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Cell number and chondrogenesis in human mesenchymal stem cell aggregates is affected by the sulfation level of heparin used as a cell coating

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Abstract

For particular cell-based therapies, it may be required to culture mesenchymal stem cell (MSC) aggregates with growth factors to promote cell proliferation and/or differentiation. Heparin, a negatively charged glycosaminoglycan (GAG) is known to play an important role in sequestration of positively charged growth factors and, when incorporated within cellular aggregates, could be used to promote local availability of growth factors. We have developed a heparin-based cell coating and we believe that the electrostatic interaction between native heparin and the positively charged growth factors will result in (1) higher cell number in response to fibroblast growth factor-2 (FGF-2) and 2) greater chondrogenic differentiation in response to transforming growth factor-β1 (TGF-β1), compared to a desulfated heparin coating. Results revealed that in the presence of FGF-2, by day 14, heparin-coated MSC aggregates increased in DNA content 8.5 ± 1.6 fold compared to day 1, which was greater than noncoated and desulfated heparin-coated aggregates. In contrast, when cultured in the presence of TGF-β1, by day 21, desulfated heparin-coated aggregates upregulated gene expression of collagen II by 86.5 ± 7.5 fold and collagen X by 37.1 ± 4.7 fold, which was higher than that recorded in the noncoated and heparin-coated aggregates. These observations indicate that this coating technology represents a versatile platform to design MSC culture systems with pairings of GAGs and growth factors that can be tailored to overcome specific challenges in scale-up and culture for MSC-based therapeutics.

Keywords

mesenchymal stem cells; heparin; chondrocytic differentiation; cell coatings; growth factor

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Additional Supporting Information may be found in the online version of this article.

INTRODUCTION

Mesenchymal stem cells (MSCs) are currently being utilized in over 350 National Institute of Health-registered clinical trials treating pathologies ranging from graft versus host disease and diabetes to bone and cartilage injuries.[1,2] Their ability to differentiate down multiple lineages and/or secrete trophic factors make MSCs a promising cell source to regenerate tissue and treat a variety of diseases.[3–5] However, in these trials, one to five doses of 2,000,000– 8,000,000 cells are required for administration; thus, for these multipotent cells to be a viable option, mass expansion is needed.[2] Additionally, for certain applications, such as cartilage repair, precise control of differentiation of these multipotent cells is required to ensure that a homogenous cell population is delivered.[6,7] To achieve levels of cell proliferation and chondrogenic differentiation that may be required for cell-based therapies for cartilage repair, growth factors such as fibroblast growth factor-2 (FGF-2; proliferation),[8,9] and transforming growth factor-β1 (TGF-β1; chondrogenesis)[10–12] can be employed.

In addition to growth factor supplementation, different MSC culture platforms can affect both MSC proliferation and differentiation. For example, pellet systems (25,000 cells) have traditionally been used to promote chondrogenic differentiation of MSCs, however, recent interest in smaller MSC aggregates (500–1000 cells) with smaller diameters has increased because of their ability to be used as injectable therapies treatment of cartilage injuries using a standard-size needle.[6,7,13] Additionally, it has been shown that MSCs aggregated into spheroid form ranging from 500 to 250,000 cells have the ability to attenuate inflammatory cytokine secretion and secrete higher levels of anti-inflammatory molecules when compared to their monolayer counterparts, which could also be advantageous to promote a pro-healing environment after cartilage injury.[14,15] However, the challenge of lack of proliferation in spheroid culture may hinder the scale-up of MSC aggregate systems.[16] Additionally, due to the 3D structure of formed aggregates, exogenous growth factors supplemented to the culture medium may not be exposed the entire cell population, especially the cells found on the interior of the aggregate.

To address these potential limitations in MSC aggregate culture, incorporation of sulfated glycosaminoglycans (GAGs), such as heparin, within an aggregate can be used as a potential vehicle to promote growth factor availability due to the GAGs' high negative charge that facilitates local electrostatic interaction with positively charged proteins.[17–21] Such a concept would mimic the actions of heparin sulfate proteoglycans (HSPGs) typically found on the plasma membrane of a cell and within the ECM.[22,23] HSPGs play a role in regulation of signaling factor activity and can act as co-receptors for various growth factor receptors to lower receptor activation or alter duration of the signaling reactions.[22,23] Because the interaction between heparin and its growth factors depends on the presence of the negatively charged sulfate groups, desulfation of the GAG can modulate the interaction with a positively charged protein.[21] Specifically, it has been seen that desulfation of heparin can affect affinity for FGF-2 and TGF-β1 and the growth factors' subsequent bioactivity,[20,24,25] but it remains unclear what effects GAGs of different sulfation levels may have on aggregate cell culture with growth factor supplementation.

To incorporate GAGs into a 3D aggregate, we have previously developed and characterized a GAG cell coating that sequesters positively charged proteins onto cell surfaces.[26] We have shown that through layer-by-layer deposition of biotin and avidin, biotinylated heparin has been grafted onto cell surfaces at different concentrations without negatively affecting cell viability and inherent anti-inflammatory properties of MSC aggregates. When loaded onto aggregates of heparin-coated cells, TGF-β1 remains bioactive and has the ability to signal to surrounding cells upon release.[26] While characterization of this heparin coating has been performed, effects of a heparin coating on cell response to growth factors in long-term culture have not been explored. Moreover, because the electrostatic interactions between heparin and growth factors are necessary for binding and signaling, decreasing the sulfation level of the coating may provide insight into how these interactions can play a role in the subsequent cellular response.

Thus, the objective of this article is to characterize heparin MSC coatings of two different sulfation levels (native sulfation and fully desulfated) and study the effect of these coatings on MSC response in the presence of two different growth factors supplemented to the culture media, as would be found in traditional cell culture approaches for stem cell therapies. To evaluate the coating effect, MSC aggregates were coated with either heparin or desulfated heparin and then cultured in serum-free media containing FGF-2 or TGF-β1. Over 21 days *in vitro*, cell morphology was characterized using histological staining, cell number was determined using a DNA assay, and chondrogenic differentiation was evaluated using quantitative reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemical (IHC) staining for chondrogenic extracellular (ECM) components. In these studies, we hypothesize that the strong electrostatic interaction between heparin and the positively charged growth factors in the vicinity of the cells will promote growth factor availability, and therefore, the fully sulfated heparin coating will result in higher cell number in response to FGF-2 and to greater chondrogenic differentiation in response to TGF-β1, compared to the desulfated heparin coating and a noncoated control.

MATERIALS AND METHODS

Heparin derivative synthesis

Desulfation of heparin was performed using a previously published protocol.[27] Briefly, heparin was mixed at 5 mg/mL in methanol (VWR, Radnor, PA) containing 0.5% v/v acetyl chloride (Thermo Fisher Scientific, Grand Island, NJ). A methyl ester of heparin product was synthesized by acidic methanol treatment for 6 days. The product was dissolved in H_2O and precipitated in an excess of 95% ethanol on ice. The methyl ester product was then precipitated in ethyl ether (Thermo Fisher Scientific) and vacuum dried using lyophilization (−40°C at 0.120 mmHg). Demethylation was performed by 0.1*M* potassium hydroxide treatment for 24 h to produce the final desulfated heparin, which was precipitated in ethanol and ethyl ether and vacuum dried via lyophilization. To produce biotinylated GAGs, heparin was dissolved in water at 2 mg/mL and conjugated with biotin by reacting N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) (Sigma–Aldrich, St. Louis, MO), hydroxybenzotriazole (HoBT) (VWR) and biotin-hydrazide (Sigma–Aldrich) at a molar excess compared to heparin of 0.4 for all reagents for 4 h at pH 5. Desulfated heparin was

dissolved at 2 mg/mL and reacted with EDC, HoBT, and biotinhydrazide at a molar of 3:3:8, respectively, for 4 h at pH 5. Each reaction solution was dialyzed for 2 days in 3500 MWCO dialysis tubing (Spectrum, Rancho Dominguez, CA) followed by flash freezing and vacuum drying via lyophilization for 2 days. All heparin products were stored at −20°C.

Proton nuclear magnetic resonance (^{1}H NMR) characterization

^{1}H NMR was used to assess level of sulfation after solvolytic desulfation of heparin and used to determine conjugation efficiency following biotinylation of heparin derivatives. Approximately 5 mg/mL of each product was dissolved in deuterated water and ^{1}H NMR experimented were conducted on a Bruker Avance III 400 spectrometer at 400 MHz. The resulting spectra were analyzed with ACD NMR Processor software (Version 12).

MSC expansion

MSCs derived from human bone marrow aspirates were obtained from the Texas A&M Health Sciences Center. Cryopreserved MSCs from three different donors (two males, one female; ages 22, 24, and 37) were thawed and expanded in α-minimum essential medium (Mediatech, Herndon, VA) containing 16.5% fetal bovine serum (FBS, Atlanta Biological, Atlanta, GA), 2 mM L-glutamine (Mediatech), 100 U/mL penicillin and 100 μg/mL streptomycin (Mediatech) under normoxic conditions (37°C, 5% CO_2, and 20% O_2). Media were changed every 2–3 days until 80% confluence and were used at passage 3.

