Chondrogenesis of Adult Stem Cells from Adipose Tissue and Bone Marrow: Induction by Growth Factors and Cartilage-Derived Matrix

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Objectives: Adipose-derived stem cells (ASCs) and bone marrow-derived mesenchymal stem cells (MSCs) are multipotent adult stem cells with potential for use in cartilage tissue engineering. We hypothesized that these cells show distinct responses to different chondrogenic culture conditions and extracellular matrices, illustrating important differences between cell types.

Methods: Human ASCs and MSCs were chondrogenically differentiated in alginate beads or a novel scaffold of reconstituted native cartilage-derived matrix with a range of growth factors, including dexamethasone, transforming growth factor β 3, and bone morphogenetic protein 6. Constructs were analyzed for gene expression and matrix synthesis.

Results: Chondrogenic growth factors induced a chondrocytic phenotype in both ASCs and MSCs in alginate beads or cartilage-derived matrix. MSCs demonstrated enhanced type II collagen gene expression and matrix synthesis as well as a greater propensity for the hypertrophic chondrocyte phenotype. ASCs had higher upregulation of aggrecan gene expression in response to bone morphogenetic protein 6 (857-fold), while MSCs responded more favorably to transforming growth factor β 3 (573-fold increase).

Conclusions: ASCs and MSCs are distinct cell types as illustrated by their unique responses to growth factor-based chondrogenic induction. This chondrogenic induction is affected by the composition of the scaffold and the presence of serum.

Introduction

CARTILAGE TISSUE ENGINEERING SEEKS to combine cells, biomaterial scaffolds, and bioactive signals to create functional tissue replacements to treat cartilage injuries or osteoarthritis.^{1,2} Primary chondrocytes expanded *in vitro* are one cell source that has been used for autologous chondrocyte implantation,³ but there has been growing interest in alternative cell sources for cartilage tissue engineering. Adult stem cells derived from adipose tissue [adipose-derived stem cells (ASCs)]^{4,5} and bone marrow (bone marrow-derived mesenchymal stem cells, MSCs)^{6,7} have shown significant chondrogenic potential for such a tissue engineering approach.^{8–11} ASCs have attracted interest due to ease of isolation procedure and relative abundance of cells available as compared to MSCs,^{12,13} but remain less well characterized.

While many studies tend to refer to these cell types using similar terminology, that is, adipose-derived MSCs,^{14–17} a growing number of studies have shown human ASCs and MSCs to be very similar but not identical cell types in

monolayer culture with regard to morphology, proliferation, gene expression, and cell surface markers.^{14–33} Some differences include ASCs being smaller,¹⁶ ASCs achieving higher passage numbers before senescence,^{15,23,26} differential expression of genes related to proliferation,^{26,32} and ASCs having reduced or absent transforming growth factor β (TGF- β) receptor ALK-5²¹ and cell surface marker vascular cell adhesion molecule 1 (CD106).^{15,16,19,29} In addition to these biological characterizations, many studies have compared the chondrogenic potential of the two cell types. With the exception of a few studies,^{15,20,23,26} it has been observed that under standard chondrogenic differentiation conditions, MSCs have an enhanced potential for chondrogenesis as compared to ASCs by measures such as glycosaminoglycans (GAG) production, type II collagen gene expression and deposition, pellet size, and consistency among donors for differentiation.^{14,16–18,22,27–31,33}

However, it is important to note that these studies have used identical culture conditions for ASCs and MSCs, typically utilizing TGF- β and dexamethasone (DEX) to induce

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chondrogenesis, with some studies adding additional growth factors.^{14,17,23,31} The tacit assumption in such studies is that culture conditions optimized for MSCs will also be optimal for ASCs. However, emerging evidence suggests that any comparison study between ASCs and MSCs will be affected by the specific culture conditions used. ASCs have been shown to be more efficiently induced toward a chondrogenic lineage by a high dose of bone morphogenetic protein-6 (BMP-6) than by TGF- β or other cocktails.³⁴ Hennig *et al.* demonstrated that the addition of BMP-6 to a TGF- β culture medium resulted in robust chondrogenesis of ASCs similar to MSCs with TGF- β .²¹ Kim and Im demonstrated that a higher concentration of growth factors was able to overcome initial differences in chondrogenic differentiation between ASCs and MSCs.²⁴ The response of adult stem cells to soluble factors that induce differentiation is therefore one method of identifying differences among tissue sources. A study design that incorporates multiple chondrogenic media conditions may be able to better assess these divergent responses to growth factors than previous single-condition studies. Our first hypothesis was that ASCs and MSCs are distinct cell types with unique responses to growth factors or other chondroinductive culture conditions.

In addition to the growth factor conditions, the extracellular environment can also influence cellular growth and differentiation. Pellet culture has been used extensively as a model system to compare chondrogenesis in MSCs and ASCs because it recapitulates the condensation that occurs during cartilage development and maintains the potential for cell-cell interaction.³⁵ Alginate bead culture is a model system that encourages a rounded cell phenotype⁸ to induce stem cells toward a chondrocyte-like lineage. Recent studies have shown that scaffolds consisting of reconstituted native cartilage-derived matrix (CDM) can induce the chondrogenesis of ASCs³⁶ or MSCs,³⁷ potentially through the establishment of interactions between cell surface receptors and extracellular matrix ligands present on the native tissue proteins. Such cell-matrix interactions are important in cartilage development³⁸ and homeostasis,³⁹ as well as in collagen remodeling by MSCs.⁴⁰ Studies using tissues such as heart,⁴¹ bladder,⁴² tendon,⁴³ and, recently, the clinical transplantation of a donor airway⁴⁴ have also shown the value in using native tissue architecture to provide instructive cues for tissue engineering.⁴⁵ Using the cell environment to induce chondrogenesis in place of or in addition to growth factors allows for further understanding of the role of the extracellular matrix in regulating chondrogenesis. Thus, our second hypothesis was that chondrogenesis in ASCs and MSCs is affected by the cell microenvironment (alginate or CDM).

