Chondroitin Sulfate Microparticles Modulate Transforming Growth Factor- β_1 -Induced Chondrogenesis of Human Mesenchymal Stem Cell Spheroids

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Key Words

Chondrogenesis · Chondroitin sulfate · Mesenchymal stem cell spheroids · Microparticles · Transforming growth factor- β_1

Abstract

Mesenchymal stem cells (MSCs) have been previously explored as a part of cell-based therapies for the repair of damaged cartilage. Current MSC chondrogenic differentiation strategies employ large pellets; however, we have developed a technique to form small MSC aggregates (500–1,000 cells) that can reduce transport barriers while maintaining a multicellular structure analogous to cartilaginous condensations. The objective of this study was to examine the effects of incorporating chondroitin sulfate methacrylate (CSMA) microparticles (MPs) within small MSC spheroids cultured in the presence of transforming growth factor (TGF)- β_1 on chondrogenesis. Spheroids with MPs induced earlier increases in collagen II and aggrecan gene expression (chondrogenic markers) than spheroids without MPs, although no large differences in immunostaining for these matrix molecules were observed by day 21 between these groups. Collagen I and X were also detected in the extracellular matrix (ECM) of all spheroids by immunostaining. Interestingly, his-

tology revealed that CSMA MPs clustered together near the center of the MSC spheroids and induced circumferential alignment of cells and ECM around the material core. This study demonstrates the use of CSMA materials to further examine the effects of matrix molecules on MSC phenotype as well as potentially direct differentiation in a more spatially controlled manner that better mimics the architecture of specific musculoskeletal tissues.

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Introduction

Osteoarthritis, a disease marked by the degeneration of articular cartilage, affects up to 27 million adults each year [Murphy et al., 2008] and chondral lesions were observed in ~60% of patients undergoing arthroscopies [Widuchowski et al., 2007], indicating the high prevalence of cartilage injuries in the US. Due to the limited intrinsic repair capacity of articular cartilage, numerous tissue-engineering approaches for cartilage restoration

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Abbreviations used in this paper

CS	chondroitin sulfate
CSMA	chondroitin sulfate methacrylate
ECM	extracellular matrix
GAG	glycosaminoglycan
HE	hematoxylin/eosin
hMSC	human mesenchymal stem cell
IHC	immunohistochemistry
MSC	mesenchymal stem cell
MP	microparticle
PEG	poly(ethylene glycol)
PLGA	poly(lactic-co-glycolic acid)
RT-PCR	reverse transcription polymerase chain reaction
α -SMA	α -smooth muscle actin
TGF- β	transforming growth factor- β

have been explored [Mahmoudifar and Doran, 2012]. However, regenerative medicine approaches to repair cartilage have been hampered by the difficulty in acquiring sufficient numbers of chondrocytes [Mahmoudifar and Doran, 2012]. Therefore, alternative methods such as differentiating multipotent mesenchymal stem cells (MSCs) toward a chondrogenic phenotype have been widely explored due to the relative ease of acquiring MSCs from different tissue sources, such as bone marrow and adipose tissue [Richardson et al., 2010; Mahmoudifar and Doran, 2012]. However, a robust means to promote differentiation of a large number of MSCs to a stable articular chondrocyte phenotype has yet to be achieved.

Current MSC chondrogenic differentiation protocols involve the culture of large cellular pellets (>250,000 cells/pellet) [Mackay et al., 1998]. The pellet culture allows high-density cell-cell contact that mimics the cartilaginous condensations found in embryonic development [DeLise et al., 2000]. Typically, MSC pellets are cultured with soluble factors like transforming growth factor (TGF)- β and dexamethasone, which have been shown to promote production of articular cartilage extracellular matrix (ECM), such as collagen II and aggrecan [Mackay et al., 1998]. Although evidence of a chondrocyte-like phenotype and matrix deposition has been observed in MSC pellets, inherent limitations exist with this culture system, including both the low-throughput nature of the culture, which traditionally has required individual culture in large conical tubes [Mackay et al., 1998], as well as heterogeneity in the phenotype of the resulting cells [Mackay et al., 1998; Pelttari et al., 2006; Richardson et al., 2010].

In particular, studies have shown that diffusional limitations are pronounced in aggregates greater than 150 μ m in diameter [Kinney et al., 2011]. Spatial heterogeneity in MSC differentiation has been demonstrated in standard pellet culture, which generates aggregates approximately 2 mm in diameter [Markway et al., 2010]. Recently, we have described a forced aggregation technique to form three dimensional aggregates (spheroids) of MSCs composed of less than 1,000 cells each (spheroid diameter ~100–150 μ m) [Bratt-Leal et al., 2011]. Therefore, small spheroids of MSCs using this technique were employed in this study to mimic the cell-cell contact found in cartilaginous condensations that is needed to induce chondrogenesis [DeLise et al., 2000]. Recently, chondrogenic differentiation of smaller human MSC (hMSC) micropellets (170 cells) demonstrated increased aggrecan and collagen II mRNA levels relative to standard MSC pellets [Markway et al., 2010].

