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Correlation Between Compositional and Mechanical Properties of Human Mesenchymal Stem Cell-Collagen Microspheres During Chondrogenic Differentiation

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Mesenchymal stem cell (MSC)-based engineering is promising for cartilage repair. However, the compositional mechanical relationship of the engineered structures has not been extensively studied, given the importance of such relationship in native cartilage tissues. In this study, a novel human MSC-collagen microsphere system was used to study the compositional mechanical relationship during *in vitro* chondrogenic differentiation using histological and biochemical methods and a microplate compression assay. The mechanical property was found positively correlating with newly deposited cartilage-relevant matrices, glycosaminoglycan, and type II collagen, and with the collagen crosslinker density, in agreement with the presence of thick collagen bundles upon structural characterization. On the other hand, the mechanical property negatively correlates with type I collagen and total collagen, suggesting that the initial collagen matrix scaffold of the microsphere system was being remodeled by the differentiating human MSCs. This study also demonstrated the application of a simple, sensitive, and nondestructive tool for monitoring the progression of chondrogenic differentiation of MSCs in tissue-engineered constructs and therefore contributes to future development of novel cartilage repair strategies.

Introduction

CARTILAGE INJURIES are very common. Owing to the acellular and avascular nature, cartilage itself has very limited ability to repair and regenerate.¹⁻⁵ Current treatments have obvious deficiencies. Arthroscopic techniques alleviate pain instead of regenerating the cartilage,⁴⁻⁸ and microfracture techniques lead to the formation of more vulnerable fibrocartilage.^{4-7,9,10} Autologous chondrocyte implantation^{5,7,11} and autogenic or allogenic tissue transplantation^{4,5,7,11-13} have donor availability problem and donor-site morbidities. Therefore, better treatment options such as autologous stem cell-based therapies are needed.

Bone marrow mesenchymal stem cells (MSCs) are attractive cell source for tissue engineering. This is because of their relatively safe and easy accessibility from the donor, the ability of self-renewal and differentiating into multiple lineages of cells,^{14,15} genetic stability, immunologically privileged status,^{14,16} and better social and ethics acceptability than embryonic stem cells. In MSC-based cartilage tissue engi-

neering, these cells are expected to differentiate into chondrogenic lineages and then remodel the biodegradable scaffolds into a tissue structure simulating the native cartilage.

The compositional and mechanical relationship of native cartilage tissues has long been studied.¹⁷⁻¹⁹ The mechanical function performed by cartilage mainly associates with its resistance to compression. Compressive modulus, shear modulus, and Poisson's ratio of native cartilage have been found to associate with the amount of extracellular matrix (ECM), including collagen,^{1,2,17,18,20-22} collagen crosslinks,^{17,23-26} and proteoglycans,^{1,5,27,28} of the tissue. Such relationship is equally important in stem cell-based engineered tissues for better understanding of the mechanisms of matrix remodeling in different scaffold systems. Nevertheless, despite a large amount of studies using MSCs in different scaffolds for cartilage repair,²⁹⁻³¹ the compositional mechanical relationship via matrix remodeling of engineered constructs are not extensively studied.

Previously, we have established a novel collagen microencapsulation technique³² to entrap cells such as MSCs,^{33,34} embryonic stem cells,³⁵ chondrocytes,³⁶ and HEK 293 cells^{37,38}

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in microspheres made of dense fibrous type I collagen meshwork, which provides a suitable 3D matrix environment for cells to survive, proliferate, and migrate.³³ Human MSCs (hMSCs) that resided in these microspheres were also proven to retain stem cell characteristics, including their immunophenotype, multipotent differentiating potential, and self-renewal capacity.³³ Moreover, this microsphere system provides a template collagen fiber meshwork for both mature cells such as chondrocytes³⁶ and differentiating stem cell such as embryonic stem cells³⁵ and MSCs^{34,39} to remodel upon chemical induction.

In this study, we hypothesize that the temporal changes of various compositional parameters would closely associate with the change in the mechanical parameter, namely, the elastic modulus of the microsphere during chondrogenic differentiation of the entrapped hMSCs. Specifically, we aim to characterize the *in vitro* chondrogenic differentiation of hMSCs in collagen microspheres and establish the association among different compositional parameter with the mechanical parameter. Specifically, reduced elastic modulus (E_r) of the microspheres will be measured using a microplate compression technique established in our lab.⁴⁰ Cartilage-relevant ECM components, including collagen type II and glycosaminoglycans (GAGs), and pyridinoline (PYD) crosslink density will be assessed using both histological and biochemical methods. Correlation coefficients between various biochemical composition parameters and the mechanical parameter will be obtained. The significance of the established association between biochemical composition and mechanical property of the microsphere would help us to better understand how hMSC remodel the scaffold to form a cartilage-like structure during chondrogenic differentiation and therefore may contribute to future novel cartilage repair strategy.

Materials and Methods

Overall research design

hMSCs were microencapsulated in collagen microspheres and induced to differentiate toward chondrogenic lineages using a chemically defined culture medium. At different time points postdifferentiation, the microsphere populations were divided, pooled, and processed for histological, biochemical, structural, and mechanical characterization. Apart from routine histological staining, histochemical staining for GAGs and immunohistochemical staining for type I and II collagen were conducted. Quantitative measurements of the biochemical compositions of the microspheres, namely, hydroxyproline (HYP) for total collagen content, uronic acids for GAG content, type I and II collagen, and PYD collagen crosslinker, have been conducted using either chemical or immunochemical methods. Scanning electron microscopy (SEM) was also conducted to reveal the fibrous structures of the microspheres during chondrogenic differentiation. A microplate step-change compression assay has been used to measure the E_r of the microspheres. Finally, correlation coefficients between the biochemical compositional parameters and the mechanical parameter of the differentiating microspheres have been calculated.

Culture of hMSCs

hMSCs from bone marrow from three donors (male, age 47–49) were kindly provided by Dr. G.C.F. Chan from the Department of Paediatrics and Adolescent Medicine of the

same institution. hMSCs were isolated and cultured as previously described.⁴¹ All procedures were approved by the Combined Clinical Ethics Committee of the University of Hong Kong and Hong Kong West Cluster Hospitals of Hospital Authority. In brief, the cells were cultured in the growth medium containing Dulbecco's modified Eagle's medium–low glucose (Gibco), 10% fetal bovine serum (Gibco), 100 U/mL penicillin (Gibco), 100 µg/mL streptomycin (Gibco), and 2 mM L-glutamine (Gibco), incubated at 37°C in a humidified atmosphere with 5% CO₂. The culture medium was replaced every 3–4 days. The cells were subcultured up to passage 6 for fabrication of microspheres. Immunophenotype, self-renewal capacity, and multidifferentiating potential of the hMSCs were characterized in a previous study.³³

Fabrication of collagen–hMSC microspheres

Collagen–hMSC microspheres were fabricated as previously described³³ and fabrication parameters optimized as reported elsewhere.³⁴ In brief, cell suspension was mixed with neutralized rat tail type I collagen solution (BD Biosciences) in an ice bath. The cell–collagen mixture was diluted by the culture medium to a final cell density of 1×10^6 cells/mL and a final collagen concentration of 1 mg/mL. Droplets of the mixture in volumes of 50 µL and 100 µL were pipetted onto UV-irradiated parafilm placed in separate Petri dishes. The 100 µL droplets were used to produce microspheres of larger sizes to ease the sample preparation process for SEM analysis. Gelation of the collagen–hMSC droplets was brought about by incubation at 37°C in a humidified atmosphere consisting of 5% CO₂ for 45 min. The gelled droplets were then cultured in the same medium for 5 days to allow for cell-induced contraction. Microspheres made from 50 µL droplets were used for subsequent biomechanical, biochemical, and histological studies, whereas those made from 100 µL droplets were used for ultrastructural study.

