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Engineered Cartilage via Self-Assembled hMSC Sheets with Incorporated Biodegradable Gelatin Microspheres Releasing Transforming Growth Factor- β 1

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Abstract

Self-assembling cell sheets have shown great potential for use in cartilage tissue engineering applications, as they provide an advantageous environment for the chondrogenic induction of human mesenchymal stem cells (hMSCs). We have engineered a system of self-assembled, microsphere-incorporated hMSC sheets capable of forming cartilage in the presence of exogenous transforming growth factor β 1 (TGF- β 1) or with TGF- β 1 released from incorporated microspheres. Gelatin microspheres with two different degrees of crosslinking were used to enable different cell-mediated microsphere degradation rates. Biochemical assays, histological and immunohistochemical analyses, and biomechanical testing were performed to determine biochemical composition, structure, and equilibrium modulus in unconfined compression after 3 weeks of culture. The inclusion of microspheres with or without loaded TGF- β 1 significantly increased sheet thickness and compressive equilibrium modulus, and enabled more uniform matrix deposition by comparison to control sheets without microspheres. Sheets incorporated with fast-degrading microspheres containing TGF- β 1 produced significantly more GAG and GAG per DNA than all other groups tested and stained more intensely for type II collagen. These findings demonstrate improved cartilage formation in microsphere-incorporated cell sheets, and describe a tailorable system for the chondrogenic induction of hMSCs without necessitating culture in growth factor-containing medium.

Keywords

tissue engineering; biomaterial; mesenchymal stem cell; transforming growth factor; microsphere

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Introduction

Osteoarthritis (OA) is a degenerative disease of the articular cartilage affecting millions of people worldwide [1]. As no current treatment can fully and consistently restore normal joint function to patients afflicted with OA [2], there is a significant clinical need for alternative therapies for cartilage regeneration. Many approaches to the tissue engineering of articular cartilage involve the use of cells in combination with soluble bioactive factors and biomaterials that may provide specific microenvironmental cues for chondrogenic induction [3–5]. Mesenchymal stem cells (MSCs) from bone marrow have been shown to be a promising cell source for these cartilage tissue engineering strategies, as they can be expanded in culture without losing multipotency, and can differentiate into many cell types of the connective tissue lineage including chondrocytes under appropriate conditions [6]. Specifically, two important factors for the *in vitro* chondrogenic induction of MSCs are high initial cell density and exposure to transforming growth factor β (TGF- β) [7–9].

Several *in vitro* culture methods have been developed for MSC chondrogenesis, including aggregate or pellet culture [9–11], micromass culture [12, 13], and self-assembling cell sheet systems [7, 8, 14, 15]. These culture systems take advantage of the abundant cell-cell interactions that occur in 3D high density culture, without the potential interference of a biomaterial scaffold. In particular, self-assembling cell sheets show promise for use in cartilage tissue engineering applications, as they may form larger constructs with much greater surface areas and volumes than aggregates or tiny micromass cultures [8, 15]. Unlike spherical cell aggregates, which are limited in size by the diffusion distance of nutrients into the center of the sphere, flat sheets of various dimensions can be formed without necessitating a proportional increase in construct thickness, enabling nutrient diffusion to all regions of the tissue. Upon surgical evaluation, chondral defects in the knee have an area of at least 0.5 cm², with over a third of the defects having areas of at least 1 cm² [16]. Self-assembling sheets could be clinically practical for the treatment of these defects, as sheets of the appropriate size could be formed and then implanted into a defect as an intact piece. This is in contrast to smaller cell constructs, which may not be as readily applied for the clinical treatment of cartilage defects since a number of constructs would be required to fill a single lesion. It may be difficult to localize multiple constructs to a defect, and in order to repair the damaged cartilage, the individual cell constructs would have to integrate with each other as well as with the surrounding host tissue.

Though MSC sheets of adequate size can be formed through self-assembly methods, mechanical stability can be a problem in high density cell systems, particularly at early time points *in vitro*. An ideal engineered cartilage construct would have the strength necessary to withstand mechanical forces in the joint until the regenerated cartilage gains adequate mechanical properties to support the tissue [17]. Additionally, constructs should be sturdy enough to be easily manipulated and implanted without losing their shape [18]. A major advantage of “scaffold-free” cell systems over traditional polymer scaffold-based constructs is the lack of excess amounts of polymer material, which eliminates problems including slow polymer degradation, potential toxicity, and interference with cell-cell contacts [7, 15, 19, 20]. However, “scaffold-free” construct approaches lack some crucial benefits of polymer scaffolds including shape, structure, and mechanical stability. During the first few weeks of culture, before abundant ECM components are produced, densely cellular constructs are typically fragile and exhibit poor mechanical properties [18, 21]. The mechanical properties of these tissues may improve over extended periods of *in vitro* culture with continuous supplementation of TGF- β [7], but the lengthy culture requirements are expensive and time-intensive, and may be prohibitive to the clinical translation of this technology.

