of 1.4 ± 0.2 MPa while MSC-laden constructs achieved a lower value of 0.8 ± 0.1 MPa ($P < 0.0001$).

Rate of production of biochemical constituents and mechanical properties

When the rate of change of these biochemical and mechanical parameters was considered (Table I), differences were observed between groups. In general, the maximum rate of change in measured parameters was more robust and/or occurred at earlier time points for chondrocyte-laden disks cultured in CM+ than for those cultured under any other condition. For example, while a similar maximal rate of production of collagen and sGAG in chondrocyte- and MSC-laden CM+ disks was observed, these maxima occurred at later time points for the MSC-laden disks. For sGAG content, although these constructs reached a similar maximum rate of production, similar levels of sGAG content were not achieved. This finding indicates that while possessed of the capacity for quickly generating cartilaginous matrix, MSCs were unable to do so over the entire time course of the study.

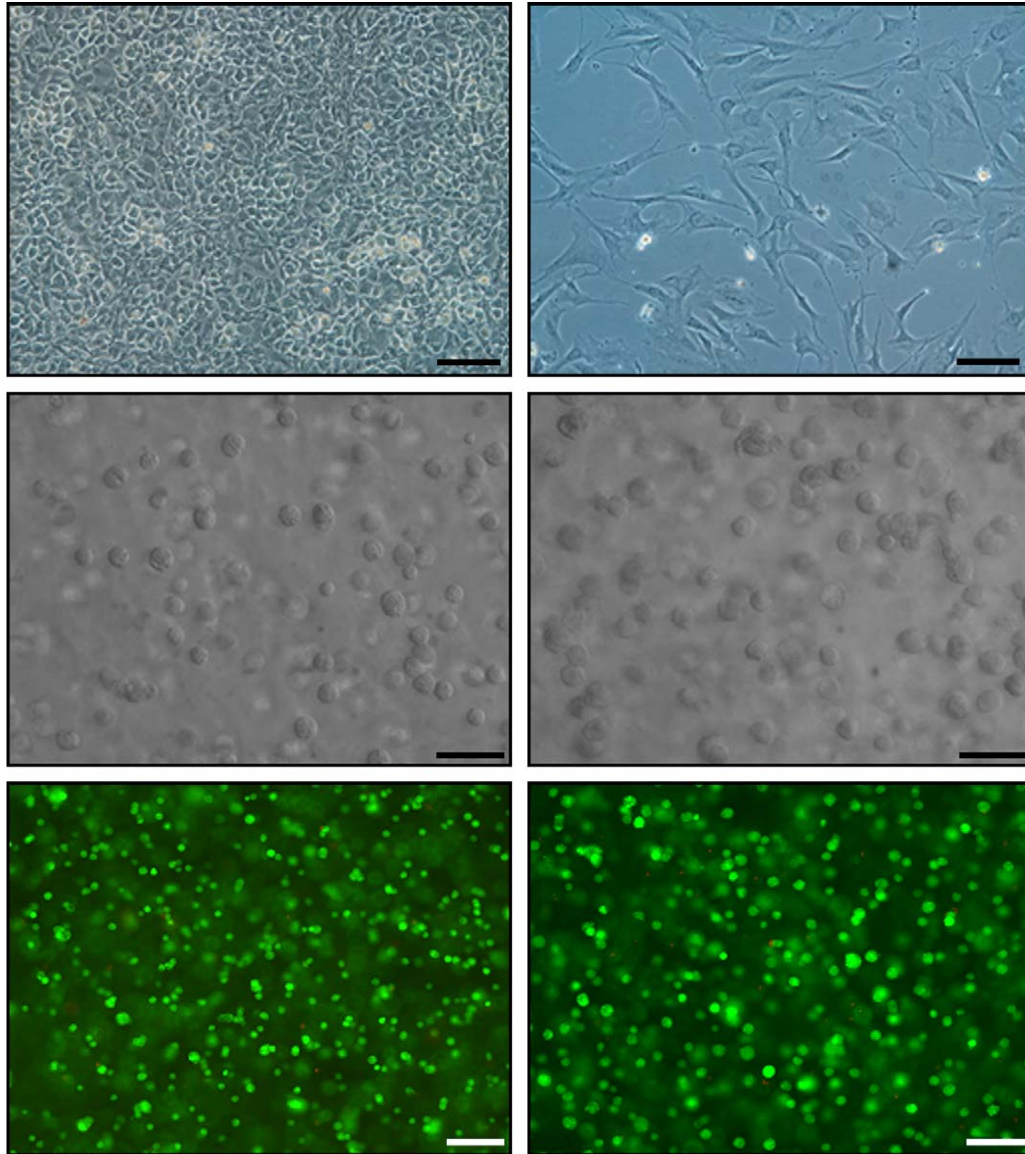

Fig. 3. Primary high-density culture of bovine chondrocytes (top left) and expansion of bovine bone marrow derived MSCs (top right) demonstrating differences in cellular morphology. Scale bar: 100 μm. Bovine chondrocytes and MSCs in 3D agarose culture take on a rounded morphology and remain viable after encapsulation. Bright-field (middle) and LIVE/DEAD (bottom) images of bovine chondrocytes (left) and MSCs (right) 24 h after encapsulation at a concentration of 20 million cells/mL. Scale bar: 50 μm (middle) or 100 μm (bottom).

Discussion