To further enhance chondrogenesis and address issues of phenotype inhomogeneity, MPs have been cultured within MSC pellets in order to introduce differentiation cues in a more uniform manner [Fan et al., 2008; Solorio et al., 2010; Ravindran et al., 2011; Ansboro et al., 2014]. Prior experiments have investigated the effects of poly(lactic-co-glycolic acid) (PLGA), poly(ethylene glycol) (PEG), hyaluronic acid MPs or gelatin MPs on chondrogenesis of MSC pellets [Fan et al., 2008; Solorio et al., 2010; Ravindran et al., 2011; Ansboro et al., 2014]. The incorporation of gelatin [Fan et al., 2008] and PEG MPs [Ravindran et al., 2011] induced glycosaminoglycans (GAG) and collagen II production comparable to pellets lacking MPs, while PLGA MPs promoted more homogeneous GAG deposition [Solorio et al., 2010]. In addition, PEG MPs reduced collagen I and X gene expression, which are markers of nonarticular chondrocyte phenotypes. MSC pellets with incorporated hyaluronic acid MPs and soluble TGF- β_3 enhanced GAG synthesis compared to pellets cultured without MPs and soluble TGF- β_3 only [Ansboro et al., 2014]. In contrast to these previous reports, this study investigated the chondrogenesis of smaller MSC spheroids containing chondroitin sulfate (CS) MPs.

While a variety of biomaterials may be used in the fabrication of MPs for enhanced chondrogenesis [Fan et al., 2008; Solorio et al., 2010; Ravindran et al., 2011; Ansboro et al., 2014], GAGs such as CS are of particular interest since they are found in cartilaginous condensations during embryonic development and CS is a major component of mature articular cartilage [DeLise et al., 2000]. CS is negatively charged due to the presence of

sulfate groups on the disaccharide units and, thus, it can bind positively charged growth factors electrostatically and provide compressive strength to cartilage through ionic interactions with water [Poole et al., 2001]. CS has been combined previously with other polymers in hydrogels and fibrous scaffolds to enhance chondrogenic differentiation of MSCs and chondrocytes [Varghese et al., 2008; Coburn et al., 2012; Steinmetz and Bryant, 2012; Lim and Temenoff, 2013]. CS-based scaffolds promoted GAG and collagen production [Varghese et al., 2008], and collagen II, SOX9 (sex-determining region Y-box 9) and aggrecan gene expression of caprine MSCs in vitro, and proteoglycan and collagen II deposition in vivo [Coburn et al., 2012] compared to scaffolds without CS. CS-based scaffolds have also induced aggrecan deposition by hMSCs compared to PEG materials [Steinmetz and Bryant, 2012], and hydrogels containing a desulfated CS derivative enhanced collagen II and aggrecan gene expression by hMSCs compared to natively sulfated CS [Lim and Temenoff, 2013]. Although the specific mechanism(s) underlying the chondrogenic effects of CS on MSCs remain unknown, these findings suggest that direct cell-GAG interactions or binding of CS with growth factors, such as TGF- β , in cell culture media are responsible for enhancing biochemical properties [Varghese et al., 2008; Lim and Temenoff, 2013].

In this study, the influence of CS-based MPs incorporated in hMSC spheroids on chondrogenic differentiation was investigated when the cells were exposed to soluble TGF- β ₁. Due to the ability of CS-based hydrogel scaffolds to promote chondrogenesis in MSCs [Varghese et al., 2008; Lim and Temenoff, 2013], we hypothesized that the incorporation of CS-based MPs in the presence of TGF- β ₁ would more effectively promote cartilaginous ECM deposition and organization in hMSC spheroids. Specifically, MSC spheroids with or without incorporated CS MPs were cultured in media containing soluble TGF- β ₁ for 21 days under hypoxic conditions (3% O₂). Changes in spheroid volume, cell morphology and GAG deposition were analyzed with image analysis and histology. Gene expression of chondrogenic markers (SOX9, collagen II and aggrecan) was determined with quantitative reverse transcription polymerase chain reaction (RT-PCR), and chondrogenic ECM protein production was confirmed by immunohistochemistry (IHC). This novel culture platform yielded new insights into the effects of GAG MPs on the production and organization of cartilage-related ECM molecules.

Materials and Methods

CS Methacrylate MP Fabrication

CS methacrylate (CSMA) was synthesized by reacting CS-A (bovine trachea) with methacrylic anhydride (Sigma-Aldrich, St. Louis, Mo., USA) and sodium hydroxide in order to conjugate methacrylate groups to the native hydroxyl groups that are present on the N-acetylgalactosamine of the CS [Lim et al., 2011]. CSMA MPs ~10 μ m in diameter were prepared using a water-in-oil, single emulsion technique, as described previously [Lim et al., 2011]. CSMA (55.6 mg) was dissolved in 440 μ l of PBS and mixed with ammonium persulfate (30 μ l, 0.3 M; Sigma-Aldrich) and tetramethylethylenediamine (30 μ l, 0.3 M; Sigma-Aldrich). The mixture was added dropwise to corn oil (60 ml) with 2 ml of Tween 20 and homogenized at 3,800 rpm for 5 min. The mixture was then stirred and heated to 50°C under N₂ purging for cross-linking. After 30 min, the mixture was centrifuged at 3,000 rpm at 4°C to isolate the MPs. Following the removal of the corn oil, the MPs were washed 3 times with ddH₂O. Prior to incorporation in MSC spheroids, the MPs were incubated in 90% ethanol on the rotary at 4°C for 1 h and washed with ddH₂O. The supernatant was removed from the MPs before lyophilization.

