Driving Cartilage Formation in High-Density Human Adipose-Derived Stem Cell Aggregate and Sheet Constructs Without Exogenous Growth Factor Delivery

Phuong N. Dang, BS,¹ Loran D. Solorio, PhD,¹ and Eben Alsberg, PhD^{1,2}

An attractive cell source for cartilage tissue engineering, human adipose-derived stem cells (hASCs) can be easily expanded and signaled to differentiate into chondrocytes. This study explores the influence of growth factor distribution and release kinetics on cartilage formation within 3D hASC constructs incorporated with transforming growth factor- β 1 (TGF- β 1)-loaded gelatin microspheres. The amounts of microspheres, TGF- β 1 concentration, and polymer degradation rate were varied within hASC aggregates. Microsphere and TGF- β 1 loading concentrations were identified that resulted in glycosaminoglycan (GAG) production comparable to those of control aggregates cultured in TGF- β 1-containing medium. Self-assembling hASC sheets were then engineered for the production of larger, more clinically relevant constructs. Chondrogenesis was observed in hASC-only sheets cultured with exogenous TGF- β 1 at 3 weeks. Importantly, sheets with incorporated TGF- β 1-loaded microspheres achieved GAG production similar to sheets treated with exogenous TGF- β 1. Cartilage formation was confirmed histologically via observation of cartilage-like morphology and GAG staining. This is the first demonstration of the self-assembly of hASCs into high-density cell sheets capable of forming cartilage in the presence of exogenous TGF- β 1 or with TGF- β 1-releasing microspheres. Microsphere incorporation may bypass the need for extended *in vitro* culture, potentially enabling hASC sheets to be implanted more rapidly into defects to regenerate cartilage *in vivo*.

Introduction

ARTICULAR CARTILAGE is a highly resilient connective tissue that supports and distributes forces generated during high mechanical joint loading and promotes frictionless movement to prevent wear or degradation of the joint.¹ However, it has limited capacity for self-repair due to its slow extracellular matrix (ECM) turnover, sparse distribution of nondividing chondrocytes, and avascularity.² Without a vasculature, there are limited numbers of progenitor cells from the blood or bone marrow that can access the defect site, differentiate into chondrocytes, and secrete a reparative matrix. As a result, even minor defects may lead to osteoarthritis, a degenerative joint disease that is a major cause of disability worldwide.³ Unfortunately, while popular current treatments can promote pain relief and improve joint function, they are often not able to fully restore the structure and biomechanical properties of native articular cartilage.⁴ Surgical strategies to stimulate subchondral bone marrow, such as subchondral drilling and microfracture, can result in poor integration with native cartilage and formation of fibrocartilage, which has

inferior structure, composition, and mechanical properties compared with native articular cartilage and may degenerate with time.⁵ Transplantation-based strategies, such as autograft, allograft, and autologous chondrocyte transplantation (ACT), take a more biological approach to treat cartilage lesions. However, they too are not without disadvantages. Autografts face issues of donor site morbidity and long recovery time.⁶ Graft rejection and risk of disease transmission may occur with the use of allografts. In addition, limited donor graft availability is also a drawback of using both autografts and allografts.⁵ ACT, a cell-based therapy in which the patient's own chondrocytes are transplanted, requires two surgeries: healthy cartilage harvest followed by implantation of cultured chondrocytes.⁷ Extensive monolayer culture, a popular method used for chondrocyte expansion, can result in chondrocyte dedifferentiation into a fibroblastic phenotype.⁸

With the incidence of osteoarthritis rising due to aging and obesity,⁹ much focus has been placed on tissue engineering solutions to meet the significant clinical need for cartilage repair and regeneration. Recently, human adipose-derived stem cells (hASCs) have emerged as an attractive cell source

Departments of ¹Biomedical Engineering and ²Orthopaedic Surgery, Case Western Reserve University, Cleveland, Ohio.

for cartilage tissue engineering. The most abundant and accessible of the adult stem cells, they can be isolated with high yield from adipose tissue following a simple surgical procedure with minimal donor site morbidity. Most importantly, they are able to differentiate down various mesenchymal cell lineages, including that leading to chondrocytes, and can be expanded in culture without losing their multipotency.^{10–12} Unlike human bone marrow-derived mesenchymal stem cells (hMSCs), which can gradually lose their chondrogenic differentiation capabilities over several passages in culture,¹³ it has been reported that the chondrogenic potential of hASCs may actually increase with extended passaging through passage 9.¹⁴ hASCs can also maintain their proliferative and differentiation capacities after long-term cryopreservation in liquid nitrogen, adding to their appeal as a cell source for tissue engineering applications.¹⁵

hASC chondrogenesis has been demonstrated most successfully in three-dimensional culture environments using transforming growth factor- β (TGF- β) and/or bone morphogenetic protein (BMP) as chondrogenic factors.^{16,17} hASCs have been encapsulated in various biomaterials, including alginate, gelatin, and agarose, and cultured in media containing chondrogenic growth factors that facilitate cartilage formation.^{18–21} In addition, scaffold-free hASC aggregate/pellet and micromass cultures, in which high-cell density constructs are formed via spontaneous aggregation or centrifugation in the absence of a biomaterial scaffold, have also been pursued.^{15,22–27} These scaffold-free approaches provide a high-density 3D cell environment that is advantageous for inducing chondrogenesis in stem cells.^{28,29}

In the past decade, cell sheet technology has gained popularity in cartilage tissue engineering.^{30–34} Similar to aggregate cultures,³⁵ cell sheets also provide a high-cell density 3D environment that is favorable for cartilage formation. However, self-assembled cell sheets have the potential for greater clinical applicability in cartilage repair than aggregate cultures, as sheets of uniform thickness can be engineered with varying diameters and can be engineered to fit larger defects, while spherically shaped aggregates are restricted in size due to diffusional limitations. Self-assembling sheets made from mature chondrocytes have been successfully developed for cartilage regeneration^{32,33,36} and have been shown to maintain a cartilaginous phenotype when implanted *in vivo*.³⁶ However, as discussed earlier, the use of chondrocytes is not ideal due to their limited supply and difficult expansion.

Sheets comprising MSCs have been engineered with TGF- β 1 exogenously supplemented in the culture medium for cartilage tissue engineering.^{37,38} However, repeated TGF- β 1 supplementation in the medium can be tedious and costly, as several weeks of *in vitro* culture are required for a neocartilaginous ECM to develop. Our lab addressed this by incorporating TGF- β 1-releasing gelatin microspheres with tunable degradation rates within self-assembled hMSC sheets.³⁹ Gelatin is a hydrolyzed form of collagen, a main component of connective tissues that is widely used in tissue engineering applications. It can form biodegradable and biocompatible hydrogels that are capable of releasing loaded growth factors via cell-mediated proteolytic degradation of its network.^{40,41} With regard to high-density culture, gelatin microspheres were chosen as they have been shown to enhance biological functions when incorporated within MSC aggregates⁴² and to deliver TGF- β 1 locally to promote growth factor-induced

MSC chondrogenesis.^{39,43} In addition to providing sustained local delivery of growth factor to the cells, gelatin microspheres incorporated within cell sheets can degrade over time to provide space for ECM accumulation. Their degradation rates can be adjusted to obtain degradation and growth factor release profiles that induce hMSC chondrogenesis at levels equivalent to or greater than those of sheets cultured in growth factor-containing medium.³⁹

In this study, we demonstrate the utility of this microsphere-incorporated cell sheet technology to form self-assembled cartilage constructs with hASCs, another promising cell source for tissue engineering applications. First, the influence of growth factor spatial distribution and release kinetics on chondrogenesis within hASC aggregates incorporated with TGF- β 1-releasing gelatin microspheres was determined by varying microsphere amount, growth factor concentration, and polymer degradation rate. Self-assembling hASC sheets were then engineered with the goal to develop a system capable of inducing hASC chondrogenesis of a more clinically relevant size. We tested the hypotheses that (i) hASCs could self-assemble to form high-cell-density sheets and undergo chondrogenesis in the presence of exogenous chondrogenic growth factor and (ii) growth factor released from gelatin microspheres within these sheets could induce chondrogenesis, forming stable neocartilage constructs without the need for extended *in vitro* culture. Cartilage formation within hASC sheets without microspheres cultured in TGF- β 1-supplemented media and sheets with TGF- β 1-containing microsphere formulations determined optimal in the aggregate study was analyzed. hASCs have been shown to successfully self-assemble into sheets for skin and adipose tissue engineering,³¹ but this is the first report of hASC sheets for cartilage tissue engineering. Importantly, this hASC-based system, which provides controlled local delivery of growth factors from incorporated microspheres to drive chondrogenesis, has the potential to enable rapid *in vivo* application to treat cartilage defects.