Materials and Methods

Cell culture and chondrogenic differentiation

Human ASCs were obtained from subcutaneous abdominal adipose tissue (Zen-Bio, Durham, NC). ASCs from seven women (average age, 41 years) were combined after initial expansion to make a superlot. Cells were cultured at 8000 cells/cm² through four passages in Dulbecco's modified Eagle's medium (DMEM)/F12 (BioWhittaker, Walkersville, MD) containing 0.25 ng/mL TGF- β 1 (R&D Systems, Minneapolis, MN), 5 ng/mL EGF (Roche Diagnostics, Indianapolis, IN), and 1 ng/mL basic fibroblast growth factor (bFGF; Roche

Diagnostics), as well as 10% fetal bovine serum (FBS; Atlas Biologicals, Ft. Collins, CO) as previously described.⁴⁶ Human MSCs were obtained from the posterior superior iliac crest of donors as approved by the Institutional Review Board as previously described.⁴⁷ MSCs from three women (average age 27 years) were combined in a superlot after initial expansion. Cells were cultured at 5000 cells/cm² through four passages in DMEM-low glucose (Gibco, Grand Island, NY) containing 1 ng/mL bFGF and 10% FBS (Sigma-Aldrich, St. Louis, MO).

ASCs and MSCs were either resuspended in 1.2% alginate (5×10^6 cells/mL) and dropped in 102 mM calcium chloride solution with a 1 mL pipette to form beads, or seeded onto 6-mm-diameter CDM scaffolds (500,000 cells in a 30 μ L medium added for 1 h before the culture medium added). CDM was prepared by homogenizing porcine articular cartilage at a concentration of 0.1 g wet weight/mL distilled water and then lyophilizing for 24 h as previously described.³⁶

Alginate and CDM constructs were cultured for 14 or 28 days. Low-attachment 24-well plates (Corning Life Sciences, Corning, NY) were used with 1 mL of the culture medium (changed every other day). The culture medium contained DMEM-high glucose (Gibco), 1% penicillin-streptomycin (Gibco), 37.5 μ g/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich), 40 μ g/mL L-proline (Sigma-Aldrich), and 1% ITS + Premix (Collaborative Biomedical-Becton Dickinson, Bedford, MA) plus combinations of the following chondroinductive agents (Figs. 1 and 3): 100 nM DEX (Sigma-Aldrich), 10 ng/mL TGF- β 3 (R&D Systems), and 10 or 500 ng/mL BMP-6 (R&D Systems). A subset of the alginate bead conditions was used for CDM constructs. Day 14 constructs were evaluated with quantitative real-time reverse transcriptase-polymerase chain reaction (qPCR), and day 28 constructs were either digested for biochemical analysis or prepared for immunohistochemistry as described below.

RNA isolation and qPCR

Fourteen-day qPCR samples were prepared for RNA isolation ($n=3$ independent samples per group). CDM constructs were snap-frozen in liquid nitrogen and pulverized using a mortar and pestle, while alginate beads were treated with 150 mM NaCl and 55 mM Na citrate to release the cells. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and quantified with spectrophotometry (Nanodrop ND-1000, Wilmington, DE). The RNA was reverse transcribed with SuperScript VILO (Invitrogen) and analyzed for gene expression using Express qPCR SuperMix Universal (Invitrogen) on an iCycler (Bio-Rad, Hercules, CA). Primer probes (Applied Biosystems, Foster City, CA) were used to determine transcript levels in triplicate for a housekeeping gene and four different genes of interest: 18S ribosomal RNA (endogenous control; assay ID Hs99999901_s1), aggrecan (AGC1; assay ID Hs00153936_m1), type I collagen (COL1A1; assay ID Hs00164004_m1), type II collagen (COL2A1; custom assay: forward primer, 5-GAGACAGCATGACGCCGAG-3; reverse primer, 5-GCGGATGCTCTCAATCTGGT-3; probe 5-FAM-TGGATGCCCACTCAAGTCCCTCAAC-TAMRA-3),²⁸ and type X collagen (COL10A1; assay ID Hs00166657_m1). The standard curve method was used to determine starting transcript quantity (copy number) for each gene using plasmids containing the gene of interest. Data were analyzed by calcu-

FIG. 1. Day 14 reverse transcriptase–polymerase chain reaction for (A) alginate bead and (B) cartilage-derived matrix (CDM) constructs seeded with adipose-derived stem cells (ASCs) or mesenchymal stem cells (MSCs) (as labeled). Data presented as fold differences from day 0 cells for *AGC1*, *COL2A1*, *COL10A1*, and *COL1A1*. Error bars represent standard error of the mean. Groups not sharing a letter are significantly different by Fisher protected least significant difference (PLSD) *post hoc*. Asterisk indicates that the medium condition is significantly different from control by analysis of variance (ANOVA).

lating the fold difference compared to day 0 cells of the same type, with each sample first normalized to its own 18S value.