Cell coating, aggregate formation, and culture

After lifting with 0.05% trypsin (Mediatech), MSCs were washed in phosphate buffered saline (PBS, Life Technologies, Grand Island, NY) two times and then modified with a glycosaminoglycan by multilayer assembly of biotin and avidin layers, as previously described.[26] Briefly, cells were first cultured in 4 mM EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) in PBS, followed by 0.5 mg/mL avidin in PBS (Life Technologies, Carlsbad, CA), and lastly 5 mg/mL biotin-conjugated heparin (Hep) or biotin-conjugated desulfated heparin (Hep-) in PBS (process outlined in Fig. 1A). Each incubation step with cells was performed in a 24-well ultra-low attachment surface plate (Corning) for 30 min at 37°C on a rotary orbital shaker plate at 65 rpm. Once cells were coated with their respective glycosaminoglycan layers, 2000 cells in 200 μL were pipetted into each well of a 5% pluronic-coated 96-well V-bottom plate to form individual aggregates. Plates were spun down at 200 rcf to promote spheroid formation in serum free media composed of Dulbecco's modified eagle medium, 1% nonessential amino acids, 1% antibiotic/antimycotic, 1% insulin, human transferrin, and selenous acid premix (BD Biosciences, San Jose, CA), and 50 μg/mL ascorbate-2-phosphate (Sigma–Aldrich). Aggregates were cultured in serum free conditions in the V-bottom wells with either 10 ng/mL of FGF-2 (R&D Systems, Minneapolis, MN) for DNA content assay or 10 ng/mL or TGF-β1 (Peprotech, Rocky Hill, NJ) and 100 nM dexamethasone (Sigma–Aldrich) for chondrogenic differentiation assays. Aggregates for chondrogenic differentiation were cultured under hypoxic conditions (37°C, 5% CO_2, and 3% O_2 and N_2).

Chromatography analysis of cell coating solutions

To quantify amount of GAG grafted to cell surfaces, supernatants were obtained immediately after GAG incubation of single cells prior to aggregate formation. Quantification of Hep or Hep- that was remaining in the supernatant was analyzed using a high-performance liquid chromatography system (Shimazdu, Columbia, MD). Duplicate samples were run in a 150 mM magnesium sulfate and 10 mM Tris base buffer through a TSK-GEL G4000PWX1 column (Tosoh Bioscience, King of Prussia, PA) for 30 min at a flow rate of 1 mg/mL. Eluted samples were detected using a UV detector at the wavelength of 256 nM. GAG concentration in each sample was calculated using a standard of known concentrations ranging from 0.05 to 5 mg/mL for both Hep and Hep- polymers. The amount of Hep or Hep- grafted onto cells was normalized to the cell number for that coating group ($n = 3$).

Histological staining

At appropriate time points, MSC aggregates were collected and washed with PBS to remove excess media. Aggregates were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific) for 15 min and embedded in Histogel (Thermo Fisher Scientific) before subsequent incubation in 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 frozen in mixture of dry ice and 100% ethanol.[28] Samples were stored at −80°C and cryosectioned at 10 μm thickness (Cryostar NX70; Thermo Fisher Scientific) prior to staining with hematoxylin & eosin (H&E).

DNA quantification

At appropriate time points, MSC aggregates were collected and washed with PBS three times to remove excess media. Aggregates were dissociated by incubation in 200 μL 0.05% trypsin for 20 min and mechanical disruption by pipetting. Samples were spun down at 6000 rcf and collected cells were washed with PBS 1x before storing in 500 μL of water containing 0.1% Triton-X100 (Sigma– Aldrich) at −20°C. Samples were subjected to three freeze-thaw cycles, in which samples were placed in a sonicating bath containing ice for 30 min, followed by freezing in the −80°C freezer for 1 h. Upon removal from the freezer, samples were thawed to room temperature for 30 min before repeating sonication and freezing cycle. Once samples were ready, DNA content was assayed using a CyQUANT® Cell Proliferation Assay (Life Technologies), according to manufacturer's protocol. Samples were read at excitation 485 nm, emission 525 nm by a plate reader (Biotek Synergy H4 Hybird Multi-Mode Microplate Reader, Winooski, VT) and DNA amount was determined using a standard curve of DNA. Data is reported as the DNA amount normalized to each group's initial amount at day 1 ($n = 8$).

Gene expression analysis (RT-PCR)

Over the course of 21 days, MSC aggregates were collected for gene expression analysis and lysed with RLT lysis buffer (Qiagen, Hilden, Germany). Each sample contained three

individually cultured aggregates to provide enough RNA for analysis. Cell lysates were filtered with QIAshredder tissue homogenizer and RNA was extracted with RNAeasy kit (Qiagen). Reverse transcription was performed using SuperScript III reverse transcriptase (Invitrogen) with Oligo$(dT)_{15}$ primers and nucleotides (Promega, Madison, WI). Primers were custom designed to target human mRNA for β-actin, 18s ribosomal protein, Sox9, collagen II, aggrecan, collagen I, collagen X, Runx2, and PPARγ2 (Table I). Quantitative RT-PCR amplification for each gene was performed on a StepOnePlus System (Applied Biosystems, Carlsbad, CA) with SYBR Green Master Mix (Applied Biosystems). Raw amplification values were processed in Lin-Reg software (v13.1, Amsterdam, Netherlands) to individually determine PCR efficiency and fold regulation relative to noncoated day 1 samples was determined for each sample with β-actin and 18s used as housekeeping gene controls ($n = 3–4$).

Immunofluorescent staining for matrix molecules

Immunostaining for ECM deposition in cryosectioned samples was performed using primary monoclonal antibodies for collagen II, aggrecan, collagen X, and collagen I (Abcam, Cambridge, UK; collagen X from Sigma–Aldrich). Antigen retrieval was performed for all sections by incubating in 20 μg/mL proteinase K (Sigma–Aldrich) for 10 min at 37°C. 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 overnight at 4°C (1:20 dilution for all primary antibodies). Secondary antibody binding with Alexa Fluor 488-conjugated goat polyclonal anti-mouse IgG (Molecular Probes, Carlsbad, CA) at room temperature for 1 h (1:200 dilution). Lastly, samples were stained with Hoechst (Sigma–Aldrich) for 5 min at room temperature to visualize cell nuclei ($n = 12$).

Statistical analysis—Quantitative data was transformed to fit a normal distribution using Box-Cox transformations, followed by a one-way and two-factor analysis of variance with Tukey's *post hoc* multiple comparisons test ($p < 0.05$) to determine statistical significance between samples in Minitab (v.15.1, State College, PA). Quantitative data are reported as mean ± standard deviation.

RESULTS

Coating characterization on MSC aggregates

Using ^{1}H NMR analysis, it was confirmed that solvolytic treatment of heparin resulted in removal of all sulfate groups. Furthermore, post biotinylation conjugation, ^{1}H NMR also determined that both heparin and desulfated heparin species were both biotinylated with a conjugation efficiency of approximately 20%. Based on chromatography analysis, at a coating concentration of 5 mg/mL of both heparin species, $9.89 \times 10^{-7} \pm 1.37 \times 10^{-7}$ mg GAG/cell of Hep and $8.53 \times 10^{-7} \pm 2.63 \times 10^{-8}$ mg GAG/cell of Hep-was grafted onto cell surfaces.

Histological staining of MSC aggregates

H&E staining revealed that the morphology of coated aggregates is distinct from noncoated aggregates. MSCs within aggregates coated with Hep and Hep- exhibited rounded cell morphology (Fig. 1B, black arrows). This behavior persisted through day 14, at which rounded cell morphologies were still observed. Coated aggregates cultured in media containing the growth factors FGF-2 or TGF-β1 also exhibited similar rounded cell morphology that persisted through 14 days in culture (data not shown).

Effect of GAG coatings on DNA content in MSC aggregates

DNA content assay of noncoated and coated aggregates revealed that the addition of FGF-2 caused an initial increase in DNA amount 7.2 ± 1.9 fold for Hep coated aggregates, which was significantly higher than the other two groups (2.54 ± 0.3 fold and 4.11 ± 0.8 fold for Hep- and noncoated aggregates, respectively) at day 4 (Fig. 2). At day 14 with FGF-2 exposure, while noncoated aggregates only had a 2.1 ± 0.9 fold increase in amount DNA, Hep coated aggregates increased 8.5 ± 1.6 fold, and Hep- coated aggregates increased 4.3 ± 1.4 fold, both of which were significantly higher compared to their respective group without FGF-2 and their respective group at day 1. At day 14, MSCs cultured without FGF-2 had DNA increases of 2.1 ± 0.9 fold in noncoated aggregates, 3.6 ± 1.6 fold in Hep coated aggregates and 1.1 ± 0.5 in Hep- coated aggregates, all of which were not significantly higher compared to their respective day 1 value.