Materials and Methods

hASC isolation and expansion

First-passage hASCs from four female donors (42.75 ± 7.93 years old with a body mass index of $25.34 \pm 4.45 \text{ kg/m}^2$) were generously provided in frozen vials from the Pennington Biomedical Research Center (Baton Rouge, LA), where they were isolated as previously described.⁴⁴ Briefly, hASCs were isolated from the stromal vascular fraction of human adipose tissue by an enzymatic digestion method. Liposuction waste tissue was digested with 200 units/mg collagenase type I (Worthington Biochemical Products, Lakewood, NJ) for 40 min at 37°C. The stromal fraction was then isolated by density centrifugation, and the stromal cells were plated at 3500 cells/cm² on tissue culture plastic in Dulbecco's modified Eagle's medium (DMEM)-F12 (BioWhittaker, Suwanee, GA) with 10% defined fetal bovine serum (FBS; HyClone, Logan, UT), 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin (BioWhittaker, Suwanee, GA). Passage 1 (P1) hASCs were cryopreserved in liquid nitrogen in medium containing 80% FBS, 10% DMEM, and 10% dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO).

On thawing, cells were expanded by plating at 8000 cells/cm² in medium containing DMEM-F12 (HyClone), 10%

FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (MP Biomedicals, Solon, OH), and 10 ng/mL fibroblast growth factor-2 (FGF-2; R&D Systems, Minneapolis, MN). Culture medium was supplemented with FGF-2, as it has been reported to enhance proliferation and chondrogenic potential of hASCs at this concentration.⁴⁵ After 80–90% confluency was reached, they were trypsinized and frozen in liquid nitrogen in the same cryopreservation medium described earlier. Cells were used at P3.

hASC donor screening

Chondrogenic differentiation of P3 hASCs from the four female donors was induced in aggregate culture with exogenously supplemented TGF-β1 (Peprotech, Rocky Hill, NJ).^{22,46,47} Cells were expanded in monolayer culture until they were ~90% confluent. Cells from each donor were then trypsinized and suspended at a concentration of 1.25×10^6 cells/mL in a chemically defined medium containing DMEM-HG (Sigma-Aldrich) with 1% ITS+Premix (insulin, transferrin, and selenious acid; BD Biosciences, Sparks, MD), 37.5 µg/mL ascorbate-2-phosphate (Wako USA, Richmond, VA), 10^{-7} M dexamethasone (MP Biomedicals), 1% nonessential amino acids (HyClone), and 1% sodium pyruvate (HyClone). Cell suspension aliquots (200 µL) were centrifuged at 500 *g* for 5 min in sterile V-bottom polypropylene plates to form free-floating aggregates.⁴⁸ Aggregates were cultured with or without 10 ng/mL of TGF-β1 supplemented in the medium. Medium was changed every other day to give the cells enough time to differentiate and form a well-developed neocartilaginous ECM. At the end of culture, the aggregates were harvested and analyzed to identify the most chondrogenic donor as indicated by glycosaminoglycan (GAG) production normalized to DNA.

Gelatin microsphere synthesis and characterization

Gelatin microspheres were synthesized as previously described.³⁹ Briefly, an aqueous solution of 11.1% w/v acidic gelatin (Sigma-Aldrich) was added dropwise into 250 mL of preheated (45°C) olive oil (GiaRussa, Coitsville, OH) and stirred on a magnetic stirring plate at 500 RPM for 10 min. The solution temperature was then lowered to 4°C with constant stirring. One hundred milliliters of chilled acetone (4°C) was added to the stirring solution after 30 min and again 1 h later. The solution was then stirred for 5 min at 1000 RPM. The resulting microspheres were collected by filtration, washed with acetone to remove residual olive oil, and dried overnight at room temperature (RT).

Microspheres were then crosslinked at RT in an aqueous solution of 1% w/v genipin (“Gp”; Wako USA) for either 2 h (“low Gp”) or 24 h (“high Gp”) to produce microspheres with different crosslinking densities. Crosslinked microspheres were collected by filtration, washed several times with ultrapure deionized water (diH₂O), and lyophilized. After lyophilization, crosslinked microspheres were stored at 4°C until use.

Growth factor loading was performed on the day of hASC aggregate or sheet production. Crosslinked microspheres were UV sterilized for 10 min and then soaked in a small volume of a solution containing TGF-β1 in phosphate-buffered saline (PBS) at pH 7.4 for 2 h at 37°C to enable the positively charged TGF-β1 to complex with the negatively

charged gelatin.⁴⁹ A small volume of growth factor solution (less than the equilibrium swelling volume of the microspheres) was used to ensure complete absorption. Empty microspheres without growth factor were hydrated with equivalent volumes of PBS only.

Crosslinked microspheres were characterized as previously described.³⁹ Microspheres were hydrated in PBS, and their diameters were measured from light photomicrographs using Image J (National Institute of Health, Bethesda, MD) analysis software ($n=295$, “low Gp”; $n=256$, “high Gp”). Crosslinking densities were determined by a ninhydrin assay ($n=3$) as previously described.⁵⁰ Briefly, 3 mg of microspheres were hydrated in 100 µL diH₂O and incubated for 1 h at RT. Ninhydrin solution was prepared by mixing 1.05 g citric acid, 0.4 g NaOH, and 0.04 g SnCl₂·2H₂O in 25 mL diH₂O with 1 g ninhydrin in 25 mL 2-methoxyethanol on a stir plate for 45 min away from light, and 1 mL was added to the hydrated microspheres. Samples and glycine standards were incubated at 100°C for 4 min, after which 5 mL of 50% isopropanol was added. The absorbance of 200 µL aliquots was read at 570 nm on a SpectraMax M3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA). The concentration of free amino groups left within the gelatin microspheres was determined from the glycine standard and normalized by the mass of the sample. The degree of crosslinking was defined by the percentage of amino groups that reacted with genipin during the crosslinking process by comparison to uncrosslinked microspheres with 100% free amino groups.

Microsphere-incorporated hASC aggregate formation

UV-sterilized crosslinked microspheres were rehydrated with PBS or a PBS solution with TGF-β1 (400 or 1200 ng/mg microspheres) as described earlier. Varying amounts of microspheres with or without TGF-β1 were suspended in chemically defined medium with P3 cells from the most chondrogenic donor from the screen (donor A) as defined by highest GAG production normalized to DNA at 3 weeks (1.25×10^6 cells/mL). Two hundred microliter aliquots were centrifuged at 500 *g* for 5 min in sterile V-bottom polypropylene plates to form free-floating aggregates. The aggregates were cultured for 2 weeks with medium changed every other day. Nine conditions were studied, in which microsphere loading amount, growth factor loading concentration, and degree of crosslinking were varied (Table 1).

hASC sheet formation

P3 cells from donors A and B were prepared to form self-assembling hASC sheets with or without incorporated microspheres under conditions similar to those previously described with hMSCs.³⁹ Sheets without microspheres and/or growth factor were prepared. Similar microsphere and growth factor loading conditions that resulted in the highest GAG and GAG/DNA production in the aggregate study were utilized to form microsphere-incorporated sheets. Briefly, crosslinked microspheres were UV sterilized and soaked in PBS or a PBS solution with TGF-β1 (400 ng/mg microspheres). 2.4 mg microspheres with or without TGF-β1 were suspended with 4×10^6 hASCs in 500 µL of the chemically defined medium and allowed to settle onto the membranes of 12 mm Transwell inserts (3 µm pore size; Corning, New York City, NY) for 48 h in a humidified

TABLE 1. CONDITIONS FOR HUMAN ADIPOSE-DERIVED STEM CELL AGGREGATE STUDY

Group	Microsphere type	Microsphere loading (mg/aggregate)	TGF- β 1 loading (ng/mg microsphere)	Exogenous TGF- β 1 (ng/mL)
1	None	0	0	10
2	Low Gp	0.15 (3 $\times$)	0	10
3	High Gp	0.15 (3 $\times$)	0	10
4	Low Gp	0.15 (3 $\times$)	400 (1 $\times$)	0
5	High Gp	0.15 (3 $\times$)	400 (1 $\times$)	0
6	Low Gp	0.05 (1 $\times$)	400 (1 $\times$)	0
7	High Gp	0.05 (1 $\times$)	400 (1 $\times$)	0
8	Low Gp	0.05 (1 $\times$)	1200 (3 $\times$)	0
9	High Gp	0.05 (1 $\times$)	1200 (3 $\times$)	0

Gp, genipin; TGF- β 1, transforming growth factor- β 1.

incubator at 37°C with 5% CO₂. Two milliliters of medium was added outside of each Transwell insert. TGF- β 1 (10 ng/mL) was added only to the media of hASC-only sheets and sheets containing unloaded microspheres. The medium was changed every other day for 3 weeks with TGF- β 1 supplemented in the specified conditions (Table 2).