Biochemical analysis

Day 28 biochemical samples ($n=3$ independent samples per group) were analyzed for double-stranded DNA (dsDNA) and sulfated GAG. Both alginate and CDM constructs were digested for 16 h with 1 mL of 125 $\mu\text{g}/\text{mL}$ papain. The PicoGreen fluorescent dsDNA assay (Molecular Probes, Eugene, OR) with λ DNA standard curve was used to calculate μg of dsDNA as a surrogate for cell number.³⁴ The 1,9-dimethylmethylene blue assay (DMMB)⁴⁸ with pH adjusted to 1.5 was used to quantify total sulfated GAG against a chondroitin-4-sulfate standard curve.⁴⁹

Immunohistochemistry and histology

Day 28 immunohistochemistry samples were fixed overnight at 4°C in a pH 7.4 solution containing 4% paraformaldehyde, 100 mM sodium cacodylate, and 50 mM BaCl_2 . Both alginate and CDM constructs were taken through a series of increasing ethanol solutions and xylene steps to clear the constructs. Samples were then embedded in paraffin and cut into 5 μm sections. Monoclonal antibodies to type I collagen (ab6308; Abcam, Cambridge, MA), type II collagen (II-16B3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), type X collagen (C7974; Sigma-Aldrich), and chondroitin 4-sulfate (2B6 antibody, gift from Dr. Virginia Kraus) were used. Sections for collagen staining were treated with Pepsin (Digest-All; Zymed, San Francisco,

CA) and sections for chondroitin 4-sulfate were treated with trypsin, soybean trypsin inhibitor, and chondroitinase ABC (all from Sigma-Aldrich) to expose the epitopes. The anti-mouse IgG secondary antibody (Product No. B7151; Sigma-Aldrich) was linked to horseradish peroxidase and reacted with aminoethyl carbazole using the Histostain-Plus ES Kit (Zymed). General histological staining using 0.1% aqueous safranin-O, 0.02% fast-green, and hematoxylin was also performed on xylene-cleared sections. Human osteochondral plugs were prepared in the same manner as samples and were used as positive controls for each antibody. Negative controls without primary antibody were also prepared for each slide.

Statistical analysis

Two-factor analysis of variance (ANOVA) and Fisher's protected least significant difference (PLSD) *post hoc* test ($\alpha = 0.05$) were used to determine significance for cell type and culture condition. Outliers were removed according to the Chauvenet's criterion.⁵⁰

Results

Gene expression is presented as the fold difference in copy number from day 0 values for each cell type (Fig. 1). For the alginate beads, the main effects of cell type and culture condition, as well as the interaction term, were statistically significant by ANOVA for all genes studied ($p < 0.001$) (Fig. 1A). *AGC1* expression was enhanced in control conditions for MSCs (17-fold increase over day 0 cells) but not ASCs. The presence of only DEX caused a decrease in MSC *AGC1* expression as compared to control ($p < 0.001$) but had no effect on ASCs. Both ASCs and MSCs responded robustly to growth factor induction, with all three growth factor groups significantly increased relative to control ($p < 0.001$). ASCs had the highest upregulation of *AGC1* with 500 ng/mL BMP-6 (857-fold increase), and MSCs had the highest upregulation with 10 ng/mL TGF- β 3 and DEX (573-fold increase). With both BMP-6 and TGF- β present in the culture medium, no differences were observed between the cell types in terms of *AGC1* upregulation ($p > 0.05$).

COL2A1 expression in ASCs was only significantly upregulated as compared to control when both TGF- β 3 and BMP-6 were administered ($p < 0.001$), whereas *COL2A1* expression in MSCs was significantly upregulated in all growth factor groups ($p < 0.001$). The highest expression was seen in the TGF- β 3 plus DEX group, with a 130,450-fold increase from day 0 values.

COL10A1 expression was significantly higher in MSCs as compared to ASCs for each culture condition tested ($p < 0.001$). For ASCs, *COL10A1* expression remained below day 0 values in every group except those containing TGF- β 3. For MSCs, *COL10A1* expression was downregulated compared to control in both the DEX and 500 ng/mL BMP-6 groups ($p < 0.001$) and was strongly upregulated in conditions containing TGF- β 3 (1720 and 2319-fold increases over day 0 cells for TGF- β 3 and dual cocktail of TGF- β 3 and BMP-6, respectively).

COL1A1 expression was significantly increased in all three growth factor groups as compared to control in both cell types ($p < 0.01$). In each culture condition, MSCs had a higher fold increase over day 0 values than ASCs ($p < 0.001$).

For the CDM constructs, the main effects of cell type and culture condition were statistically significant by ANOVA ($p < 0.001$) for each gene studied with the exception of the effect of culture condition on *COL1A1* expression (Fig. 1B). The interaction term of cell type and culture condition was only significant for *COL2A1*. The two growth factor groups investigated were a subset of those studied in the alginate bead system and both included 10 ng/mL TGF- β 3 plus 100 nM DEX, with one group also containing 10 ng/mL BMP-6.

In CDM constructs, *AGC1* upregulation was higher in MSCs than in ASCs ($p < 0.05$) and was significantly higher in the growth factor conditions as compared to control ($p < 0.001$), with no difference between the two groups. The highest *AGC1* upregulation over day 0 cells was the MSC TGF- β 3-only group with a 217-fold increase. *COL2A1* expression was enhanced in the growth factor groups over control conditions for both cell types ($p < 0.001$), but to a much greater degree in MSCs with an average increase of 23,927-fold over day 0 cells for MSCs and 74-fold for ASCs.

For *COL10A1* expression, MSCs had significantly higher upregulation than ASCs in CDM constructs ($p < 0.001$) and the growth factors induced higher *COL10A1* expression as compared to the control conditions ($p < 0.001$). Finally, *COL1A1* expression was higher in MSCs than in ASCs ($p < 0.001$), but there was no difference among the medium conditions ($p > 0.05$).

Figure 2 depicts the gross appearance of the CDM scaffolds after 28 days of culture. The texture of the scaffolds in the growth factor groups is altered and is smoother than the seeded constructs cultured in control conditions or the unseeded construct. There was contraction of the CDM scaffolds as compared to the 6-mm-diameter starting scaffold, with the most contraction occurring in growth factor-treated groups.