Chondrogenic differentiation of hMSCs in collagen microspheres

Chondrogenic differentiation of hMSCs encapsulated in the collagen microspheres was brought about by culturing the collagen–hMSC microspheres in the chondrogenic differentiation induction medium, defined as Dulbecco's modified Eagle's medium–high glucose, with the supplementation of 10 ng/mL recombinant human TGF-β3 (Preprotech), 100 nM dexamethasone (Sigma-Aldrich), 0.1 nM L-ascorbic acid 2-phosphate (Fluka), 6 mg/mL insulin (Merck), 6 µg/mL transferrin (Sigma), 1 mM sodium pyruvate (Gibco), 0.35 mM L-proline (Merck), and 1.25 mg/mL bovine serum albumin (Sigma). The medium was changed every 2 days and lasted for 3 weeks. Microspheres were harvested on days 0, 7, 14, and 21 upon the addition of chondrogenic differentiation induction medium for subsequent analyses. Cell-free collagen microspheres can also be used as the control, but these microspheres disintegrated and therefore weakened over time such that subsequent analyses could not be performed.

Histological, histochemical, and immunohistochemical staining of ECM components

Harvested microspheres were fixed in 4% phosphate-buffered saline (PBS)–buffered paraformaldehyde (BDH),

paraffin embedded, and sectioned at 5 μm thickness. Routine hematoxylin and eosin staining was performed to reveal cell morphology. Alcian blue histochemical staining was performed to observe the presence of GAGs. Immunohistochemical analysis was performed for type I and type II collagen, using primary mouse monoclonal antibodies against type I collagen (Sigma) and type II collagen (EMD), respectively. These antibodies exhibited cross-reactivity with human samples. After overnight incubation with the primary antibodies at 4°C, sections were incubated with anti-mouse secondary antibodies (Dako). The incubation lasted for 30 min at room temperature, followed by another 30 min with avidin-biotin-peroxidase complex (Vector Laboratories). Immunostaining was detected colorimetrically using diaminobenzidine (Dako). Sections were counterstained with hematoxylin.

Quantitative measurement of ECM components

Uronic acid analysis was adopted instead of the widely used 1,9-dimethylmethylene blue (DMMB) assay. Uronic acid is a regular component of disaccharide repeating units of chondroitin sulfates and hyaluronans, major GAGs found in the ECM of articular cartilage.⁴² This assay offers an advantage over the DMMB assay because it is independent of the GAG size and the degree of sulfation.⁴³ In brief, each microsphere was digested overnight at 60°C with 300 $\mu\text{g}/\text{mL}$ papain (Sigma) in 50 mM phosphate buffer (Sigma; pH 6.5) containing 5 mM L-cysteine (Calbiochem) and 5 mM ethylenediaminetetraacetic acid (Sigma), followed by sequential precipitation at 4°C in 0.5% cetylpyridinium chloride (Sigma) in 0.025 M acetate buffer (pH 5.8; Sigma), and then sodium acetate-saturated ethanol. The dried GAG product was dissolved in a defined volume distilled water for the assay.⁴⁴ Procedures for uronic acid analysis were described elsewhere.⁴³ Briefly, 200 μL of 0.025 M di-sodium tetraborate 10-hydrate (Merck) in concentrated sulfuric acid was added to each precooled test GAG solution and then heated at 100°C for 10 min. Twenty microliters of 0.125% carbazole (Eastman Kodak) in absolute ethanol was then added to each of the cooled mixtures and heated at 100°C for another 15 min to produce the colored product. After cooling to room temperature, the optical density of the product was read at 530 nm with a microplate reader. Optical densities of standard glucuronic acid (Sigma) with different concentrations were plotted in a standard curve. Hexuronic acid content of the test sample was determined from the standard curve after correction for dilution effects.

HYP assay was used to determine the content of HYP, which is the marker for total collagen, presented in the microspheres as described previously.^{45,46} In brief, 10 microspheres at each time point were pooled together and hydrolyzed in 200 μL of 6 N hydrochloric acid (Sigma) at 110°C for 18 h in a hydrolysis tube (Pierre) after being flushed with nitrogen gas for 30 s. The acid hydrolyzate was neutralized by 6 N sodium hydroxide (NaOH). One hundred microliters of neutralized acid hydrolysate (pH 6–7) was added to a 96-well plate and oxidized by 50 μL 0.05 M chloramine T (Sigma) for 20 min, followed by incubating with 50 μL 3.15 M perchloric acid (Sigma) for 5 min. Oxidized samples were then mixed with 50 μL *p*-dimethylaminobenzaldehyde (20%, w/v; Sigma) and incubated at 60°C for 20 min to

produce coloured products. The optical densities of the assayed samples were measured at 557 nm with a microplate reader. HYP content was computed by linear interpolation using trans-4-hydroxy-L-Proline (Sigma) as standard.

Collagen type II enzyme-linked immunosorbent assay (ELISA; MD Bioscience) was used to determine the amount of type II collagen presented in the microspheres. Twenty microspheres at each time point were pooled together and digested by pepsin in 0.5 N acetic acid (Sigma; collagen: pepsin = 10:1, w/w) to extract type II collagen. Pretreatment with 0.2 M NaOH was performed for samples harvested on days 14 and 21 at 4°C for 24 h to remove any proteoglycans as it has been demonstrated to improve the collagen extraction efficiency. Pepsin was digested at 20°C for 18 h with constant stirring. Insoluble residues were separated by centrifugation at 15,000 rpm for 15 min and supernatants were collected.⁴⁷ They were adjusted to pH 8.0 with 1 M NaOH, followed by the digestion using 1/10 starting volume of pancreatic elastase solution (1 mg/mL in 1 \times TBS, pH 7.8–8.0; Sigma) at 4°C overnight. Extracted type II collagen was measured according to the manufacturer's instructions. In brief, samples and standards were added to the supplied 96-well plate and incubated with the capture antibodies for 2 h. The wells were then washed and loaded with biotinylated antibodies for another 2 h, followed by a wash and incubation with streptavidin-horseradish peroxidase (HRP) for 30 min. After all, the plate was washed again and incubated with tetramethylbenzidine (TMB) solution for 20 min. Sulfuric acid was added as the final step to stop the reaction and obtain color products. Absorbance was read at 450 nm. All the incubation steps were carried out at room temperature. Type II collagen was quantified by linear interpolation with the standard.