Biochemical analysis

At each time point, aggregates and sheets were harvested for analysis. Two 5 mm-diameter punches from each sheet were frozen until analysis: one punch for biochemical analysis and one for thickness measurement and mechanical testing ($n \geq 3$). Four aggregates from each group and one 5 mm-diameter punch obtained from each sheet were used for DNA and GAG quantification. Samples were digested with papain (Sigma-Aldrich) at 65°C for 2 h, and DNA and GAG content of digests was quantified with PicoGreen (Invitrogen, Carlsbad, CA)⁵¹ and dimethylmethylene blue (DMMB) dye (Sigma-Aldrich)⁵² assays, respectively.

Histology and immunohistochemistry

Sheet portions designated for histological examination ($n = 4$ for all, except $n = 3$ for exo. TGF- β 1) were fixed in formalin and paraffin embedded. Five micrometer sections were stained as previously described for GAG content via Safranin O (Acros Organic, Geel, Belgium)⁴⁸ and chondroitin-6-sulfate (C-6-S)⁴⁴ with a Fast Green counterstain. Sections were deparaffinized, rehydrated with decreasing concentrations of ethanol, and washed with PBS. Their endogenous peroxidase activity was quenched using H₂O₂ (30% v/v) and methanol at a ratio of 1:9. Sections for collagen types I and II staining were digested with

pronase (Sigma-Aldrich). Sections for C-6-S staining were also treated with chondroitinase ABC (Sigma-Aldrich) to further expose chondroitin sulfate epitopes. Anti-C-6-S (Millipore, Billerica, MA), anti-collagen type I (Sigma-Aldrich) or anti-collagen type II (Developmental Studies Hybridoma Bank, Iowa City, IA) was used as the primary antibody, and mouse IgG (Vector Laboratories, Burlingame, CA) was used instead of primary antibody as a negative control. The Histostain-Plus Bulk kit (Invitrogen) containing 10% goat nonimmune blocking serum, biotinylated secondary antibody, and enhanced horse-radish peroxidase (HRP) conjugated streptavidin was used in accordance to the manufacturer's instructions. Aminoethyl carbazole (AEC), an HRP substrate/chromogen (Invitrogen), was added and made to react with the enhanced HRP on the secondary antibody. HRP catalyzes the AEC substrate and converts it to a red deposit. Slides were mounted with glycerol vinyl alcohol (GVA) (Invitrogen) and imaged using an Axio Observer Z1 (Zeiss, Thornwood, NY) inverted fluorescent microscope equipped with a C10600 digital camera (Hamamatsu, Bridgewater, NJ).

Thickness measurements and mechanical testing

Using a computer-controlled testing system (TestResources, Shakopee, MN), step strain stress-relaxation testing in unconfined compression was performed with a slight modification to a previously described protocol.³⁹ Briefly, frozen sheet punches from donor A ($n \geq 3$) were thawed and equilibrated in PBS for 1 h at RT. Sample thicknesses were measured using a handheld gauge. A prestress of 0.02 N followed by a 5% step strain with a ramp displacement rate of 0.001 mm/s were first applied to the samples, which were immersed in PBS. The ramp displacement was held until the

TABLE 2. CONDITIONS FOR HUMAN ADIPOSE-DERIVED STEM CELL SHEET STUDY

Group	Microsphere type	Microsphere loading (mg/sheet)	TGF- β 1 loading (ng/mg microsphere)	Exogenous TGF- β 1 (ng/mL)
exo. TGF- β 1	None	0	0	10
Low Gp + exo.	Low Gp	1.5	0	10
High Gp + exo.	High Gp	1.5	0	10
Low Gp + TGF- β 1	Low Gp	1.5	400	0
High Gp + TGF- β 1	High Gp	1.5	400	0
Negative control	None	0	0	0

exo., exogenous.

FIG. 1. Human adipose-derived stem cell (hASC) donor screen. (A) DNA, (B) glycosaminoglycan (GAG), and (C) GAG/DNA contents at 3 weeks for four different donors. ■ Significantly lower ($p < 0.001$) than “exogenous transforming growth factor-β1 (TGF-β1)” group from the same donor. ▲ Significantly lower ($p < 0.001$) than “exogenous TGF-β1” group from donor A. ◆ Significantly lower ($p < 0.01$) than “exogenous TGF-β1” group from donor A. ● Significantly lower ($p < 0.05$) than “exogenous TGF-β1” group from donor A.

change in reaction force was < 0.005 N for 250 s, and this equilibrium was considered the zero stress and strain state. Two sequential 5% step strains were then applied at the same rate with both held until the change in reaction force was < 0.005 N for 250 s. The magnitudes of the equilibrium stresses measured after each step strain were then plotted and fit to a line forced through the origin. The slope of this best-fit line was calculated to yield the equilibrium compressive modulus (E) for each sample. The coefficient of determination, R^2 , was ≥ 0.95 for each best-fit line.

Statistical analysis

Statistical analysis was performed using InStat 3.06 software (GraphPad Software, Inc., La Jolla, CA). Microsphere diameters were compared using a nonparametric Mann–Whitney test. All other analyses were performed using one-way ANOVA with Tukey’s *post hoc* tests. Values of $p < 0.05$ were considered statistically significant.

Results

DNA and GAG analysis of hASC donor screen

A donor screen was performed to examine the chondrogenic potential of hASCs from four female donors, A–D. After 3 weeks of culture, DNA, GAG, and GAG/DNA

contents were significantly higher for aggregates from donor A cultured with exogenously supplemented TGF-β1 than aggregates cultured in control media without TGF-β1 and those from the other donors (Fig. 1). No significant differences in GAG and GAG/DNA contents, however, were found with or without TGF-β1 for donors B, C, and D. Interestingly, there was baseline GAG production for aggregates from all donors, even in the absence of TGF-β1, indicating that the chemically defined media alone may be enough for low-level chondrogenic induction of this cell source. This finding corroborates reports from other groups that found measurable GAG content in hASC aggregates⁵³ and hASCs encapsulated within alginate beads¹⁸ which were cultured in growth factor-free media containing ITS+, ascorbate-2-phosphate, and dexamethasone. The ability of these three factors to induce baseline GAG production by hASCs in the absence of a chondrogenic growth factor may be attributed to their roles in enhancing expression of mRNA for cartilage markers (e.g., *Sox9*, aggrecan, and collagen type II) in chondrocyte cultures.^{54–56}

Microsphere characterization

Hydrated microspheres were roughly spherical with similar average diameters (60.9 ± 50.1 μm, “low Gp”; 54.3 ± 47.9 μm, “high Gp”) and non-Gaussian size distributions (Fig. 2). The

FIG. 2. Photomicrographs of low Gp (A) and high Gp (B) crosslinked gelatin microspheres hydrated in phosphate-buffered saline, and (C) microsphere size histograms showing the frequency of microsphere size in 20 μm intervals. Scale bar = 50 μm.

microspheres that were incubated with genipin for 2 h were $28.8\% \pm 6.8\%$ crosslinked and appeared as a light blue color. Those that were incubated with genipin for 24 h were $64.9\% \pm 9.9\%$ crosslinked and were a dark blue color.