A minimum 3-week culture requirement is typical for high density MSC systems, which need extended periods of growth factor supplementation in order to differentiate and develop a neocartilaginous extracellular matrix (ECM). The ECM growth and maturation occurring during the culture period is beneficial to the mechanical stability of the construct, however, new problems arise as the tissue volume increases. Non-uniform spatial growth factor delivery occurs due to diffusional limitations of TGF- β from the culture media to cells in central regions of the tissue as well as growth factor uptake by cells in the exterior tissue regions. Uneven delivery of growth factor can lead to non-uniform patterns of differentiation throughout the tissue bulk, and in some cases necessitates an increase in growth factor concentration in the culture medium to achieve chondrogenesis in the construct interior [10].

To address many of the problems with current high density MSC systems, we have developed a system of self-assembling MSC sheets incorporated with growth factor releasing hydrogel microspheres. The inclusion of biodegradable gelatin microspheres within MSC sheets could balance the need for quickly-degrading scaffolds of limited mass with the structural advantages of an incorporated biomaterial. Gelatin is uniquely suited for this application because it is a biocompatible, biodegradable hydrogel that facilitates sustained delivery of certain growth factors including TGF- β 1 at rates adjustable by controlling the rate of polymer degradation, which in turn can be controlled by the degree of polymer crosslinking [22–24]. When distributed within self-assembling MSC sheets, TGF- β 1 loaded gelatin microspheres could uniformly deliver chondrogenic growth factor directly to the interior regions of the sheets over a sustained period, enabling spatially homogenous differentiation at rates tailorable by adjusting the microsphere crosslinking levels. Additionally, this system could potentially reduce *in vitro* culture time, as the need for extended periods of exogenous growth factor supplementation would be eliminated.

Here, we describe a system of self-assembled, microsphere-incorporated human MSC (hMSC) sheets capable of forming cartilage in the presence of exogenous TGF- β 1 or with TGF- β 1 released from the incorporated microspheres. Our hypothesis was that the incorporation of gelatin microspheres with or without growth factor into hMSC sheets could improve both the mechanical properties and spatial distribution of neocartilage matrix. We also hypothesized that TGF- β 1 loaded microspheres could enable enhanced chondrogenesis in hMSC sheets without requiring exogenous growth factor supplementation. Gelatin microspheres with two different degrees of genipin crosslinking enabled elucidation of the roles of different microsphere degradation and growth factor release rates on chondrogenesis within the system.

Materials & Methods

hMSC isolation and culture

Bone marrow aspirates from the posterior iliac crest of healthy donors were obtained under a protocol approved by the University Hospitals of Cleveland Institutional Review Board and processed by the Skeletal Research Center Mesenchymal Stem Cell Core Facility as previously described [25]. Briefly, the aspirates were washed with growth medium, which was comprised of low glucose Dulbecco's modified Eagle's medium (DMEM-LG; Sigma) containing 10% pre-screened fetal bovine serum (FBS) [26]. Mononucleated cells were isolated via centrifugation with a Percoll (Sigma) density gradient. Isolated cells were seeded at a density of 1.8×10^5 cells/cm² in growth medium and cultured at 37°C with 5% CO₂ in a humidified incubator. After 4 days, nonadherent cells were removed by a medium change. Subsequently, medium was changed every 3 days. After approximately 2 weeks, primary cultures were subcultured and plated at 5×10^3 cells/cm². Cells were used at passage 2 or 3.

Gelatin microsphere synthesis

Gelatin microspheres were synthesized according to a previously established method [27] with slight modifications. All chemicals used in these studies were from Fisher Chemical unless otherwise noted. Briefly, an aqueous solution of 11.1 wt% acidic gelatin (Sigma) was preheated to 45°C, added dropwise into 250 ml of olive oil (Gia Russa) at 45°C and stirred at 500 RPM for 10 min. The solution temperature was lowered to 4°C with constant stirring to facilitate gelation. After 30 min, 100 ml chilled acetone (4°C) was added to the stirring solution. After 1 hr, an additional 100 ml of acetone was added to the emulsion and stirred for 5 min at 1000 RPM. The resulting microspheres were collected by filtration, washed with acetone to remove residual olive oil, and air dried. Dry microspheres were crosslinked at 37°C in an aqueous solution of 1 wt% genipin (“Gp”; Wako USA) for either 2 hrs (“low Gp”) or 21 hrs (“high Gp”) to produce microspheres with different crosslinking densities [28]. Genipin covalently binds primary amine residues on the gelatin, forming intramolecular and short-range intermolecular crosslinks [29]. Crosslinked microspheres were collected by filtration, washed 3 times with ultrapure deionized water (diH₂O), and lyophilized. Growth factor-loaded microspheres were prepared by soaking crosslinked microspheres in a solution of TGF-β1 (Peprotech) in phosphate buffered saline (PBS) at pH 7.4 for 2 hrs at 37°C. At pH 7.4, complexation occurs between positively charged TGF-β1 (IEP of 9.5) and acidic gelatin (IEP of 5.0) [23]. To ensure complete absorption during loading, the volume of TGF-β1 solution added was much less than the equilibrium swelling volume of the microspheres [30]. Unloaded microspheres without growth factor were hydrated similarly with PBS.