Effect of GAG coatings on chondrocytic gene expression in MSC aggregates

Gene expression revealed that when noncoated and Hep and Hep aggregates were cultured with TGF-β1 under hypoxia, MSCs from Hep- coated aggregates demonstrated an 86.5 ± 7.5 fold upregulation of collagen II expression, which was significantly greater than the 37.7 ± 10.6 fold increase for Hep coated aggregates and the 15.1 ± 4.7 fold increase in noncoated aggregates at day 21 (Fig. 3A). At day 14, collagen II expression in Hep- coated aggregates was significantly increased compared to non-coated aggregates, while this was not observed Hep coated aggregates. Aggrecan expression was significantly upregulated compared to the day 1 level at day 7 in Hep coated aggregates only (8.2 ± 64.3 fold), however, it did not significantly increase further over time (19.3 ± 5.4 fold at day 21). For Hep- coated aggregates, aggrecan expression was significantly upregulated (15.9 ± 7.7 fold) only at day 21 (Fig. 3B) and was not significantly different than the expression levels observed in Hep coated aggregates. Interestingly, Sox9 expression was not detected over the course of the 21 days for all samples (data not shown).

Collagen I expression, indicative of fibroblastic differentiation, initially increased by day 7 but by day 21 was only expressed at 1.2 ± 0.1 fold in noncoated groups, 1.8 ± 0.5 fold in Hep coated groups and 0.8 ± 0.6 fold in Hep- group (Fig. 3D), all of which were not significantly different from each other or compared to day 1. At day 7, Hep and Hep- coated aggregates had significantly upregulated expression of collagen I (3.14 ± 0.5 fold and 3.68 ± 0.2 fold, respectively) when compared to noncoated aggregates (2.37 ± 0.2 fold). Collagen X expression, an ECM marker of hypertrophic chondrocytes, gradually increased over time for all groups but was significantly upregulated in Hep-coated aggregates over both noncoated and Hep coated aggregates at day 21. Hep- coated aggregates exhibited a 37.1

± 4.7 fold upregulation on day 21 compared to an 18.4 ± 8 fold regulation in Hep coated and 11.8 ± 4.4 fold regulation in noncoated (Fig. 3C). By day 14, all groups had significantly upregulated expression of collagen X compared to its respective day 1 level (13.1 ± 1.8 fold for noncoated, 15.91 ± 0.3 for Hep coated, and 28.9 ± 7.7 for Hep- coated), however, only Hep- coated aggregates exhibited significant upregulation compared to the other two groups at that time point and its day 1 level as early at day 7 (14.28 ± 1.4 fold). No trends in expression levels were observed for Runx2, an osteogenic marker, and PPARγ2, an adipogenic marker, over time for all groups (data not shown).

Effect of GAG coatings on chondrocytic ECM deposition in MSC aggregates

Immunostaining was performed to visualize specific ECM component deposition. On day 14, coated groups exhibited similar levels of staining for collagen II and appeared slightly stronger compared to noncoated aggregates (Fig. 4). This increase in deposition for coated groups was observed as early as day 14. Additionally, at day 14, there appeared to be increased aggrecan staining in all samples, however, at day 14 and 21, both Hep and Hep- coated groups seemed to demonstrate stronger positive staining compared to non-coated samples (Fig. 5). Positive staining for collagen X was obvious for all groups by day 14, at which time, differences could already be discerned between noncoated and coated aggregates. By day 21, Hep- coated aggregates exhibited pockets of pericellular collagen X, which was not observed for noncoated and Hep coated aggregates (Fig. 6, insets). Collagen I staining was detected throughout all noncoated and coated aggregates, however, the staining intensity was not observed to change over the course of 21 days within each group (Supplementary Material Fig. 4).

DISCUSSION

In this study, we have demonstrated the ability to coat MSC surfaces with different heparin species (Hep and Hep-) using an established layer-by-layer technology. Via chromatography analysis, the same GAG concentration (5 mg/mL) in the coating solution resulted in similar amounts of each heparin species grafted onto cell aggregates. Moreover, using confocal microscopy imaging, over time, both fluorescent coatings decreased at similar rates (Supplementary Material Fig. 1). Therefore, we believe that similar amounts of Hep or Hep- are grafted on cell surfaces within the aggregate at any time point and that the different effects observed between heparin species are thus not a result of differing amounts of GAGs present in the system.

After coating, H&E staining over time revealed rounded cell morphology within coated aggregates (Fig. 1B). This phenomenon is specific to the presence of a GAG layer, as seen by the lack of rounded cell morphology in aggregates coated only with biotin and avidin (Supplementary Material Fig. 2). Additionally, when soluble heparin is introduced into the media, the morphology looks similar to that of the noncoated group (Supplementary Material Fig. 3A). While the mechanism is unknown, we conclude that Hep and Hep- coatings on cell surfaces can affect the organization and packing of the cells within the aggregate to produce the unique rounded cellular morphology observed in areas of the aggregate.

It is known that MSCs have limited proliferation capacity when cultured as aggregates in long-term suspension systems.[29] Because cell-based therapies require high dosages, developing a system that utilizes the mitogenic growth factor FGF-2 to improve proliferation may be necessary. Increased cell number over time was observed in MSC aggregates when both the Hep coating and FGF-2 were present in the system. Because heparin is known to preserve the bioactivity of FGF-2,[30,31] it is possible that the presence of a Hep coating may simply be maintaining higher levels of active growth factor in the culture media. However, because this effect was not observed in MSC aggregates cultured with FGF-2 and heparin added to the culture media (Supplementary Material Fig. 3B), the response of increased cell number is unique to the pairing of the cell surface presentation of heparin and the presence of the mitogenic growth factor.

FGF-2 signaling occurs when dimerization of the growth factor and cell surface receptor, facilitated by heparin interactions between the 2-O and N-sulfate groups and growth factor, occurs.[32–34] Thus, the presence of the Hep coating could facilitate the sequestration of FGF-2 within the aggregate, as well as could promote signaling through dimerization of the receptor, together resulting in the increased cell number observed in these studies (Fig. 2). Similar results have been observed when heparin was immobilized onto 2D surfaces and cultured with exogenous growth factors found in serum. The cultured cells on this 2D system exhibited increased proliferation when compared to MSCs cultured on surfaces without heparin.[35] Additionally, when heparin is cross-linked into a 3D hydrogel and loaded with FGF-2, the encapsulated MSCs undergo increased angiogenesis and proliferation when compared to hydrogels not containing heparin and loaded FGF-2.[9,35] While the Hep-coating may still be able to sequester FGF-2 locally in the aggregate, due to an overall negative charge that exists because of the remaining carboxyl groups, it may lack the sulfate groups necessary for signaling of FGF-2 to cause cell proliferation,[20] resulting in DNA content more similar to the noncoated controls in this system. Taken together, these results suggest that a heparin coating combined with mitogenic growth factors in the media may help address the reduced proliferation capacity and thus could be used as a system for expansion of MSC aggregates for subsequent administration in cell-based therapies such as treating graft versus host or autoimmune diseases.

However, for applications of MSCs in cartilage repair, cell expansion alone may not be sufficient, and systems that aim to differentiate MSCs down a chondrogenic pathway may be required to develop effective therapies. TGF-β1 is a potent growth factor known to induce chondrogenic differentiation.[36] Thus, the effects of our GAG coatings on the response of MSC aggregates to TGF-β1 in the media was examined to better understand how this system can be utilized for stem cell-based therapies to treat cartilage injury or disease. When coated and cultured in the presence of TGF-β1, Hep-coated aggregates exhibited more upregulation of the chondrogenic gene marker collagen II (nearly 90 fold) by day 21 compared to both noncoated and Hep coated aggregates, indicating that a Hep- coating can promote increased chondrocytic differentiation in MSC aggregates. Additionally, both noncoated and Hep coated aggregates had significantly higher collagen II expression at day 21 when compared to day 1, thus while the Hep coating may not promote high expression levels of collagen II, as seen with Hep- samples, it does not diminish the ability of the MSC aggregates to differentiation down a chondrogenic lineage. Overall increase in gene expression was

matched by an apparent increase in staining for collagen II over time, however, intensity for the two coated groups looked similar at day 21 (Fig. 4).

Gene expression for aggrecan, another chondrogenic ECM molecule, was upregulated in Hep coated aggregates at day 14, followed by upregulation in Hep- coated aggregates at day 21 [Fig. 3(B)]. In contrast, increase in aggrecan deposition was seen as early as day 14 for all groups (Fig. 5) via immunostaining, and both types of coated aggregates appeared to exhibit stronger positive staining for aggrecan at day 21, compared to noncoated aggregates. The differences observed in gene expression and immunostaining are somewhat similar to previous work in our laboratory with chondroitin sulfate microparticles incorporated in human MSCs aggregates, in which differences in aggrecan gene expression, but not immunostaining, were observed between groups treated with GAG versus untreated.[28] This could be due, in part, to post-transcriptional regulation of ECM production that was not captured in the PCR results.[37] Although Sox9 expression was not detected in this system, it has been shown that collagen II expression is not correlated to levels of Sox9 expression in adult chondrocytes[38] and that Sox9 expression is a regulator of chondrogenic differentiation typically expressed early during the differentiation process.[39] Therefore, it may be possible that differentiation in this system is occurring during the 21 day culture time and end points earlier than day 7 may need to be performed to capture the initial upregulation of Sox9 expression.