The developmental history of the chondrocyte results in a cell whose biosynthetic activities are optimized to maintain the concentration and structural organization of the cartilaginous matrix. This specialization of cellular activity, which is tuned to the mechanical loading environment, ensures the formation of a mature tissue whose ECM can function in a mechanically demanding *in vivo* environment. To engineer this unique tissue, numerous studies (using fully differentiated chondrocytes) have monitored the generation of these functional cartilaginous properties with changes in experimental inputs, such as modulations of the mechanical loading environment *in vitro*[10,14,50,56–58]. As a first step in extending these studies to incorporate multipotential MSCs in place of chondrocytes, this study directly tested the matrix forming capacity of bovine MSCs compared to that of chondrocytes isolated from the same group of animals in long-term free swelling agarose culture. We posited that MSCs would undergo chondrogenesis in agarose, but would require additional time to begin producing high levels of functional ECM to match the activity of fully differentiated chondrocytes.

This study first demonstrated the possibility of the isolation of a pool of adherent multipotential MSCs in bovine bone marrow that could be induced to differentiate along adipogenic, osteogenic, and chondrogenic lineages, using standard assay techniques[49]. In these studies, we further demonstrated positive chondrogenesis with accompanying increases in mechanical properties for these bovine MSCs seeded in agarose hydrogels at 20 million cells/mL over a 10-week time course. This positive chondrogenesis confirms previous reports of chondrogenesis in agarose using both human and rabbit marrow derived MSCs[29,59], as well