Microsphere characterization

Hydrated microspheres were imaged via light microscopy on a TMS microscope (Nikon) with a Coolpix995 camera (Nikon) and their diameters were measured using Image J analysis software (N=245, “low Gp”; N=230, “high Gp”). Microsphere crosslinking densities were determined by a ninhydrin assay as previously described [28]. Briefly, 3 mg microspheres were hydrated in 100 μl diH₂O and 1 ml of a ninhydrin solution (1.05 g citric acid, 0.4 g NaOH, 0.04 g SnCl₂·2H₂O, 1 g ninhydrin, 25 ml 2-methoxyethanol, and 25 ml diH₂O) was added (N=4). Samples and glycine standards were incubated at 100°C for 20 min, 5 ml of 50% isopropanol was added to each, and 200 μl aliquots were added to the wells of a 96-well plate. The absorbance was read at 570 nm on a Safire microplate reader (Tecan, Durham, NC). The concentration of free amino groups was determined by comparison to glycine standards. Degree of crosslinking was defined as the percentage of free amino groups within gelatin microspheres that were reacted with the crosslinking agent.

To determine growth factor release from crosslinked microspheres in cell culture media without proteases, microspheres were loaded with 100 ng TGF-β1 per mg and suspended in a concentration of 5 mg of microspheres per 1 ml of DMEM-LG. One ml samples of the suspensions were added to microcentrifuge tubes, and then the tubes were placed on a rotary shaker at 40 RPM and 37°C (N=4). At various points over a period of 16 days, samples were centrifuged, the supernatant was collected, and fresh release medium was added. TGF-β1 release was quantified using an ELISA kit (R&D Systems). To determine microsphere degradation rates in protease-containing media, microspheres were hydrated in PBS then suspended at a concentration of 5 mg/ml in PBS containing 10 ng/ml Type II Collagenase (Worthington) and incubated at 37°C (N=3). The buffer solution was changed every 3–4 days in all samples. At days 0, 1, 4, 8, 14, and 21, microsphere samples were collected via centrifugation and the supernatant was discarded. The microsphere samples were frozen and lyophilized to dryness, and weighed to determine mass loss over time.

Microsphere-incorporated hMSC sheet production

Crosslinked microspheres were UV sterilized for 10 min, then soaked with PBS or loaded with 400 ng TGF- β 1 per mg microspheres. 1.5 mg microspheres with or without TGF- β 1 were combined with 2×10^6 hMSCs and suspended in 500 μ l of a 1:1 mixture of growth medium and chemically defined medium (DMEM-HG with 1% ITS+ Premix (BD Biosciences), 37.5 μ g/mL ascorbate-2-phosphate (Wako USA), 10^{-7} M dexamethasone (MP Biomedicals), 1% nonessential amino acids (HyClone), and 1% sodium pyruvate (HyClone)) and allowed to settle onto the membranes of 12 mm Transwell inserts (Corning) for 48 hrs in a humidified incubator at 37°C with 5% CO₂. Control sheets without microspheres were also prepared. 10 ng/ml TGF- β 1 was added only to the media of control sheets without microspheres and sheets containing unloaded microspheres. After 48 hrs, the media was replaced with 2.5 ml chemically defined media, was and subsequently changed every other day with TGF- β 1 supplemented in the specified conditions. Negative control sheets incorporated with unloaded microspheres were also prepared and cultured in media without growth factor.

Sheet harvest and biochemical analysis

Sheets were harvested for analysis after 3 wks in culture and their wet masses were determined. Two 5 mm-diameter punches were taken from each sheet for DNA and glycosaminoglycan (GAG) quantification (N=4) or frozen for subsequent thickness measurements and mechanical testing (N $\geq$ 3). Remaining sheet portions were processed for immunohistologic evaluation (N=4). Punches designated for biochemical analysis were digested with papain (Sigma) at 65°C for 2–3 hrs, and the digests were assayed for DNA and GAG content with PicoGreen (Invitrogen) [31] and dimethylmethylene blue (DMMB) dye (Sigma) [32], respectively.