DNA and GAG analysis of microsphere-incorporated hASC aggregates

TGF- β 1-loaded gelatin microspheres were then incorporated within hASC aggregates from donor A. Microsphere amount, growth factor concentration, and polymer degradation rate were varied to study the influence of growth factor distribution and release kinetics on hASC chondrogenesis (Table 1). DNA content after 1 week was not significantly different among the nine groups analyzed (Fig. 3A). Groups 1 through 5 had similar DNA content at 1 and 2 weeks, with the exception of group 3, which had significantly higher DNA than that of group 4 at 2 weeks.

FIG. 3. hASC (donor A) aggregate study. (A) DNA, (B) GAG, and (C) GAG/DNA content of hASC aggregates at 1 (dark gray) and 2 (light gray) weeks. ● Significantly different ($p < 0.05$) from week 2 time point of group. ■ Significantly different ($p < 0.01$) from week 2 time point of group. ▲ Significantly different ($p < 0.001$) from week 2 time point of group. ◇ Significantly higher ($p < 0.01$) than group 4 at week 2. ○ Significantly higher ($p < 0.01$) than group 8 at week 2. □ Significantly higher ($p < 0.001$) than groups 6, 7, and 9 at week 2. Δ Significantly higher ($p < 0.001$) than groups 6 through 9 at week 2. ◆ Significantly higher ($p < 0.01$) than groups 6 through 9 at week 2. # Significantly higher ($p < 0.05$) than groups 6 and 7 at week 2. Ω Significantly higher ($p < 0.01$) than groups 8 and 9 at week 2.

However, DNA levels for groups 6 through 9 were significantly lower at week 2 than at week 1. They were also significantly lower than the DNA contents of groups 1 through 5 at week 2. In groups 6 and 7, a lower microsphere loading concentration was used with the same TGF- β 1 loading concentration per microsphere as groups 4 and 5, resulting in a lower total amount of TGF- β 1 per aggregate. This decrease in microsphere mass led to lower cell viability by week 2 compared with groups 1 through 5. Maintaining the lower microsphere loading concentration while increasing the TGF- β 1 loading concentration by a factor of 3 resulted in a similar decrease in DNA content at 2 weeks for groups 8 and 9.

GAG and GAG/DNA contents at week 1 were similar for all groups (Fig. 3B, C). In groups 1 through 5, GAG content was higher at week 2 than week 1, with week 2 GAG content for groups 1 and 3 significantly higher than those at week 1 (Fig. 3B). Average GAG/DNA values at week 2 were also higher than the respective values at week 1 for groups 1 through 5, with statistical significance found for group 2 only (Fig. 3C). There were no significant differences in GAG and GAG/DNA contents among these groups at week 2. Importantly, groups 4 and 5, in which TGF- β 1-loaded microspheres were incorporated into the aggregates, were able to induce GAG production at levels similar to control groups in which TGF- β 1 was exogenously supplemented (groups 1 through 3). However, GAG and GAG/DNA contents in aggregates of groups 6 through 9 did not increase between weeks 1 and 2 and were significantly lower than the GAG and GAG/DNA levels in groups 1 through 5 after 2 weeks (Fig. 3B, C). Groups 6 through 9 had fewer incorporated microspheres, and even though the TGF- β 1 loading concentration in groups 8 and 9 was tripled to yield the same total amount of TGF- β 1 per aggregate as in groups 4 and 5, GAG production was still significantly lower than in groups 1 through 5. DNA, GAG, and GAG/DNA contents for groups 6 through 9 were similar at both time points despite the difference in TGF- β 1 loading concentration. No significant differences in DNA, GAG, and GAG/DNA contents were found between low Gp and high Gp groups with matching microsphere and growth factor loading concentrations.

Biochemical analysis of microsphere-incorporated hASC sheets

The positive results from the aggregate study led to the development of a larger, more clinically relevant model. Self-assembled hASC sheets cultured in TGF- β 1-supplemented medium were engineered. hASC sheets with incorporated TGF- β 1-releasing microspheres were also produced. The conditions are summarized in Table 2. After 3 weeks in culture, sheets were harvested and their DNA and GAG contents were quantified. DNA content was similar for all groups, and no significant differences were observed in GAG and GAG/DNA contents among the conditions tested (Fig. 4). Negative control sheets lacking both microspheres and exogenous growth factor supplementation were also prepared but completely dissociated during the 3 weeks of culture due to the lack of necessary growth factor in the serum-free media and, thus, could not be analyzed.

FIG. 4. hASC (donor A) sheet study. (A) DNA, (B) GAG, and (C) GAG/DNA content of hASC sheet constructs after 3 weeks in culture.

Histology and immunohistochemistry of microsphere-incorporated hASC sheets

Cartilage formation within hASC sheets was further confirmed via histologic evaluation. Regions with the most intense Safranin O staining of GAG are shown in Figure 5. Some degree of GAG staining was present in all groups. Regions with round chondrocytic morphology could be observed in all groups. In most areas that lacked metachromatic staining, cell phenotype was elongated and fibroblastic. High Gp microspheres (dark blue) in the high Gp+exo. and high Gp+TGF-β1 groups appeared to be in the process of degrading, while the low Gp microspheres initially incorporated into the low Gp+exo. and low Gp+TGF-β1 groups were mostly degraded by 3 weeks.

Immunohistochemistry for C-6-S, a sulfated GAG that is a major component of cartilage, was performed to further visualize the presence and spatial distribution of GAG as this is a more sensitive method than histochemical staining^{57,58} (Fig. 6). Positive staining for C-6-S occurred throughout all sheet groups. As with the Safranin O images, incorporated high Gp microspheres (Fig. 6, “high Gp+exo.” and “high Gp+TGF-β1”) were still visible while incorporated low Gp microspheres (Fig. 6, “low Gp+exo.” and “low Gp+TGF-β1”) could not be seen throughout the constructs. Immunohistochemistry against collagen type II, another important ECM component of articular cartilage, was also performed. However, staining for collagen type II was minimal with the most intense staining in a region of the exo. TGF-β1 sheets (Fig. 6). No staining was observed for collagen type I and negative control mouse IgG (not shown).

Thickness measurements and mechanical testing of microsphere-incorporated hASC sheets

Thickness measurements of sheets from donor A were not statistically different among the conditions (Table 3). Step

strain stress-relaxation testing yielded equilibrium compressive moduli (E) that were also similar for all conditions (Table 3). Sheets incorporated with high Gp microspheres had slightly higher average E than that of sheets in the other conditions; however, no statistical significance was observed.

Discussion

This study aimed at exploring the influence of growth factor distribution and release kinetics on cartilage formation within high-density hASC aggregates incorporated with TGF-β1-containing microspheres and at engineering larger, more clinically relevant cartilage constructs via the production of self-assembled hASC sheets with or without TGF-β1-loaded microspheres. Incorporation of growth factor releasing biodegradable polymer microspheres within high-density hASC constructs may circumvent the need for exogenous growth factor supplementation during *in vitro* culture by providing spatiotemporal delivery of growth factor to cells within the constructs. Genipin-crosslinked gelatin microspheres were used in this study, because they have been shown to locally deliver growth factor at varying rates based on the degree of crosslinking and to promote growth factor-induced cell differentiation and tissue regeneration.⁵⁰ Genipin, a natural cross-linking agent, stabilizes gelatin through the covalent binding of primary amine groups⁵⁹ with minimal toxic residue production.⁶⁰

After gelatin microspheres with two different crosslinking levels were produced, they were incorporated with or without loaded TGF-β1 within hASC aggregates from the most chondrogenic donor in response to exogenous TGF-β1 as identified in the donor screen. The different degrees of crosslinking were used to regulate cell-mediated degradation and growth factor release profiles. Microsphere and growth factor loading concentrations were varied to determine the

FIG. 5. Photomicrographs of histologic sections of hASC sheets from donor A stained for Safranin O. Scale bars = 100 µm. Color images available online at www.liebertpub.com/tea

FIG. 6. Photomicrographs of histologic sections of hASC sheets from donor A immunohistochemically stained for chondroitin-6-sulfate (C-6-S) and collagen type II (Col-II). Scale bars = 100 μ m. Color images available online at www.liebertpub.com/tea

influence of growth factor distribution on chondrogenesis within microsphere-incorporated hASC aggregates. When TGF- β 1 was supplemented in the medium, the presence of microspheres without loaded growth factor did not negatively affect cell viability or GAG production (Fig. 3). DNA, GAG, and GAG/DNA contents in groups 1 (positive control without microspheres), 2, and 3 were similar at each time point and increased GAG production between week 1 and 2 was observed in each group. These results indicate that the incorporated microspheres did not affect cell viability or hASC chondrogenesis.