While collagen I expression was upregulated for coated groups at days 7 and 14 and decreased by day 21 (Fig. 3C), minimal differences in positive staining for ECM deposition was observed over time in each group (Supplementary Material Fig. 5). This discrepancy can be explained by the fact that the magnitude of fold regulation increase in the system (around threefold) may not be enough to elicit a visual increase in deposition of the ECM protein. Additionally, the level of collagen I expression and staining is consistent with what has previously been shown in our laboratory with this cell type,[28] and may be a result of basal-level production of this molecule throughout culture. When the collagen II/collagen I ratio, a measure used to assess chondrocytic differentiation in MSCs,[40] was calculated, all aggregates exhibited an upregulation (18.2 ± 3.23 fold for noncoated, 19.7 ± 8.0 fold for Hep coated, 182.2 ± 40 fold for Hep- coated) at day 21. While collagen I is expressed in this system, the increased ratio between collagen II to collagen I indicates that MSCs in this system are favoring a chondrocytic pathway, rather than a more fibroblastic pathway.

Expression of collagen X, a marker for MSC hypertrophic chondrogenic differentiation, increased over time for all groups, however, it was the greatest for Hep- aggregates by day 21 [Fig. 3(D)]. This was also observed in the staining for ECM deposition, in which bright pockets of pericellular collagen X were observed in Hep- coated aggregates on day 21 (Fig. 6). Taken together, this indicates that coated MSC aggregates may become hypertrophic in our system, a result that has been previously observed in our laboratory[10,28] and reported in other studies with human MSCs, including those with larger cell aggregates.[11,41] This demonstrates that while many different platforms of MSC chondrogenesis exist, preventing hypertrophy remains a key challenge in cartilage tissue engineering, and additional culture methods, such as exposure to parathyroid hormones or co-culture with chondrocytes may be required to prevent hypertrophic differentiation during *in vitro* culture.[42–44] Although

collagen X upregulation was measured in this MSC aggregate system, gene expression of other lineage markers, Runx2 (osteogenic), and PPARγ2 (adipogenic) were minimally changed or not expressed (data not shown), suggesting that those differentiation pathways were not favored in this system.

Although Hep coatings were seen to promote proliferation in response to the growth factor FGF-2 in MSC aggregates, upregulated chondrogenic marker expression was observed in Hep- coated aggregates in response to TGF-β1. It has previously been shown that the addition of GAG species with decreased sulfation level compared to heparin (such as hyaluronan and chondroitin sulfate) can activate or bind CD44, a cell surface receptor known to complex with the TGF-β1 receptor and activate downstream effector functions without the presence of TGF-β1.[45–47] This demonstrates that multiple GAG species have potential to elicit TGF-β1 signaling in a manner that does not require binding between the GAG and growth factor. Another factor that can play a role when negatively charged GAGs are introduced is the change in osmotic swelling pressure within the aggregate. Previous studies have shown that increasing osmolarity can increase chondrogenic marker expression and matrix synthesis in MSC and progenitor cell systems undergoing chondrogenic differentiation.[48,49]

While these possibilities may play a role in the observed effects with the Hep- coating, it was seen that coated aggregates cultured without TGF-β1 did not exhibit chondrogenic differentiation (data not shown), suggesting that the effects observed are a result of the coating interacting with the chondrogenic growth factor to promote a cellular response. It has been shown that the 6-O and N-sulfate groups play a role in the interaction of heparin with TGF-β1, and upon desulfation of certain groups, the affinity for the growth factor decreases.[25] We speculate that in this coating system, the strong binding of TGF-β1 to heparin may have prevented growth factor interaction with its receptor, resulting in reduced chondrogenic effects when compared to Hep-coated MSCs. The effect of desulfation has also been observed in another system in our laboratory, in which in the presence of TGF-β1, MSCs encapsulated in desulfated chondroitin hydrogels had significantly greater expression of collagen II, aggrecan, and collagen X when compared to MSCs in natively sulfated chondroitin sulfate hydrogels.[10] This provides evidence that although the desulfated forms of different GAGs may have a lower affinity for TGF-β1, their effect on cellular response is not dictated by that binding interaction. While these results support the concept that GAG cell coatings can be used to improve the effect and presentation of growth factors in culture, it is important to consider that a “one GAG fits all” strategy may not be optimal for all MSC culture applications and that non-native sulfation patterns may have the capability to potentiate the activity of specific growth factors. While, specifically, for cartilage applications, an additional dissociation and coating step may need to be included to achieve both enhanced proliferation and enhanced chondrogenesis, overall, these results suggest that GAG-based coatings may be extremely useful for one or both steps in this process, depending on the needs of the supplier. Thus, our coating technology represents a versatile platform to design MSC culture systems with pairings of GAGs and growth factors that can be tailored to overcome specific challenges in scale-up and culture for MSC-based therapeutics.

CONCLUSION

These studies have demonstrated that GAG coatings have the ability to modulate growth factor interactions with MSC aggregates. By using layer-by-layer technology, biotinylated GAGs can be grafted onto cells within the MSC aggregate at similar amounts, thus enabling control over both the amount and sulfation level of heparin at cell surfaces. When cultured in the presence of the mitogenic growth factor FGF-2, natively sulfated Hep coatings were able to increase cell number over the culture period of 2 weeks greater than both Hep- coated and noncoated aggregates. On the contrary, when cultured in the presence of the chondrogenic growth factor TGF-β1, Hep- coated aggregates exhibited the greatest expression of collagen II and collagen X, gene markers for chondrogenic and hypertrophic differentiation. The finding that heparin coatings of two different sulfation levels can result in different responses to two distinct growth factors indicates that this novel cell coating platform that enables specific pairings of growth factors with GAG sulfation patterns could be a potent future means of modifying cell response to address a specific limitation during scale-up culture of stem cells for cell-based therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Mendicino M, Bailey AM, Wonnacott K, Puri RK, Bauer SR. MSC-based product characterization for clinical trials: An FDA perspective. Cell Stem Cell. 2014; 14:141–145. [PubMed: 24506881]
2. Trounson A, McDonald C. Stem cell therapies in clinical trials: Progress and challenges. Cell Stem Cell. 2015; 17:11–22. [PubMed: 26140604]
3. Ankrum J, Karp JM. Mesenchymal stem cell therapy: Two steps forward, one step back. Trends Mol Med. 2010; 16:203–209. [PubMed: 20335067]
4. Ayerst Bethanie I, Day Anthony J, Nurcombe V, Cool Simon M, Merry Catherine LR. New strategies for cartilage regeneration exploiting selected glycosaminoglycans to enhance cell fate determination. Biochem Soc Trans. 2014; 42:703–709. [PubMed: 24849240]
5. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006; 98:1076–1084. [PubMed: 16619257]
6. Winter A, Breit S, Parsch D, Benz K, Steck E, Hauner H, Weber RM, Ewerbeck V, Richter W. Cartilage-like gene expression in differentiated human stem cell spheroids: A comparison of bone marrow-derived and adipose tissue-derived stromal cells. Arthritis Rheum. 2003; 48:418–429. [PubMed: 12571852]

