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Chondrogenic Differentiation of Adipose-Derived Stromal Cells in Combinatorial Hydrogels Containing Cartilage Matrix Proteins with Decoupled Mechanical Stiffness

Tianyi Wang, MS,[1] Janice H. Lai, MS,[2] Li-Hsin Han, PhD,[3] Xinming Tong, PhD,[3] and Fan Yang, PhD[1,3]

Adipose-derived stromal cells (ADSCs) are attractive autologous cell sources for cartilage repair given their relative abundance and ease of isolation. Previous studies have demonstrated the potential of extracellular matrix (ECM) molecules as three-dimensional (3D) scaffolds for promoting chondrogenesis. However, few studies have compared the effects of varying types or doses of ECM molecules on chondrogenesis of ADSCs in 3D. Furthermore, increasing ECM molecule concentrations often result in simultaneous changes in the matrix stiffness, which makes it difficult to elucidate the relative contribution of biochemical cues or matrix stiffness on stem cell fate. Here we report the development of an ECM-containing hydrogel platform with largely decoupled biochemical and mechanical cues by modulating the degree of methacrylation of ECM molecules. Specifically, we incorporated three types of ECM molecules that are commonly found in the cartilage matrix, including chondroitin sulfate (CS), hyaluronic acid (HA), and heparan sulfate (HS). To elucidate the effects of interactive biochemical and mechanical signaling on chondrogenesis, ADSCs were encapsulated in 39 combinatorial hydrogel compositions with independently tunable ECM types (CS, HA, and HS), concentrations (0.5%, 1.25%, 2.5%, and 5% [w/v]), and matrix stiffness (3, 30, and 90 kPa). Our results show that the effect of ECM composition on chondrogenesis is dependent on the matrix stiffness of hydrogels, suggesting that matrix stiffness and biochemical cues interact in a nonlinear manner to regulate chondrogenesis of ADSCs in 3D. In soft hydrogels ($\sim$3kPa), increasing HA concentrations resulted in substantial upregulation of aggrecan and collagen type II expression in a dose-dependent manner. This trend was reversed in HA-containing hydrogels with higher stiffness ($\sim$90 kPa). The platform reported herein could provide a useful tool for elucidating how ECM biochemical cues and matrix stiffness interact together to regulate stem cell fate, and for rapidly optimizing ECM-containing scaffolds to support stem cell differentiation and tissue regeneration.

Introduction

Osteoarthritis (OA) represents one of the most common joint degenerative diseases in the western world, affecting $\sim$50% of the population above the age of 65.[1] Cartilage is an avascular tissue and has limited potential to self-repair.[2] There is a strong clinical need to treat cartilage loss at an early stage of degeneration, reducing the incidences of disease progression to late stage OA. Stem cells are promising cell sources for cartilage repair given their self-renewal capacity and potential to differentiate into chondrocytes.[3] Among various stem cell sources, autologous stem cells are particularly attractive for treating cartilage degeneration and can be harvested from various adult tissues.[4] Most previous studies have focused on chondrogenesis using bone marrow-derived stromal cells (BMMSCs), while the use of adipose-derived stromal cells (ADSCs) for cartilage repair is still at an early stage. Compared with BMMSCs, ADSCs are more abundant, easier to isolate, and represent an alternative promising cell source for cartilage repair.[5–7]

To realize the potential of stem cells for articular cartilage repair, it is important to understand how stem cell fates are regulated in response to complex niche cues, including soluble factors, insoluble biochemical ligands, and matrix stiffness. The effects of soluble factors on stem cell chondrogenesis have been extensively studied, and most current protocols for promoting stem cell chondrogenesis rely on soluble factors.[8–12] To mimic the biochemical ligands present in the extracellular matrix (ECM), ECM-derived peptides can be incorporated into three-dimensional (3D) scaffolds to promote desirable stem cell differentiation.[13–15] Alternatively, decellularized cartilage matrices have also been explored for promoting stem cell chondrogenesis and cartilage repair.[16] Compared to peptides, ECM molecules often convey a higher

Departments of [1]Bioengineering and [2]Mechanical Engineering, and [3]Orthopaedic Surgery, Stanford University, Stanford, California.