However, when TGF- β 1 was not exogenously supplemented, the amount of incorporated microspheres and concentration of growth factor within the microspheres affected cell survival and GAG production. DNA, GAG, and GAG/DNA contents were similar in all groups after 1 week, but these contents in groups 1 through 5 were significantly higher than those in groups 6 through 9, in which the microsphere and growth factor loading concentrations were varied, after 2 weeks. Increased GAG and GAG/DNA contents from week 1 to 2 were observed in groups 4 and 5 in which 0.15 mg microspheres loaded with 400 ng TGF- β 1/mg microspheres were incorporated into each aggregate (Fig. 3, groups 4 and 5). DNA, GAG, and GAG/DNA contents in groups 4 and 5 did not differ significantly from those in aggregates cultured in growth factor containing medium (groups 1–3) at either time point, indicating that the incorporated growth factor-loaded microspheres had the potential to induce hASC chondrogen-

esis at levels equivalent to those of groups 1 through 3 in which TGF- β 1 was exogenously supplemented.

When the amount of microspheres was reduced by a factor of 3 from 0.15 to 0.05 mg but the growth factor concentration per mg microspheres stayed at 400 ng/mg (Fig. 3, groups 6 and 7), DNA level significantly decreased after 1 week and little GAG was produced. Groups 6 and 7 also exhibited significantly lower DNA, GAG, and GAG/DNA content than groups 1 through 5 at week 2. Since there are less microspheres in these groups, the average distance from a cell to its nearest microsphere is greater. The lower overall DNA and GAG content could have resulted because cells further from the microspheres may receive less growth factor as it would have to diffuse a greater distance and, in addition, it might bind with the ECM or be taken up by cells along this longer path. The lower biochemical content in groups 6 and 7 may also be a result of the reduction in the total amount of TGF- β 1 that was available to the cells. A study by Awad *et al.* showed that hASC survival, a balance between cell proliferation and apoptosis, was dependent on TGF- β 1 concentration when ITS+, instead of FBS, was supplemented in the culture medium.¹⁸ Similarly, these results suggest that there may be a dose-dependent effect of TGF- β 1 on cell survival and that the decrease in DNA content at week 2 may be a result of greater cell apoptosis rate compared with proliferation rate within aggregates exposed to less total TGF- β 1.

To determine whether a higher growth factor loading concentration per microsphere while maintaining the lower microsphere mass would result in higher cell survival and GAG production, TGF- β 1 loading concentration was increased by a factor of 3 from 400 ng TGF- β 1/mg microspheres to 1200 ng TGF- β 1/mg microspheres. After 2 weeks, DNA, GAG, and GAG/DNA content was significantly lower than that in groups 1 through 5 (Fig. 3, groups 8 and 9) even though the total amount of growth factor per aggregate was the same as in groups 4 and 5. It is also possible that the high local concentrations of TGF- β 1 from the microspheres had a negative impact on chondrogenesis, as it has been reported that high concentrations of TGF- β 1 can inhibit the chondrogenic differentiation of hASCs.²⁴

TABLE 3. THICKNESS MEASUREMENTS AND EQUILIBRIUM COMPRESSIVE MODULI FROM STEP STRAIN STRESS-RELAXATION TESTING OF HUMAN ADIPOSE-DERIVED STEM CELL SHEETS FROM DONOR A

Condition	Thickness (μ m)	E (kPa)
Exo. TGF- β 1	449 $\pm$ 32	4.96 $\pm$ 0.94
Low Gp + exo. TGF- β 1	495 $\pm$ 89	6.04 $\pm$ 0.33
High Gp + exo. TGF- β 1	492 $\pm$ 28	6.87 $\pm$ 3.07
Low Gp + TGF- β 1	470 $\pm$ 79	4.87 $\pm$ 0.74
High Gp + TGF- β 1	498 $\pm$ 12	8.51 $\pm$ 1.15

Results from groups 6 through 9 compared with groups 4 and 5 provide evidence that the amount of growth factor-loaded microspheres, growth factor loading concentration per microsphere, and/or total amount of growth factor per aggregate may be critical design variables to maintain cell viability and achieve hASC chondrogenesis in this system.

No significant differences were found between low Gp and high Gp groups with matching microsphere and growth factor loading concentrations (groups 2 and 3, groups 4 and 5, groups 6 and 7, and groups 8 and 9). This is especially interesting, because microspheres with two different levels of crosslinking were used, resulting in different cell-mediated degradation rates. Since the low Gp microspheres have a lower degree of crosslinking, their enzyme-mediated degradation should be quicker than that of the high Gp microspheres.³⁹ Since TGF- β 1 release is mediated by microsphere degradation, more TGF- β 1 may have been released from the low Gp microspheres than from high Gp microspheres by 2 weeks. One would then expect that a higher GAG level would result in the TGF- β 1-loaded low Gp group compared with the other groups as has been observed in a related system with hMSCs.³⁹ However, this was not the case for these hASC aggregates. It is possible that this difference in TGF- β 1 exposure is not enough to significantly affect hASC chondrogenesis for this culture period, but the more sustained delivery of TGF- β 1 from the high Gp microspheres may potentially result in improved cartilage formation at later time points.

To develop constructs of a more clinically relevant size for cartilage regeneration, self-assembling hASC sheets were then engineered. To our knowledge, this is the first report of hASC sheet constructs for cartilage tissue engineering. hASC sheets were created containing cells only and cultured in TGF- β 1 supplemented medium, or incorporated with growth factor releasing hydrogel microspheres. Culture time was extended to 3 weeks to give the cells more time to differentiate and secrete a neocartilaginous ECM. For cells-only sheets cultured in TGF- β 1-containing medium, quantitative biochemical measurements indicated that the constructs remained viable throughout culture as shown by stable DNA content and high GAG content. GAG and GAG/DNA values were comparable to the largest values that have been reported for *in vitro* hASC chondrogenesis.⁶¹

Microsphere-incorporated hASC sheets were also produced (Table 2) by utilizing similar conditions of TGF- β 1-loaded microsphere incorporation to those that promoted the best chondrogenesis in aggregates (from Table 1, groups 4 and 5). DNA, GAG, and GAG/DNA were similar for all conditions, including hASC-only sheets. The equivalent levels of GAG production in low Gp+TGF- β 1 sheets and high Gp+TGF- β 1 sheets is particularly noteworthy. Since the low Gp microspheres have a lower degree of crosslinking, their enzyme-mediated degradation occurs more rapidly than that of the high Gp microspheres.³⁹ Since TGF- β 1 release is mediated by microsphere degradation, more loaded TGF- β 1 may have been released from the low Gp microspheres than the high Gp microspheres by the end of culture. In the comparable system of microsphere-incorporated hMSC sheets, this rapid degradation and complete growth factor release led to increased GAG production in the low Gp+TGF- β 1 sheets after 3 weeks.³⁹ However, this was not the case for the microsphere-incorporated hASC sheets. While

the reasoning behind this is unclear, it is possible that this difference in TGF- β 1 release is not significant enough to result in different levels of chondrogenesis by the hASCs. It should also be noted that hASCs are a different cell type which responds differently to TGF- β 1,^{25,62,63} and this study further demonstrates the differences between hMSC and hASC responses under similar culture conditions. Overall, these results demonstrate that the presence of microspheres without loaded TGF- β 1 had no apparent effect on chondrogenesis, while sheets containing TGF- β 1-loaded microspheres achieved levels of chondrogenesis equivalent to those cultured with medium containing TGF- β 1.

Negative control hASC sheets with empty microspheres and no exogenous growth factor supplementation were formed but did not survive during the culture period. This indicated that GAG production in the sheets containing TGF- β 1-loaded microspheres (low Gp+TGF- β 1 and high Gp+TGF- β 1) was due to the growth factor released from the incorporated microspheres rather than the presence of the microspheres themselves.