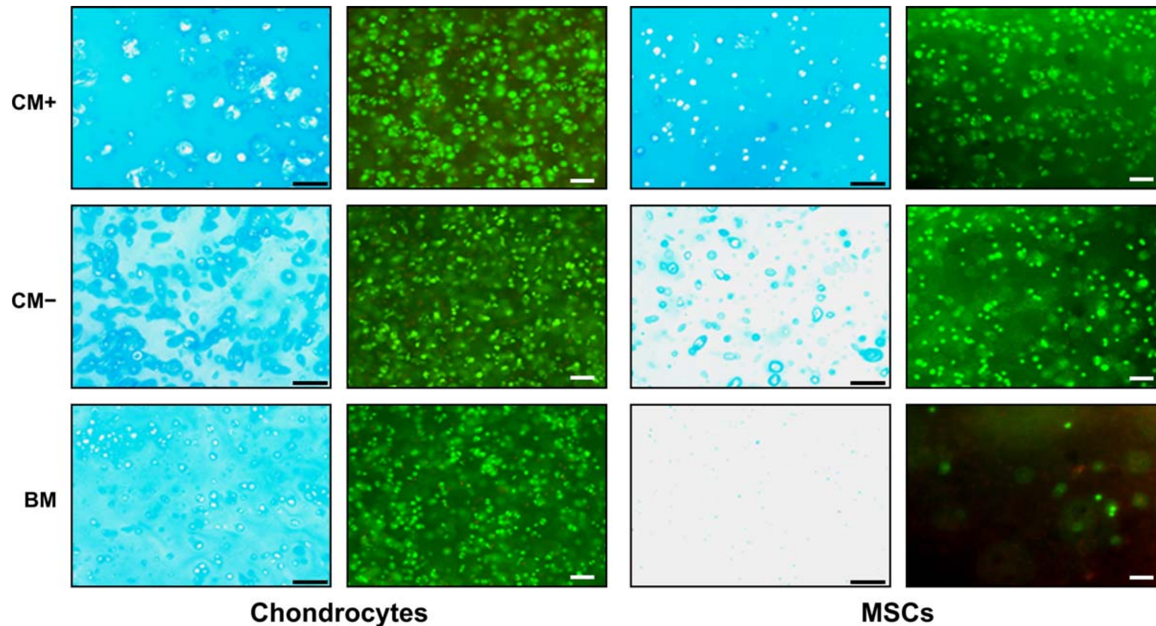

Fig. 4. Alcian Blue staining of Day 70 constructs reveals the formation of a proteoglycan-rich cartilaginous matrix in both chondrocyte- (first column) and MSC- (third column) laden constructs with differing medium compositions. Scale bar: 100 μm. Cell viability of constructs analyzed by LIVE/DEAD staining of chondrocyte- (second column) and MSC- (fourth column) laden constructs after 11 weeks of culture with differing medium compositions. Chondrocytes are viable in every culture condition, with evidence of cell division in CM+. Bovine MSCs remain viable in 3D culture only when cultured in CM. Scale bar: 100 μm.

as human adipose derived adult stem (ADAS) cells[28]. Interestingly, in this latter study, ADAS cells seeded in agarose at 10 million cells/mL failed to accumulate mechanical properties over a 28-day time period[28], countering the findings of the current study using a seeding density of 20 million cells/mL. This discrepancy suggests a difference in functional chondrogenic potential depending on the origin (marrow vs adipose tissue) and/or age of the stem cell source[60], as well as a possible dependence on seeding density, as has been observed for chondrocytes seeded in both hydrogels and fibrous matrices[8,10,61,62].

To prove the clinical potential to form cartilaginous construct using multipotential MSCs, direct comparisons of the matrix forming potential of these cells to differentiated chondrocytes must be undertaken. In this study, due to the possibility that components of the CM might enhance the growth of chondrocytes in agarose, both chondrocytes and MSC-laden gels were cultured in both BM and CM, with (CM+) and without (CM−) the addition of TGF-β3. The marked growth of chondrocyte-laden gels in CM+ provides a positive control for MSC-laden gels, and indicates (when compared to CM− medium) that TGF-β3 has a profound effect on chondrocyte-laden construct growth. Indeed, the mechanical properties of chondrocyte-laden disks in this medium (CM+) far surpassed those of disks maintained in BM in this and previous studies[61]. This finding indicates that a chemically defined serum-free medium can be successfully employed for cartilage tissue engineering studies, as has been suggested in studies examining chondrocytes seeded in self-assembling peptide hydrogels[63], particularly when combined with positive modulators of matrix biosynthesis.

Bovine MSCs cultured under the same chondrogenic conditions (CM+) showed significant accumulations of cartilaginous matrix, although the mechanical properties achieved in these gels were two- to three-fold lower than that produced by chondrocytes cultured under the same conditions. This finding suggests a limited matrix forming capacity of MSCs in this 3D culture. These findings are consistent with the recent findings of reduced MSC potential in studies in alginate hydrogels[64] using a mixed bovine chondrocyte/equine model system. These results suggest for the first time that there may exist an inherently reduced efficacy in matrix forming potential in MSCs that have undergone chondrogenic differentiation that is independent of the hydrogel employed, which must be overcome to optimize their clinical applicability.

When initiating these studies, we hypothesized that a delay (during which chondrogenesis and commitment to phenotype proceeds) would be apparent for MSC-laden gels that would slow their maturation compared to chondrocyte-laden gels similarly maintained. To address this possibility, we explored the maximum rate of change of the measured parameters over the 10-week time course. This analysis indicated that over the entire time course, nearly every metric had a higher rate of change for chondrocyte-laden gels compared to MSC-laden gels. When similar in magnitude, these maxima occurred earlier for chondrocyte-laden gels compared to MSC-laden gels. Moreover, several important parameters, such as the sGAG content and equilibrium and dynamic moduli, appear to have reached a plateau in MSC-laden gels, but continue to increase in chondrocyte-laden gels similarly maintained. The one noted exception was collagen content, which was not different between chondrocyte- and MSC-laden gels in CM+ medium on Day 70. Preliminary characterization of collagen type revealed a similar amount of collagen type II in gels seeded with both cell types. Conversely, the marked increase in DNA content observed in chondrocyte-laden constructs in CM+ resulted in a three-fold increase in cell number over MSCs over the 10-week time course. This finding is consistent with staining for cell viability, with