The viability and cell proliferation was measured by using dsDNA as a surrogate and is expressed as the percentage of each cell type's starting DNA (Fig. 3). The amount of sulfated GAG was measured using the DMMB assay and is presented both in terms of total GAG and GAG per DNA (Fig. 3). In both the alginate bead and CDM systems, MSCs had significantly higher DNA values as compared to ASCs under each culture condition ($p < 0.05$). The highest values in alginate beads were seen in the TGF- β 3 and BMP-6 group, with 126% of day 0 DNA in MSCs and 46% in ASCs, and the highest values in the CDM were seen in the TGF- β 3-only group, with 277% in MSCs and 98% in ASCs. Total GAG production in the alginate beads was higher in the MSCs than in ASCs for both conditions containing TGF- β 3 ($p < 0.001$), but was higher in the ASCs for the 500 ng/mL BMP-6 group ($p < 0.001$). Total GAG production in the CDM was higher in the MSCs under all conditions ($p < 0.05$), and was highest in the TGF- β 3-only group (316 μ g for MSCs and 134 μ g for ASCs). The trends were slightly altered in the alginate beads when GAG production was normalized to DNA content, as GAG per DNA was higher in ASCs than in MSCs for the TGF- β 3-only group in addition to the BMP-6 group ($p < 0.001$).

The immunohistochemical staining of day 28 CDM scaffolds for type I, II, and X collagens, as well as chondroitin-4-sulfate, is shown in Figure 4. All scaffolds demonstrate staining for type II collagen and chondroitin-4-sulfate with minimal staining for type I and X collagens. The native porcine matrix is still present at 28 days and partially contrib-

FIG. 2. Gross appearance of CDM scaffolds seeded with ASCs or MSCs under control, transforming growth factor β (TGF- β), and TGF- β plus bone morphogenetic protein 6 (BMP-6) conditions (as labeled) at day 28. An unseeded CDM construct at day 0 and an unseeded construct cultured for 28 days are also shown. Scale bar is 2 mm. Color images available online at www.liebertonline.com/ten.

FIG. 3. Percentage of day 0 DNA, total glycosaminoglycan (GAG) content (μ g), and glycosaminoglycan content per DNA (μ g/ μ g) for (A) alginate bead and (B) CDM constructs seeded with ASCs or MSCs (as labeled). Groups not sharing a letter are significantly different by Fisher PLSD *post hoc*. Asterisk indicates that the medium condition is significantly different from control by ANOVA. Error bars represent standard error of the mean.

FIG. 4. Immunohistochemistry for type II collagen, chondroitin-4-sulfate, type X collagen, and type I collagen. CDM seeded with ASCs or MSCs under control, TGF- β , and TGF- β plus BMP-6 conditions (as labeled) at day 28. A human osteochondral plug is the positive control and an unseeded CDM construct cultured for 28 days is provided as an additional control. Pictures are 4 $\times$ magnification with 200- μ m scale bar. Color images available online at www.liebertonline.com/ten.

utes to the positive staining for the extracellular matrix proteins of cartilage. Cell-seeded scaffolds demonstrated enhanced retention of native matrix as compared to the unseeded CDM scaffold. Neotissue was synthesized by both ASCs and MSCs in response to growth factor conditions and was clearly distinguished from native matrix by intensity of staining as well as texture. The neotissue was more abundant in MSC-seeded scaffolds as compared to ASCs, with matrix

staining positive for type II collagen and chondroitin-4-sulfate completely filling in the CDM in response to TGF- β 3. The two growth factor groups, TGF- β 3 and TGF- β 3 plus BMP-6, elicited generally similar matrix deposition from both cell types, with the exception that MSC synthesis of chondroitin-4-sulfate appeared to be stronger in the TGF- β 3-only group. Type I collagen staining was detected in the neotissue at similar levels for each scaffold but was not nearly

as abundant as type II collagen. No significant staining for type X collagen was observed. High-magnification images of safranin-O/fast-green-stained sections indicated that the cell morphology of MSCs had become rounded in response to growth factors, while the ASCs appeared to remain spindle shaped (Fig. 5).

Experiments were performed to evaluate the effect of serum on ASCs and MSCs during differentiation (Fig. 6). In the CDM system, the extent of new matrix production was not significantly altered by serum (Fig. 6B), but gross appearance suggested that 10% serum did affect the culture system by enhancing cell-mediated contraction (Fig. 6A). In the alginate bead system, ASCs produced more type II collagen in response to TGF- β when 10% serum was included (Fig. 6C). MSCs produced less type II collagen in response to TGF- β when serum was present.

Discussion

Adult stem cells from adipose tissue (ASCs) and bone marrow (MSCs) underwent chondrogenic induction by growth factors in both alginate bead culture and CDM scaffolds, demonstrating upregulation of cartilage-specific genes and the synthesis of cartilaginous proteins. Under the conditions used in this study, MSCs demonstrated an overall greater

chondrogenic response than ASCs as indicated by higher *COL2A1* upregulation and more extensive matrix synthesis across all medium conditions in both alginate beads and CDM. However, the level of induction of chondrogenic genes was highly dependent on the exact culture conditions used in each experiment. Thus, the conclusion that MSCs are inherently more chondrogenic than ASCs is not warranted from this work or similar comparison studies due to the strong dependence of results on the monolayer expansion,⁵¹ three-dimensional culture conditions,^{21,24} and time point of analysis.^{18,28,33} Optimal conditions for both cell types remain to be discovered over time, but this study was not designed for that goal. Instead, we sought to demonstrate the unique nature of MSCs and ASCs through divergent responses to chondrogenic growth factors and extracellular matrices. Our findings also show that CDM may provide a novel culture system for the study of stem cell chondrogenesis, alone or in combination with exogenous growth factors.

