

# Evaluation of Adult Equine Bone Marrow- and Adipose-Derived Progenitor Cell Chondrogenesis in Hydrogel Cultures

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**ABSTRACT:** Bone marrow mesenchymal stem cells (BM-MSCs) and adipose-derived progenitor cells (ADPCs) are potential alternatives to autologous chondrocytes for cartilage resurfacing strategies. In this study, the chondrogenic potentials of these cell types were compared by quantifying neo-tissue synthesis and assaying gene expression and accumulation of extracellular matrix (ECM) components of cartilage. Adult equine progenitor cells encapsulated in agarose or self-assembling peptide hydrogels were cultured in the presence or absence of TGF $\beta$ 1 for 3 weeks. In BM-MSCs-seeded hydrogels, TGF $\beta$ 1 stimulated ECM synthesis and accumulation 3–41-fold relative to TGF $\beta$ 1-free culture. In ADPC cultures, TGF $\beta$ 1 stimulated a significant increase in ECM synthesis and accumulation in peptide (18–29-fold) but not agarose hydrogels. Chromatographic analysis of BM-MSC-seeded agarose and peptide hydrogels cultured in TGF $\beta$ 1 medium showed extensive synthesis of aggrecan-like proteoglycan monomers. ADPCs seeded in peptide hydrogel also synthesized aggrecan-like proteoglycans, although to a lesser extent than seen in BM-MSC hydrogels, whereas aggrecan-like proteoglycan synthesis in ADPC-seeded agarose was minimal. RT-PCR analysis of TGF $\beta$ 1 cultures showed detectable levels of type II collagen gene expression in BM-MSC but not ADPC cultures. Histological analysis of TGF $\beta$ 1-cultured peptide hydrogels showed the deposition of a continuous proteoglycan- and type II collagen rich ECM for BM-MSCs but not ADPCs. Therefore, this study showed both protein and gene expression evidence of superior chondrogenesis of BM-MSCs relative to ADPCs. © 2007 Orthopaedic Research Society.

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**Keywords:** chondrogenesis; progenitor cells; tissue engineering

## INTRODUCTION

The development of cell-based tissue engineering strategies for articular cartilage resurfacing has focused predominantly on the use of chondrocytes isolated from autologous tissue. Whereas utilizing differentiated chondrocytes would ensure that the transplanted cell system is phenotypically similar to the surrounding cartilage, practical concerns related to autologous cartilage harvest and subsequent cell preparation must also be considered. These concerns include: the need for a surgical procedure to obtain cartilage biopsies<sup>1</sup>; limitations

on the amount of cartilage that can be biopsied from noncritical joint surfaces, therefore requiring culture expansion to increase cell numbers<sup>1</sup>; and chondrocyte dedifferentiation to a fibroblastic phenotype that occurs with culture expansion.<sup>2</sup> Progenitor cells have emerged as a potential alternative.<sup>3,4</sup> Tissues such as bone marrow,<sup>5</sup> adipose tissue,<sup>6</sup> muscle,<sup>7</sup> bone,<sup>8</sup> and synovium<sup>9</sup> contain multipotent cells that can differentiate along a chondrogenic lineage. While present in low density in the native tissue, progenitor cells may be expanded in vitro while retaining an uncommitted phenotype. Subsequent chondrogenesis can be induced by exposure to selected growth factors.<sup>10</sup> Therefore, progenitor cell- and chondrocyte-based repair strategies contain similarities in the required ex vivo processing. However, progenitor cells may be harvested using minimally invasive techniques from noncritical locations.

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Bone marrow and adipose tissue have emerged as candidate sources of progenitor cells due to the low morbidity associated with harvest and their renewable nature. Bone marrow can be collected from the iliac crest via aspiration<sup>11</sup> with a low morbidity rate.<sup>12,13</sup> Bone marrow-derived mesenchymal stem cells (BM-MSCs) can be isolated and culture-expanded with the potential to produce millions of cells from 10 ml of aspirate.<sup>11</sup> Adipose tissue can be harvested from subcutaneous locations<sup>14</sup> with an associated morbidity generally accepted to be less than that of bone marrow harvests. After collagenase digestion, adipose-derived progenitor cells (ADPCs) can also be culture-expanded to obtain millions of cells.

The chondrogenic potential of BM-MSCs and ADPCs has been compared in vitro.<sup>15–18</sup> Human cells maintained in aggregate culture<sup>5</sup> demonstrated greater chondrogenesis for BM-MSCs. The aggregate culture system is an established model for evaluating chondrogenesis, but results in high cell density and extensive cell–cell contact that may not fully represent cartilage repair strategies. For example, tissue engineering strategies target a lower density of cells distributed within a 3D scaffold that serves as a template for neo-tissue accumulation. Chondrogenesis of BM-MSC- or ADPC-seeded constructs has been characterized separately in numerous scaffolds; however, limited information has been collected for parallel cultures.<sup>16</sup>