Histology & Immunohistochemistry (IHC)

Portions of the sheets designated for immunohistological analysis were fixed in formalin and paraffin-embedded. Five μ m sections were stained for GAG content via Safranin O (Acros Organics) with a Fast Green counterstain or type I and II collagen as previously described [33]. Briefly, sections designated for IHC were deparaffinized, pronase-digested, and blocked with 5% BSA. Primary antibodies anti-Collagen Type I (Col-1, Sigma) and anti-Collagen Type II (Cat# II-II6B3, Developmental Studies Hybridoma Bank) or control mouse IgG (Vector Laboratories) were applied to adjacent sections. FITC-conjugated goat anti-Mouse IgG (MP Biomedicals) was used as the secondary antibody. Hoechst 33258 (Sigma) was used as a nuclear counterstain. Slides were mounted with Fluoromount (Sigma) and imaged using an Axio Observer Z1 (Zeiss) inverted fluorescent microscope equipped with a C10600 digital camera (Hamamatsu).

Mechanical testing

Step strain stress-relaxation testing in unconfined compression was performed using a computer-controlled testing system (TestResources, Shakopee, MN, USA) with modifications to a previously described protocol [34]. Prior to mechanical testing, frozen 5 mm diameter sheet punches were thawed and equilibrated in PBS for 2 hrs at room temperature, and thickness measurements were taken with a micrometer. Samples were immersed in PBS, where they remained throughout the testing process. Three sequential 5% step strains were applied to the specimens with a ramp displacement rate of 0.001 mm/s. Each ramp displacement was held until the measured reaction force reached equilibrium, which was defined as a force change of less than 0.005 N in 250 s. Equilibrium stress-strain curves were generated based on the magnitude of the equilibrium forces measured after each step strain. An equilibrium compressive modulus (E) value for each sample was obtained

from the slope of the equilibrium stress-strain curve, which was considered linear for strains below 15% [35].

Statistical analysis

All values are reported as mean $\pm$ standard deviation. Statistical analyses were performed on all groups using one-way ANOVA with Tukey's *post hoc* tests, except for microsphere diameters which were compared via a nonparametric Mann-Whitney test. Statistical analyses were performed using GraphPad InStat 3.06 software, with values of $p < 0.05$ considered statistically significant.

Results

Microsphere characterization

Hydrated gelatin microspheres were roughly spherical, with smooth surfaces (Fig. 1). The diameters of low and high Gp microspheres did not significantly differ, while the degrees of crosslinking between microsphere groups were significantly different, with the low Gp group $28.3 \pm 7.2\%$ crosslinked and the high Gp group $67.6 \pm 4.5\%$ crosslinked (Table 1). High Gp microspheres were a dark blue color as a result of the genipin crosslinking reaction [36]. Growth factor release from crosslinked microspheres was measured over 16 days in DMEM (Fig. 2).

By day 16, only a small fraction of the total incorporated TGF- β 1 was released from the microspheres, with $4.14 \pm 0.23\%$ release from low Gp and $0.55 \pm 0.17\%$ release from high Gp. From previous reports, it is likely that most of the incorporated TGF- β 1 remained entrapped within the microspheres, retained via charge interactions [23]. In protease-containing media, low Gp microspheres degraded more quickly, and were completely degraded after 7 days (Fig. 3). High Gp microspheres degraded more slowly, with a remaining undegraded mass of $50.0 \pm 0.1\%$ after 3 wks.

Harvest of microsphere-incorporated hMSC sheets

After 3 wks of *in vitro* culture, sheets were easily harvested by peeling from Transwell membranes and photographed (Fig. 4). Sets of sheets cultured in media supplemented with exogenous TGF- β 1 containing hMSCs only, unloaded low Gp microspheres, or unloaded high Gp microspheres were designated control, low Gp + exo., and high Gp + exo., respectively. Sheets cultured without exogenous growth factor containing low Gp microspheres loaded with TGF- β 1 or high Gp microspheres loaded with TGF- β 1 were designated low Gp + TGF- β 1, and high Gp + TGF- β 1, respectively. The dark blue color in Fig. 4C and 4E is due to the presence of undegraded high Gp microspheres. All sheets initially incorporated with microspheres had significantly greater wet masses than the control sheets, but there were no significant differences among the microsphere-containing sheets (Fig. 5A). Negative control sheets incorporated with unloaded microspheres and cultured in media without TGF- β 1 were originally prepared, but they did not undergo chondrogenic differentiation due to lack of necessary growth factor supplementation in the serum-free media. As a result, these negative controls did not survive the 3 wk culture period and were not available for histologic, biochemical, or mechanical analyses.