To further confirm cartilage formation within sheets, histologic evaluation was performed. Although Safranin O staining for GAG was not uniform throughout the sheets, some regions of positive staining and cartilage-like morphological structure were observed in all groups (Fig. 5). Immunohistochemistry against C-6-S was performed to better visualize GAG production, as it is a more specific and more sensitive method than histochemical staining.^{57,58} As expected, intense staining for C-6-S was observed in all groups (Fig. 6). The C-6-S staining serves as additional evidence that the hASCs within this system were able to undergo chondrogenesis and produce proteoglycan-rich ECM. When immunohistochemistry was performed against collagen type I, no positive staining was observed (not shown), providing evidence that fibrocartilage was not formed. However, minimal or no positive staining for collagen type II was also observed even though it is an important marker of articular cartilage (Fig. 6). While unfortunate, minimal or lack of staining for this marker was not surprising, as multiple reports found that collagen type II staining was either negative or at best weak in high-density hASC constructs cultured in TGF- β -containing media for as long as 42 days.^{23,26,64,65} In a study by Winter *et al.*, staining in just one region of hASC aggregates was observed after 4 weeks.²³ In addition, Afizah *et al.* and Hildner *et al.* showed no positive staining in hASC aggregates after 28 and 35 days, respectively,^{26,65} and Hennig *et al.* reported that only hASC aggregates from one out of nine hASC donors stained positively for collagen type II after 42 days.⁶⁴

Incorporated low Gp microspheres within the low Gp+exo. and low Gp+TGF- β 1 sheet constructs appeared to be completely degraded by 3 weeks. The space left by the degraded low Gp microspheres seemed to have been filled in by cells and ECM. On the other hand, incorporated high Gp microspheres, stained blue in Figures 5 and 6, within the high Gp+exo. TGF- β 1 and high Gp+TGF- β 1 groups were still present after 3 weeks. These visual differences confirmed the difference in degradation rates between the low Gp and high Gp microspheres.

The histology of the constructs presented here were not as similar to native cartilage as hMSC-generated cartilage

sheets,³⁹ confirming the findings of several other studies utilizing these cell populations in different systems. For example, one study found that toluidine blue staining of sulfated proteoglycans in exogenously TGF- β 1-treated hASC aggregates was weak compared with that of hMSC aggregates.²⁵ In a study by Diekman *et al.*, the histological Safranin O GAG staining of hASCs cultured for 4 weeks in a cartilage-derived matrix with TGF- β 3 and BMP-6 was weak compared with similarly cultured hMSC constructs and the positive control of a human osteochondral plug.⁶¹ An elongated phenotype was described for the hASCs, while a more rounded morphology was seen in hMSC-generated cartilage. These findings suggest that more work is needed to identify better soluble factors to drive hASC chondrogenesis. However, such efforts may prove worthwhile, as the high availability of adipose tissue and ability to obtain high numbers of easily expanded hASCs, which can maintain their differentiation capacity, make this an excellent candidate cell source for tissue engineering and regeneration.

Due to the importance of articular cartilage's stiffness under compression as a load-bearing tissue, hASC sheets from donor A were subjected to step strain stress-relaxation testing in unconfined compression, a commonly used method for characterizing the biomechanical properties of tissue engineered cartilage^{66–68} as articular cartilage exhibits a stress-relaxation response to deformation.^{69,70} As with thickness measurements, the average equilibrium compressive moduli were similar for all groups (Table 3). Sheets incorporated with high Gp microspheres had slightly higher but not significantly different E values, potentially due to the presence of residual high Gp microspheres that may have contributed to the higher average mechanical stiffness of these sheets. It is important to note that the TGF- β 1 delivered from both low Gp- and high Gp-incorporated microspheres were capable of inducing chondrogenic differentiation that resulted in constructs which were as stiff as those treated with exogenously supplemented TGF- β 1. While no significant biochemical (aggregates and sheets) or mechanical (sheets) differences were observed between the growth factor-loaded low Gp and high Gp conditions, cartilage formation may improve to a greater extent at later time points within constructs incorporated with high Gp microspheres compared with those with low Gp microspheres, as the high Gp microspheres may continue to degrade and deliver additional loaded growth factor over time. More sustained chondrogenic growth factor delivery could potentially result in improved chondrogenesis at later time points to yield cartilage constructs with enhanced mechanical properties.

To demonstrate reproducibility of this microsphere-incorporated self-assembling sheet technology, hASC sheets from a second donor (donor B from the donor screen) were also produced and analyzed (Supplementary Data; Supplementary Data are available online at www.liebertpub.com/tea). As with donor A, DNA, GAG, and GAG/DNA content for donor B was similar for all conditions (Supplementary Fig. S1). Safranin O staining for GAG revealed nonuniform staining throughout the constructs for each condition. Positively stained regions for each condition are shown in Supplementary Figure S2. However, positive immunohistochemical staining for C-6-S was observed throughout each construct (Supplementary Fig. S3). While the negative staining for collagen type I (not shown) provides evidence that these constructs were not fibrocartilage, staining for

collagen type II was also negative (Supplementary Fig. S3). Comparing the two donors, GAG and GAG/DNA content in sheets from donor B were lower than those for sheets from donor A, and GAG staining for donor A was more intense than that for donor B. The difference in GAG production between these two donors is likely due to donor-to-donor variability. Donor A was more chondrogenic than donor B in the donor screen (Fig. 1), so it is not surprising that sheets from the latter contain less GAG and GAG/DNA content than the former. Nevertheless, similar trends in the results from both donors demonstrated the repeatability of this technology with hASCs from multiple donors. While cells from one donor outperformed the other in this study, this system still has strong clinical applicability because the immunomodulatory properties of hASCs may enable patients whose hASCs are not chondrogenic to be treated with highly chondrogenic allogeneic donor hASCs.^{71–73}

Overall, this system of growth factor-releasing biodegradable polymer microspheres incorporated within self-assembled hASC sheets shows great promise for use in cartilage tissue engineering applications. Although significant differences in GAG production between the different types of crosslinked microspheres used in this study were not observed in this system, different growth factors could be used for which the degree of microsphere crosslinking and proteolytic degradation rate can be tuned along with microsphere loading density and growth factor loading concentrations to potentially achieve varying rates and levels of cartilage formation in hASC sheets. Since our lab has recently demonstrated the efficacy of TGF- β 1 in a related system using hMSCs,³⁹ TGF- β 1 was selected for this proof-of-principle study. It is possible that alternative chondrogenic growth factors or a combination of growth factors, more specifically TGF- β 3 and BMP-6⁶⁴ or TGF- β 1 and BMP-2,⁷⁴ may result in enhanced chondrogenesis compared with any of the factors alone.¹⁶ This approach can be explored in future studies to enhance hASC chondrogenesis within this system.

Conclusion

This proof-of-principle study is the first to report the production of hASC sheets for cartilage tissue engineering. It was demonstrated that TGF- β 1-loaded gelatin microspheres can be incorporated within high-cell-density hASC constructs, including aggregates and self-assembled cell sheets. In the cell aggregates, it was found that microsphere and growth factor loading concentrations influenced the degree of chondrogenesis achieved with specific conditions inducing chondrogenesis at levels equivalent to control aggregates cultured with exogenously supplemented TGF- β 1, as demonstrated by GAG production at 2 weeks. A larger, more clinically relevant model was then explored via the engineering of self-assembling hASC sheets. hASC sheets cultured in TGF- β 1-containing medium for 3 weeks developed into neocartilaginous constructs as confirmed by biochemical and histological analyses. hASC sheets with incorporated growth factor-loaded microspheres were also produced. In these sheets, microsphere-mediated TGF- β 1 release induced GAG production at levels equivalent to sheets without microspheres cultured in TGF- β 1 supplemented medium at 3 weeks. The

inclusion of TGF- β 1-releasing gelatin microspheres provides sustained, localized growth factor delivery by cell-mediated degradation, without the time and cost inefficiencies of exogenously supplementing growth factor in the medium. Future studies could investigate the influence of other chondrogenic growth factors, microsphere loading density, and growth factor loading concentration on cartilage formation in self-assembled hASC sheets. This simple system has great clinical potential to enable hASC chondrogenesis and cartilage formation *in vivo* without previous extended *in vitro* culture.