Rat collagen type I ELISA (Chondrex) was used to determine the amount of type I collagen presented in the microspheres. Five microspheres at each time point were pooled together for collagen extraction using the same protocol as stated above. Extracted type I collagen was accessed according to the manufacturer's instructions. In brief, samples and standards were added to the supplied 96-well plate precoated with capture antibodies and were incubated for 2 h. The wells were then washed and loaded with detection antibodies for another 2 h, followed by a wash and incubation with streptavidin peroxidase for 1 h. Afterward, the plate was washed again and incubated with orthophenylene-diamine (OPD) solution for 30 min. Sulfuric acid was added as the final step to stop the reaction and obtain colored products. Absorbance was read at 490 nm. All the incubation steps were carried out at room temperature. Rat type I collagen was quantified by linear interpolation with the standard.

Metra[®] PYD ELISA kit (Quidel Corporation) was used to quantify the amount of PYD, which is one of the major collagen crosslinkers,^{23,48} presented in the microspheres. The acid hydrolyzates obtained previously in HYP assay was used for PYD quantification, following the procedures instructed by the manufacturer. In brief, samples or standards were added to each well of the coated strips, followed by the addition of anti-pyridinium crosslinks antibodies conjugated to alkaline phosphate. The mixtures were incubated at 4°C for 3 h in dark. Afterward, each well was washed and substrate solution containing ethanolamine and magnesium chloride was added. Incubation at room temperature for another 1 h was carried out and the substrate reaction was

stopped afterward by NaOH. Color products were produced and the absorbance was read at 405 nm. The PYD amount was determined from the standard curve obtained.

Measurement of elastic modulus of the microspheres

A microplate rate-jump compression method⁴⁰ was used to determine the E_r of the collagen-hMSC microspheres at different time points during chondrogenic differentiation. E_r is chosen because it reflects the elastic components and therefore the resilience of the structures, which is characteristic to cartilage tissue. In brief, custom-made rigid and flexible microplates were mounted onto the metal holders of two separately controlled micromanipulators (MP-285 motorized micromanipulator; Sutter Instrument). A microsphere was attached onto the rigid microplate for around 5 min to assure firm adhesion, followed by the compression in 1×PBS by the flexible microplate, which had been calibrated for stiffness (k) using an electronic balance and a height gauge.⁴⁰ A step change compression protocol was employed to impose a sudden change in displacement rate during the process so as to eliminate the viscous component of the viscoelastic deformation of the microsphere.^{40,49} Sixty percent strain (i.e., 60% of the diameter of the microsphere in the compression axis) was set to be the displacement at the fixed end of the flexible microplate (δ). The compression started off with an initial displacement rate ($\dot{\delta}_-$) of 1 $\mu\text{m/s}$, followed by a sudden change to 2 $\mu\text{m/s}$ ($\dot{\delta}_+$) upon reaching half of δ . The whole compression process was captured by a digital camera (CoolSNAP ES; Photometrics) and the image was analyzed by image analysis software Metamorph (Molecular Devices Corporation). The free end displacement (δ') of the flexible microplate was tracked by the object tracking module of the software and the radius (a) in the contact region between the two bodies was measured. E_r was calculated using the equation $E_r = \frac{E}{1 - \nu^2} = \frac{k}{a} \left(\frac{\Delta\dot{\delta}}{\Delta\dot{\delta}'} - 1 \right)$, where

$\Delta\dot{\delta}$ and $\Delta\dot{\delta}'$ represent the displacement rate change for the fixed and free end of the flexible microplate, respectively, at the time the sudden displacement rate change was imposed.

Size measurement of microspheres during chondrogenic differentiation

To estimate the effect of cells-induced contraction, the sizes of 36–40 microspheres at each time point during differentiation were measured using Metamorph. As the microsphere was not perfectly spherical, its dimensions parallel and perpendicular to the axis of compression were recorded. The mean of the two parameters was taken to be the effective dimension of the microsphere.

SEM analysis of the microspheres

SEM was used to examine the ultra-structure of collagen-hMSC microspheres. Microspheres harvested at different time points were rinsed with 1×PBS and fixed by 2.5% glutaraldehyde (Sigma) for 2 h at room temperature. They were then dehydrated through a graded series of ethanol (50%, 70%, 80%, 90%, 95%, and 100% for twice) and then critical point dried. The dried samples were fractured using a sharp blade to expose their cross sections and were mounted onto a metal stub using carbon cement. The samples were

sputtered with gold for 20 s for electrical conductance during SEM examination.

Statistical analysis

Differences on microsphere dimension was analyzed by one-way analysis of variance (ANOVA) with Bonferroni's *post-hoc* test while those on E_r , GlcUA, HYP, PYD, and collagen type I and II of the microspheres at different time points of differentiation and in different subjects were analyzed using two-way ANOVA (General Linear Model, Univariate) with Bonferroni's *post-hoc* tests. Nonparametric Spearman's Rho correlation coefficients between various biochemical compositional parameters and the mechanical property E_r of the microspheres in each subject were used to reveal their association during differentiation. Nonparametric tests were used because sample size per subject was small. All statistical tests were performed using SPSS 17.0 (SPSS). A significance level was set at 0.05. Values were reported as mean \pm standard deviation unless otherwise specified.

Results

Reduction in size of microspheres

Upon chondrogenic differentiation, the hMSC-encapsulated collagen microspheres contracted over time (Fig. 1A–D). Their sizes decreased significantly by half on day 7 (Fig. 2A), whereas subsequent decline at 14 and 21 day postdifferentiation was no longer significant. One-way ANOVA with Bonferroni *post-hoc* test showed significant differences between all time point and day 0 ($p < 0.05$).