7. Zhang L, Su P, Xu C, Yang J, Yu W, Huang D. Chondrogenic differentiation of human mesenchymal stem cells: A comparison between micromass and pellet culture systems. Biotechnol Lett. 2010; 32:1339–1346. [PubMed: 20464452]
8. Solchaga LA, Penick K, Goldberg VM, Caplan AI, Welter JF. Fibroblast growth factor-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone-marrow-derived mesenchymal stem cells. Tissue Eng Part A. 2010; 16:1009–1019. [PubMed: 19842915]
9. Zieris A, Prokoph S, Levental KR, Welzel PB, Grimmer M, Freudenberg U, Werner C. FGF-2 and VEGF functionalization of starPEG-heparin hydrogels to modulate biomolecular and physical cues of angiogenesis. Biomaterials. 2010; 31:7985–7994. [PubMed: 20674970]
10. Lim JJ, Temenoff JS. The effect of desulfation of chondroitin sulfate on interactions with positively charged growth factors and upregulation of cartilaginous markers in encapsulated MSCs. Biomaterials. 2013; 34:5007–5018. [PubMed: 23570717]
11. Markway BD, Tan GK, Brooke G, Hudson JE, Cooper-White JJ, Doran MR. Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures. Cell Transplant. 2010; 19:29–42. [PubMed: 19878627]
12. Romo P, Madigan MC, Provis JM, Cullen KM. Differential effects of TGF-β and FGF-2 on in vitro proliferation and migration of primate retinal endothelial and Muller cells. Acta Ophthalmol. 2011; 89:e263–8. [PubMed: 20670342]
13. Carlsberg A, Pucci B, Rallapalli R, Tuan RS, Hall D. Efficient chondrogenic differentiation of mesenchymal cells in micromass culture by retroviral gene transfer of BMP-2. Differentiation. 2000; 67:128–138.
14. Bartosh TJ, Ylostalo JH, Mohammadipoor A, Bazhanov N, Coble K, Claypool K, Lee RH, Choi H, Prockop DJ. Aggregation of human mesenchymal stromal cells into 3D spheroids enhances their antiinflammatory properties. Proc Natl Acad Sci. 2010; 107:13724–12729. [PubMed: 20643923]
15. Zimmermann JA, McDevitt TC. Pre-conditioning mesenchymal stromal cell spheroids for immunomodulatory paracrine factor secretion. Cytotherapy. 2014; 16:331–345. [PubMed: 24219905]
16. Hayashi K, Tabata Y. Preparation of stem cell aggregates with gelatin microspheres to enhance biological functions. Acta Biomater. 2011; 7:2797–2803. [PubMed: 21549223]
17. Hortensius RA, Becraft JR, Pack DW, Harley BA. The effect of glycosaminoglycan content on polyethylenimine-based gene delivery within three-dimensional collagen-GAG scaffolds. Biomater Sci. 2015; 3:645–654. [PubMed: 26097698]
18. Liang Y, Kiick KL. Heparin-functionalized polymeric biomaterials in tissue engineering and drug delivery applications. Acta Biomater. 2014; 10:1588–1600. [PubMed: 23911941]
19. Nie T, Baldwin A, Yamaguchi N, Kiick KL. Production of heparin-functionalized hydrogels for the development of responsive and controlled growth factor delivery systems. J Control Release. 2007; 122:287–296. [PubMed: 17582636]
20. Lundin L, Larsson H, Kreuger J, Kanda S, Lindahl U, Salmivirta M, Claesson-Welsh L. Selectively desulfated heparin inhibits fibroblast growth factor-induced mitogenicity and angiogenesis. J Biol Chem. 2000; 275:24653–24660. [PubMed: 10816596]
21. Miller T, Goude MC, McDevitt TC, Temenoff JS. Molecular engineering of glycosaminoglycan chemistry for biomolecule delivery. Acta Biomater. 2014; 10:1705–1719. [PubMed: 24121191]
22. Bishop JR, Schuksz M, Esko JD. Heparan sulphate proteoglycans fine-tune mammalian physiology. Nature. 2007; 446:1030–1037. [PubMed: 17460664]
23. Sarrazin S, Lamanna WC, Esko JD. Heparan sulfate proteoglycans. Cold Spring Harb Perspect Biol. 2011; 3:1–33.
24. Nakamura S, Ishihara M, Obara K, Masuoka K, Ishizuka T, Kanatani Y, Takase B, Matsui T, Hattori H, Sato T, Kariya Y, Maehara T. Controlled release of fibroblast growth factor-2 from an injectable 6-O-desulfated heparin hydrogel and subsequent effect on in vivo vascularization. J Biomed Mater Res A. 2006; 78:364–371. [PubMed: 16673389]
25. Lee J, Wee S, Gunaratne J, Chua J, Smith R, Ling L, Fernig D, Nurcombe V, Cool SM. Structural determinants of heparin-transforming growth factor-β1 interactions and their effects on signaling. Glycobiology. 2015; 25:1491–1504. [PubMed: 26306634]

26. Lei J, McLane LT, Curtis JE, Temenoff JS. Characterization of a multilayer heparin coating for biomolecule presentation to human mesenchymal stem cell spheroids. Biomater Sci. 2014; 2:666–673. [PubMed: 25126416]
27. Seto SP, Miller T, Temenoff JS. Effect of selective heparin desulfation on preservation of bone morphogenetic protein-2 bioactivity after thermal stress. Bioconjug Chem. 2015; 26:286–293. [PubMed: 25621929]
28. Goude MC, McDevitt TC, Temenoff JS. Chondroitin sulfate micro-particles modulate transforming growth factor-β1-induced chondrogenesis of human mesenchymal stem cell spheroids. Cells Tissues Organs. 2014; 199:117–130. [PubMed: 25413333]
29. Sart S, Tsai AC, Li Y, Ma T. Three-dimensional aggregates of mesenchymal stem cells: Cellular mechanisms, biological properties, and applications. Tissue Eng Part B Rev. 2014; 20:365–380. [PubMed: 24168395]
30. Zomer Volpato F, Almodovar J, Erickson K, Popat KC, Migliaresi C, Kipper MJ. Preservation of FGF-2 bioactivity using heparin-based nanoparticles, and their delivery from electrospun chitosan fibers. Acta Biomater. 2012; 8:1551–1559. [PubMed: 22210184]
31. Gospodarowicz D, Cheng J. Heparin protects basic and acidic FGF from inactivation. J Cell Physiol. 1986; 128:475–484. [PubMed: 3528177]
32. Esko, JD., Linhardt, RJ. Proteins that bind sulfated glycosaminoglycans. In: Varki, A.Cummings, RD., Esko, JD., editors. Essentials of Glycobiology. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2009.
33. Baird A, Schubert D, Ling N, Guillemin R. Receptor- and heparin-binding domains of basic fibroblast growth factor. Proc Natl Acad Sci. 1988; 85:2324–2328. [PubMed: 2832850]
34. Bellosta P, Iwahori A, Plotnikov AN, Eliseenkova AV, Basilico C, Mohammadi M. Identification of receptor and heparin binding sites in fibroblast growth factor 4 by structure-based mutagenesis. Mol Cell Biol. 2001; 21:5946–5957. [PubMed: 11486033]
35. Hudalla GA, Koepsel JT, Murphy WL. Surfaces that sequester serum-borne heparin amplify growth factor activity. Adv Mater. 2011; 23:5415–5418. [PubMed: 22028244]
36. Orth P, Rey-Rico A, Venkatesan JK, Madry H, Cucchiarini M. Current perspectives in stem cell research for knee cartilage repair. Stem Cells Cloning. 2014; 7:1–17. [PubMed: 24520197]
37. Lodish H. Post-translational modification of proteins. Enzyme Microb Technol. 1981; 3:178–188.
38. Aigner T, Gebhard PM, Schmid E, Bau B, Harley V, PûÑschl E. SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes. Matrix Biol. 2003; 22:363–372. [PubMed: 12935820]
39. Akiyama H. Control of chondrogenesis by the transcription factor Sox9. Modern Rheumatol. 2008; 18:213–219.
40. Marlovits S, Hombauer M, Vescei V, Schlegel W. Changes in the ratio of type-I and type-II collagen expression during monolayer culture of human chondrocytes. J Bone Joint Surg Br. 2004; 86:286–295. [PubMed: 15046449]
41. Steinmetz N, Bryant S. Chondroitin sulfate and dynamic loading alter chondrogenesis of human MSCs in PEG hydrogels. Biotechnol Bioeng. 2012; 109:2671–2682. [PubMed: 22511184]
42. Chen S, Fu P, Cong R, Wu H, Pei M. Strategies to minimize hypertrophy in cartilage engineering and regeneration. Genes Diseases. 2015; 2:76–95. [PubMed: 26000333]
43. Fischer J, Aulmann A, Dexheimer V, Grossner T, Richter W. Intermittent PTHrP(1-34) exposure augments chondrogenesis and reduces hypertrophy of mesenchymal stromal cells. Stem Cells Dev. 2014; 23:2513–2523. [PubMed: 24836507]
44. Fischer J, Dickhut A, Rickert M, Richter W. Human articular chondrocytes secrete parathyroid hormone-related protein and inhibit hypertrophy of mesenchymal stem cells in coculture during chondrogenesis. Arthritis Rheum. 2010; 62:2696–2706. [PubMed: 20496422]
45. Bourguignon LY, Singleton PA, Zhu H, Zhou B. Hyaluronan promotes signaling interaction between CD44 and the transforming growth factor β receptor I in metastatic breast tumor cells. J Biol Chem. 2002; 277:39703–39712. [PubMed: 12145287]
46. Hascall, V., Esko, JD. Essentials of Glycobiology. In: Cummings, RD., Esko, JD., editors. Hyaluronan. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2009.