DNA and GAG analysis

DNA content among all conditions did not significantly differ, indicating that the presence of microspheres did not affect cell viability or proliferation after 3 wks (Fig. 5B). Total GAG content in the low Gp + TGF- β 1 group was significantly higher than that of the control and all other experimental groups (Fig. 5C), but no other significant differences in total GAG content were found. The GAG per DNA in the low Gp + TGF- β 1 group was

significantly higher than that of all other conditions (Fig. 5D). Additionally, both the control and low Gp + exo. groups had significantly higher GAG per DNA than the high Gp + exo. group.

Safranin O/Fast Green histology

Histological sections were stained with Safranin O to indicate the presence and distribution of GAG in the center (Fig. 6A–E) and edge (6F–J) regions of sheets after 3 wks.

Dark blue high Gp microspheres are indicated by black arrows in the high Gp + exo. (Fig. 6C, H) and high Gp + TGF- β 1 (Fig. 6E, J) sections. High Gp microspheres appeared to be in the process of degrading. Black arrows in the low Gp + exo. (Fig. 6B, G) and low Gp + TGF- β 1 (Fig. 6D, I) sections indicate areas that appear to have been occupied by low Gp microspheres which subsequently degraded. These areas had vague circular outlines and were filled in by cells and GAG-containing matrix.

Control sheets appeared thinner than all other groups of sheets initially incorporated with microspheres. While the control sheets were thinner in the central region than at the peripheral edge, sheets from the microsphere-incorporated groups had a more uniform thickness throughout the diameter of the constructs. Additionally, the microsphere-incorporated sheets displayed more uniform GAG production throughout the thickness of each construct. This was particularly noticeable in the central region of control sheets (Fig. 6A), where there was a clear gradient from intense orange stain on the side of the sheet cultured in contact with the Transwell membrane to counterstained fibrous tissue on the opposite surface. Though there was some fibrous tissue present in the microsphere-incorporated groups, they did not exhibit an obvious gradient of GAG production. This may have been due to increased nutrient diffusion within the tissues incorporated with gelatin microspheres [37]. The low Gp + TGF- β 1 sheets displayed more uniformly intense GAG staining than all other groups (Fig. 6D, I). Morphologically, neocartilage produced in the low Gp + TGF- β 1 group appeared most similar to native articular cartilage, with relatively low cell density, rounded chondrocyte morphology, and large amounts of GAG-containing matrix.

Collagen immunostaining

Histological sections were stained for type I and II collagen to determine relative amounts and distribution of collagen within the sheets (Fig. 7).

It was noted that both high Gp microsphere-containing groups contained degrading microspheres (Fig. 7C, E, H, J), which appear bright green due to autofluorescence of the crosslinked gelatin [38]. Sheets from all 5 conditions were weakly positive for type I collagen, with the most intense staining visible in the control group (Fig. 7A). Type II collagen staining was weakly positive in the control group (Fig. 7F) and both groups incorporated with unloaded microspheres (Fig. 7G, H). The most intense type II collagen staining was observed in the low Gp + TGF- β 1 group.

Thickness measurements and mechanical testing

When measured via micrometer, all sheets initially incorporated with microspheres were significantly thicker than control sheets after 3 wks (Table 2), which was in agreement with the histological findings.

The low Gp + TGF- β 1 group was significantly thicker than both the control group and the unloaded microsphere groups. In step strain stress-relaxation testing, equilibrium compressive moduli (E) for control samples could not be determined because there was no

detectable change in equilibrium force with each sequential strain up to 15% strain. All groups initially incorporated with microspheres demonstrated measurable E values (Table 2). Among experimental groups, low Gp + TGF- β 1 sheets had a significantly higher modulus than the low Gp + exo. group. This was the only significant difference among the microsphere-incorporated groups. These results reflected our observations, as the low Gp + TGF- β 1 sheets were more firm and resistant to deformation during handling than all of the other groups, while control sheets appeared thin and fragile by comparison to all microsphere-incorporated sheets.

Discussion

The aim of this study was to engineer self-assembling hMSC constructs containing biodegradable microspheres with or without chondrogenic growth factor, enabling improved neocartilage matrix formation and mechanical properties without requiring exogenous growth factor supplementation. In contrast to our previous work involving hMSC aggregates incorporated with TGF- β 1 releasing PLGA microspheres [39], this system of gelatin microsphere-incorporated sheets enables the formation of larger constructs with increased GAG production and the potential for greater utility for the treatment of cartilage defects. Within this system, genipin was used as the microsphere crosslinking agent as it is less cytotoxic than the more commonly used crosslinker glutaraldehyde [40]. Although the mean diameters of the 2 crosslinked microsphere formulations did not differ significantly, the microspheres had a wide, non-Gaussian size distribution. This is thought to be a result of the microsphere formation process, which employed a single-emulsion stirring technique.