Decrease in type I collagen and total collagen

Type I collagen has great homology across different species. The antibody used for immunohistochemistry reacts with both human and rat type I collagen. As a result, type I collagen detected by immunohistochemistry include both rat type I collagen in the template and the newly synthesized human type I collagen if any. As shown in Figure 1E–H, immunopositive staining was found at all time points. Type I collagen was largely homogeneous throughout the microsphere before chondrogenic differentiation (Fig. 1E). Thick bundle-like immunonegative structures were detected at day 7 and 14 postdifferentiation (Fig. 1F, F1, G), whereas nodules with immunonegative staining could be found throughout the microsphere in these samples. At 21 days postdifferentiation, further increase in regions with immunonegative staining was detected particularly at the center (Fig. 1H). This decreasing trend was revealed by quantitative measurement of type I collagen in all three subjects using ELISA (Fig. 2B). Two-way ANOVA showed that the time factor ($p = 0.001$) but not the subject factor ($p = 0.107$) significantly affected type I collagen. Bonferroni's *post-hoc* tests on the time factor showed that day 21 was significantly different from day 0 and 7 ($p \leq 0.009$) and day 14 was significantly different from day 7 ($p = 0.009$). HYP is the marker for total collagen including type I collagen used for fabrication of the microspheres and other types of collagen newly synthesized by the differentiating hMSCs. As shown in Figure 2C, the total collagen content decreases progressively during the chondrogenic differentiation

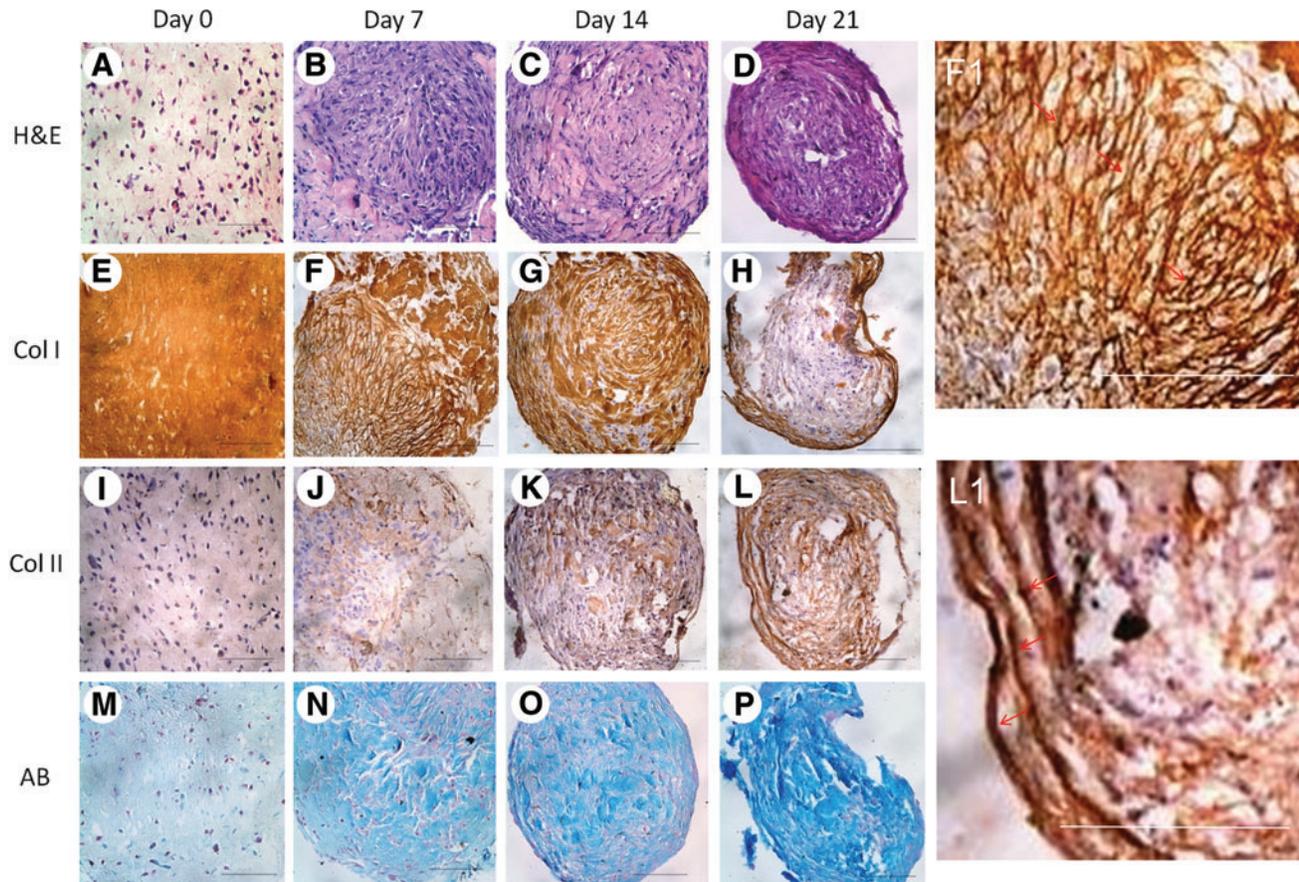


FIG. 1. Histological, histochemical, and immunohistochemical characterization of collagen-hMSC microspheres at different time points during chondrogenic differentiation. (A–D) H&E staining. (E–H) Immunohistochemistry of type I collagen (Col I). (I–L) Immunohistochemistry of type II collagen (Col II). (M–P) Alcian blue (AB) histochemical staining for glycosaminoglycans. F1, magnified view of F; L1, magnified view of L. Arrows: thick collagen bundles. Scale bar: 100 μ m. hMSC, human mesenchymal stem cells; H&E, hematoxylin and eosin. Color images available online at www.liebertonline.com/ten.