MSC Expansion

All cell culture reagents were acquired from Mediatech unless otherwise noted. Human bone marrow MSCs from 3 donors, 7,071 (male, 22 years), 7,076 (female, 37 years) and 7,078 (male, 24 years), were obtained from the Texas A&M Health Science Center (Temple, Tex., USA). Passage 2 MSCs from each donor were plated separately at low density (100 cells/cm²) and expanded in growth medium composed of minimal essential medium- α , 16.3% fetal bovine serum (Atlanta Biologicals, Lawrenceville, Ga., USA), 1% antibiotic/antimycotic and 1% L-glutamine until confluency under normoxia (37°C at 5% CO₂ and 20% O₂). Passage 3 MSCs were then trypsinized and cells from all 3 donors were pooled prior to spheroid formation.

MSC Spheroid Formation

MSC spheroids were formed as previously described by forced aggregation within 400 $\times$ 400 μ m agarose microwell inserts [Ungrin et al., 2008; Bratt-Leal et al., 2011]. A single cell suspension of MSCs (4.2 $\times$ 10⁶ cells/ml) was added to the microwell inserts and centrifuged at 200 g for 5 min to deposit cells into the individual wells. The cells were incubated for 18 h to allow aggregation under normoxia (37°C at 5% CO₂ and 20% O₂). The MSC spheroids were removed from the inserts using a wide-bore pipette for subsequent alginate encapsulation. MSC spheroids containing CSMA MPs were formed similarly; a premixed suspension of MPs and cells (3:1 ratio) was added to the agarose microwell inserts followed by a similar centrifugation and overnight incubation.

Spheroid Culture and Retrieval

After formation, MSC spheroids were suspended in 1.5% sodium alginate (Spectrum Chemical, Gardena, Calif., USA) that was cross-linked in a 100-mm Petri dish using a precut filter paper (75 mm in diameter) to uniformly distribute 100 mM calcium chloride (EMD, Darmstadt, Germany) across the surface, resulting in a thin layer (75 mm in diameter and ~1 mm thick) that remained immobilized on the dish surface throughout the study. Approximately 2,000 spheroids (700 cells with or without CSMA MPs) were cul-

tured in each alginate layer, resulting in a density of ~450 spheroids/ml of alginate. Alginate encapsulation was necessary to prevent agglomeration of MSC spheroids during extended culture periods (>4 days).

MSC spheroids suspended in alginate were cultured in serum-free medium containing high-glucose Dulbecco's modified Eagle's medium, 1% nonessential amino acids, 1% antibiotic/antimycotic, 1% insulin, human transferrin and selenous acid premix (BD Biosciences, San Jose, Calif., USA), 50 µg/ml ascorbate-2-phosphate (Sigma-Aldrich) and 100 nM dexamethasone (Sigma-Aldrich) under hypoxic conditions (37°C at 5% CO₂, 3% O₂ and N₂) for 21 days as the untreated group. For chondrogenic culture, 10 ng/ml TGF-β₁ (PeproTech, Rocky Hills, N.J., USA) were added to the medium of spheroids with or without CSMA MPs and designated as +TGF-β and +MP+TGF-β, respectively, in subsequent sections.

During culture, the alginate layers were dissociated with 55 mM sodium citrate (Sigma-Aldrich) and reformed using the aforementioned method every 7 days of culture to minimize degradation of alginate. At experimental time points, the alginate layers were dissociated with sodium citrate and washed with phosphate buffer solution in order to collect samples for subsequent analysis on days 1, 7, 14 and 21.

Spheroid Volume Analysis

MSC spheroids were imaged on days 1 and 21 using a phase-contrast microscope (Nikon Eclipse TE2000-U; Nikon, Tokyo, Japan). A minimum of 5 images with multiple spheroids per field (~10 spheroids/field) were taken ($n_{\text{spheroid}} = 150$) for each experimental replicate ($n_{\text{population}} = 3$). Spheroid diameters were measured using the ImageJ (v. 1.47) straight line selection tool and used to calculate the volume, assuming perfect spheres.