As has been previously demonstrated, basic growth factors ionically immobilized in an acidic gelatin hydrogel can only be released by biodegradation of the hydrogel matrix, and their release is highly correlated with cell-mediated polymer degradation [23]. Gelatin does not undergo hydrolytic degradation, so as a result, gelatin microspheres are not degraded under *in vitro* conditions without the presence of proteases [24]. As expected, microspheres in non-protease containing media exhibit a small burst release, followed by very little further TGF- β 1 release over a period of 16 days (Fig. 2). The initial growth factor burst is thought to be due to uncomplexed TGF- β 1 molecules on the microsphere surface [23]. Total burst release from the high Gp microspheres was less than that from the low Gp microspheres, as has been reported in other studies [24].

Microsphere degradation behavior was examined in collagenase-containing medium (Fig. 3) to demonstrate the differences in degradation rates resulting from varied microsphere crosslinking densities under specific proteolytic conditions. A concentration of 10 ng/ml type II collagenase was selected to achieve degradation rates that roughly approximated those observed in the cellular sheets. Microspheres degraded over time at rates dependent on the level of crosslinking. At the selected protease concentration, microsphere degradation behavior was comparable to that observed in the histological images, which indicated that the low Gp microspheres within hMSC sheets were completely degraded after 3 wks and the high Gp microspheres were only partially degraded, with many microspheres still visibly present (Fig. 6). It is important to note, however, that the selected collagenase type and concentration only approximated the microsphere degradation rate. Release of growth factors from gelatin microspheres is dependent on both protease concentration and type [24, 41], and multiple protease concentrations and types could be present in the microenvironment within the microsphere-incorporated cell sheets. Therefore, an accurate representation of cell-mediated TGF- β 1 release is difficult to obtain. TGF- β 1 released from microspheres caused chondrogenic differentiation comparable to exogenous supplementation indicating that the growth factor released via cell-mediated polymer degradation was bioactive.

One of the hypotheses of this study was that the inclusion of biodegradable microspheres within neocartilage sheets could improve the spatial distribution of ECM. After 3 wks, all sheets initially incorporated with microspheres were thicker than cell-only control sheets (Fig. 5A). This was also true in the low Gp microsphere-incorporated groups, in which the gelatin microspheres were completely degraded by the time of harvest. This suggests that the incorporated microsphere volume was not solely responsible for the observed increase in thickness, but instead the degrading microspheres may have acted as spacing elements within the neocartilage tissue, allowing room for cell migration and matrix elaboration in spaces where the hydrogel degraded. This theory is supported by the biochemical and immunohistochemical results (Fig. 7), particularly in the examination of the low Gp + exo. group. The low Gp + exo. sheets had GAG and GAG/DNA levels equivalent to Control sheets without microspheres, and both groups exhibited similar staining for type II collagen. However, the low Gp + exo. sheets were thicker, had a higher wet mass, and had a lower cell density as was evident in the histological images by comparison to the control. This spacing effect was not observed in the high Gp + exo. group, which contained microspheres that degraded more gradually. The high Gp + exo. sheets had significantly less GAG per DNA than the other groups supplied with exogenous TGF- β 1 (control and low Gp + exo.). While the exact cause of this result is unclear, it could be due to less room for GAG-containing matrix deposition in sheets containing slow-degrading microspheres.

Since the release of ionically complexed growth factor from gelatin hydrogels is governed by hydrogel biodegradation, microspheres with a faster degradation rate could deliver more growth factor to the surrounding cells during the time course of this investigation, potentially improving chondrogenic differentiation. This appeared to be the case for the low Gp + TGF- β 1 sheets. The low Gp + TGF- β 1 sheets were thicker than the control group and both groups of sheets containing unloaded microspheres. Additionally, they had significantly more GAG and GAG per DNA than any of the other experimental conditions, and they stained more strongly for type II collagen. This was likely due to the fact that hMSCs within the low Gp + TGF- β 1 sheets had access to more growth factor, as the microspheres underwent more cell-mediated degradation than those in the High Gp + TGF- β 1 sheets. Negative control sheets incorporated with unloaded microspheres cultured in media without TGF- β 1 did not undergo differentiation and fell apart during the 3 wk culture period. This indicated that the cartilage formation observed in groups incorporated with growth factor loaded microspheres was not due to the incorporated microspheres themselves, but due to bioactive growth factor released from the microspheres.