47. Lo YL, Sung KH, Chiu CC, Wang LF. Chemically conjugating polyethylenimine with chondroitin sulfate to promote CD44-mediated endocytosis for gene delivery. Mol Pharm. 2013; 10:664–676. [PubMed: 23281918]

48. Bertram K, Krawetz R. Osmolarity regulates chondrogenic differentiation potential of synovial fluid derived mesenchymal progenitor cells. Biochem Biophys Res Commun. 2012; 422:455–461. [PubMed: 22579684]

49. Caron M, van der Windt A, Emans P, van Rhijn L, Jahr H, Welting T. Osmolarity determines the in vitro chondrogenic differentiation capacity of progenitor cells via nuclear factor of activated T-cells 5. Bone. 2013; 53:94–102. [PubMed: 23219947]

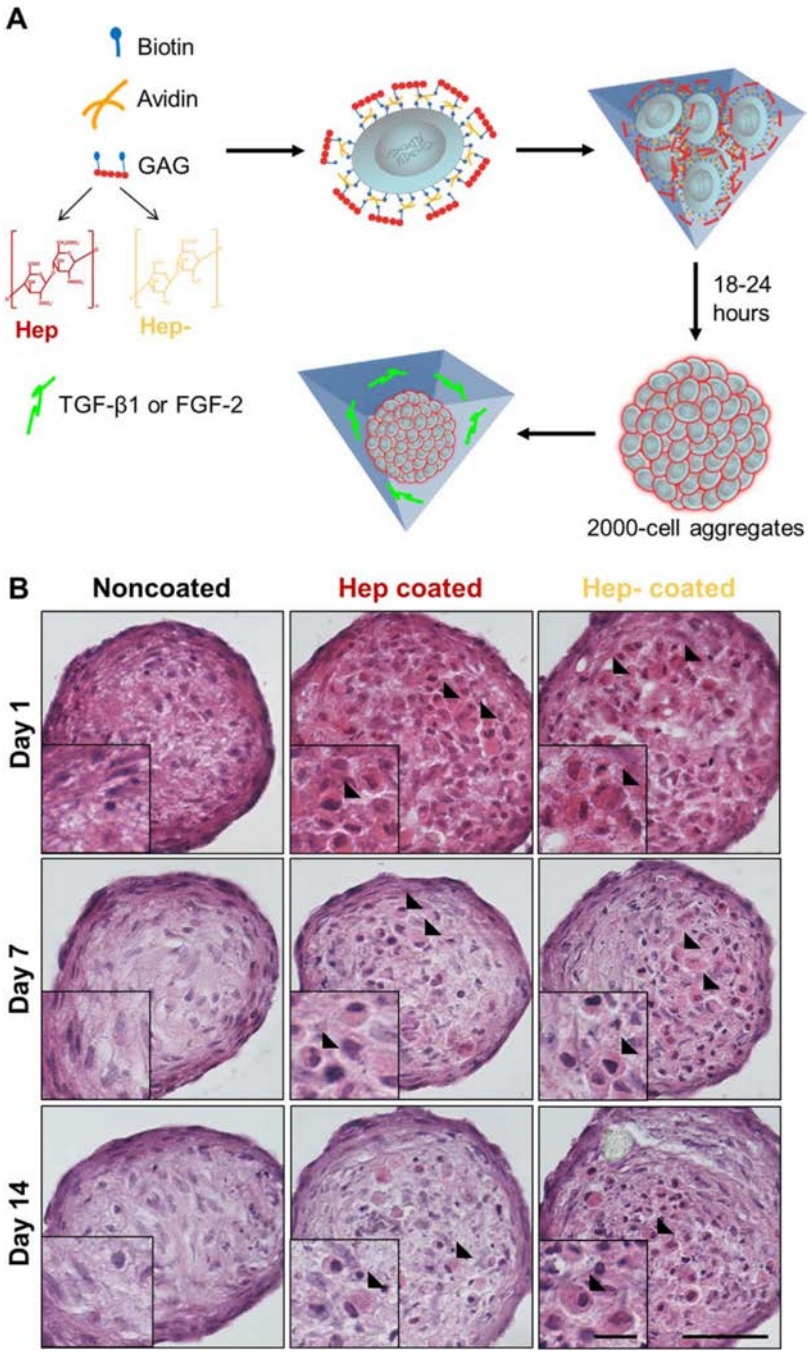

FIGURE 1.

Hep and Hep- coated cells exhibit rounded cell morphology within aggregates. (A) Schematic of layer-by-layer coating technology and MSC aggregate formation. (B) H&E staining demonstrates rounded cell morphology observed in coated aggregates at days 1, 7, and 14. Arrowheads indicate cells with rounded morphology within the aggregate. Scale bar = 100 μm, inset scale bar = 25 μm, $n = 8$.

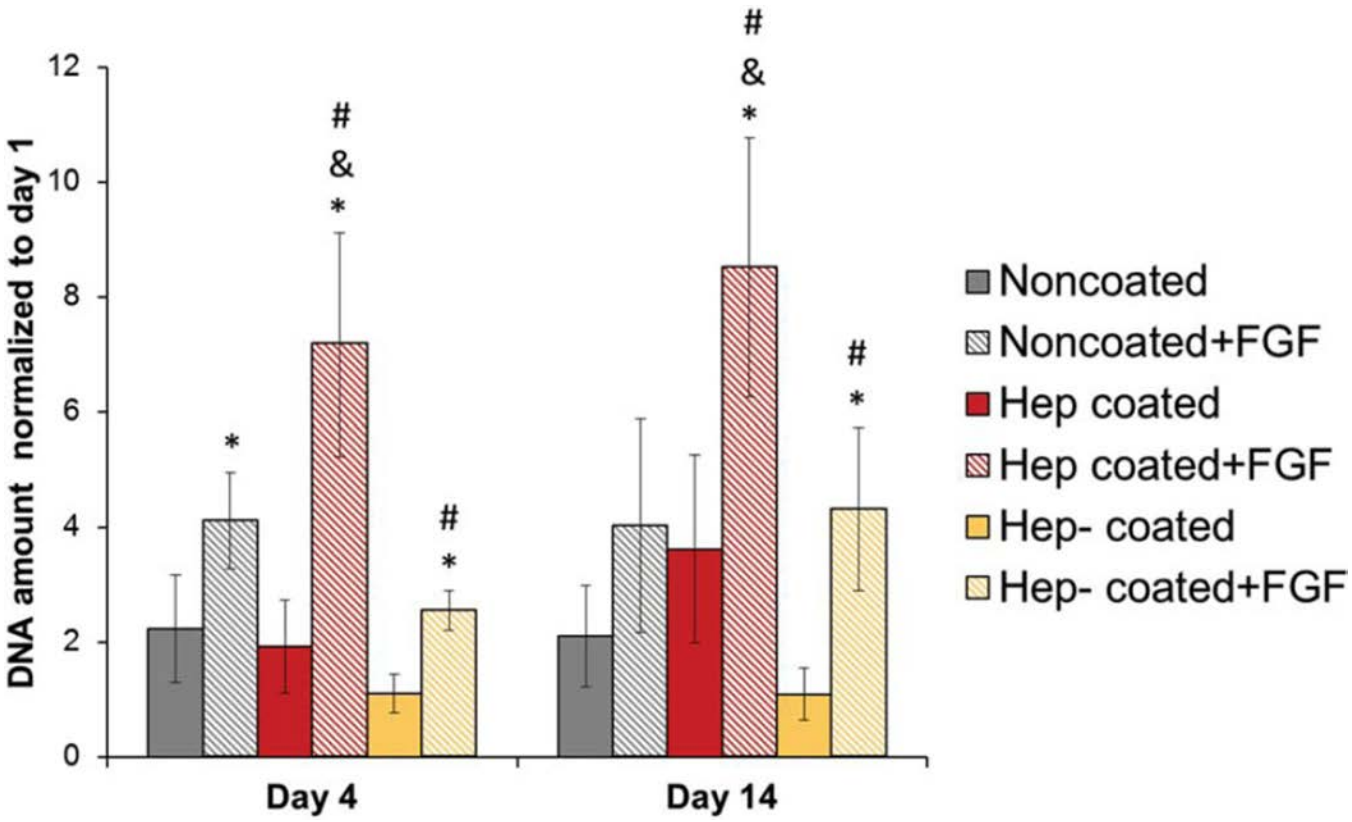

FIGURE 2.

DNA content of Hep coated aggregates cultured with FGF-2 is significantly greater at days 4 and 14 compared to other groups cultured with FGF-2. * indicates significant difference from respective coating group without FGF on same day; & indicates significant difference from noncoated and Hep- coated cultured with FGF on same day; # indicates significant difference from same group at day 1. Data reported as average mean ± standard deviation, $p < 0.05$, $n = 8$.