While ASCs and MSCs exhibited many of the same trends in response to chondrogenic induction, some distinct responses to the specific growth factors used in alginate bead culture were observed. ASCs had significantly higher *AGC1* upregulation in response to BMP-6 than to TGF- β , while the opposite was true for MSCs (Fig. 1). This trend was supported by GAG content when normalized for DNA content

FIG. 5. Safranin-O/fast-green staining with hematoxylin counterstain. CDM seeded with ASCs or MSCs under control, TGF- β , and TGF- β plus BMP-6 conditions (as labeled) at day 28. A human osteochondral plug is the positive control, and an unseeded CDM construct cultured for 28 days is provided as an additional control. The area for detailed view was selected from the middle of construct with the most intense proteoglycan staining and visible cell morphology. Pictures are 40 $\times$ magnification with 20- μ m scale bar. Color images available online at www.liebertonline.com/ten.

FIG. 6. CDM and alginate constructs cultured in the presence of 10% fetal bovine serum or in serum-free conditions (as labeled) for 28 days. **(A)** Gross appearance of ASC- or MSC-seeded CDM constructs in control conditions containing 100 nM Dex. **(B)** Type II collagen immunohistochemistry of ASC- or MSC-seeded CDM constructs in control conditions containing 100 nM Dex. **(C)** Type II collagen immunohistochemistry of ASC- or MSC-seeded alginate bead constructs cultured with 10 ng/mL TGF- β 3 and 100 nM Dex. Scale bars are 2 mm for **(A)** and 200 μ m for **(B)** and **(C)**. Color images available online at www.liebertonline.com/ten.

but not when total GAG content was used due to the low cell viability in the ASCs BMP-6 group (Fig. 3). The large response of ASCs to high-dose BMP-6 supports previous work.³⁴

MSCs showed a stronger propensity than ASCs toward a hypertrophic chondrocyte phenotype, as observed by the upregulation of *COL1A1* and *COL10A1* without the addition

of growth factors. However, both cell types showed similar upregulation of these genes in response to growth factor supplementation as compared to control conditions (Fig. 1). Enhanced *COL10A1* in MSCs as compared to ASCs has been seen by others.^{22,27,28} Interestingly, higher *COL10A1* upregulation in MSCs appears to be in response to three-dimensional conditions and not monolayer expansion, as we saw a threefold higher *COL10A1* copy number in ASCs than MSCs at day 0 (data not shown). A high dose of BMP-6 in addition to TGF- β has been shown to cause upregulation of *COL10A1* in MSCs,⁵² whereas BMP-6 alone caused a down-regulation of *COL10A1* in ASCs.³⁴ Our data in alginate beads illustrate that 500 ng/mL BMP-6 without TGF- β resulted in *COL10A1* expression similar to control values in ASCs and slightly downregulated compared to control for MSCs, but that TGF- β induced significant *COL10A1* upregulation in both ASCs and MSCs (Fig. 1A).

An important finding of this study was the comparison of the chondrogenic potential of ASCs and MSCs in a scaffold derived from native cartilage. Immunohistochemistry for type II collagen and chondroitin-4-sulfate indicated that MSCs seeded in CDM synthesized abundant new cartilaginous matrix that filled in any open areas of the native porcine cartilage scaffold (Fig. 4). New matrix was also seen in ASC constructs but had not fully filled in the CDM scaffold by the 28 day time point (Fig. 4). Part of the enhanced matrix synthesis may be explained by the increased proliferation of MSCs as compared to ASCs, although GAG/DNA measures indicate significantly higher GAG synthesis when controlled for cell number as well as total GAG content (Fig. 3). MSCs in growth factor conditions adopted a spherical morphology among the neotissue, while ASCs retained the elongated phenotype characteristic of monolayer culture (Fig. 5). The cell type differences in immunohistochemical results correlated to growth factor-induced gene expression data at day 14, as significantly greater upregulation in *COL2A1* and *AGC1* was seen in MSCs than in ASCs (Fig. 1).

In general, similar results were seen in terms of gene expression and biochemical assays between the alginate bead system and CDM, although viability/cell proliferation was enhanced in the CDM. MSCs had a different response to growth factors in the two model systems. In the alginate beads, both percentage of day 0 DNA and total GAG content were significantly higher in the dual cocktail of TGF- β and BMP-6 as compared to TGF- β alone, whereas TGF- β alone had higher viability and higher (although not statistically significant) GAG as compared to the dual cocktail in CDM (Fig. 3). The CDM also limited MSC upregulation of *COL10A1* in response to conditions containing TGF- β (33.36 average fold increase over control in CDM vs. 57.76 in alginate beads) without decreasing the upregulation of *COL2A1* (35,187 average fold increase over control in CDM vs. 14,031 in alginate beads). This was supported by the absence of extensive staining for type X collagen in the CDM scaffolds at day 28 (Fig. 4). The observation that cell-matrix interactions may limit the hypertrophic phenotype during MSC chondrogenesis could be important for future work. The hypertrophic chondrocyte phenotype during chondrogenic differentiation has been well documented for MSCs,^{53,54} and ongoing work is attempting to address it.²⁵ A recent study demonstrated that adult stem cells from bone marrow, adipose tissue, and synovium all demonstrated some degree of