Microspheres incorporated within low Gp + TGF- β 1 sheets contained a total 600 ng of incorporated growth factor, which is roughly twice the amount of total growth factor supplied to the exogenously treated sheet groups over 3 wks. As the low Gp + TGF- β 1 microspheres rapidly degraded, presumably all of the entrapped growth factor became available to the surrounding cells. High Gp + TGF- β 1 microspheres were only partially degraded after 3 wks, so the total amount of incorporated growth factor was not released from those microspheres. If only a fraction of the total incorporated growth factor was released from the partially degraded high Gp + TGF- β 1 microspheres, the surrounding cells may have had access to an amount of TGF- β 1 comparable to that supplied to the sheets treated with exogenous growth factor over the first 3 wks of culture. The GAG numbers reflected this pattern, as the low Gp + TGF- β 1 group had significantly more GAG and GAG per DNA than exogenously treated groups while the high Gp + TGF- β 1 group had equivalent GAG and GAG per DNA to exogenously treated groups. In addition to the total growth factor concentration, the temporal profile of delivery may also have an effect on chondrogenic differentiation. Within this study, it appears that the rapid delivery of growth factor by the low Gp + TGF- β 1 microspheres over the course of the first 3 weeks had a positive effect on chondrogenesis. However, the more sustained growth factor delivery from

the degrading high Gp + TGF- β 1 microspheres could potentially induce improved chondrogenic differentiation at later time points.

Tissue engineered hMSC sheets were subjected to step strain stress-relaxation tests in unconfined compression, as this is a method commonly used for the biomechanical characterization of tissue engineered cartilage [20, 21, 34, 35]. The thin, easily compressed control sheets exhibited changes in equilibrium force below the threshold detectable using our mechanical testing apparatus, potentially due to limited elastic matrix within these tissues. All microsphere-incorporated sheets had improved mechanical properties by comparison to control sheets. This was true for low Gp-incorporated sheets (in which all microspheres were degraded by 3 wks) and high Gp-incorporated sheets which contained remaining undegraded microspheres. Increased stiffness of the low Gp + TGF- β 1 sheets could be attributed to the increased GAG and type II collagen contents [35]. The increased E values of the microsphere-incorporated sheets compared to the control could be partly due to the increased matrix spacing lending to improved water retention within the tissue. All sheets initially incorporated with microspheres had a greater wet mass than control sheets after 3 wks, suggesting that higher water content could be playing a role in the improved mechanical properties in the microsphere-incorporated sheets. Additionally, the presence of residual high Gp microspheres may have contributed to the enhanced mechanical stiffness of the sheets incorporated with high Gp microspheres.

This membrane-based system of self-assembling hMSCs can be compared to the “macroaggregate” system developed using human nasoseptal or auricular chondrocytes to form cartilage sheets. In the study by Naumann et al., macroaggregates were formed by centrifuging mature chondrocytes onto membranes at a cell density similar to that used in this study, and then cultured *in vitro* for 3 weeks [18]. The resultant constructs were sturdy enough to be handled at the time of harvest, however, their mechanical properties were not determinable by indentation assay, as the samples failed to reach equilibrium during testing. The authors suggested that this may be due to GAG contents much lower than those of native cartilage, as GAGs are known to contribute to increased tissue stiffness in compression [42]. This result was comparable to our findings for control hMSC sheets without microspheres, which could be peeled from Transwell membranes without tearing but did not demonstrate any change in equilibrium force during step-strain stress relaxation testing, making it impossible to obtain a measurement for E . However, microsphere-incorporated sheets cultured in exogenous TGF- β 1 had GAG levels similar to those of control sheets, yet they demonstrated improved mechanical properties in compression testing. We propose that this may be due to the favorable distribution of GAG-containing matrix leading to increased water content within microsphere-incorporated sheets by comparison to controls, particularly for the low Gp condition in which the microspheres had almost entirely degraded. Though the collagen distributions appeared similar among control and microsphere-incorporated groups, differences in total collagen amount may have also contributed to this result.

Despite the enhancement of mechanical properties by incorporation of microspheres into self-assembling hMSC sheets, these microsphere-incorporated sheets have compressive moduli that are lower than those of normal human articular cartilage [43]. As has been demonstrated in other high density cell systems, it may be possible to increase the stiffness of these constructs with increased time in culture [7, 19], in perfusion-based bioreactor systems [20, 44], or with the application of external mechanical stimuli [20, 45]. In addition, since the microsphere-incorporated sheets are easily handled at early times in culture, it may be possible to implant them *in vivo*, allowing physiological mechanical stimulation to induce cartilage formation and enhance mechanical properties of the neocartilage tissue. The sheets incorporated with TGF- β 1 loaded microspheres would be optimal for this application, as

they have the potential to induce *in vivo* differentiation without extended prior *in vitro* culture. While sheets on the size scale described here could be useful for treating partial-thickness cartilage defects such as those occurring during the early stages of osteoarthritis and also for cartilage reconstruction in the nose and ear, they may need to be thicker to fill a full-thickness chondral defect with a single sheet. To increase the applicability of these sheets for treating full-thickness defects, methods of increasing sheet thickness may include using a higher cell number and/or mass of microspheres, or bioreactor culture. Alternatively, multiple sheets could be stacked to fill a single full-thickness defect. We are currently pursuing several of these strategies to enhance the utility of these microsphere-incorporated sheets for a variety of cartilage repair applications.