In this study, we evaluated chondrogenesis of BM-MSCs and ADPCs encapsulated in two hydrogels, agarose and self-assembling peptide. Agarose has been frequently utilized as a 3D model system for studying chondrocyte biology<sup>19,20</sup> and progenitor cell chondrogenesis.<sup>21–23</sup> Self-assembling peptide is an emerging biomaterial presently under investigation for applications to cartilage,<sup>24</sup> cardiovascular,<sup>25</sup> and liver<sup>26</sup> repair. Cells were derived from skeletally mature horses to maintain relevance to established animal models of cartilage resurfacing.<sup>27</sup> Chondrogenesis was evaluated in response to TGF $\beta$ 1.<sup>5</sup> Quantitative measures of extracellular matrix (ECM) synthesis and accumulation were evaluated as indicators of the repair potential of each cell type. In addition, chondrogenesis was explored using gel filtration chromatography to identify the size distribution of synthesized proteoglycan (PG) monomers relative to native cartilage, histological analysis to identify accumulation of cartilage-like ECM, and expression of ECM genes that are hallmarks of the chondrocyte phenotype.

## METHODS

### Tissue Harvest and Cell Isolation

Adipose tissue and bone marrow were harvested from skeletally mature (2–5-year-old) mixed breed horses euthanized at Colorado State University for reasons unrelated to conditions that would affect either tissue. Unless otherwise noted, tissue samples were animal-matched in that bone marrow and adipose tissues were taken from the same animals. Three animal-matched cultures were processed for each quantitative assay that was statistically analyzed.

### BM-MSCs

From the iliac crest, 10–30 ml of bone marrow was aseptically harvested. Marrow samples were washed in PBS and then mixed with 0.8% ammonium chloride to lyse the majority of the red blood cells. The remaining nucleated cell pellet was rinsed with PBS, resuspended in low glucose DMEM (Invitrogen, Chicago, IL) containing 10% FBS, and seeded in tissue culture flasks at a concentration of  $0.66 \times 10^6$  nucleated cells/cm<sup>2</sup>. BM-MSC colony formation was observed after 6–7 days of culture, after which the colonies proliferated until locally reaching near-confluence at 10–12 days. BM-MSCs were then reseeded at  $20 \times 10^3$  cells/cm<sup>2</sup> and expanded in growth medium containing 1 ng/ml FGF-2 (R&D Systems, Minneapolis, MN).<sup>28</sup> BM-MSC cultures were passaged at a split ratio of 1:3 once prior to seeding in hydrogel scaffolds.

### ADPCs

From the tailhead fat pad, 10–20 grams of subcutaneous adipose tissue was collected. The tissue was washed with PBS, minced, then digested in low glucose DMEM containing 10% FBS and 0.1% collagenase (Worthington, Lakewood, NJ) for 3–4 h with agitation.<sup>6</sup> Nucleated adipose cells were pelleted, washed, and then seeded into tissue culture flasks at a concentration of  $3.5 \times 10^3$  nucleated cells/cm<sup>2</sup> in low glucose DMEM +10% FBS. ADPCs adhered to and proliferated on the tissue culture surface, reaching confluence in 5–6 days. The cultures were reseeded at  $20 \times 10^3$  cells/cm<sup>2</sup> in growth medium containing 1 ng/ml FGF-2, and passaged once at a split ratio of 1:3 prior to hydrogel seeding.

### Hydrogel Seeding and Culture

Culture-expanded progenitor cells were encapsulated in 2% (w/v) low melting temperature agarose (Invitrogen, Chicago, IL) or 0.36% (w/v) self-assembling peptide (Cambridge, MA) AcN-KLDLKLKLDL-CNH<sub>2</sub> (MIT Biopolymers Lab, Cambridge, MA) at a concentration of  $10 \times 10^6$  cells/ml in a 1.6-mm thick flat slab geometry.<sup>24</sup> One 12-mm disk was then punched for each medium condition. All hydrogels were cultured in high glucose DMEM (Invitrogen, Chicago, IL) supplemented with 1% ITS+ (Sigma-Aldrich, St. Louis, MO), 0.1  $\mu$ M dexamethasone (Sigma-Aldrich, St. Louis, MO), and 37.5  $\mu$ g/ml

ascorbate-2-phosphate (Wako Chemicals, Richmond, VA), with or without 10 ng/ml recombinant human TGF $\beta$ 1 (R&D Systems, Minneapolis, MN).<sup>5</sup> Cultures were maintained for 21 days, with medium changes every third day, prior to analysis.

#### Cellular Biosynthesis of ECM Macromolecules

From the 12-mm disks, 10–20-mg samples were cut, transferred to medium supplemented with 5  $\mu$ Ci/ml <sup>35</sup>S-sulfate and 10  $\mu$ Ci/ml <sup>3</sup>H-proline, and then cultured for 24 h to measure the synthesis rate of sulfated PGs and total protein, respectively.<sup>29</sup> Labeled samples were digested in proteinase K (Roche Applied Science, Indianapolis, IN)-Tris HCl solution overnight at 60°C. From the digests, radiolabel incorporation and total accumulated sulfated glycosaminoglycan (GAG) content, measured by the DMMB dye binding assay,<sup>30</sup> were noted. These data were normalized to the wet weight of the samples.