calcification *in vivo* after *in vitro* growth factor-based chondrogenesis.⁵⁵

For both ASCs and MSCs, the CDM without exogenously added growth factors induced minimal new matrix synthesis and no upregulation in chondrogenic gene expression, illustrating the importance of growth factor supplementation. Previous work using CDM indicated that significant chondrogenic induction of ASCs can be achieved without the use of growth factors.³⁶ One difference between the studies is that the current study was serum free during differentiation, whereas the previous work included 10% FBS throughout the culture period. Thus, the presence of a variety of growth factors and cytokines in serum may significantly influence, and potentially interact with, the effects of the CDM on chondrogenesis. While this study saw effects of serum on contraction (Fig. 6A) but not matrix production (Fig. 6B), serum has been shown to be inhibitory to cartilage production in synovial fibroblast^{56,57} and chondrocyte culture.⁵⁸ Serum-free chondrogenic conditions have been standard for MSC culture ever since early observations in rabbit MSCs that pellets did not form in the presence of 10% serum.³⁵ ASCs have been successfully differentiated down a chondrogenic lineage in 10% serum in alginate beads^{34,49} and in 1% serum in micromass culture.⁵ The current study demonstrated that in alginate beads treated with TGF- β , ASCs produced more type II collagen in the presence of serum, while MSCs produced more type II collagen without serum (Fig. 6C). Similarly, ASCs show enhanced *AGC1* expression in control conditions if 10% FBS is present (data not shown). While further study is needed to confirm the observation that ASCs differentiate at least as well in the presence of serum, the present findings taken together with previous literature suggest that responsiveness to serum may be another possible difference between the two cell types. A second difference between this study and that of Cheng *et al.*³⁶ is the greater degree of contraction seen during culture in the present study, even though serum-free conditions were used. The mechanical properties of the specific CDM scaffolds could affect chondrogenic differentiation, as mechanical cues such as substrate stiffness⁵⁹ and cell shape⁶⁰ have been shown to affect stem cell differentiation toward other lineages.

The guiding hypothesis that ASCs and MSCs are unique cell types that respond to different culture conditions led us to choose to expand each cell type in monolayer conditions that have been shown to be effective at priming that particular cell type for chondrogenesis instead of using identical culture conditions.^{51,61} For example, lot-selected FBS specific to each cell type was used, with the MSC serum chosen by a rigorous selection process described elsewhere.⁴⁷ Since bFGF has been demonstrated to have substantial effects on downstream chondrogenesis in both MSCs and ASCs,^{61,62} both cell types were expanded in the presence of 1 ng/mL bFGF.

In summary, MSCs demonstrated more robust chondrogenesis and a greater tendency to display the hypertrophic chondrocyte phenotype under the specific conditions studied, although these results do not speak to the intrinsic chondrogenic potential that is ultimately possible for the two cell types. This study supports previous work illustrating the distinct nature of ASCs and MSCs by their responses to different growth factors and culture conditions. This work also establishes the potential for CDM scaffolds as a valuable

model for comparing stem cell chondrogenesis across different tissue sources.

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Disclosure Statement

No competing financial interests exist.

References

- Guilak, F., Butler, D.L., and Goldstein, S.A. Functional tissue engineering: the role of biomechanics in articular cartilage repair. *Clin Orthop Relat Res* **391**, S295, 2001.
- Song, L., Baksh, D., and Tuan, R.S. Mesenchymal stem cell-based cartilage tissue engineering: cells, scaffold and biology. *Cytotherapy* **6**, 596, 2004.
- Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., and Peterson, L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med* **331**, 889, 1994.
- Gimble, J., and Guilak, F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* **5**, 362, 2003.
- Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P., and Hedrick, M.H. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* **7**, 211, 2001.
- Caplan, A.I. Mesenchymal stem cells. *J Orthop Res* **9**, 641, 1991.
- Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science (NY)* **284**, 143, 1999.
- Erickson, G.R., Gimble, J.M., Franklin, D.M., Rice, H.E., Awad, H., and Guilak, F. Chondrogenic potential of adipose tissue-derived stromal cells *in vitro* and *in vivo*. *Biochem Biophys Res Commun* **290**, 763, 2002.
- Guilak, F., Awad, H.A., Fermor, B., Leddy, H.A., and Gimble, J.M. Adipose-derived adult stem cells for cartilage tissue engineering. *Biorheology* **41**, 389, 2004.
- Mackay, A.M., Beck, S.C., Murphy, J.M., Barry, F.P., Chichester, C.O., and Pittenger, M.F. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng* **4**, 415, 1998.
- Yoo, J.U., Barthel, T.S., Nishimura, K., Solchaga, L., Caplan, A.I., Goldberg, V.M., and Johnstone, B. The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J Bone Joint Surg* **80**, 1745, 1998.
- Aust, L., Devlin, B., Foster, S.J., Halvorsen, Y.D., Hicok, K., du Laney, T., Sen, A., Willingmyre, G.D., and Gimble, J.M. Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy* **6**, 7, 2004.
- Varma, M.J., Breuls, R.G., Schouten, T.E., Jurgens, W.J., Bontkes, H.J., Schuurhuis, G.J., van Ham, S.M., and van