RT-PCR

MSC spheroids were collected for gene expression on days 1, 7, 14 and 21 and lysed with RLT lysis buffer (Qiagen, Hilden, Germany). The cell lysates were further filtered with the QIAshredder tissue homogenizer (Qiagen) and RNA was extracted with the RNeasy kit (Qiagen). RT was performed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, Calif., USA) using the T100 thermal cycler (Bio-Rad). Primers (Invitrogen) were custom designed to target human mRNA for β-actin, SOX9, collagen II, aggrecan, collagen I, collagen X, myoD and RUNX2 (runt-related transcription factor 2), as shown in online supplementary table 1 (see www.karger.com/doi/10.1159/000365966 for all online suppl. material). Quantitative PCR was performed using the SYBR Green Master Mix (Life Technologies, Carlsbad, Calif., USA). The raw fluorescence data were first processed in LinRegPCR software to more accurately determine individual PCR efficiency and mRNA starting concentration (v13.1; <http://www.hartfaalcentrum.nl>) [Ramakers et al., 2003]. Fold regulation relative to the untreated day 1 control was determined for each sample with 18S ribosomal protein and β-actin used as endogenous housekeeping controls.

Histological Staining

Intact MSC spheroids were retrieved from the alginate hydrogels on days 1, 7, 14 and 21 and fixed in a 10% formaldehyde solution for 30 min for histological analysis. The fixed spheroids were embedded in Histogel and immersed in 5% w/v sucrose solution (EMD), before subsequently being replaced with increasing sucrose solution concentrations up to 15% under vacuum (~25 in

Hg). Samples were then vacuum infiltrated with increasing concentrations of 20% sucrose:optimal cutting temperature compound solutions (4:1 to 1:2 volume ratios). After overnight infiltration, samples were embedded in optimal cutting temperature compound and allowed to solidify for 10 min in a mixture of dry ice and 100% ethanol. Samples were stored at -80°C and cryosectioned at 10-µm thickness (Cryostar NX70; Thermo Scientific) prior to staining with either hematoxylin/eosin (HE) or safranin O.

Immunofluorescent Staining

Immunostaining for ECM deposition in cryosectioned samples was performed using primary monoclonal antibodies for type I, II and X collagen, aggrecan and α-smooth muscle actin (α-SMA). Antigen retrieval was performed for all sections by incubating in 20 µg/ml proteinase K (Sigma-Aldrich) for 10 min at 37°C immediately prior to staining. Samples for aggrecan and collagen X immunostaining were deglycosylated with 0.75 U/ml chondroitinase ABC (Sigma-Aldrich) for 1.5 h at 37°C. Samples were blocked with Image-iT FX signal enhancer (Life Technologies) and incubated with the primary antibodies (for dilutions and vendor information, see online suppl. table 2) overnight at 4°C. Secondary antibody binding with Alexa Fluor 488-conjugated goat polyclonal anti-mouse IgG (Molecular Probes, Carlsbad, Calif., USA) or IgM (Molecular Probes) was performed at room temperature for 1 h. The samples were stained with Hoechst (Sigma-Aldrich) to visualize the nuclei. Isotype controls were similarly stained using a monoclonal mouse IgG1 (Abcam) or IgM (Abcam) isotype antibody (minimal signal was observed with isotype controls; data not shown).

Statistical Analysis

First, Box Cox transformations were performed on the spheroid volume and PCR amplification results to create normally distributed data [Box and Cox, 1964]. Subsequently, a two-factor analysis of variance with Tukey's post hoc multiple comparison test ($p \leq 0.05$) was performed on the transformed data to determine statistical significance between samples using Minitab software (v15.1; Minitab, State College, Pa., USA).

Results

Effect of TGF-β and MPs on MSC Spheroid Size

The incorporation efficiency (~80%) of CSMA MPs in MSC spheroids was independent of the initial number loaded up to a 3:1 MP:cell ratio (online suppl. fig. 2). The highest ratio (3:1) that yielded ~1,600 MPs per spheroid was used for this study in order to best observe any potential chondrogenic effects of the CSMA MPs without compromising the formation of multicellular aggregates. Our previous studies indicated that incorporation of MPs in embryonic and mesenchymal stem cell aggregates at these MP:cell ratios did not adversely impact intercellular adhesion formation, and MPs were relatively uniformly incorporated within aggregates [Bratt-Leal et al., 2011;

Fig. 1. Changes in MSC spheroid volumes in response to MP incorporation and TGF- β . Differences in MSC spheroid sizes for each culture condition were observed for 21 days in representative phase images (a–h). On day 1, the volume of +MP+TGF- β spheroids was significantly greater than that of all other groups (i). On

day 21, the volume of all groups differed significantly from each other (j). $n_{\text{population}} = 3$, $n_{\text{spheroid}} = 150$. * $p < 0.05$ vs. same treatment on day 1. + $p < 0.05$ vs. +TGF group. & $p < 0.05$ vs. all other groups. Scale bars = 200 μm . The minimum, lower quartile, mean, upper quartile and maximum are reported in the box plots.