#### Superose 6 Chromatography

Only TGF $\beta$ 1 cultures were analyzed via chromatography. Samples of 20–30 mg hydrogel were cultured in medium supplemented with 50  $\mu$ Ci/ml <sup>35</sup>S-sulfate for 24 h. Proteins were extracted from minced hydrogel samples in 4 M guanidine solution containing 0.3 M amino-caproic acid, 50 mM sodium acetate, 15 mM benzamidine, 5 mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride, 10 mM MES, 5 mM iodoacetic acid, and 1  $\mu$ g/ml pepstatin A, pH 6.5<sup>31</sup> for 48 h at 4°C with agitation. The extracted proteins were desalted on a PD-10 column (Amersham Biosciences, Piscataway, NJ), lyophilized, and then resuspended in 500 mM ammonium acetate for separation of proteoglycan monomers on a Superose 6 column (Amersham Biosciences, Piscataway, NJ).

#### Real-Time PCR

Cell-seeded hydrogels were flash frozen in liquid nitrogen, pulverized, and then subjected to a QIAshredder (Qiagen, Valencia, VA) cell lysis kit. RNA was extracted (RNeasy mini kit, Qiagen, Valencia, CA) and quantified using a Nanodrop spectrophotometer (Agilent Technologies, Santa Clara, CA), where the purities of the samples ranged from 1.7 to 2.0 260/280 nm. Samples were treated with DNase digestion (Fermentas, Glen Burnie, MD) prior to cDNA synthesis. Absorbance measurements at 260 nm were used to normalize each sample to total RNA content for use in reverse transcription (Superscript III RT kit, Invitrogen, Chicago, IL). Real-time PCR of equal quantities of total RNA (250 ng) was performed using an ABI 7000 and TaqMan 2X PCR Master Mix (Applied Biosystems, Foster City, CA). Expression of ECM genes (type II collagen, type I collagen, aggrecan) was quantified using equine primers and probes.<sup>32</sup> Final quantification was done using the comparative Ct (cycle number at which amplification is detected) method and reported as relative transcription.

Expression is reported as the *n*-fold difference relative to the cDNA for the housekeeping gene GAPDH.

#### Histological Analysis

##### Sample Preparation

Cell-seeded peptide samples were fixed in 10% neutral buffered formalin overnight at 4°C and then embedded in paraffin. Five-micrometer thick sections were spread on charged slides, de-paraffinized, and then rehydrated prior to staining.

##### H&E

Sections were incubated in aqueous hematoxylin solution (Richard-Allan Scientific, Kalamazoo, MI) for 5 min, then alcoholic eosin solution (Richard-Allan Scientific, Kalamazoo, MI) for 2 min.

##### Toluidine Blue Staining

Sections were incubated in an aqueous, 0.04% Toluidine Blue O solution (American Master\*Tech Scientific Inc., Lodi, CA) for 5 min, then rinsed briefly in de-ionized water and left to air dry.

##### Type II Collagen Immunostaining

Sections were treated with a primary, mouse anti-collagen-type II IgG (Developmental Studies Hybridoma Bank, University of Iowa, II-II6B3 1:10 in TBS)<sup>32</sup> for 15 min, rinsed in TBS, and then incubated in biotinylated rabbit anti-mouse IgG (HRP-conjugated, Dako P-0260, Glostrup, Denmark; in TBS) for 5 min. The sections were then stained with Fast Red/Streptavidin Alkaline phosphatase (Enhanced Alk. Phos. Red Detection Kit, Ventana Medical Systems 760-031, Tucson, AZ) and counterstained with hematoxylin (Ventana Medical Systems 760-2021, Tucson, AZ). Equine tracheal tissue containing cartilage and fibrous tissue was processed in parallel to verify specificity of the primary antibody.

#### Statistical Analysis

For biosynthesis and aggrecan/type I collagen expression data, a mixed model ANOVA with a fixed effect of cell source and TGF $\beta$ 1 was applied separately to agarose and peptide cultures. Individual comparisons were made using least square means procedure. For type II collagen data, chi squared comparison was used to test the association for categorical variables defined by the presence or absence of expression. A *p*-value <0.05 was considered significant.

## RESULTS

### Cell Aggregation and Hydrogel Contraction in Agarose

After trypsinization from monolayer expansion cultures, the cells assumed a spherical morphology

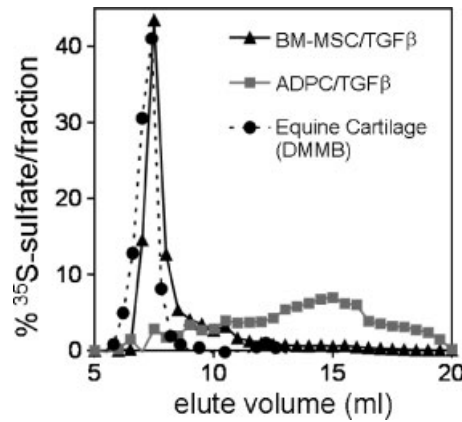
that was retained during hydrogel seeding. For BM-MSC- and ADPC-seeded agarose, distinguishable changes in cell morphology were not observed over the 21-day culture period (data not shown). No contraction of the agarose hydrogels was observed for either cell type.