- Milligen, F.J. Phenotypical and functional characterization of freshly isolated adipose tissue-derived stem cells. *Stem Cells Dev* **16**, 91, 2007.
14. Im, G.I., Shin, Y.W., and Lee, K.B. Do adipose tissue-derived mesenchymal stem cells have the same osteogenic and chondrogenic potential as bone marrow-derived cells? *Osteoarthritis Cartilage/OARS, Osteoarthritis Res Soc* **13**, 845, 2005.
 15. Kern, S., Eichler, H., Stoeve, J., Kluter, H., and Bieback, K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells (Dayton)* **24**, 1294, 2006.
 16. Rider, D.A., Dombrowski, C., Sawyer, A.A., Ng, G.H., Leong, D., Hutmacher, D.W., Nurcombe, V., and Cool, S.M. Autocrine fibroblast growth factor 2 increases the multipotentiality of human adipose-derived mesenchymal stem cells. *Stem Cells (Dayton)* **26**, 1598, 2008.
 17. Sakaguchi, Y., Sekiya, I., Yagishita, K., and Muneta, T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum* **52**, 2521, 2005.
 18. Afizah, H., Yang, Z., Hui, J.H., Ouyang, H.W., and Lee, E.H. A comparison between the chondrogenic potential of human bone marrow stem cells (BMSCs) and adipose-derived stem cells (ADSCs) taken from the same donors. *Tissue Eng* **13**, 659, 2007.
 19. De Ugarte, D.A., Alfonso, Z., Zuk, P.A., Elbarbary, A., Zhu, M., Ashjian, P., Benhaim, P., Hedrick, M.H., and Fraser, J.K. Differential expression of stem cell mobilization-associated molecules on multi-lineage cells from adipose tissue and bone marrow. *Immunol Lett* **89**, 267, 2003.
 20. De Ugarte, D.A., Morizono, K., Elbarbary, A., Alfonso, Z., Zuk, P.A., Zhu, M., Dragoo, J.L., Ashjian, P., Thomas, B., Benhaim, P., Chen, I., Fraser, J., and Hedrick, M.H. Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* **174**, 101, 2003.
 21. Hennig, T., Lorenz, H., Thiel, A., Goetzke, K., Dickhut, A., Geiger, F., and Richter, W. Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6. *J Cell Physiol* **211**, 682, 2007.
 22. Huang, J.I., Kazmi, N., Durbhakula, M.M., Hering, T.M., Yoo, J.U., and Johnstone, B. Chondrogenic potential of progenitor cells derived from human bone marrow and adipose tissue: a patient-matched comparison. *J Orthop Res* **23**, 1383, 2005.
 23. Izadpanah, R., Trygg, C., Patel, B., Kriedt, C., Dufour, J., Gimble, J.M., and Bunnell, B.A. Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. *J Cell Biochem* **99**, 1285, 2006.
 24. Kim, H.J., and Im, G.I. Chondrogenic differentiation of adipose tissue-derived mesenchymal stem cells: greater doses of growth factor are necessary. *J Orthop Res* **27**, 612, 2009.
 25. Kim, Y.J., Kim, H.J., and Im, G.I. PTHrP promotes chondrogenesis and suppresses hypertrophy from both bone marrow-derived and adipose tissue-derived MSCs. *Biochem Biophys Res Commun* **373**, 104, 2008.
 26. Lee, R.H., Kim, B., Choi, I., Kim, H., Choi, H.S., Suh, K., Bae, Y.C., and Jung, J.S. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell Physiol Biochem* **14**, 311, 2004.
 27. Liu, T.M., Martina, M., Hutmacher, D.W., Hui, J.H., Lee, E.H., and Lim, B. Identification of common pathways mediating differentiation of bone marrow- and adipose tissue-derived human mesenchymal stem cells into three mesenchymal lineages. *Stem Cells (Dayton)* **25**, 750, 2007.
 28. Mehlhorn, A.T., Niemeyer, P., Kaiser, S., Finkenzeller, G., Stark, G.B., Sudkamp, N.P., and Schmal, H. Differential expression pattern of extracellular matrix molecules during chondrogenesis of mesenchymal stem cells from bone marrow and adipose tissue. *Tissue Eng* **12**, 2853, 2006.
 29. Noel, D., Caton, D., Roche, S., Bony, C., Lehmann, S., Casteilla, L., Jorgensen, C., and Cousin, B. Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials. *Exp Cell Res* **314**, 1575, 2008.
 30. Rebelatto, C.K., Aguiar, A.M., Moretao, M.P., Senegaglia, A.C., Hansen, P., Barchiki, F., Oliveira, J., Martins, J., Kuligovski, C., Mansur, F., Christofis, A., Amaral, V.F., Brofman, P.S., Goldenberg, S., Nakao, L.S., and Correa, A. Dissimilar differentiation of mesenchymal stem cells from bone marrow, umbilical cord blood, and adipose tissue. *Exp Biol Med (Maywood)* **233**, 901, 2008.
 31. Segawa, Y., Muneta, T., Makino, H., Nimura, A., Mochizuki, T., Ju, Y.J., Ezura, Y., Umezawa, A., and Sekiya, I. Mesenchymal stem cells derived from synovium, meniscus, anterior cruciate ligament, and articular chondrocytes share similar gene expression profiles. *J Orthop Res* **27**, 435, 2009.
 32. Wagner, W., Wein, F., Seckinger, A., Frankhauser, M., Winkler, U., Krause, U., Blake, J., Schwager, C., Eckstein, V., Ansorge, W., and Ho, A.D. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol* **33**, 1402, 2005.
 33. Winter, A., Breit, S., Parsch, D., Benz, K., Steck, E., Hauner, H., Weber, R.M., Ewerbeck, V., and Richter, W. Cartilage-like gene expression in differentiated human stem cell spheroids: a comparison of bone marrow-derived and adipose tissue-derived stromal cells. *Arthritis Rheum* **48**, 418, 2003.
 34. Estes, B.T., Wu, A.W., and Guilak, F. Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. *Arthritis and rheumatism* **54**, 1222, 2006.
 35. Johnstone, B., Hering, T.M., Caplan, A.I., Goldberg, V.M., and Yoo, J.U. *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* **238**, 265, 1998.
 36. Cheng, N.C., Estes, B.T., Awad, H.A., and Guilak, F. Chondrogenic differentiation of adipose-derived adult stem cells by a porous scaffold derived from native articular cartilage extracellular matrix. *Tissue Eng Part A*, **15**, 231, 2009.
 37. Yang, Q., Peng, J., Guo, Q., Huang, J., Zhang, L., Yao, J., Yang, F., Wang, S., Xu, W., Wang, A., and Lu, S. A cartilage ECM-derived 3-D porous acellular matrix scaffold for *in vivo* cartilage tissue engineering with PKH26-labeled chondrogenic bone marrow-derived mesenchymal stem cells. *Biomaterials* **29**, 2378, 2008.
 38. Shakibaei, M. Inhibition of chondrogenesis by integrin antibody *in vitro*. *Exp Cell Res* **240**, 95, 1998.
 39. Svoboda, K.K. Chondrocyte-matrix attachment complexes mediate survival and differentiation. *Microsc Res Tech* **43**, 111, 1998.
 40. Chang, C.F., Lee, M.W., Kuo, P.Y., Wang, Y.J., Tu, Y.H., and Hung, S.C. Three-dimensional collagen fiber remodeling by mesenchymal stem cells requires the integrin-matrix interaction. *J Biomed Mater Res* **80**, 466, 2007.
 41. Ott, H.C., Matthiesen, T.S., Goh, S.K., Black, L.D., Kren, S.M., Netoff, T.I., and Taylor, D.A. Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nat Med* **14**, 213, 2008.