Acknowledgments

The authors would like to thank Amad Awadallah and Dr. Joseph Mansour for technical assistance and Dr. Jeffrey Gimble for providing the hASCs. The authors gratefully acknowledge funding from the National Institutes of Health (AR007505, AR063194, and AR061265), a Biomedical Research and Technology Transfer Grant 09-071 from the Ohio Department of Development, a New Scholar in Aging grant from the Ellison Medical Foundation, and a National Science Foundation Graduate Research Fellowship (PND).

Disclosure Statement

No competing financial interests exist.

References

- Cohen, N.P., Foster, R.J., and Mow, V.C. Composition and dynamics of articular cartilage: structure, function, and maintaining healthy state. *J Orthop Sports Phys Ther* **28**, 203, 1998.
- Wang, Y., Kim, U.J., Blasioli, D.J., Kim, H.J., and Kaplan, D.L. *In vitro* cartilage tissue engineering with 3D porous aqueous-derived silk scaffolds and mesenchymal stem cells. *Biomaterials* **26**, 7082, 2005.
- Martel-Pelletier, J., Wildi, L.M., and Pelletier, J.P. Future therapeutics for osteoarthritis. *Bone* **51**, 297, 2012.
- Redman, S.N., Oldfield, S.F., and Archer, C.W. Current strategies for articular cartilage repair. *Eur Cell Mater* **9**, 23, 2005; discussion 23–32.
- Hunziker, E.B., Driesang, I.M., and Saager, C. Structural barrier principle for growth factor-based articular cartilage repair. *Clin Orthop Relat Res* **391 Suppl**, S182, 2001.
- Ahmed, T.A., and Hincke, M.T. Strategies for articular cartilage lesion repair and functional restoration. *Tissue Eng Part B Rev* **16**, 305, 2010.
- Batty, L., Dance, S., Bajaj, S., and Cole, B.J. Autologous chondrocyte implantation: an overview of technique and outcomes. *ANZ J Surg* **81**, 18, 2011.
- Zhang, L., Hu, J., and Athanasiou, K.A. The role of tissue engineering in articular cartilage repair and regeneration. *Crit Rev Biomed Eng* **37**, 1, 2009.
- Bijlsma, J.W., Berenbaum, F., and Lafeber, F.P. Osteoarthritis: an update with relevance for clinical practice. *Lancet* **377**, 2115, 2011.
- Gimble, J., and Guilak, F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* **5**, 362, 2003.
- Ronziere, M.C., Perrier, E., Mallein-Gerin, F., and Freyria, A.M. Chondrogenic potential of bone marrow- and adipose tissue-derived adult human mesenchymal stem cells. *Biomed Mater Eng* **20**, 145, 2010.
- Hildner, F., Albrecht, C., Gabriel, C., Redl, H., and van Griensven, M. State of the art and future perspectives of articular cartilage regeneration: a focus on adipose-derived stem cells and platelet-derived products. *J Tissue Eng Regen Med* **5**, e36, 2011.
- Solchaga, L.A., Penick, K., Goldberg, V.M., Caplan, A.I., and Welter, J.F. Fibroblast growth factor-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone-marrow-derived mesenchymal stem cells. *Tissue Eng Part A* **16**, 1009, 2010.
- 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.
- Gonda, K., Shigeura, T., Sato, T., Matsumoto, D., Suga, H., Inoue, K., *et al.* Preserved proliferative capacity and multipotency of human adipose-derived stem cells after long-term cryopreservation. *Plastic Reconstr Surg* **121**, 401, 2008.
- Puetzer, J.L., Petitte, J.N., and Lobo, E.G. Comparative review of growth factors for induction of three-dimensional *in vitro* chondrogenesis in human mesenchymal stem cells isolated from bone marrow and adipose tissue. *Tissue Eng Part B Rev* **16**, 435, 2010.
- Estes, B.T., and Guilak, F. Three-dimensional culture systems to induce chondrogenesis of adipose-derived stem cells. *Methods Mol Biol* **702**, 201, 2011.
- 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.
- Awad, H.A., Wickham, M.Q., Leddy, H.A., Gimble, J.M., and Guilak, F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials* **25**, 3211, 2004.
- 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.
- 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 Rheum* **54**, 1222, 2006.
- Xu, Y., Balooch, G., Chiou, M., Bekerman, E., Ritchie, R.O., and Longaker, M.T. Analysis of the material properties of early chondrogenic differentiated adipose-derived stromal cells (ASC) using an *in vitro* three-dimensional micromass culture system. *Biochem Biophys Res Commun* **359**, 311, 2007.
- Winter, A., Breit, S., Parsch, D., Benz, K., Steck, E., Hauner, H., *et al.* 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.
- Huang, J.I., Zuk, P.A., Jones, N.F., Zhu, M., Lorenz, H.P., Hedrick, M.H., *et al.* Chondrogenic potential of multipotential cells from human adipose tissue. *Plastic Reconstr Surg* **113**, 585, 2004.
- 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.
26. 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.
 27. Bunnell, B.A., Flaatt, M., Gagliardi, C., Patel, B., and Ripoll, C. Adipose-derived stem cells: isolation, expansion and differentiation. *Methods* **45**, 115, 2008.
 28. 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.
 29. Yoo, J.U., Barthel, T.S., Nishimura, K., Solchaga, L., Caplan, A.I., Goldberg, V.M., *et al.* The chondrogenic potential of human bone-marrow-derived mesenchymal progenitor cells. *J Bone Joint Surg Am* **80**, 1745, 1998.
 30. Yang, J., Yamato, M., Shimizu, T., Sekine, H., Ohashi, K., Kanzaki, M., *et al.* Reconstruction of functional tissues with cell sheet engineering. *Biomaterials* **28**, 5033, 2007.
 31. Labbe, B., Marceau-Fortier, G., and Fradette, J. Cell sheet technology for tissue engineering: the self-assembly approach using adipose-derived stromal cells. *Methods Mol Biol* **702**, 429, 2011.
 32. Gilpin, D.A., Weidenbecher, M.S., and Dennis, J.E. Scaffold-free tissue-engineered cartilage implants for laryngotracheal reconstruction. *Laryngoscope* **120**, 612, 2010.
 33. Tani, G., Usui, N., Kamiyama, M., Oue, T., and Fukuzawa, M. *In vitro* construction of scaffold-free cylindrical cartilage using cell sheet-based tissue engineering. *Pediatr Surg Int* **26**, 179, 2010.
 34. Yang, J., Yamato, M., Nishida, K., Ohki, T., Kanzaki, M., Sekine, H., *et al.* Cell delivery in regenerative medicine: the cell sheet engineering approach. *J Control Release* **116**, 193, 2006.
 35. Solorio, L.D., Fu, A.S., Hernandez-Irizarry, R., and Alsberg, E. Chondrogenic differentiation of human mesenchymal stem cell aggregates via controlled release of TGF-beta1 from incorporated polymer microspheres. *J Biomed Mater Res A* **92**, 1139, 2010.
 36. Kaneshiro, N., Sato, M., Ishihara, M., Mitani, G., Sakai, H., and Mochida, J. Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage. *Biochem Biophys Res Commun* **349**, 723, 2006.
 37. Murdoch, A.D., Grady, L.M., Ablett, M.P., Katopodi, T., Meadows, R.S., and Hardingham, T.E. Chondrogenic differentiation of human bone marrow stem cells in transwell cultures: generation of scaffold-free cartilage. *Stem Cells* **25**, 2786, 2007.
 38. Mayer-Wagner, S., Schiergens, T.S., Sievers, B., Docheva, D., Schieker, M., Betz, O.B., *et al.* Membrane-based cultures generate scaffold-free neocartilage *in vitro*: influence of growth factors. *Tissue Eng Part A* **16**, 513, 2010.
 39. Solorio, L.D., Vieregge, E.L., Dhimi, C.D., Dang, P.N., and Alsberg, E. Engineered cartilage via self-assembled hMSC sheets with incorporated biodegradable gelatin microspheres releasing transforming growth factor-beta1. *J Control Release* **158**, 224, 2012.
 40. Ikada, Y., and Tabata, Y. Protein release from gelatin matrices. *Adv Drug Deliv Rev* **31**, 287, 1998.
 41. Young, S., Wong, M., Tabata, Y., and Mikos, A.G. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. *J Control Release* **109**, 256, 2005.
 42. Hayashi, K., and Tabata, Y. Preparation of stem cell aggregates with gelatin microspheres to enhance biological functions. *Acta Biomater* **7**, 2797, 2011.
 43. Ogawa, T., Akazawa, T., and Tabata, Y. *In vitro* proliferation and chondrogenic differentiation of rat bone marrow stem cells cultured with gelatin hydrogel microspheres for TGF-beta1 release. *J Biomater Sci Polym Ed* **21**, 609, 2010.
 44. Estes, B.T., Diekmann, B.O., Gimble, J.M., and Guilak, F. Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. *Nat Protoc* **5**, 1294, 2010.
 45. 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.
 46. Han, Y., Wei, Y., Wang, S., and Song, Y. Enhanced chondrogenesis of adipose-derived stem cells by the controlled release of transforming growth factor-beta1 from hybrid microspheres. *Gerontology* **55**, 592, 2009.
 47. Merceron, C., Vinatier, C., Portron, S., Masson, M., Amiaud, J., Guigand, L., *et al.* Differential effects of hypoxia on osteochondrogenic potential of human adipose-derived stem cells. *Am J Physiol Cell Physiol* **298**, C355, 2010.
 48. Penick, K.J., Solchaga, L.A., and Welter, J.F. High-throughput aggregate culture system to assess the chondrogenic potential of mesenchymal stem cells. *Biotechniques* **39**, 687, 2005.
 49. Yamamoto, M., Ikada, Y., and Tabata, Y. Controlled release of growth factors based on biodegradation of gelatin hydrogel. *J Biomater Sci Polym Ed* **12**, 77, 2001.
 50. Solorio, L., Zwolinski, C., Lund, A.W., Farrell, M.J., and Stegemann, J.P. Gelatin microspheres crosslinked with genipin for local delivery of growth factors. *J Tissue Eng Regen Med* **4**, 514, 2010.
 51. McGowan, K.B., Kurtis, M.S., Lottman, L.M., Watson, D., and Sah, R.L. Biochemical quantification of DNA in human articular and septal cartilage using PicoGreen and Hoechst 33258. *Osteoarthritis Cartilage* **10**, 580, 2002.
 52. Farndale, R.W., Buttle, D.J., and Barrett, A.J. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. *Biochim Biophys Acta* **883**, 173, 1986.
 53. Yang, X., Shang, H., Katz, A., and Li, X. A modified aggregate culture for chondrogenesis of human adipose-derived stem cells genetically modified with growth and differentiation factor 5. *Biores Open Access* **2**, 258, 2013.
 54. Chua, K.H., Aminuddin, B.S., Fuzina, N.H., and Ruszymah, B.H. Insulin-transferrin-selenium prevent human chondrocyte dedifferentiation and promote the formation of high quality tissue engineered human hyaline cartilage. *Eur Cell Mater* **9**, 58, 2005; discussion.
 55. Sandell, L.J., and Daniel, J.C. Effects of ascorbic acid on collagen mRNA levels in short term chondrocyte cultures. *Connect Tissue Res* **17**, 11, 1988.
 56. Sekiya, I., Koopman, P., Tsuji, K., Mertin, S., Harley, V., Yamada, Y., *et al.* Dexamethasone enhances SOX9 expression in chondrocytes. *J Endocrinol* **169**, 573, 2001.
 57. Marques, M.E., Coelho, K.I., Sotto, M.N., and Bacchi, C.E. Comparison between histochemical and immunohistochemical methods for diagnosis of sporotrichosis. *J Clin Pathol* **45**, 1089, 1992.