**Quantification of ECM Synthesis in Agarose**

In BM-MSC-seeded agarose, TGFβ1 stimulated 10- and 41-fold increases in <sup>3</sup>H-proline and <sup>35</sup>S-sulfate incorporation, respectively, relative to TGFβ1-free cultures (Fig. 1, *p* < 0.002). Total GAG accumulation in BM-MSC-seeded agarose cultured in TGFβ1 was ~11-fold higher than TGFβ1-free culture (*p* < 0.002). In ADPC-seeded agarose, TGFβ1 did not have a significant effect on <sup>3</sup>H-proline (*p* = 0.59) or <sup>35</sup>S-sulfate (*p* = 0.89) incorporation. Likewise, total GAG accumulation in ADPC-seeded agarose was similar for both TGFβ1 culture conditions (*p* = 0.95). **A comparison of BM-MSC and ADPC-seeded agarose cultured in TGFβ1 medium showed that <sup>3</sup>H-proline and <sup>35</sup>S-sulfate incorporation for BM-MSCs was 5- and 28-fold higher than for ADPCs, respectively (*p* < 0.002).** Total GAG accumulation in TGFβ1-cultured BM-MSC-seeded agarose was 10-fold higher than TGFβ1-cultured ADPCs (*p* < 0.002).

**Superose 6 Chromatography in Agarose**

PG monomers extracted from adult equine stifle cartilage were analyzed to establish an aggrecan reference profile (Fig. 2). Separation of cartilage protein extract resulted in a single peak PG centered on the void volume of ~7.4 ml.<sup>33</sup> Chromatographic analysis was performed for PG monomers

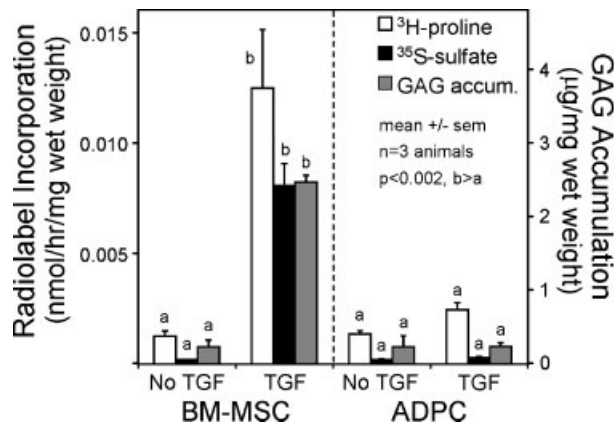


**Figure 2.** Superose 6 chromatography of TGFβ1-cultured samples in agarose. Newly synthesized PGs were labeled for analysis with <sup>35</sup>S-sulfate over the final 24 h of the 21-day culture period. An aggrecan monomer reference profile was established by analyzing PGs from adult equine cartilage via DMMB dye binding. Aggrecan monomer was found to elute at the void volume (7.4 ml). For BM-MSC cultures, the majority of incorporated <sup>35</sup>S-sulfate eluted as an aggrecan-like peak centered on the void volume. ADPCs synthesized a broad range of PGs that lacked a distinguishable aggrecan-like peak.

extracted from TGFβ1 cultures (Fig. 2). BM-MSC-synthesized <sup>35</sup>S-PGs that largely eluted at the void volume. A second, smaller peak was centered on the 10.5-ml elute volume, corresponded to a *K<sub>av</sub>* = 0.20 that has been associated with the small PG decorin,<sup>33</sup> suggesting that BM-MSCs synthesized small PGs.<sup>34</sup> Small PGs may also have been created during culture by secreted catabolic enzymes associated with the chondrocyte phenotype.<sup>35</sup> ADPC cells synthesized sulfated PGs with a broad size-range, suggesting either small or degraded PGs. The eluted profile lacked a clear peak at the void volume, with maximum <sup>35</sup>S-sulfate detected at the 15-ml elute volume (*K<sub>av</sub>* = 0.45). Additional non-animal-matched TGFβ1 cultures (one BM-MSC, two ADPCs) demonstrated similar chromatography profiles to those observed in Figure 2 for each cell type.

**Type II Collagen Gene Expression in Agarose**

Type II collagen expression was detected in all BM-MSC cultures (data not shown, *n* = 3 animals). Type II collagen/GAPDH expression was 0.136 ± 0.067 (mean ± SEM) and 0.007 ± 0.003 in TGFβ1 and TGFβ1-free conditions, respectively. In ADPC cultures, type II collagen expression was detected in one TGFβ1-free culture and no TGFβ samples. **The difference in type II collagen expression between BM-MSCs and ADPCs was significant (*p* = 0.0005).**



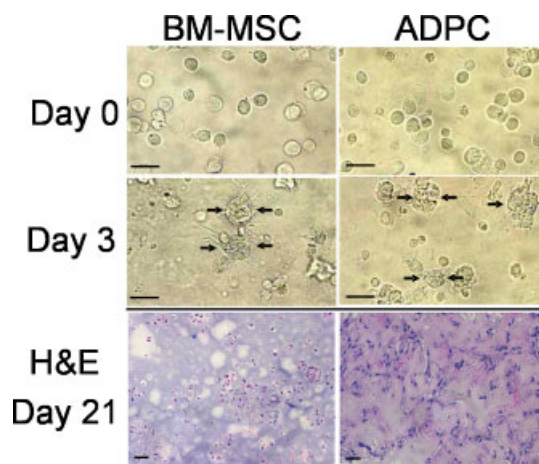
**Figure 1.** ECM synthesis and accumulation after 21 days of culture in agarose. Data for each cell type and TGFβ1 condition were calculated from cultures established from three animals. Significant differences among conditions are denoted by “a” and “b.”