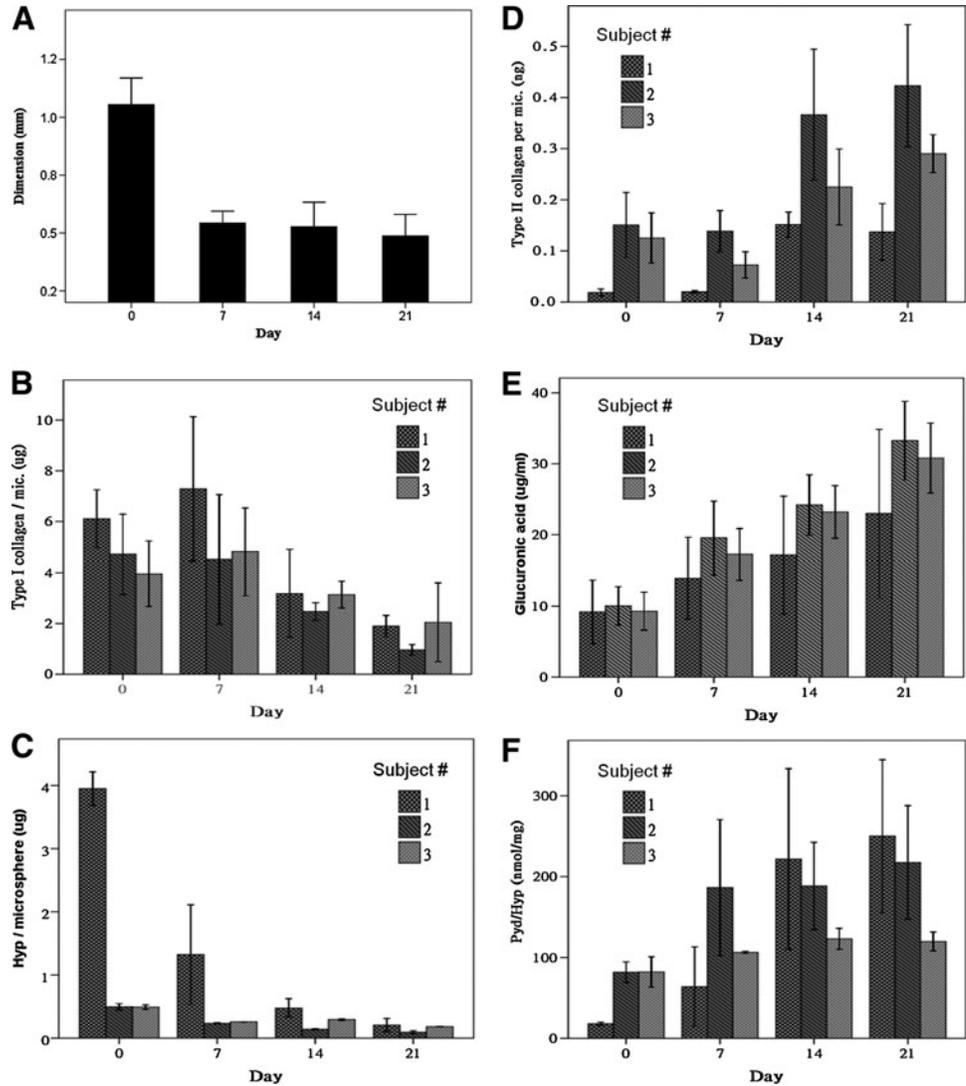
process in all three subjects, but the change was more dramatic in subject 1. Two-way ANOVA showed that both the time factor ($p < 0.001$) and the subject factor ($p < 0.001$) significantly affected HYP. Bonferroni's *post-hoc* tests on the time factor showed that day 21 was significantly different from all other time points ($p \leq 0.038$), day 14 was significantly different from day 0 ($p < 0.001$) and day 21 ($p = 0.038$), day 7 was significantly different from day 0 ($p = 0.002$), and day 21 ($p < 0.001$) and day 0 were significantly different from all other time points ($p \leq 0.002$). Moreover, Bonferroni's *post-hoc* test on subject factor showed that subject 1 was significantly different from the other two subjects ($p < 0.001$).

Increase in type II collagen and GAGs

Type I collagen was the only scaffold material entrapping the hMSCs within the microsphere. Therefore, any matrix components other than type I collagen should be newly synthesized by the entrapped cells. ECM markers for chondrogenic lineages include type II collagen and proteoglycans. Figure 1I–L shows the immunohistochemical staining of type II collagen in microspheres during chondrogenic differentiation. Negative staining was found before differentiation (Fig. 1I). Slight immunopositive staining of type II collagen

was found at day 7 postdifferentiation (Fig. 1J) followed up by an increase in both area and intensity of the staining at day 14 and 21 postdifferentiation (Fig. 1K, L, L1). Quantitative analysis of type II collagen using ELISA confirmed the increasing trend particularly on day 14 and 21 postdifferentiation in all three subjects (Fig. 2D). Two-way ANOVA showed that both the time factor ($p < 0.001$) and the subject factor ($p < 0.001$) significantly affected type II collagen. Bonferroni's *post-hoc* tests on the time factor showed that both day 21 and day 14 were significantly different from days 7 and 0 ($p \leq 0.001$). Moreover, Bonferroni's *post-hoc* tests on the subject factor showed that all three subjects are significantly different from one another ($p \leq 0.009$). Minimal staining for GAGs was found in microspheres before chondrogenic differentiation was induced (Fig. 1M). Both area and intensity of the GAG staining were found increased after 7 days (Fig. 1N). Intensive staining for GAG was found at day 14 and 21 postdifferentiation (Fig. 1O, P). Quantitative analysis of GlcUA, which is the regular component of GAG chains, confirmed the trend of continuous increasing proteoglycan content over time in all three subjects (Fig. 2E). Two-way ANOVA showed that both the time factor ($p < 0.001$) and the subject factor ($p = 0.02$) significantly contributed to the changes in GlcUA. Bonferroni's *post-hoc*

FIG. 2. Physical and biochemical characterization of collagen-hMSC microspheres at different time points during chondrogenic differentiation. (A) Diameter of microspheres ($n = 36-40$). (B) Type I collagen ($n = 2$ to 4, each sample is a pool of 5 microspheres). (C) Hyp content ($n = 2-3$, each sample is a pool of 10 microspheres). (D) Type II collagen ($n = 3$ to 4, each sample is a pool of 20 microspheres). (E) Uronic acid content ($n = 3$ to 9). (F) Pyl-to-Hyp ratio ($n = 2$ to 3, each sample is a pool of 10 microspheres). $N = 3$ subjects for biochemical characterization. Hyp, hydroxyproline; Pyl, pyridinoline.



tests on the time factor showed that day 21 was significantly different from all other time points ($p \leq 0.039$), day 14 was significantly different from day 0 ($p = 0.001$) and day 21 ($p = 0.039$), day 7 was significantly different from day 21 ($p < 0.001$), and day 0 was significantly different from all time points ($p \leq 0.001$) but day 7. Nevertheless, Bonferroni's *post-hoc* tests on the subject factor did not reveal any significant difference ($p \geq 0.108$).

Bundle formation and increased collagen crosslinker density

SEM analysis of the collagen-hMSC microsphere before chondrogenic differentiation showed a highly randomized meshwork of collagen (Fig. 3A). Collagen fibers were found randomly distributed and intercross with each other. Nevertheless, they become more organized as chondrogenic differentiation goes on (Fig. 3B-D). Specifically, closely packed fibers were found, forming thick bundles throughout the microspheres, particularly at days 14 and 21 post-differentiation (Fig. 3C, D). Such structural changes are highly agreeable with the bundle structures observed in immunohistochemistry of both type I (Fig. 1F, F1) and II (Fig.

1L, L1) collagen, suggesting the enhanced organization of the matrix by the differentiating hMSCs. Moreover, nonfibrous ground substances coating the fibrils were observed throughout the matrix particularly at later time points (Fig. 3C, D). Further, the density of PYD, a major nonreducible collagen crosslinker, has been found significantly increasing over time in all three subjects (Fig. 2F), suggesting an enhanced organization of collagen matrix. This correlates well with the presence of thick bundles observed in SEM (Fig. 3C, D). Two-way ANOVA showed that the time factor ($p = 0.002$) but not the subject factor ($p = 0.121$) significantly affected PYD density. Bonferroni's *post-hoc* tests on the time factor showed that day 21 ($p = 0.002$) and day 4 ($p = 0.007$) were significantly different from day 0.