Baraniak et al., 2012]. However, differences in spheroid sizes between culture conditions were observed, even after only 1 day (fig. 1). On day 1, there was no difference in volume between untreated spheroids and spheroids containing only MPs or TGF- β (fig. 1i), but the +MP+TGF- β spheroids had the largest volume (0.009 mm^3) and were almost 2 times larger than the other spheroids. After 21 days, the +MP+TGF- β spheroids had the largest volume (0.016 mm^3) and were approximately

2 times greater than those of the +MP spheroids ($\sim 0.008 \text{ mm}^3$; fig. 1j). The +TGF- β spheroids also exhibited slightly larger volumes (~ 1.2 times) than the +MP group. Regardless of MP incorporation, spheroids cultured in chondrogenic conditions exhibited a greater increase in volume (~ 2 – 3 times) compared to spheroids in non-chondrogenic conditions (~ 1.8 – 2 times) over the 3-week culture period.

Fig. 2. Histology of spheroid morphology over 21 days. Cell morphology and organization changes in response to MP or TGF- β were observed in HE staining (a-l). The presence of GAGs in the

CSMA MPs (white arrowheads) or ECM (white arrowheads) was confirmed by safranin O staining (red; m-x). Fast green was used as a cytoplasm counterstain (m-x). n = 3. Scale bars = 50 μ m.

Morphological Changes in MSC Spheroids with MPs

One day after formation, the +MP and +MP+TGF- β spheroids consisted of a mixture of cells and MPs. However, clustering of CSMA MPs near the center of the MSC spheroids was observed (with or without TGF- β) as early as day 7 in histological sections (fig. 2f, h, j, l). Particularly in the +MP+TGF- β spheroids, the cell nuclei exhibited pronounced elongation and circumferential alignment around the core of MPs on days 14 and 21 (fig. 2h, l, arrowheads). The presence of GAG was detected in the ECM of +TGF- β spheroids on days 14 and 21 (fig. 2s, w, arrowheads) by safranin O staining. In addition to positive GAG staining of the CSMA MPs, GAG presence was also observed in the region of organized cells and ECM around the MP core in +MP+TGF- β spheroids on day 21 (fig. 2x, arrowheads), but was absent in the +MP spheroids (fig. 2v, arrowheads). Due to the lack of evident biochemical response of MSCs to the CSMA MPs in the absence of TGF- β , the +MP spheroids were omitted from subsequent analysis.

TGF- β and MP Effects on MSC Gene Expression

Gene expression of the chondrogenic transcription factor SOX9 was significantly higher in the +MP+TGF- β spheroids (1.4 ± 0.3 fold increase) than the untreated group on day 7, but decreased on day 21 (0.6 ± 0.2 ; fig. 3a). The +TGF- β spheroids exhibited a gradual increase in the gene expression of aggrecan from days 7 to 21 with a 6.7 ± 0.7 fold increase on day 21 compared to the untreated group (fig. 3b). Similarly, collagen II expression in +TGF- β spheroids was increased on day 14 (1.6 ± 0.7 fold increase) and day 21 (44 ± 18 fold increase) relative to the day 1 untreated group (fig. 3c). The +MP+TGF- β spheroids also demonstrated increases in aggrecan and collagen II gene expression, but the presence of MPs resulted in earlier peaks (4.8 ± 1.4 and 101 ± 10 fold increases, respectively) by day 14 compared to the untreated spheroids.

In addition to chondrogenic markers, expression of collagen I and X, which are indicative of fibrocartilaginous or hypertrophic cartilage phenotypes, respectively, were examined. Collagen I expression increased less than 2-fold over 21 days in all groups (fig. 3d). For collagen X expression, the untreated spheroids demonstrated a gradual increase over time, reaching a 5.7 ± 0.5 fold increase on day 21 (fig. 3e). In the +TGF- β spheroids, a 52 ± 5 fold increase in collagen X regulation was observed by day 7 and persisted until day 21 (66 ± 6 fold increase compared to untreated spheroids) while the addition of MPs in the spheroids promoted a large increase (81 ± 17 fold in-

crease) by day 14 followed by a sharp reduction on day 21 (12 ± 3 fold increase) relative to the untreated spheroids. No significant difference in collagen X expression was detected between +TGF- β and +MP+TGF- β spheroids on day 14, but the addition of MPs resulted in less collagen X gene expression compared to the +TGF- β spheroids on day 21.

ECM Organization and Deposition in hMSC Spheroids

On day 14, both groups cultured in TGF- β exhibited similar levels of increased staining for aggrecan compared to the untreated group (fig. 4a–f). Collagen II staining was slightly stronger in the +TGF- β and +MP+TGF- β spheroids compared to the untreated group and there was no appreciable difference between both TGF- β -treated groups (fig. 4g–l). Collagen I appeared more organized in the +TGF- β spheroids and was distinctly aligned around the MP core in the +MP+TGF- β spheroids compared to the amorphous staining in the untreated group (fig. 4m–r, arrowheads). Some alignment of collagen X around the MP core was also seen in the +MP+TGF- β spheroids compared to the other groups on day 14 (fig. 4s–x, arrowheads). The presence of α -SMA was detected strongly at the borders of the untreated and +TGF- β spheroids with some weak pericellular staining in the center (fig. 4y–dd). However, the addition of MPs in the presence of TGF- β appeared to greatly reduce the expression of α -SMA on the spheroid surface.