### Aggrecan Expression in Agarose

Aggrecan expression was detected in all BM-MSC and ADPC/TGF $\beta$ 1 cultures (data not shown,  $n = 3$  animals). In ADPC/TGF $\beta$ 1-free cultures, aggrecan was detected in two of three samples. For each animal source, expression was higher in BM-MSC/TGF $\beta$  cultures than BM-MSC/TGF $\beta$ 1-free samples (2–24-fold) and ADPCs in the presence (~20–530-fold) and absence (3–10-fold) of TGF $\beta$ 1. However, analysis of data pooled over the three animals did not show significant differences among conditions. Aggrecan/GAPDH expression in BM-MSC cultures was  $5.81 \pm 3.98$  and  $0.62 \pm 0.24$  in TGF $\beta$ 1 and TGF $\beta$ 1-free conditions, respectively. In ADPC cultures, aggrecan/GAPDH expression was  $0.07 + 0.05$  and  $0.50 + 0.40$  in TGF $\beta$ 1 and TGF $\beta$ 1-free conditions, respectively. Given the high variability of expression in the animal-matched studies, additional data were pooled to explore animal-to-animal variability with a larger sample size. These secondary data were obtained from BM-MSCs and ADPCs that were isolated from separate animals; therefore, statistical comparison between cell types was not conducted. In two additional ADPC cultures, aggrecan/GAPDH expression was detected in TGF $\beta$ 1 cultures (0.17, 0.015) but not TGF $\beta$ 1-free samples, similar to the animal-matched data that was below or near the detection limit. Aggrecan expression was detected in four additional BM-MSC cultures, with higher expression in TGF $\beta$ 1 medium in three of four. Pooled normalized expression values over all seven BM-MSC samples were  $4.71 + 1.82$  and  $2.70 + 1.66$  for TGF $\beta$ 1 and TGF $\beta$ 1-free cultures, respectively. These pooled data were not significantly different ( $p = 0.43$ ). Therefore, this increase in sample size did not greatly affect the variability seen for animal-matched samples.

### Cell Aggregation and Hydrogel Contraction in Peptide

In TGF $\beta$ 1 medium, the initially spherical BM-MSCs and ADPCs (Fig. 3, Day 0) became irregularly shaped, with multiple filopodia extending into the peptide hydrogel within hours of seeding. Over time, the encapsulated cells contacted neighboring cells and condensed into spherical multicell aggregates that were larger than the individual cells on Day 0 (Fig. 3, Day 3, filopodia are still visible around BM-MSC aggregates). Little cell aggregation was observed in TGF $\beta$ 1-free culture. ADPC-seeded peptide hydrogels cultured in TGF $\beta$ 1 medium contracted the 12-mm disks to ~5-mm

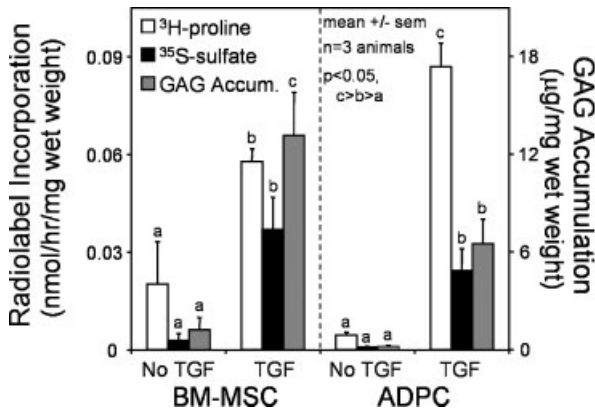


**Figure 3.** Peptide–cell aggregation in TGF $\beta$ 1: Light microscope images of encapsulated cells (Days 0 and 3). Encapsulated BM-MSCs and ADPCs were spherical immediately after hydrogel casting (Day 0). Within days, morphological changes led to cell–cell contact between neighboring cells, with spherical multicell aggregates (arrows) forming with time in culture (Day 3). Cell filopodia are still visible around BM-MSC aggregates. H&E staining (Day 21): BM-MSCs and ADPCs were largely localized in cell aggregates. Bar = 50  $\mu$ m.

diameter by day 21. BM-MSC-seeded peptide hydrogels cultured in TGF $\beta$ 1 contracted to ~10-mm diameter. Changes in hydrogel thickness were not quantified, but were estimated to be proportional to the diameter decreases. No hydrogel contraction was observed for either cell type in the absence of TGF $\beta$ 1. In TGF $\beta$ 1 medium, BM-MSCs and ADPCs appeared heterogeneously distributed throughout the hydrogel (Fig. 3, Day 21). In BM-MSC-seeded peptide, many cells were associated in aggregates. As observed for BM-MSCs, the bulk of the ADPCs appeared to be associated in cell aggregates.