Increase in E_r

Figure 4A and B shows the collagen-hMSC microsphere before and after the step change compression assay, which is a rapid, simple, and nondestructive procedure, from which the contact radius between the microsphere and the soft plate was obtained for subsequent calculation. Figure 4C shows the raw data and the linearly regressed slopes in the

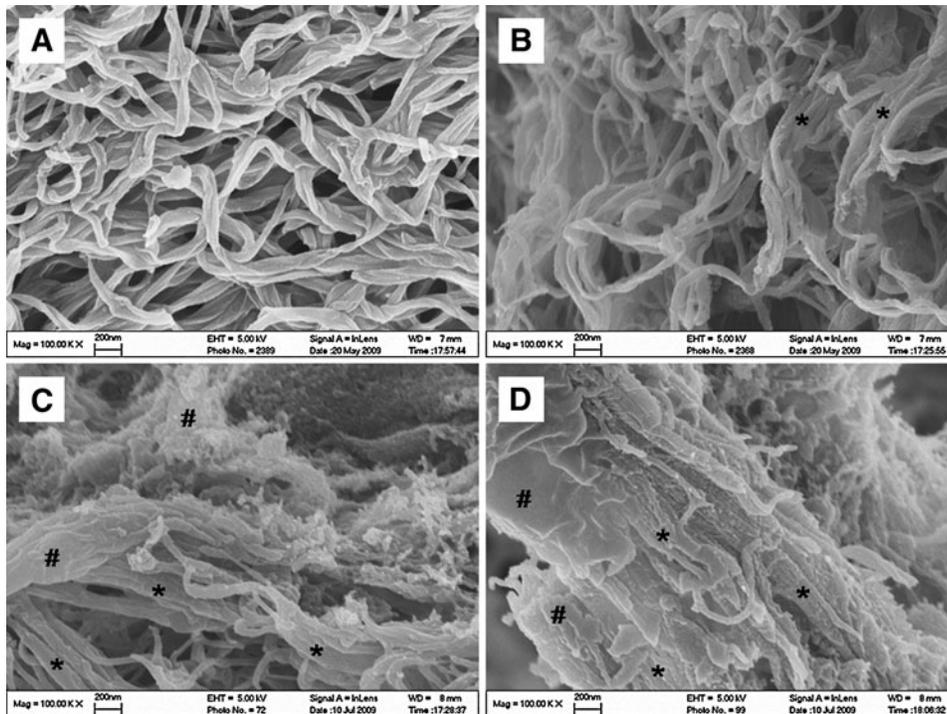


FIG. 3. Scanning electron microscopy of the collagen fibril meshwork of collagen-hMSC microspheres at different time points during chondrogenic differentiation. **(A)** Day 0. **(B)** Day 7. **(C)** Day 14. **(D)** Day 21. Magnifications: 100K \times ; *, parallel bundles; #, ground substances coating the collagen fibrils.

step-change compression protocol. From which, the difference in slopes of the two compression steps was obtained for subsequent calculation. The mean E_r with standard deviation of the microspheres of three subjects predifferentiation at day 0 was 4.2 ± 2.5 kPa. Upon chondrogenic induction, the mean E_r of the differentiating microspheres from three subjects was increased to 58 ± 38 kPa, 65 ± 44 kPa, and 96 ± 48 kPa on days 7, 14, and 21 postdifferentiation, respectively, accounting for 13.8-, 15.5-, and 22.9-fold of increase. Two-way ANOVA showed that both the time factor ($p < 0.001$) and the subject factor ($p < 0.001$) significantly affected E_r . Bonferroni's *post-hoc* tests showed that day 21 was significantly different from all other time points ($p \leq 0.02$), day 14 was significantly different from day 21 and 0 ($p < 0.02$) but not day 7, day 7 was significantly different from days 21 and 0 ($p < 0.003$) but not day 14, and day 0 was significantly different from all other time points ($p < 0.001$). Moreover, Bonferroni's *post-hoc* tests on the subject factor showed that subject 1 was significantly different from the other two subjects ($p < 0.001$).

Association between biochemical compositional parameters and mechanical parameter

Table 1 shows the nonparametric correlation coefficients with significance values between the compositional parameters and the E_r of the microspheres during differentiation. Specifically, HYP showed a strong negative correlation with E_r in all three subjects (-0.973 , -0.714 , -0.905), whereas type I collagen also showed a moderate negative correlation in all subjects (-0.582 , -0.479 , and -0.555). On the other hand, type II collagen (0.510 , 0.753 , and 0.633) and GAGs (0.469 , 0.945 , and 0.877) showed positive correlation with E_r with moderate to high coefficients, whereas PYD density showed strong positive correlation (0.733 , 0.810 , and 0.810). Corre-

lation coefficients for HYP, GAGs, and PYD density were statistically significant in all three subjects ($p < 0.05$). Correlation coefficient for type I collagen was statistically significant in one subject ($p = 0.029$) but marginal for the other two subjects ($p = 0.077$ and 0.162). Similarly, correlation coefficient for type II collagen was statistically significant in one subject ($p = 0.019$) but marginal in the other two subjects ($p = 0.062$ and 0.067).

Discussion

This study reported the histological, biochemical, structural, and mechanical characterization of collagen-hMSC microspheres during chondrogenic differentiation and demonstrated close correlation between the compositional parameters and the mechanical property of the microspheres. Our results suggested that the differentiating hMSCs are actively remodeling the template type I collagen meshwork in the microspheres, depositing new ECM, including proteoglycans and type II collagen, and organizing the fibrous matrix via collagen crosslinking. This study helps us to better understand how hMSC remodel the microsphere matrix during chondrogenic differentiation and therefore contributes to future development of novel cartilage repair strategies. Finally, this study also illustrated the application of a simple sensitive and nondestructive method monitoring the temporal change in elastic modulus of soft and viscoelastic structures.