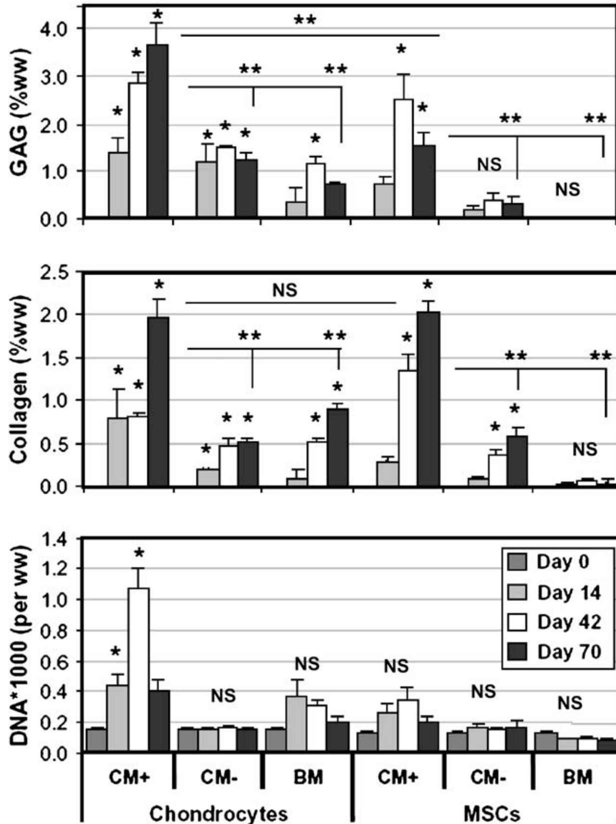

Fig. 5. sGAG content (%ww), bulk collagen content (%ww), and DNA content (DNA*1000 (per ww)) of chondrocyte- and MSC-laden constructs in free swelling culture in different media over a 10-week time course. In CM+, chondrocyte-laden constructs show larger increases in sGAG and DNA content than MSC-laden constructs, while similar bulk collagen levels are achieved in each cell type. CM+: CM with TGF-β3; CM−: CM without TGF-β3. Data represent the mean and standard deviation of three to nine samples per group. *Indicates $P < 0.05$ vs Day 0 within group; **indicates $P < 0.05$ between groups on Day 70.

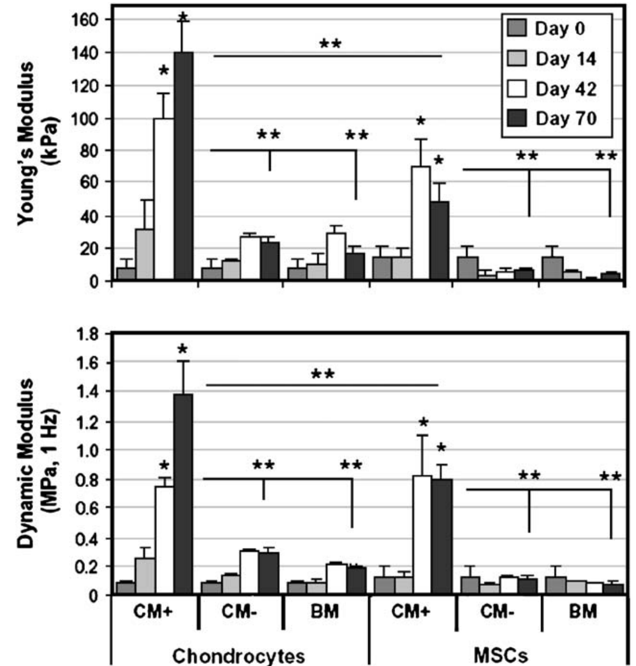

Fig. 6. Equilibrium compressive Young's modulus (E_{Y-}) and dynamic modulus (E_D, 1 Hz) of chondrocyte- and MSC-laden constructs in free swelling culture in different media over a 10-week time course. Increases in mechanical properties are most prominent in cultures maintained in CM+, with chondrocyte-laden constructs achieving a higher stiffness than MSC-laden constructs similarly maintained. CM+: CM with TGF-β3; CM−: CM without TGF-β3. Data represent the mean and standard deviation of three to nine samples per group. *Indicates $P < 0.05$ vs Day 0 within group; **indicates $P < 0.05$ between groups on Day 70.