### Quantification of ECM Synthesis in Peptide

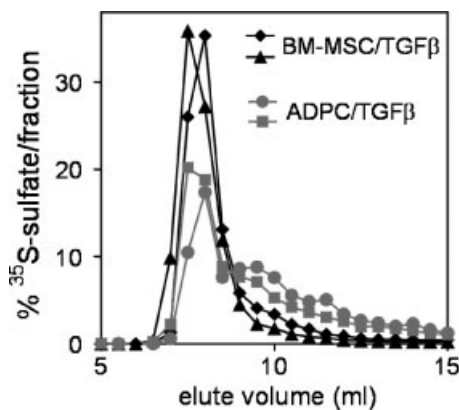
TGF $\beta$ 1 stimulated a significant increase in biosynthesis in both BM-MSC- and ADPC-seeded peptide hydrogels (Fig. 4).  $^3$ H-proline incorporation was 3- and 19-fold higher than TGF $\beta$ 1-free conditions for BM-MSCs and ADPCs, respectively ( $p < 0.05$ ). Likewise, TGF $\beta$ 1 treatment increased  $^{35}$ S-sulfate incorporation 13- and 18-fold for BM-MSCs and ADPCs, respectively ( $p < 0.05$ ). Total GAG accumulation in TGF $\beta$ 1-cultured BM-MSC and ADPC-seeded peptide was 11- and 29-fold higher ( $p < 0.05$ ), respectively. For TGF $\beta$ 1 cultures,  $^3$ H-proline incorporation for ADPCs was 50% higher than for BM-MSCs ( $p < 0.05$ ), whereas  $^{35}$ S-sulfate incorporation was not significantly different ( $p = 0.09$ ). Total GAG accumulation for BM-MSCs was two-fold higher than ADPCs ( $p < 0.05$ ).



**Figure 4.** ECM synthesis and accumulation after 21 days of culture in peptide. Data for each cell type and TGFβ1 condition were calculated from cultures established from three animals. Significant differences among conditions were denoted by “a,” “b,” and “c.”

**Superose 6 Chromatography in Peptide**

BM-MSC-synthesized PGs largely eluted as an aggrecan-like peak centered on the void volume (Fig. 5). This peak decreased to baseline levels by the 11.5-ml elute volume (Kav=0.25), demonstrating that a relatively small amount of small PGs were synthesized. ADPCs cultured in TGFβ1 produced an aggrecan-like peak and a broad peak of small PGs that approached baseline levels by the 15-ml elute volume (Kav=0.45). The magnitude and width of the peaks between 9.0- and 15-ml elute volumes (Kav=0.10–0.45, respectively) demonstrated that ADPCs synthesized a



**Figure 5.** Peptide–Superose 6 chromatography of two TGFβ1-cultured samples per cell type. Newly synthesized PGs were labeled for analysis with <sup>35</sup>S-sulfate over the final 24 h of the 21-day culture period. For BM-MSC cultures, the majority of the incorporated <sup>35</sup>S-sulfate eluted as an aggrecan-like peak centered on the void volume. ADPCs synthesized both aggrecan-like and small PGs, with a greater percent of the total incorporated <sup>35</sup>S-sulfate in small PGs than seen for BM-MSCs.

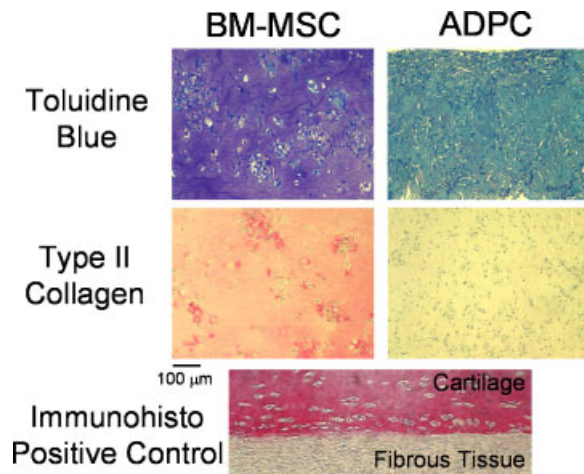
greater percentage of small PGs or aggrecan fragments than did BM-MSCs.

**Type II Collagen and Aggrecan Gene Expression in TGFβ1 Medium in Peptide**

In BM-MSC-seeded peptide, aggrecan/GAPDH expression was 3.55 + 2.79, whereas type II collagen was 0.10 + 0.07 (data not shown, n = 3 animals). In ADPC-seeded peptide, aggrecan/GAPDH expression was 0.27 + 0.14, whereas no type II collagen was detected. For each animal source, aggrecan expression was 2.5–43-fold higher in BM-MSCs relative to paired ADPC cultures. However, pooled expression levels for each cell type were not significantly different (p = 0.31).