By day 21, organized pericellular staining of aggrecan was present around elongated nuclei in +TGF- β and +MP+TGF- β spheroids (fig. 5a–f). Collagen II staining was high in +TGF- β spheroids, but slightly reduced with the incorporation of MPs (fig. 5g–l). Similar amounts of positive staining for collagen I and X were observed in the +TGF- β and +MP+TGF- β spheroids (fig. 5m–x). In the +MP+TGF- β spheroids, strong positive collagen I staining was observed on the periphery of the MP core and near the individual MPs on day 21 (fig. 5o, r, arrowheads). Organization of collagen I around the MP core was still obvious after 3 weeks of culture and was also evident in collagen X staining (fig. 5u, x, arrowheads). The presence of α -SMA on the spheroid surface was observed in all groups, but the +TGF- β spheroids exhibited additional pericellular staining in the center compared to the +MP+TGF- β group on day 21 (fig. 5y–dd).

A comparison between day 14 and 21 IHC showed no appreciable changes in aggrecan staining detected in +TGF- β spheroids or in +MP+TGF- β samples. Collagen II appeared to increase in +TGF- β spheroids over time,

Fig. 3. Expression of chondrogenic ECM gene markers by MSC spheroids in response to TGF- β and CSMA MPs normalized to the untreated group on day 1. Gene expression of SOX9 remained relatively constant over 21 days (a). The addition of TGF- β with or without CSMA MPs promoted the expression of aggrecan (b) and collagen II (c) by day 7. Slight increases in collagen I expression were observed in all groups (d), while large increases in collagen X expression occurred in all except the untreated group (e). $n = 3$, means $\pm$ SD. * $p < 0.05$ vs. same treatment on day 1. # $p < 0.05$ vs. untreated at the same time point. + $p < 0.05$ vs. +TGF group at the same time point.

Fig. 4. Immunofluorescence staining for the deposition of chondrogenic ECM molecules in MSC spheroids on day 14. Positive aggrecan and collagen II staining was detected in all except the untreated group (a–l). Similar levels of collagen I and X were pres-

ent in all groups (m–x) with some differences in ECM organization (arrowheads). Differences were observed for α -SMA levels (y–dd). Molecules of interest are in green and cell nuclei in blue. $n = 3$.

(For figure see next page.)

while little change was seen in the +MP+TGF- β spheroids. No difference was observed in collagen I and X staining between days 14 and 21 in +TGF- β spheroids or in +MP+TGF- β spheroids. An apparent reduction in the area of positive α -SMA staining on the surface of untreated and +TGF- β spheroids along with decreased pericellular staining in the center occurred between days 14 and 21. Although the +MP+TGF- β spheroids exhibited a slight increase in α -SMA staining on the surface between days 14 and 21, α -SMA staining observed on day 21 was still comparable to that of +TGF- β spheroids.

Discussion

In this study, we have demonstrated that incorporation of GAG-based MPs in hMSC spheroids promoted earlier expression of chondrogenic gene markers. Moreover, MSC spheroid volume was significantly enhanced by the combination of CSMA MPs and TGF- β , which also resulted in a unique organization of cells and ECM around the MP core. Spheroid size analysis indicated that +MP+TGF- β spheroids exhibited the largest volume on both days (1 and 21). Part of this large increase in volume could be attributed to the presence of MPs; however, calculating the sum of theoretical total MP volume and the volume of a spheroid alone cultured in TGF- β on days 1 and 21 resulted in ~ 20 and $\sim 30\%$ lower values, respectively, than in the +MP+TGF- β spheroids. Similarly, DNA analysis (see online suppl. fig. 1) reveals that a greater cell number was observed in both groups containing TGF- β by day 7, so changes in spheroid size cannot be explained by preferential cell proliferation in the +MP+TGF- β samples. In a comparable hMSC spheroid system without exogenous growth factors, size difference between spheroids with or without gelatin MPs was not observed on day 1 nor was any increase seen up to 7 days of culture [Baraniak et al., 2012].

The incorporation efficiency for CSMA MPs was $\sim 80\%$ for the 3:1 ratio and approached 100% for two oth-

er MP:cell ratios investigated (online suppl. fig. 2), suggesting that the MSCs can readily interact with CS-based materials. When PLGA, agarose or gelatin MPs were incorporated in embryonic stem cell aggregates, differences in incorporation efficiencies were attributed to the relative adhesivity of the materials [Bratt-Leal et al., 2011]. In addition to high incorporation, the CSMA MPs clustered within the MSC spheroids by day 7 and remained at the core of the aggregates for the duration of the culture as shown by histology, a phenomenon that was not observed with polystyrene MPs (online suppl. fig. 3), even though the polystyrene MPs were incorporated at similar levels as CSMA MPs (data not shown). Moreover, clustering of MPs in MSC pellet culture containing PEG, PLGA or gelatin MPs with comparable sizes to the CSMA MPs used in this study ($\sim 10 \mu\text{m}$) has not been reported previously [Fan et al., 2008; Solorio et al., 2010; Ravindran et al., 2011]. Because PLGA and PEG are synthetic materials, it might be expected that MSCs may interact with them differently than with the CS-based MPs. However, clustering of gelatin MPs was also not observed in MSC pellets [Fan et al., 2008] or in hMSC spheroids similar to the ones in this study [Baraniak et al., 2012]. The absence of a gelatin MP core and the lack of gelatin MP effects on MSC spheroid size shown previously [Baraniak et al., 2012] suggest that there may be interactions of MSCs specifically with CS-based particles that allow their movement and rearrangement in the spheroids after formation. Such interactions may affect overall cellular or ECM packing in the spheroids [Fan et al., 2008] that leads to a larger spheroid volume in the presence of TGF- β , even after only 1 day.