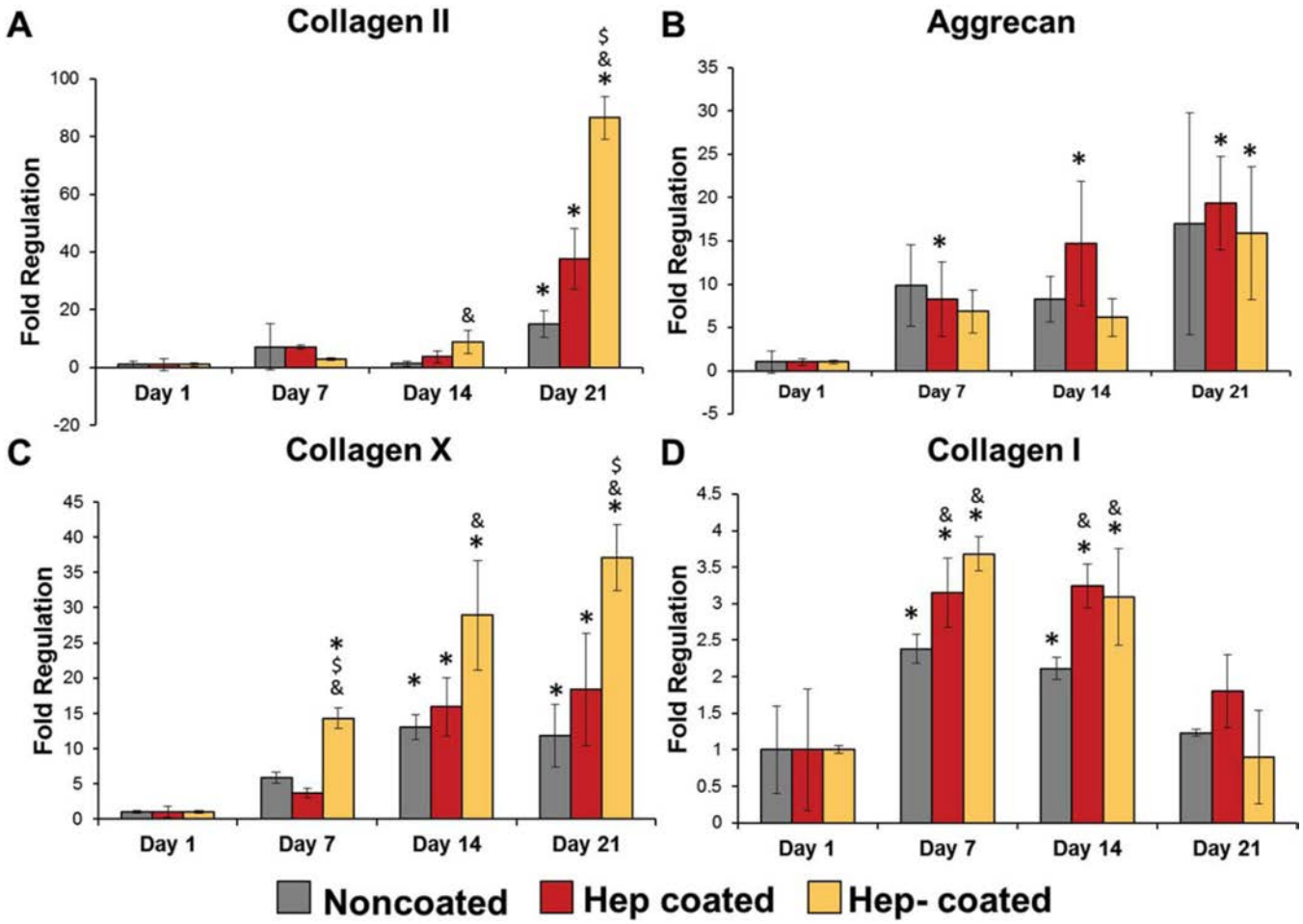

FIGURE 3.

MSC aggregates coated with Hep- upregulate gene expression for collagens II and X compared to noncoated and Hep coated controls. Summary of gene expression for (A) collagen II and (B) aggrecan gene expression, markers for chondrocytic differentiation, (C) collagen X gene expression, a marker for chondrocyte hypertrophy and (D) collagen I gene expression, a marker for fibroblast/fibrochondrocyte differentiation. * indicates significantly greater than respective coating group at day 1. & indicates significantly greater than noncoated on same day. $ indicates significantly greater than Hep coated on same day. Data reported as average mean ± standard deviation, $p < 0.05$, $n = 3–4$.

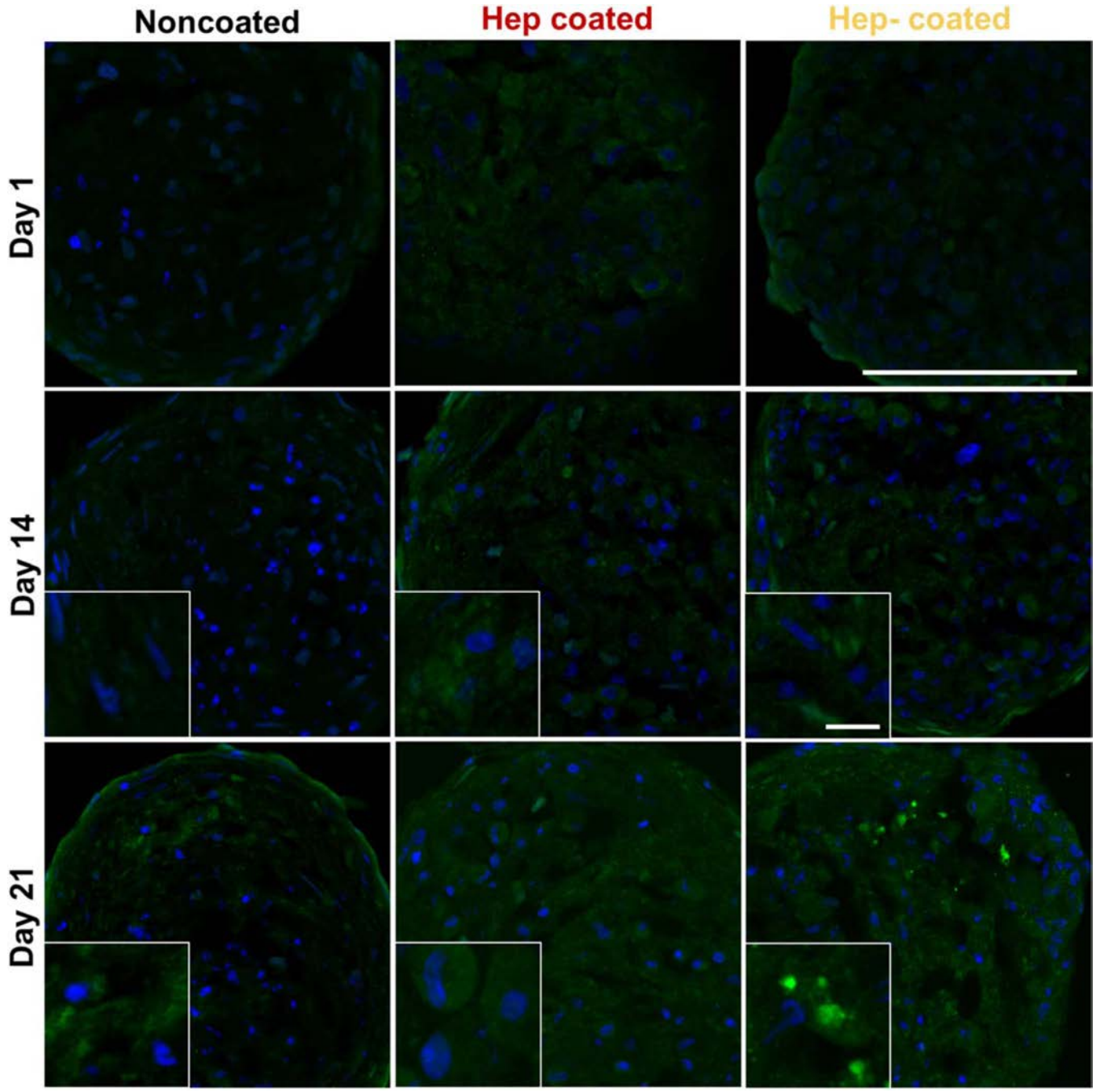

FIGURE 4.

Positive immunofluorescent staining of collagen II appeared to increase over 21 days for all aggregates; however, Hep and Hep-coated aggregates exhibited stronger staining compared to noncoated aggregates at day 21. Collagen II is seen in green and cell nuclei are stained blue. Scale bar = 100 μm, inset scale bar = 10 μm, $n = 12$.