obvious chondrocyte division in CM+ medium. Thus, one explanation for the enhanced growth of chondrocyte-laden gels compared to MSC-laden gels may be the accumulation of cells via proliferation. Recent studies exploring MSC-laden gels seeded at three times the seeding density used in this study (60 million cells/mL), however, suggest that even with DNA content similar to the final levels observed in chondrocyte-laden gels in this study, MSC-laden gels are not able to produce a similarly functional matrix[65]. These findings suggest that even under balanced conditions of cell density and medium equivalency, chondrocytes out-perform MSCs in forming a functional matrix.

The findings of this study bring forth a number of interesting ideas regarding the application of chondrogenically differentiated MSCs for tissue engineering applications. With the caveat that this is a single study, in one animal system,

Table I

*Maximal rate of change in construct parameters for chondrocytes and MSCs over a 10-week culture period**

Group	Chondrocytes			MSCs		
	CM+	CM−	BM	CM+	CM−	BM
Thickness (mm/week)	0.10 (28–42)	0.10 (28–42)	0.05 (42–56)	0.13 (56–70)	0.04 (14–28)	0.03 (42–56)
Diameter (mm/week)	0.17 (58–70)	0.06 (14–28)	0.04 (14–28)	0.03 (28–42)	0.03 (14–28)	0.06 (14–28)
Wet weight (mg/week)	6.7 (58–70)	5.3 (0–14)	8.0 (28–42)	4.8 (56–70)	4.8 (28–42)	2.0 (0–14)
GAG (%ww/week)	0.688 (0–14)	0.582 (0–14)	0.219 (28–42)	0.571 (14–28)	0.197 (14–28)	0.005 (0–14)
DNA*1000 (per ww/week)	0.228 (28–42)	0.014 (14–28)	0.109 (0–14)	0.062 (0–14)	0.058 (14–28)	0.027 (14–28)
Collagen (%ww/week)	0.40 (0–14)	0.09 (0–14)	0.14 (42–58)	0.37 (14–28)	0.18 (14–28)	0.01 (0–14)
E_Y (kPa/week)	40.10 (14–28)	5.74 (14–28)	3.48 (28–42)	17.06 (14–28)	2.72 (14–28)	1.94 (14–28)
E_D (kPa/week)	283.3 (58–70)	51.7 (28–42)	19.4 (28–42)	201.2 (28–42)	19.9 (28–42)	1.9 (28–42)
Alcian Blue (Day 70)	+++	++	+++	+++	−	−
Viability (Day 77)	+++	++	++	++	++	−

*Number in parentheses indicates time period (days) over which maximal change occurred.

based on an *in vitro* culture system, our findings show for the first time that MSCs, compared to fully differentiated chondrocytes, have a reduced potential in terms of functional matrix forming capacity. To minimize variability and possible age-related decreases in potential, this study used MSCs and chondrocytes taken from the same group of young healthy calves, a best-case scenario. Upon further consideration, it should perhaps be expected that even under these optimal conditions MSCs would under-perform chondrocytes; the chondrocyte has a developmental history which shapes its phenotype, while the MSC is being pushed towards a particular lineage using a combination of growth-factors concocted in an *in vitro* setting. Our results suggest that successful tissue engineering of cartilaginous constructs using MSCs will require the optimization of the differentiation conditions. Furthermore, engineering considerations of the properties that are necessary and sufficient for *in vivo* function must take into account the maximal rate of synthesis of newly differentiated cells, and characterize the degree to which they continue maturing once implanted *in vivo*.

Conclusions

While MSCs have been shown to undergo chondrogenic differentiation, few studies have assessed the mechanical integrity of the matrix formed by these newly differentiated cells. Furthermore, the efficacy of these cells in forming a functional matrix must be compared directly to that of healthy chondrocytes from the same donor for a proper comparison to be made. This necessitates the utilization of animal models that provide a sufficient supply of healthy cells of both types, not typically available in human samples. The results of this study show that while chondrogenic differentiation does occur in MSC-laden hydrogels, the amount of the forming matrix and measures of its mechanical properties are lower than that produced by chondrocytes from the same group of donors. Furthermore, the fact that sGAG content and mechanical properties plateau in MSC-laden constructs suggests that the results are not simply due to a delay while differentiation occurs. These results suggest that while MSC-laden constructs can develop mechanical cartilaginous properties, further optimization must be done to achieve levels similar to those produced by differentiated chondrocytes.