Importantly, the system of self-assembling, microsphere-incorporated MSC sheets reported here is versatile, and may accommodate the formation of sheets containing other cells with chondrogenic potential including adipose-derived stem cells or mature chondrocytes. Additionally, the incorporated microspheres could be made from different biopolymer formulations with other physical and biochemical properties and loaded with other bioactive factors such as alternative growth factors, plasmid DNA, and/or siRNA. Microsphere-incorporated sheets could be a new platform system for the engineering of different tissue types in addition to cartilage, based on the cell and polymer types used as well as the bioactive factors selected for delivery.

Conclusions

This study demonstrates the utility of growth factor-incorporated microspheres as a means of enhancing neocartilage tissue formation in high-density hMSC culture. As evaluated via biochemical assays, histological and immunohistochemical analysis, and biomechanical testing, incorporation of growth-factor releasing microspheres into hMSC sheets enhances the structure and function of the high density cell sheets. Beyond producing sheets with superior mechanical properties and more uniform matrix deposition, there may be further advantages of using growth factor-releasing microspheres for *in vivo* implantation. By eliminating the need for exogenous growth factor supplementation, incorporation of TGF- β 1 loaded microspheres could decrease culture time necessary prior to implantation of neocartilage constructs and may circumvent the problem of loss of the chondrogenic phenotype *in vivo* by providing prolonged local exposure of hMSCs to growth factor. Future studies will include examining the potential of the microsphere-incorporated constructs for repairing articular cartilage defects.

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Figure 1.
Light photomicrographs of low Gp (A) and high Gp (B) microspheres hydrated in PBS.
(Scale bar = 50 μ m)

Figure 2.
Diffusion-mediated release of TGF-β1 from both formulations of microspheres in non-protease containing media.

Figure 3. Mass loss over time from high Gp and low Gp microspheres in collagenase-containing media.

Figure 4.

Cartilage sheets harvested from membranes after 3 wks. Left to right: control, low Gp + exo., high Gp + exo., low Gp + TGF- β 1, and high Gp + TGF- β 1. The dark blue color in the high Gp microsphere-containing sheets is due to remaining undegraded microspheres. (Scale bar = 10 mm)

Figure 5. Wet weights (A), DNA contents (B), GAG contents (C), and GAG per DNA (D) of harvested sheets. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Figure 6. Photomicrographs of Safranin-O/Fast Green stained cross-sections from the central (A–E) and edge (F–J) regions of sheets. Orange stain indicates the presence of GAG. Arrows indicate cell and matrix-filled regions where low Gp microspheres degraded (B, G and D, I) or the presence of residual high Gp microspheres (C, H and E, J).

Figure 7.

Photomicrographs of immunofluorescence staining of cross-sections from the central regions of sheets. Green fluorescence indicates the presence of type I collagen (A–E) or type II collagen (F–J). Bright green residual high Gp microspheres can be observed in C, H and E, J due to autofluorescence of the crosslinked gelatin.

Table 1

Average diameters and degrees of crosslinking of the 2 formulations of microspheres.

Microsphere Type	Diameter (μm)	Degree of Crosslinking (%)
low Gp	61.6 ± 56.0	28.3 ± 7.2
high Gp	53.0 ± 46.7	$67.6 \pm 4.5^{***}$

p<0.001

Table 2

Thickness measurements of sheet punches and equilibrium compressive moduli from step strain stress-relaxation testing.

Condition	Thickness (μm)	E (kPa)
control	$269 \pm 21^*$	---
low Gp + exo.	371 ± 41	1.46 ± 1.91
high Gp + exo.	366 ± 18	4.21 ± 2.58
low Gp + TGF- β 1	$443 \pm 35^{**}$	$9.88 \pm 6.47^{***}$
high Gp + TGF- β 1	367 ± 30	3.52 ± 1.76

* Indicates thickness significantly less than all other groups.

** Indicates thickness significantly greater than control, low Gp + exo., and high Gp + exo.

*** Indicates E significantly greater than low Gp + exo.