In this system, it was observed that the MSC spheroids exhibited uniform circumferential organization of elongated cell nuclei and ECM around the clustered MP core as seen in HE (fig. 2h, l) and IHC staining (fig. 4r, x, 5r, x), particularly in the presence of TGF- β . Chondrocytes adopt a fibroblast-like morphology with a spread and elongated appearance in monolayer culture on two-dimensional substrates [Glowacki et al., 1983]. Concomitant with the loss of a round morphology, chondrocytes de-differentiate and decrease expression of aggrecan and collagen II, while increasing production of collagen I [Mayne et al., 1976; Stokes et al., 2002]. Despite the elongated cell morphologies observed in the +MP+TGF- β MSC spheroids, no phenotypic evidence was observed based on gene expression analysis or IHC that would suggest that fibroblastic differentiation was preferentially occurring in these samples. Instead, the unique organization around the MP core presents a pos-

Fig. 5. Immunofluorescence staining for the deposition of chondrogenic ECM molecules in MSC spheroids on day 21. Positive staining for aggrecan was detected in all groups, but collagen II staining was still absent in the untreated group (**a-l**). Collagen I levels in the untreated spheroids was low, but collagen X staining was similar between all groups (**m-x**). ECM appeared to be organized in the +MP+TGF- β spheroids (**r, x**, arrowheads). α -SMA staining was also observed across all culture conditions (**y-dd**). Molecules of interest are in green and cell nuclei in blue. $n = 3$.

sible strategy for directing the microtissue radial architecture from the inside out to emulate aspects of the zonal organization of tissues such as articular cartilage [Poole et al., 2001].

TGF- β_1 can increase α -SMA expression and contractility in human MSCs [Kinner et al., 2002] and α -SMA expression has been detected in the periphery of MSC pellets [Kinner et al., 2002; Ravindran et al., 2011]; thus, α -SMA expression within MSC spheroids was examined. A similar pattern of α -SMA expression observed at the surface of all spheroids suggests that the MSC phenotype may have resulted from the contractility exerted by the cells comprising the surface of the spheroids. Interestingly, there was a pronounced reduction in α -SMA protein on the border of +MP+TGF- β spheroids on day 14, indicating that the CSMA MPs may have the ability to prevent TGF- β from inducing α -SMA expression, perhaps by acting as a substrate that modulates cell contractility [Arora et al., 1999; Kinner et al., 2002]. A similar reduction in α -SMA staining was seen at the border of MSC pellets containing PEG MPs cultured in TGF- β_3 -supplemented media [Ravindran et al., 2011], further indicating that the physical presence of MPs may play an important role in mediating α -SMA production, possibly by disrupting cell-cell and cell-ECM interactions.

Hypoxic culture has been used for MSC chondrogenesis *in vitro* to help maintain a stable articular chondrocyte phenotype during differentiation [Duval et al., 2012; Gawlitta et al., 2012; Sheehy et al., 2012], and, accordingly, the experiments in this study were performed at 3% O₂. Although the +MP+TGF- β spheroids displayed similar levels of increased expression for chondrogenic genes (aggrecan and collagen II) as the +TGF- β spheroids, the +MP+TGF- β spheroids expressed the highest levels for collagen II and aggrecan 1 week earlier than the +TGF- β group (fig. 3b, c), which suggests that CSMA MPs modulate the temporal sequence of TGF- β -induced chondrogenesis. CS has been shown to electrostatically interact with positively charged growth factors, such as TGF- β , and to modulate growth factor signaling during cartilage morphogenesis [Willis and Kluppel, 2012], so it is possible that the MP core could impact the quantity and distribution of TGF- β_1 available to induce differentiation in our culture system, resulting in the earlier expression of cartilaginous genes by MSCs. We also noted that gene expression of the lineage markers RUNX2 (osteogenic) and MyoD (myofibroblastic) were minimally changed in all spheroids over 21 days (online suppl. fig. 4), suggesting that other differentiation pathways were not favored in these culture conditions.