Matrix remodeling activities of the differentiating hMSCs contribute to the change in mechanical property of the microspheres

The first matrix compositional change is the increase in newly deposited cartilage-relevant ECMs, including proteoglycans

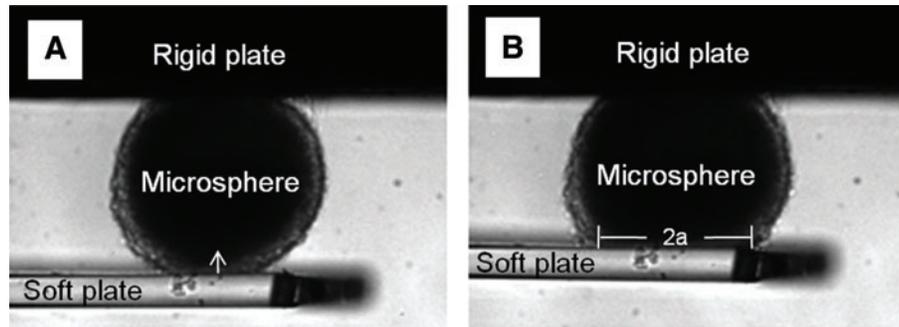
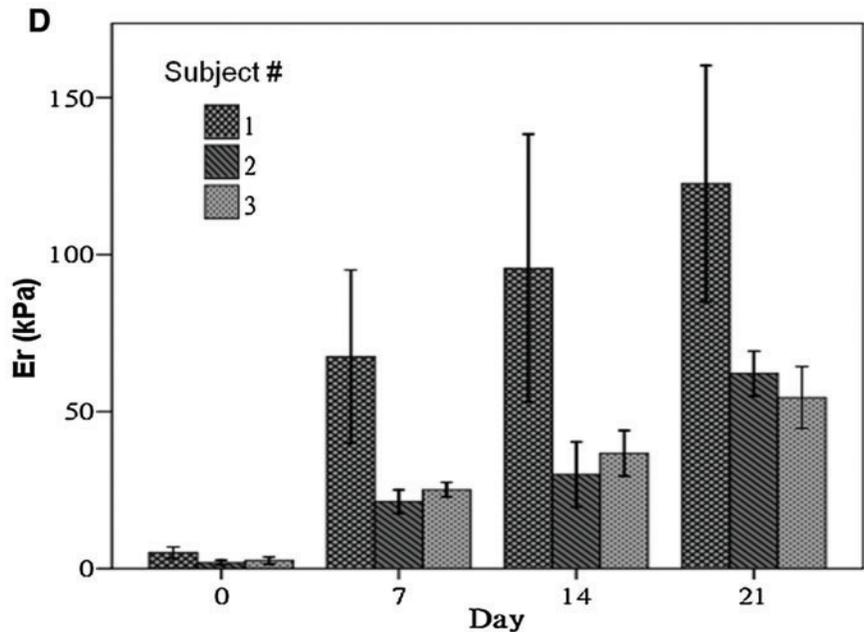
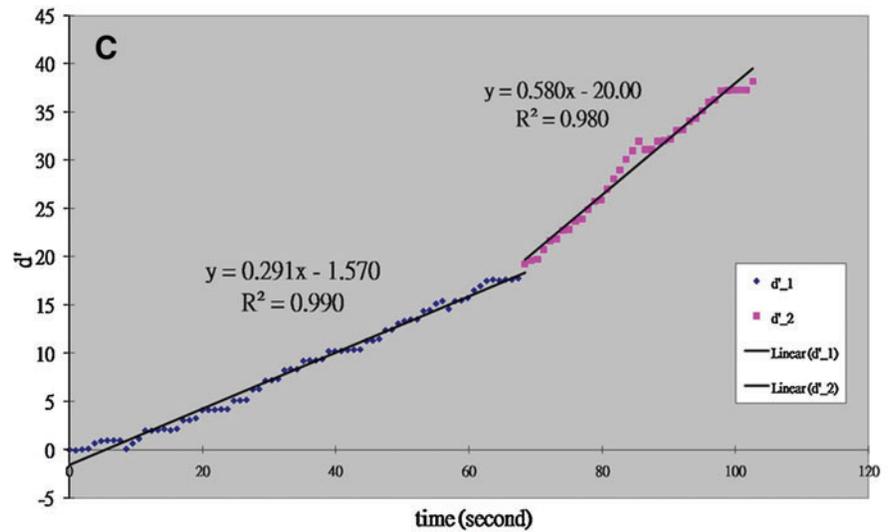


FIG. 4. Mechanical characterization of collagen-hMSC microspheres at different time points during chondrogenic differentiation. **(A, B)** Phase-contrast images showing a collagen-hMSC microsphere before **(A)** and after **(B)** the simple step-change compression assay (arrow, contact point for tracking path of the soft microplate compression; 2a, diameter of the instantaneous contact region between the microsphere and the soft microplate); **(C)** A representative displacement (micrometers) time (second) curve of collagen-hMSC microsphere during the step-change compression assay (d'_1 , raw data collected during compression with displacement rate 1; d'_2 , raw data collected during compression with displacement rate 2; linear (d'_1), best fit linear regression line for compression with displacement rate 1; linear (d'_2), best fit linear regression line for compression with displacement rate 2. **(D)** E_r of collagen-hMSC microspheres harvested at different time points post-differentiation (error bars, standard deviations; $n = 3$ to 9 per time point). E_r , reduced elastic modulus. Color images available online at www.liebertonline.com/ten.



and type II collagen. Despite the differences in types of stem cells and scaffolds used, this characteristic association has been reported in other tissue-engineered constructs.^{50–52} Awad *et al.* reported a progressive increase in equilibrium shear modulus and complex shear modulus for constructs made of agarose, alginate, and gelatin with adiposed-derived adult stem cells, whereas GAG and HYP contents were

found to increase continuously with time⁵⁰; Mauck *et al.* reported the chondrogenesis of bovine MSCs in agarose culture and demonstrated an increase in Young's modulus and dynamic modulus with collagen and GAG⁵²; the hMSCs seeded silk scaffolds reported by Hofmann *et al.* also demonstrated a significant increase in the aggregate modulus together with a progressive increase in GAG and expression

TABLE 1. NONPARAMETRIC SPEARMAN'S RHO CORRELATION COEFFICIENTS BETWEEN VARIOUS BIOCHEMICAL COMPOSITIONAL PARAMETERS AND THE REDUCED ELASTIC MODULUS (E_r) OF HUMAN MESENCHYMAL STEM CELL-COLLAGEN MICROSPHERES FABRICATED FROM THREE SUBJECTS DURING CHONDROGENIC DIFFERENTIATION

| | Subject no. 1 | | Subject no. 2 | | Subject no. 3 | |
|--|------------------------------------|---------|------------------------------------|---------|------------------------------------|---------|
| | Correlation coefficient with E_r | p-Value | Correlation coefficient with E_r | p-Value | Correlation coefficient with E_r | p-Value |
| Hydroxyproline (Hyp) | -0.937 ^a | <0.001 | -0.714 ^a | 0.047 | -0.905 ^a | 0.002 |
| Type I collagen | -0.582 ^a | 0.029 | -0.479 | 0.162 | -0.555 | 0.077 |
| Uronic acid (GlcUA) | 0.469 ^a | 0.005 | 0.945 ^a | <0.001 | 0.877 ^a | <0.001 |
| Pyridinoline to hydroxyproline ratio (Pyd/Hyp) | 0.733 ^a | 0.016 | 0.810 ^a | 0.015 | 0.810 ^a | 0.015 |
| Type II collagen | 0.51 | 0.062 | 0.753 ^a | 0.019 | 0.633 | 0.067 |

^aData with statistical significance, that is, significant correlation with E_r .