Acknowledgments

This study was supported with funding from National Institutes of Health ZO1 AR41131. The authors gratefully acknowledge Dr Lin Song for her assistance with MSC isolation and characterization of multipotentiality, and Dr Faye Chen for her assistance with assays of collagen type II content.

References

1. Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, *et al*. Osteoarthritis: new insights. Part 1: the disease and its risk factors. Ann Intern Med 2000;133(8):635–46.
2. Felson DT, Lawrence RC, Hochberg MC, McAlindon T, Dieppe PA, Minor MA, *et al*. Osteoarthritis: new insights. Part 2: treatment approaches. Ann Intern Med 2000;133(9):726–37.
3. Freed LE, Marquis JC, Nohria A, Emmanual J, Mikos AG, Langer R. Neocartilage formation *in vitro* and *in vivo* using cells cultured on synthetic biodegradable polymers. J Biomed Mater Res 1993; 27(1):11–23.
4. Freed LE, Martin I, Vunjak-Novakovic G. Frontiers in tissue engineering. *In vitro* modulation of chondrogenesis. Clin Orthop 1999;(367 Suppl):S46–58.
5. Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R, Freed LE, *et al*. Modulation of the mechanical properties of tissue engineered cartilage. Biorheology 2000;37(1–2):141–7.
6. Vunjak-Novakovic G, Obradovic B, Martin I, Bursac PM, Langer R, Freed LE. Dynamic cell seeding of polymer scaffolds for cartilage tissue engineering. Biotechnol Prog 1998;14(2):193–202.
7. Vunjak-Novakovic G, Obradovic B, Martin I, Freed LE. Bioreactor studies of native and tissue engineered cartilage. Biorheology 2002;39(1–2):259–68.
8. Chang SC, Rowley JA, Tobias G, Genes NG, Roy AK, Mooney DJ, *et al*. Injection molding of chondrocyte/alginate constructs in the shape of facial implants. J Biomed Mater Res 2001;55(4):503–11.
9. Li WJ, Laurencin CT, Caterson EJ, Tuan RS, Ko FK. Electrospun nanofibrous structure: a novel scaffold for tissue engineering. J Biomed Mater Res 2002; 60(4):613–21.
10. Mauck RL, Wang CC, Oswald ES, Ateshian GA, Hung CT. The role of cell seeding density and nutrient supply for articular cartilage tissue engineering with deformational loading. Osteoarthritis Cartilage 2003; 11(12):879–90.
11. Thirion S, Berenbaum F. Culture and phenotyping of chondrocytes in primary culture. Methods Mol Med 2004;100:1–14.
12. Stokes DG, Liu G, Coimbra IB, Piera-Velazquez S, Crowl RM, Jimenez SA. Assessment of the gene expression profile of differentiated and dedifferentiated human fetal chondrocytes by microarray analysis. Arthritis Rheum 2002;46(2):404–19.
13. Lee CR, Grodzinsky AJ, Hsu HP, Martin SD, Spector M. Effects of harvest and selected cartilage repair procedures on the physical and biochemical properties of articular cartilage in the canine knee. J Orthop Res 2000;18(5):790–9.
14. Carver SE, Heath CA. Influence of intermittent pressure, fluid flow, and mixing on the regenerative properties of articular chondrocytes. Biotechnol Bioeng 1999; 65(3):274–81.
15. Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med 2001;7(6):259–64.
16. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med 2004;8(3): 301–16.
17. Caplan AI. Mesenchymal stem cells. J Orthop Res 1991;9(5):641–50.
18. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, *et al*. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–7.
19. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71–4.
20. Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive

sub-cultivation and following cryopreservation. J Cell Biochem 1997;64:278–94.
21. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. Exp Cell Res 1998;238:265–72.
22. Awad H, Halvorsen Y-DC, Gimble JM, Guilak F. Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells. Tissue Eng 2003; 9(6):1301–12.
23. Majumdar MK, Banks V, Peluso DP, Morris EA. Isolation, characterization, and chondrogenic potential of human bone marrow-derived multipotential stromal cells. J Cell Physiol 2000;185(1):98–106.
24. Denker AE, Haas AR, Nicoll SB, Tuan RS. Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: I. Stimulation by bone morphogenetic protein-2 in high-density micromass cultures. Differentiation 1999;64(2):67–76.
25. Majumdar MK, Wang E, Morris EA. BMP-2 and BMP-9 promotes chondrogenic differentiation of human multipotential mesenchymal cells and overcomes the inhibitory effect of IL-1. J Cell Physiol 2001;189(3): 275–84.
26. Haas AR, Tuan RS. Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: II. Stimulation by bone morphogenetic protein-2 requires modulation of N-cadherin expression and function. Differentiation 1999;64(2):277–89.
27. Worster AA, Brower-Toland BD, Fortier LA, Bent SJ, Williams J, Nixon AJ. Chondrocytic differentiation of mesenchymal stem cells sequentially exposed to transforming growth factor-beta 1 in monolayer and insulin-like growth factor-I in a three-dimensional matrix. J Orthop Res 2001;19(4):738–49.
28. Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials 2004;25(16):3211–22.
29. Huang CY, Reuben PM, D'Ippolito G, Schiller PC, Cheung HS. Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. Anat Rec A Discov Mol Cell Evol Biol 2004; 278(1):428–36.
30. Erickson GR, Gimble JM, Franklin DM, Rice HE, Awad H, Guilak F. Chondrogenic potential of adipose tissue-derived stromal cells *in vitro* and *in vivo*. Biochem Biophys Res Commun 2002;290(2):763–9.
31. Ma H-L, Hung S-C, Lin S-Y, Chen Y-L, Lo W-H. Chondrogenesis of human mesenchymal stem cells encapsulated in alginate beads. J Biomed Mater Res 2003; 64A:273–81.
32. Williams CG, Kim TK, Taboas A, Malik A, Manson P, Elisseeff J. *In vitro* chondrogenesis of bone marrow-derived mesenchymal stem cells in a photopolymerizing hydrogel. Tissue Eng 2003;9(4):679–88.
33. Martin I, Padera RF, Vunjak-Novakovic G, Freed LE. *In vitro* differentiation of chick embryo bone marrow stromal cells into cartilaginous and bone-like tissues. J Orthop Res 1998;16(2):181–9.
34. Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, *et al*. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials 2005;26(6): 599–609.
35. Caterson EJ, Li WJ, Nesti LJ, Albert T, Danielson K, Tuan RS. Polymer/alginate amalgam for cartilage-tissue engineering. Ann N Y Acad Sci 2002;961:134–8.
36. Garrett JC. Osteochondral allografts for reconstruction of articular defects of the knee. Instr Course Lect 1998;47:517–22.
37. Butler DL, Goldstein SA, Guilak F. Functional tissue engineering: the role of biomechanics. J Biomech Eng 2000;122(6):570–5.
38. Guilak F, Butler DL, Goldstein SA. Functional tissue engineering: the role of biomechanics in articular cartilage repair. Clin Orthop 2001;(391 Suppl):S295–305.
39. Bosnakovski D, Mizuno M, Kim G, Ishiguro T, Okumura M, Iwanaga T, *et al*. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system. Exp Hematol 2004; 32(5):502–9.
40. Martin I, Shastri VP, Padera RF, Yang J, Mackay AJ, Langer R, *et al*. Selective differentiation of mammalian bone marrow stromal cells cultured on three-dimensional polymer foams. J Biomed Mater Res 2001; 55(2):229–35.
41. Bujia J, Pitzke P, Kastenbauer E, Wilmes E, Hammer C. Effect of growth factors on matrix synthesis by human nasal chondrocytes cultured in monolayer and in agar. Eur Arch Otorhinolaryngol 1996;253(6): 336–40.
42. Blunk T, Sieminski AL, Gooch KJ, Courter DL, Hollander AP, Nahir AM, *et al*. Differential effects of growth factors on tissue-engineered cartilage. Tissue Eng 2002;8(1):73–84.
43. Frenkel SR, Saadeh PB, Mehrara BJ, Chin GS, Steinbrech DS, Brent B, *et al*. Transforming growth factor beta superfamily members: role in cartilage modeling. Plast Reconstr Surg 2000;105(3):980–90.
44. Morales TI. Transforming growth factor-beta 1 stimulates synthesis of proteoglycan aggregates in calf articular cartilage organ cultures. Arch Biochem Biophys 1991;286(1):99–106.
45. van Osch GJ, van den Berg WB, Hunziker EB, Hauselmann HJ. Differential effects of IGF-1 and TGFbeta-2 on the assembly of proteoglycans in pericellular and territorial matrix by cultured bovine articular chondrocytes. Osteoarthritis Cartilage 1998;6(3): 187–95.
46. Mauck RL, Nicoll SB, Seyhan SL, Ateshian GA, Hung CT. Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. Tissue Eng 2003;9(4):597–611.
47. Gooch KJ, Blunk T, Courter DL, Sieminski AL, Vunjak-Novakovic G, Freed LE. Bone morphogenetic proteins-2, -12, and -13 modulate *in vitro* development of engineered cartilage. Tissue Eng 2002;8(4):591–601.
48. Watt FM. Effect of seeding density on stability of the differentiated phenotype of pig articular chondrocytes in culture. J Cell Sci 1988;89(Pt 3):373–8.
49. Song L, Baksh D, Tuan RS. Mesenchymal stem cell-based cartilage tissue engineering: cells, scaffold and biology. Cytotherapy 2004;6(6):596–601.
50. Mauck RL, Soltz MA, Wang CC, Wong DD, Chao PH, Valhmu WB, *et al*. Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng 2000; 122(3):252–60.
51. Soltz MA, Ateshian GA. Experimental verification and theoretical prediction of cartilage interstitial fluid