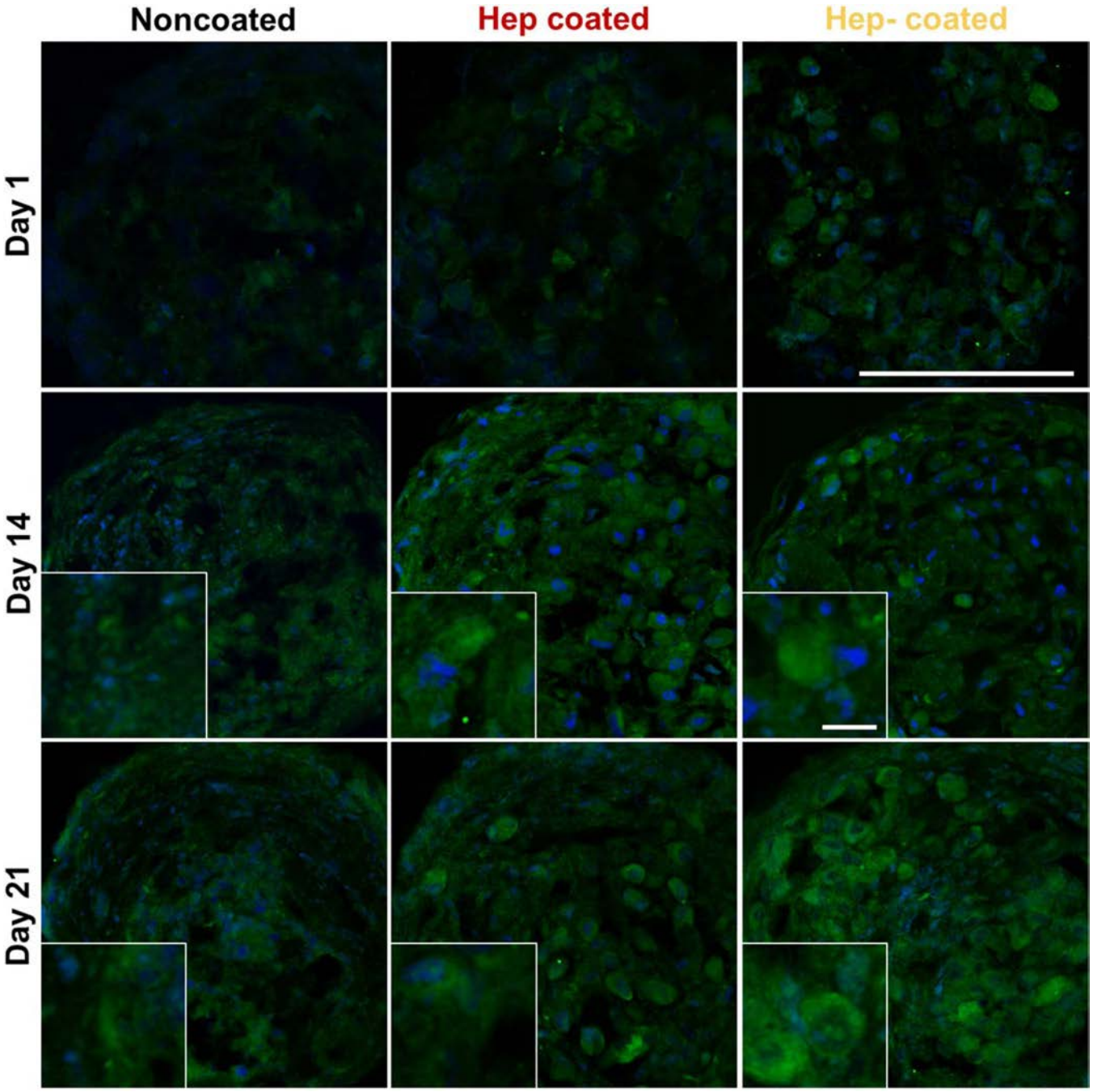

FIGURE 5.

Positive immunofluorescent staining for aggrecan appeared to increase by day 14 for all aggregates. Aggrecan is seen in green and cell nuclei are stained blue. Scale bar = 100 μm, inset scale bar = 10 μm, $n = 12$.

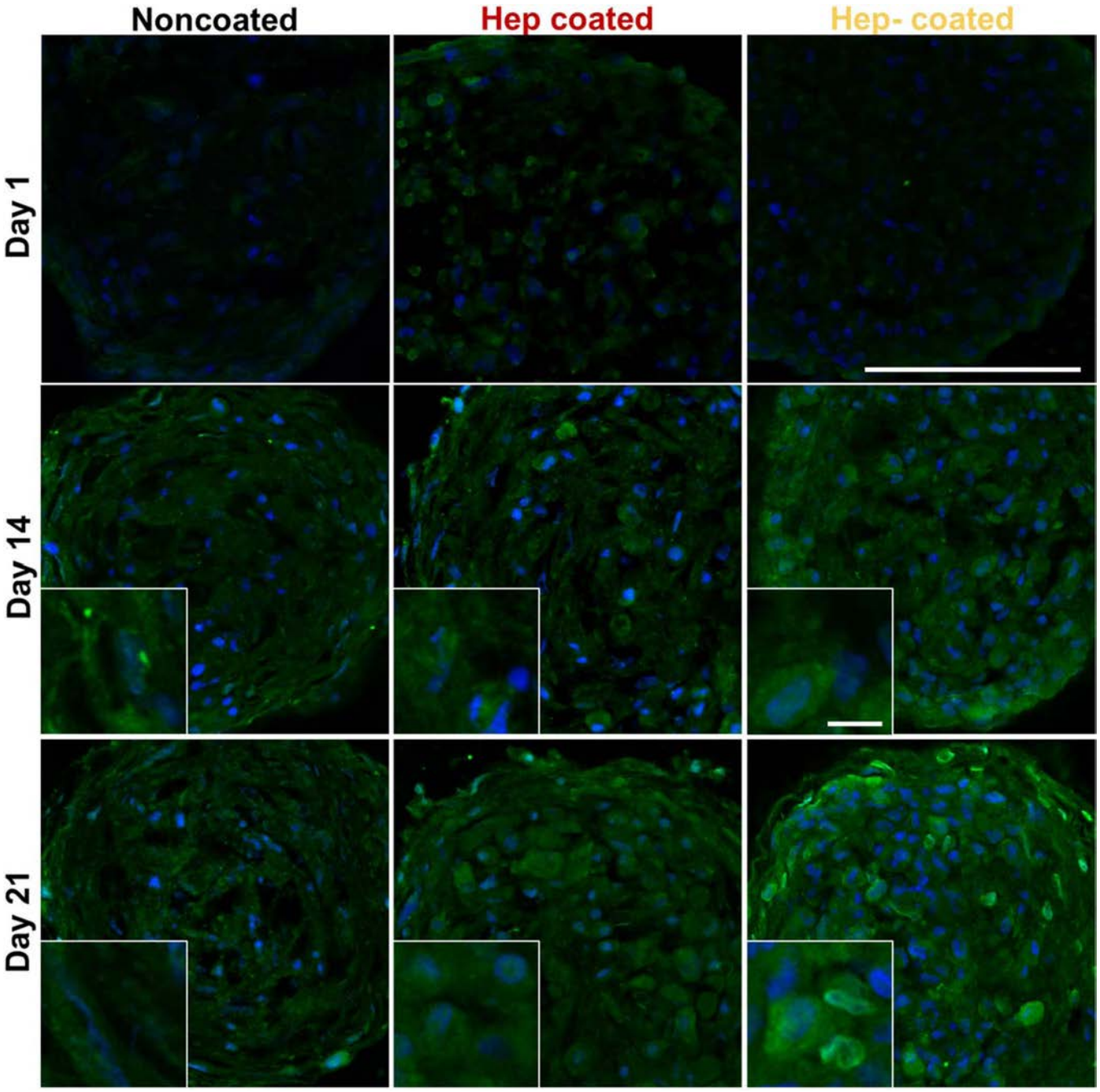

FIGURE 6.

Positive immunofluorescent staining for collagen X appeared to increase by day 14 for all aggregates, and Hep- coated aggregates exhibited stronger staining compared to Hep coated and noncoated aggregates at day 21. Collagen X is seen in green and cell nuclei are stained blue. Scale bar = 100 μm, inset scale bar = 10 μm, $n = 12$.

TABLE I

Human Primer Sequences for Quantitative PCR

Target	Marker		Primer Sequences (5′→3′)	GenBank
β-actin	Housekeeping	F	GCAGTCGGTTGGAGCGAGCATCCCC	NM_001101
		R	TCCCCTGTGTGGACTTGGGAGAGGAC	
Ribosomal 18s	Housekeeping	F	CGATGGGCGGCGGAAAATAGCCTTTGC	NM_022551
		R	CAGTGGTCTTGGTGTGCTGGCCTCGG	
Sox9	Chondrogenic	F	GCGGAGGAAGTCGGTGAAGAACGGGCA	NM_000346
		R	TGTGAGCGGGTGATGGGCGGG	
Collagen II	Chondrogenic	F	ACCCCAATCCAGCAAACGTT	NM_001844
		R	ATCTGGACGTTGGCAGTGTTG	
Aggrecan	Chondrogenic	F	ACAGCTGGGGACATTAGTGG	NM_001135
		R	GTGGAATGCAGAGGTGGTTT	
Collagen I	Fibroblastic	F	GAAAACATCCCAGCCAAGAA	NM_000089
		R	GCCAGTCTCCTCATCCATGT	
Collagen X	Hypertrophic Chondrocyte	F	GGCCCAGCAGGAGCAAAGGG	NM_000493
		R	GTGGCCCGGTGGGTCCATTG	
Runx2	Osteogenic	F	GTGCAGAGTCCAGCAAAGGT	NM_199173
		R	AGCAGAGCGACACCCTAGAC	
PPARγ2	Adipogenic	F	TCCATGCTGTTATGGGTGAA	NM_015869
		R	GGGAGTGGTCTTCCATTACG	