42. Chun, S.Y., Lim, G.J., Kwon, T.G., Kwak, E.K., Kim, B.W., Atala, A., and Yoo, J.J. Identification and characterization of bioactive factors in bladder submucosa matrix. *Biomaterials* **28**, 4251, 2007.
43. Basile, P., Dadali, T., Jacobson, J., Hasslund, S., Ulrich-Vinther, M., Soballe, K., Nishio, Y., Drissi, M.H., Langstein, H.N., Mitten, D.J., O'Keefe, R.J., Schwarz, E.M., and Awad, H.A. Freeze-dried tendon allografts as tissue-engineering scaffolds for Gdf5 gene delivery. *Mol Ther* **16**, 466, 2008.
44. Macchiarini, P., Jungebluth, P., Go, T., Asnaghi, M.A., Rees, L.E., Cogan, T.A., Dodson, A., Martorell, J., Bellini, S., Parnigotto, P.P., Dickinson, S.C., Hollander, A.P., Mantero, S., Conconi, M.T., and Birchall, M.A. Clinical transplantation of a tissue-engineered airway. *Lancet* **372**, 2023, 2008.
45. Badylak, S.F., Freytes, D.O., and Gilbert, T.W. Extracellular matrix as a biological scaffold material: structure and function. *Acta Biomater* **5**, 1, 2009.
46. Estes, B.T., Wu, A.W., Storms, R.W., and Guilak, F. Extended passaging, but not aldehyde dehydrogenase activity, increases the chondrogenic potential of human adipose-derived adult stem cells. *J Cell Physiol* **209**, 987, 2006.
47. Lennon, D.P., and Caplan, A.I. Isolation of human marrow-derived mesenchymal stem cells. *Exp Hematol* **34**, 1604, 2006.
48. Enobakhare, B.O., Bader, D.L., and Lee, D.A. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. *Anal Biochem* **243**, 189, 1996.
49. Awad, H.A., Halvorsen, Y.D., Gimble, J.M., and Guilak, F. Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells. *Tissue Eng* **9**, 1301, 2003.
50. Taylor, J.R. *An Introduction to Error Analysis*. Mill Valley, CA: University Science Books, 1982.
51. Estes, B.T., Diekman, B.O., and Guilak, F. Monolayer cell expansion conditions affect the chondrogenic potential of adipose-derived stem cells. *Biotechnol Bioeng* **99**, 986, 2008.
52. Sekiya, I., Colter, D.C., and Prockop, D.J. BMP-6 enhances chondrogenesis in a subpopulation of human marrow stromal cells. *Biochem Biophys Res Commun* **284**, 411, 2001.
53. Mueller, M.B., and Tuan, R.S. Functional characterization of hypertrophy in chondrogenesis of human mesenchymal stem cells. *Arthritis Rheum* **58**, 1377, 2008.
54. Pelttari, K., Winter, A., Steck, E., Goetzke, K., Hennig, T., Ochs, B.G., Aigner, T., and Richter, W. Premature induction of hypertrophy during *in vitro* chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis Rheum* **54**, 3254, 2006.
55. Dickhut, A., Pelttari, K., Janicki, P., Wagner, W., Eckstein, V., Egermann, M., and Richter, W. Calcification or dedifferentiation: requirement to lock mesenchymal stem cells in a desired differentiation stage. *J Cell Physiol* **219**, 219, 2009.
56. Bilgen, B., Orsini, E., Aaron, R.K., and Ciombor, D.M. FBS suppresses TGF-beta1-induced chondrogenesis in synovio-cyte pellet cultures while dexamethasone and dynamic stimuli are beneficial. *J Tissue Eng Regen Med* **1**, 436, 2007.
57. Kurth, T., Hedbom, E., Shintani, N., Sugimoto, M., Chen, F.H., Haspl, M., Martinovic, S., and Hunziker, E.B. Chondrogenic potential of human synovial mesenchymal stem cells in alginate. *Osteoarthritis Cartilage/OARS, Osteoarthritis Res Soc* **15**, 1178, 2007.
58. Ballock, R.T., and Reddi, A.H. Thyroxine is the serum factor that regulates morphogenesis of columnar cartilage from isolated chondrocytes in chemically defined medium. *J Cell Biol* **126**, 1311, 1994.
59. Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. Matrix elasticity directs stem cell lineage specification. *Cell* **126**, 677, 2006.
60. McBeath, R., Pirone, D.M., Nelson, C.M., Bhadriraju, K., and Chen, C.S. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell* **6**, 483, 2004.
61. Solchaga, L.A., Penick, K., Porter, J.D., Goldberg, V.M., Caplan, A.I., and Welter, J.F. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. *J Cellular Physiol* **203**, 398, 2005.
62. Chiou, M., Xu, Y., and Longaker, M.T. Mitogenic and chondrogenic effects of fibroblast growth factor-2 in adipose-derived mesenchymal cells. *Biochem Biophys Res Commun* **343**, 644, 2006.

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