In order to determine the relative amount and spatial location of deposited ECM molecules, IHC staining was performed. In contrast to the gene expression data, which indicated earlier onset of differentiation for the MP-laden group, both sets of TGF- β -treated spheroids (with or without MPs) exhibited similar levels of staining for aggrecan and collagen II protein deposition on days 14 and 21 (fig. 4, 5e, f, k, l). In addition, GAG staining in +TGF- β spheroids was observed earlier than the +MP+TGF- β group (fig. 2w, x). hMSC pellet culture has also resulted in increases in collagen II and aggrecan gene expression that was not reflected in protein production [Khan et al., 2010]. Post-transcriptional regulation, as well as differences in production and degradation rates of mRNA and proteins, may lead to a low correlation between mRNA and protein levels [Vogel and Marcotte, 2012]. Increased protease activity in the +MP+TGF- β spheroids may provide another potential explanation for the differences in ECM staining versus gene expression as CS has been shown to increase stability and activity of cathepsin K, which cleaves collagen I and II [Li et al., 2000].

In addition to enhancing chondrogenic gene and ECM markers, a critical goal of *in vitro* MSC chondrogenesis is to avoid differentiation to fibrocartilaginous and/or hypertrophic phenotypes, which can be detrimental for long-term articular cartilage restoration [Peltari et al., 2006; Farrell et al., 2009]. While fibrocartilaginous collagen I gene expression did not change greatly over time (fig. 3d), strong positive IHC staining was observed throughout the ECM at all time points in the spheroids as seen in hMSC micropellets [Markway et al., 2010] and larger MSC pellets without MPs [Mackay et al., 1998; Markway et al., 2010] or with PEG [Ravindran et al., 2011] and gelatin MPs [Fan et al., 2008]. Despite the reported ability of hypoxic culture to delay or suppress hypertrophy in pellets or encapsulated MSCs [Duval et al., 2012; Gawlitta et al., 2012; Sheehy et al., 2012], an ~80-fold increase in collagen X gene expression by day 14 was found in TGF- β -treated spheroids with or without MPs, and collagen X production was confirmed by IHC staining (fig. 3e). Even under hypoxic conditions, increases in collagen X levels during chondrogenesis have been reported in MSCs cultured in various formats [Zscharnack et al., 2009; Markway et al., 2010; Meretoja et al., 2013], reflecting the difficulty in preventing hypertrophy *in vitro*. Mixed results were observed in previous work with MSC pellets containing MPs, where the incorporation of gelatin MPs led to collagen I mRNA levels similar to those seen in no MP controls [Fan et al., 2008], but PEG MPs reduced both collagen I and X gene expression [Ravin-

dran et al., 2011]. Hypoxic culture of MSC pellets with hyaluronic acid MPs and soluble TGF- β_3 also induced similar levels of collagen X gene expression as no MP controls [Ansboro et al., 2014]. Such findings show varying levels of effectiveness in suppressing collagen I and X expression between independent studies, which implies that other factors, such as aggregate size and MP type, may play a role in modulating MSC phenotype. Thus, culture conditions for our system could be further optimized to reduce the fibroblastic and hypertrophic differentiation of MSCs.

While the gene expression results are intriguing, the presence of the CSMA MPs did little to enhance deposition of cartilaginous ECM in this spheroid culture. There could be several explanations for this, including that an insufficient amount of CS was available to interact with cells. Despite the large number of CSMA MPs present within a spheroid, due to the clustering effect, only a number of MPs on the surface of the core are available for direct cell-GAG interaction. Moreover, there was little degradation seen over the course of the experiment based on histological staining, further reducing the 'dose' of GAG available to cells comprising the spheroid. Also because CSMA MPs have been previously shown to sequester TGF- β with minimal release and without degradation [Lim et al., 2011], any growth factors sequestered by the MPs may have remained concentrated within the MP core and unlikely to be released. In the future, developing CSMA MPs with the ability to more readily undergo partial degradation may allow a more homogeneous distribution of GAGs and sustained release of any sequestered growth factors throughout spheroid culture to better promote chondrogenesis, as has been explored previously with degradable gelatin and PLGA MPs in MSC pellets [Fan et al., 2008; Solorio et al., 2010]. Alternatively, the

use of smaller CSMA MPs (1–5 μm in diameter) in the spheroids may also promote more uniform dispersal throughout the aggregate ECM as observed previously with embryonic stem cell aggregates containing smaller PLGA MPs (1 μm in diameter) compared to larger ones (11 μm in diameter) [Carpenedo et al., 2010]. Together, such a spheroid system would more closely mimic the native ECM by achieving a more homogeneous distribution of GAGs among cells [Wang et al., 2008] rather than being localized to discrete foci within the pellet/spheroid.

In these studies, we have demonstrated that the incorporation of CSMA MPs in hMSC spheroids did not adversely affect TGF- β_1 -mediated chondrogenesis and that MPs promote earlier gene expression of chondrogenic markers compared to spheroids without MPs. In addition, the clustering of CSMA MPs at the core of MSC spheroids resulted in unique cellular and ECM alignment that may provide a means to promote zonal organization and cellular alignment within microtissues. As GAGs are found in a wide variety of tissue types, these results indicate that this culture system can serve as a novel platform both to further examine the effects of GAGs and growth factors on MSC phenotype as well as potentially direct differentiation in a more spatially controlled manner that better mimics the architecture of specific target tissues.

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