**Histological Evaluation of ECM Accumulation in Peptide Hydrogels in TGFβ1 Medium in Peptide**

Metachromatic staining was uniformly present throughout BM-MSC-seeded peptide hydrogels (Fig. 6). In ADPC cultures, trace levels of metachromatic staining were visible in relatively acellular areas of the hydrogel. In BM-MSC cultures, positive type II collagen staining was present throughout the hydrogel, with the greatest staining found within and around cell clusters (Fig. 6). In ADPC cultures, no positive type II collagen staining was present.



**Figure 6.** (A,B) ECM accumulation in peptide hydrogels cultured in TGFβ1. PGs were identified by Toluidine blue staining while immunohistochemical staining (fast red/streptavidin alk. phos.) was performed to detect type II collagen accumulation. Tracheal tissue was processed in parallel to verify specificity of the primary antibody. Positive staining was present in BM-MSC cultures, while little to no staining was detected in ADPC cultures. Bar = 100 µm.

### Type I Collagen Gene Expression for Agarose and Peptide Cultures

Type I collagen expression was detected for all cultures (data not shown,  $n = 3$  animals for each hydrogel).

#### Agarose

For each of the animal sources, type I collagen expression in BM-MSCTGF $\beta$ 1 cultures were lower than two BM-MSCTGF $\beta$ 1-free (no change,  $\sim$ 17-, 21-fold decrease) and all ADPC cultures ( $\sim$ 2–63-fold decrease). When pooled across the three animal sources, type I collagen/GAPDH expression for BM-MSCTGF $\beta$ 1 cultures was  $0.35 \pm 0.19$  and  $2.22 + 1.37$  in TGF $\beta$ 1 and TGF $\beta$ 1-free conditions, respectively. In ADPC cultures, type I collagen/GAPDH expression was  $7.19 + 5.25$  and  $4.29 + 3.33$  in TGF $\beta$ 1 and TGF $\beta$ 1-free conditions, respectively. No significant differences were found among conditions.

#### Peptide Cultured in TGF $\beta$ 1 Medium

For each animal, type I collagen expression in ADPCs was 4–36-fold higher than matched BM-MSCTGF $\beta$ 1 cultures. Type I collagen/GAPDH expression in ADPCs was  $9.1 + 5.60$ , whereas expression in BM-MSCTGF $\beta$ 1 cultures was  $0.41 + 0.08$ , although these results were not significantly different ( $p = 0.20$ ).

## DISCUSSION

With the exception of tritiated proline incorporation in peptide samples, adult equine BM-MSCTGF $\beta$ 1 cultures encapsulated in hydrogel scaffolds synthesized and accumulated ECM in response to TGF $\beta$ 1 at a higher rate than did ADPC-seeded hydrogels. Furthermore, chromatographic, type II collagen expression, and histological analyses demonstrated greater TGF $\beta$ 1-mediated chondrogenesis of BM-MSCTGF $\beta$ 1 cultures than of ADPCs. These data are consistent with previously reported trends of chondrogenesis from human BM-MSCTGF $\beta$ 1 aggregate cultures,<sup>15–18</sup> with the emphasis that the expression and synthesis of cartilage-like ECM correlated with high levels of neo-tissue synthesis. The present study also demonstrated that the material environment provided by a tissue engineering scaffold may be an important factor when evaluating the chondrogenic potential of progenitor cells, since for ADPCs, a significant effect of TGF $\beta$ 1 treatment was observed for peptide (Fig. 5) but not agarose (Fig. 1) culture, and rates of ECM synthesis and accumulation in BM-MSCTGF $\beta$ 1-seeded

peptide were  $\sim$ 4–6-fold higher than in agarose culture.

TGF $\beta$ 1 stimulation of ECM synthesis of BM-MSCTGF $\beta$ 1 seeded in agarose was consistent with previously reported data for other BM-MSCTGF $\beta$ 1-seeded scaffolds.<sup>23,36</sup> In addition, GAG accumulation in BM-MSCTGF $\beta$ 1-seeded agarose cultured in TGF $\beta$ 1 medium was on the order of that seen for immature bovine BM-MSCTGF $\beta$ 1 encapsulated in agarose<sup>23</sup> and other BM-MSCTGF $\beta$ 1-seeded scaffolds.<sup>36–38</sup> For adult equine ADPC-seeded agarose, the lack of stimulation of ECM synthesis and accumulation with TGF $\beta$ 1 was consistent with results of human ADPCs seeded in elastin-like polypeptide<sup>39</sup> and alginate hydrogel<sup>40</sup> beyond the first week of culture. GAG accumulation was similar to that for human ADPCs encapsulated in agarose hydrogel and cultured in TGF $\beta$ 1 medium.<sup>21</sup> Therefore, adult equine BM-MSCTGF $\beta$ 1 and ADPCs seem to share a similar potential to synthesize ECM in response to TGF $\beta$ 1 with human and other animal sources.

Differences in the rates of biosynthesis between peptide and agarose hydrogels may have arisen from factors associated with the structure and composition of each material.<sup>41,42</sup> However, the most obvious difference was the degree of cell aggregation, a factor that may be critical for the induction of progenitor cell chondrogenesis,<sup>5</sup> that occurred in peptide (Fig. 4) but not agarose hydrogel. Peptide hydrogel is characterized by compressive properties an order of magnitude less than those of agarose.<sup>24</sup> Therefore, the major difference between the two hydrogels may have been the ability of the progenitor cells to displace peptide but not agarose.