pressurization at an impermeable contact interface in confined compression. J Biomech 1998;31(10):927–34.
52. Park SY, Hung CT, Ateshian GA. Mechanical response of bovine articular cartilage under dynamic unconfined compression loading at physiological stress levels. J Biomech 2003;12(1):391–400.
53. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883(2):173–7.
54. Stegemann H, Stalder K. Determination of hydroxyproline. Clin Chim Acta 1967;18(2):267–73.
55. Vunjak-Novakovic G, Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R, *et al*. Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. J Orthop Res 1999;17(1):130–8.
56. Kisiday JD, Jin M, DiMicco MA, Kurz B, Grodzinsky AJ. Effects of dynamic compressive loading on chondrocyte biosynthesis in self-assembling peptide scaffolds. J Biomech 2004;37(5):595–604.
57. Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Kandel RA. Long-term intermittent shear deformation improves the quality of cartilaginous tissue formed *in vitro*. J Orthop Res 2003;21(4):590–6.
58. Carver SE, Heath CA. Increasing extracellular matrix production in regenerating cartilage with intermittent physiological pressure. Biotechnol Bioeng 1999; 62(2):166–74.
59. Huang CY, Hagar KL, Frost LE, Sun Y, Cheung HS. Effects of cyclic compressive loading on chondrogenesis of rabbit bone-marrow derived mesenchymal stem cells. Stem Cells 2004;22(3):313–23.
60. Murphy JM, Dixon K, Beck S, Fabian D, Feldman A, Barry F. Reduced chondrogenic, adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis Rheum 2002;46(3):704–13.
61. Mauck RL, Seyhan SL, Ateshian GA, Hung CT. Influence of seeding density and dynamic deformational loading on the developing structure/function relationships of chondrocyte-seeded agarose hydrogels. Ann Biomed Eng 2002;30(8):1046–56.
62. Puelacher WC, Kim SW, Vacanti JP, Schloo B, Mooney D, Vacanti CA. Tissue-engineered growth of cartilage: the effect of varying the concentration of chondrocytes seeded onto synthetic polymer matrices. Int J Oral Maxillofac Surg 1994;23(1):49–53.
63. Kisiday J, Jin M, Kurz B, Hung H, Semino C, Zhang S, *et al*. Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: implications for cartilage tissue repair. Proc Natl Acad Sci U S A 2002;99(15):9996–10001.
64. Gleghorn JP, Dines ME, Cox M, Ayala P, Bonassar LJ. Comparison of chondrogenesis by MSCs and chondrocytes in alginate: evaluation of biochemical composition and mechanical properties (Abstract). Trans Orthop Res Soc 2005. Poster #1782.
65. Mauck RL, Yuan X, Tuan RS, On the ability of mesenchymal stem cells to form functional cartilaginous tissues in three dimensional agarose culture (Abstract). Proc 2005 ASME Summer Bioeng Conf. Paper b0295876.