of type II collagen.⁵¹ The second matrix compositional change is related to the structural organization of collagen fibers as demonstrated by both the increase in the density of the collagen crosslinker PYD and the presence of thick collagen bundles in SEM analysis. PYD is generally known to contribute to the tensile properties of tendons^{46,53} and ligaments,^{54,55} and has been demonstrated to associate with both the compressive¹⁷ and tensile properties⁵⁶ of articular cartilage. The significance of PYD in contributing to the mechanical properties of tissue-engineered constructs has been demonstrated by the significantly lower equilibrium modulus and secant modulus of chondrocyte-seeded alginate beads when exposed to prolonged crosslink inhibition.⁵⁷ The positive association between PYD and mechanical properties in engineered tissue constructs has previously been reported in chondrocyte-seeded polylactic acid and polyglycolic acid.⁵⁸ Moreover, the positive association between HYP normalized PYD and E_r in the microspheres is very relevant to cartilage tissue engineering as type II collagen is more heavily crosslinked than other types.⁴⁸ This may also partially explain the net and significant increase in E_r of the microspheres even though the amount of type II collagen being deposited is minute. A third compositional change is the net decrease in type I collagen and total collagen (HYP). This negative association between E_r and type I collagen or total collagen should be a result of the degradation activity of the differentiating hMSCs. The scaffold material used in fabricating the microspheres is type I collagen, which serves as a degradable template meshwork for hMSCs to remodel as they differentiate. Although the differentiating hMSCs may also synthesize type I collagen, the overall decreasing trend of type I collagen and total collagen suggests that the degradation of the template material outweighs the newly deposited type I collagen if any. Involvement of various enzymes or proteases in matrix remodeling during chondrogenesis has been previously reported.⁵⁹⁻⁶³ Matrix metalloproteinase 13 is one of the enzymes known to degrade collagen type I, II, III, and IV⁵⁹⁻⁶¹ and has been shown up-regulated during chondrogenesis.^{62,63} The amount of type I collagen initially used in fabricating the microsphere is way higher (tens of microgram level) than that deposited (nanogram level for type II collagen and microgram level for type I collagen). As a result, the degradation rate of the template material outweighs the deposition rate of any collagen subtype if any. A fourth factor contributing to the increase in E_r of the microspheres is the reduction in size and thus the

volume of the microsphere due to hMSC-induced matrix contraction. This leads to a higher matrix density and results in a more compact and solid structure in the microsphere. Nevertheless, the size has reduced only less than half during differentiation, whereas the increase of the E_r was >24-fold, suggesting that the compaction effect due to size reduction could not explain the tremendous increase in E_r of the microspheres.

Advantages and limitations of the current study

The microplate compression technique established in our lab⁴⁰ presents a simple method in measuring elastic modulus of small soft and viscoelastic structures particularly those hydrogel-based including the commonly used alginate and agarose (data not shown). Utilizing calibrated microplates made of different materials and with different bending stiffness, this method is able to measure structures with a wide range of elastic modulus, ranging from ~100 Pa for cell-free collagen microspheres⁴⁰ to hundreds of kPa for microspheres with chondrogenic differentiated hMSCs.⁴⁰ The close correlations between the compositional changes of the microspheres and their elastic modulus established in this work suggest that the microplate method can be used to monitor the progression of chondrogenic differentiation particularly with matrix remodeling activity of hMSCs. Specifically, we can use this method to determine the end point of *in vitro* manipulation of the microspheres before implantation. Other advantages of this method include its nondestructive nature and the small amount of samples required. The assay is a two-step compression protocol using a very soft and flexible microplate, which will not damage the microsphere at all. Moreover, a single microsphere made of as little as 2.5 μ L of cell-gel mixture containing hundreds of cells is able to yield a data point, comparing with the biochemical assays where pooling tens of microspheres is required to yield one detectable data point, suggesting the excellent sensitivity of this microplate compression assay. This work also reveals the subject variability in using human cells and suggests that multiple subjects need to be used in this kind of studies. Specifically, significant subject variability was detected in HYP, type II collagen, and E_r but not in GAG, type I collagen, and PYD density. Moreover, the compositional mechanical correlation study also revealed the subject variability in type I and II collagen but not in HYP, PYD, and GAGs. The subject variability could be inherent to

the subjects, and may also be due to changes induced in the cells upon subcultures, insensitivity of certain biochemical assays, and other random errors. One limitation of the current study is that the sample microsphere cannot be used to measure all biochemical and mechanical parameters. On the other hand, for some biochemical tests, pooling of microspheres is required to yield a single data point. As a result, it is impossible to build a statistical model specifically multiple regression, which describes how do the biochemical parameters contribute to the mechanical property of the microspheres. Therefore, separate correlation coefficients between the mechanical property and each of the biochemical parameter were calculated to reveal the associations between each biochemical parameter and the mechanical property. Nevertheless, the relative importance of different biochemical parameters in contributing to the mechanical property of the microspheres cannot be determined.

Elastic modulus of the differentiating collagen-hMSC microspheres is approaching although not achieving that of the native cartilage

The microplate compression technique was also applied to measure the Young's modulus (E) of rabbit articular cartilage. The mean value was 511 ± 411 kPa, which is comparable to values reported elsewhere.⁶⁴⁻⁶⁷ The equation for E_r of

the microspheres is $E_r = \frac{E}{1 - \nu^2} = \frac{k}{a} \left(\frac{\Delta \delta}{\Delta \dot{\delta}} - 1 \right)$, which is related to Young's modulus E via $E = E_r \times (1 - \nu^2)$. For positive-

Poisson-ratio materials, Poisson's ratio, ν , is between 0 and 0.5, and so E should be smaller than E_r . The mean E_r of the differentiated collagen-hMSC microspheres on day 21 was 96 kPa and the corresponding E would have a value smaller than that, say, 87 kPa if ν is assumed to be 0.3. Thus, the mean E of the differentiated microspheres was only around 20% of that of native cartilage. As a result, further stimuli for chondrogenesis and new matrix production via prolonged culture in the chondrogenic medium or mechanical stimulation⁶⁸⁻⁷⁰ may be needed to further stiffen the engineered microspheres before implantation.

Conclusion

The compositional mechanical relationship of collagen-hMSC microspheres during chondrogenic differentiation has been characterized. Particularly, the temporal change of the reduced elastic modulus of the microspheres was positively associated with newly deposited ECM components, including GAGs and type II collagen, and with increasing matrix organization in terms of collagen crosslinker density during differentiation. The temporal changes in type I collagen and total collagen were negatively associated with the reduced elastic modulus, suggesting the matrix remodeling activity of the differentiating hMSCs. This work illustrates the application of the microplate compression method as a simple sensitive and nondestructive monitoring tool for stem cell differentiation in engineered constructs. This study contributes to future development of stem cell-based cartilage repair.

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Disclosure Statement

No competing financial interests exist.

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