The significant differences in PG synthesis and accumulation with cell type and TGF $\beta$ 1 conditions were not conclusively reflected in pooled aggrecan core protein gene expression due to high animal-to-animal variability. Likewise, the pooling of seven BM-MSCTGF $\beta$ 1 agarose samples did not result in significant differences with TGF $\beta$ 1 treatment. Therefore, aggrecan expression was a weak indicator of chondrogenesis relative to measures of PG synthesis. Others have reported disparities between aggrecan core protein gene expression and PG synthesis for BM-MSCTGF $\beta$ 1<sup>34</sup> and ADPC<sup>43</sup> cultures. Also, it is generally accepted that the presence of mRNA does not ensure protein translation, as posttranscriptional regulation is important in cartilage ECM synthesis.<sup>44</sup> Furthermore, while a high degree of sulfation is characteristic of aggrecan, reduced sulfation of the core protein might contribute to underestimating protein synthesis using the assays in this study. Further exploration

into the relationship between aggrecan core protein gene transcription and protein translation may be of interest. However, these data support the need for quantification of ECM synthesis to support claims of differentiation into a phenotype capable of high levels of cartilage-like, neo-tissue synthesis.

The presence of type I collagen transcripts was an expected outcome based on previous studies that have shown its persistent expression with time in BM-MSC cultures.<sup>5,23,34,45,46</sup> Type I collagen gene transcripts may be partly attributed to flattened cells of a fibroblastic morphology that specifically populate the surfaces of aggregate and scaffold cultures.<sup>5,34,36,45</sup> Type I collagen expression was also detected in progenitor cells that concurrently expressed type II collagen transcripts.<sup>34</sup> The ubiquitous nature of type I collagen gene expression complicates the interpretation of its presence as a definitive measure of a fibroblastic, as opposed to a chondrocytic, phenotype. In this study, the general trend of increased type I collagen mRNA expression in ADPCs relative to BM-MSCs in TGF $\beta$ 1 culture may be sufficient to suggest a difference in phenotypic differentiation. Also, the relatively strong contractile behavior in ADPC peptide cultures may suggest a fibroblastic phenotype.<sup>47</sup> These data may hold greater significance when considering the lack of type II collagen expression in ADPCs, suggesting that the increase in type I collagen mRNA expression may have been an independent event that was not associated with TGF $\beta$ 1-mediated chondrogenesis in ADPC cultures.

The selection of progenitor cells over autologous chondrocytes for cartilage resurfacing may be partly motivated by clinical considerations; however, the benefits of a simplified harvest from noncritical tissues must be justified by a repair potential comparable to chondrocytes. Chondrocytes synthesize ~50% more ECM than BM-MSCs when comparing cells from skeletally immature animal sources.<sup>23,48</sup> These data demonstrate that significant differences may exist in the tissue repair potential of progenitor cells and chondrocytes for a given animal source and scaffold. In the absence of an adult chondrocyte comparison here, neo-tissue synthesis of progenitor cells encapsulated in peptide hydrogel may be compared to previous studies utilizing newborn bovine chondrocytes.<sup>49</sup> Chondrocyte-seeded peptide hydrogel seeded at  $30 \times 10^6$  cells/ml accumulated 13.5 mg GAG/mg wet weight after 21 days of culture, with protein and PG synthesis rates of 0.031 and 0.026 nmol/h/mg wet weight, respectively, values similar to those measured in this study for adult progenitor

cells in peptide hydrogel despite an initial cell seeding density of  $10 \times 10^6$  cells/ml. Given that newborn chondrocytes possess a greater capacity for synthesizing neo-tissue than chondrocytes from skeletally mature animals in hydrogel culture,<sup>20</sup> adult equine progenitor cells should compare favorably to adult equine chondrocytes. This comparison is especially encouraging for BM-MSCs as the observed 20% hydrogel contraction only modestly influenced wet weight normalization. Whereas ADPCs had high rates of ECM synthesis as a function of wet weight, the ~4-fold aggregation of cells with contraction suggest a repair potential per cell that is significantly lower than BM-MSCs.

Both protein and gene expression evidence of advanced chondrogenesis of BM-MSCs relative to ADPCs suggest that BM-MSCs are more promising for cartilage repair. In addition, the low levels of ECM synthesis in TGF $\beta$ 1-free cultures support the need for including chondrogenic factors in progenitor cell repair strategies. Continuous growth factor treatment over several weeks of culture prior to implantation represents one option, but other strategies that do not require extensive culturing may improve upon the clinical feasibility of implementing progenitor cell therapies.<sup>25,48,50</sup> Furthermore, chondrogenic growth factors such as BMPs,<sup>43,50–52</sup> IGF-1,<sup>48</sup> or combinations of growth factors<sup>43,48</sup> represent other options for inducing chondrogenesis, although such strategies have not been proven to stimulate higher rates of neo-tissue synthesis than TGF $\beta$ 1 alone. Such parameters merit ongoing consideration to explore the potential of progenitor cells for cartilage repair within practical clinical guidelines that would allow for the widespread application of a successful strategy.

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