58. Obert, L.A., Sobocinski, G.P., Bobrowski, W.F., Metz, A.L., Rolsma, M.D., Altrogge, D.M., *et al.* An immunohistochemical approach to differentiate hepatic lipidosis from hepatic phospholipidosis in rats. *Toxicol Pathol* **35**, 728, 2007.
59. Liang, H.C., Chang, Y., Hsu, C.K., Lee, M.H., and Sung, H.W. Effects of crosslinking degree of an acellular biological tissue on its tissue regeneration pattern. *Biomaterials* **25**, 3541, 2004.
60. Sung, H.W., Huang, R.N., Huang, L.L., and Tsai, C.C. *In vitro* evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation. *J Biomater Sci Polym Ed* **10**, 63, 1999.
61. Diekman, B.O., Rowland, C.R., Lennan, D.P., Caplan, A.L., and Guilak, F. Chondrogenesis of adult stem cells from adipose tissue and bone marrow: induction by growth factors and cartilage-derived matrix. *Tissue Eng Part A* **16**, 523, 2010.
62. Mehlhorn, A.T., Niemeyer, P., Kaiser, S., Finkenzeller, G., Stark, G.B., Sudkamp, N.P., *et al.* Differential expression pattern of extracellular matrix molecules during chondrogenesis of mesenchymal stem cells from bone marrow and adipose tissue. *Tissue Eng* **12**, 2853, 2006.
63. Kisiday, J.D., Kopesky, P.W., Evans, C.H., Grodzinsky, A.J., McIlwraith, C.W., and Frisbie, D.D. Evaluation of adult equine bone marrow- and adipose-derived progenitor cell chondrogenesis in hydrogel cultures. *J Orthop Res* **26**, 322, 2008.
64. Hennig, T., Lorenz, H., Thiel, A., Goetzke, K., Dickhut, A., Geiger, F., *et al.* 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.
65. Hildner, F., Peterbauer, A., Wolbank, S., Nurnberger, S., Marlovits, S., Redl, H., *et al.* FGF-2 abolishes the chondrogenic effect of combined BMP-6 and TGF-beta in human adipose derived stem cells. *J Biomed Mater Res A* **94**, 978, 2010.
66. Lima, E.G., Bian, L., Ng, K.W., Mauck, R.L., Byers, B.A., Tuan, R.S., *et al.* The beneficial effect of delayed compressive loading on tissue-engineered cartilage constructs cultured with TGF-beta3. *Osteoarthritis Cartilage* **15**, 1025, 2007.
67. Kelly, D.J., Crawford, A., Dickinson, S.C., Sims, T.J., Mundy, J., Hollander, A.P., *et al.* Biochemical markers of the mechanical quality of engineered hyaline cartilage. *J Mater Sci Mater Med* **18**, 273, 2007.
68. Tran, S.C., Cooley, A.J., and Elder, S.H. Effect of a mechanical stimulation bioreactor on tissue engineered, scaffold-free cartilage. *Biotechnol Bioeng* **108**, 1421, 2011.
69. Sophia Fox, A.J., Bedi, A., and Rodeo, S.A. The basic science of articular cartilage: structure, composition, and function. *Sports Health* **1**, 461, 2009.
70. Mansour, J.M. Biomechanics of cartilage. In: Oatis, C., ed. *Kinesiology: The Mechanics and Pathomechanics of Human Movement*. Philadelphia, PA: Lippincott Williams and Wilkins, 2003. pp. 66–79.
71. Puissant, B., Barreau, C., Bourin, P., Clavel, C., Corre, J., Bousquet, C., *et al.* Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. *Br J Haematol* **129**, 118, 2005.
72. Melief, S.M., Zwaginga, J.J., Fibbe, W.E., and Roelofs, H. Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts. *Stem Cells Transl Med* **2**, 455, 2013.
73. Yoo, K.H., Jang, I.K., Lee, M.W., Kim, H.E., Yang, M.S., Eom, Y., *et al.* Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. *Cell Immunol* **259**, 150, 2009.
74. Mehlhorn, A.T., Niemeyer, P., Kaschte, K., Muller, L., Finkenzeller, G., Hartl, D., *et al.* Differential effects of BMP-2 and TGF-beta1 on chondrogenic differentiation of adipose derived stem cells. *Cell Prolif* **40**, 809, 2007.

Address correspondence to:

Eben Alsberg, PhD

Departments of Biomedical Engineering

and Orthopaedic Surgery

Case Western Reserve University

Wickenden 309

10900 Euclid Avenue

Cleveland, OH 44106

E-mail: eben.alsberg@case.edu

Received: September 10, 2012

Accepted: May 28, 2014

Online Publication Date: July 3, 2014