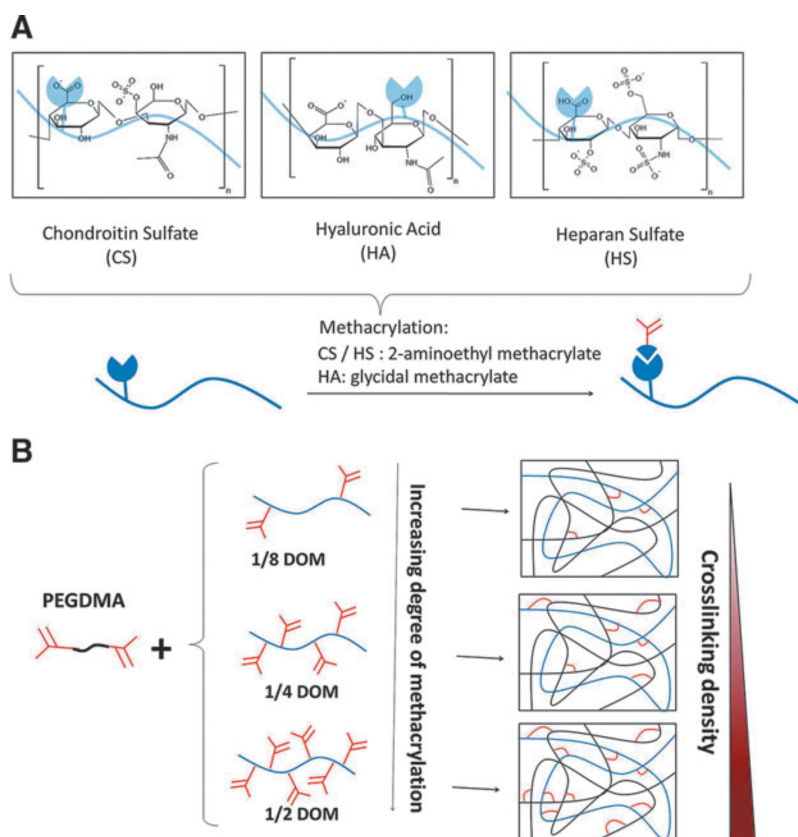

FIG. 1. **(A)** Chemical structures of three extracellular matrix (ECM) molecules used in the study, including chondroitin sulfate (CS), hyaluronic acid (HA) and heparan sulfate (HS). All ECM molecules were modified with methacrylate end-group to allow photocrosslinking. **(B)** ECM molecules were modified with different degree of methacrylation (DOM), with higher DOM resulting in higher crosslinking density within hydrogel network. Color images available online at www.liebertpub.com/tea

level of biological activity and may further promote tissue regeneration through serving as a binding reservoir for paracrine factors or modulating matrix turnover.[17] Various cartilage-derived ECM molecules, such as chondroitin sulfate (CS) and hyaluronic acid (HA), have been employed as 3D hydrogels to promote chondrogenesis *in vitro* and *in vivo*.[18,19] However, given the large molecular weight of natural ECM molecules, increasing the ECM molecule concentration to vary biochemical cues often results in simultaneous changes in the matrix stiffness.[20–22] This makes it difficult to elucidate the relative contribution of a biochemical ligand or matrix stiffness on stem cell fate using ECM-containing hydrogels. Recent studies have highlighted the importance of matrix stiffness alone in directing stem cell differentiation in both 2D and 3D culture.[23,24] To minimize the effects of varying ECM biochemical concentrations on the scaffold stiffness, previous studies used cartilage ECM molecules at concentrations that are orders of magnitude lower than their physiological range in the native tissues.[25,26] ECM molecules have also been physically entrapped in 3D scaffolds, but often suffer from inhomogeneous distribution and are subject to rapid loss through diffusion.[27,28] The goal of this study is to develop an ECM-containing hydrogel platform with largely decoupled biochemical and mechanical cues, for elucidating the effects of interactive niche signaling on chondrogenesis of human adipose-derived stromal cells (hADSCs) in 3D. We hypothesize that by tuning the degree of methacrylation (DOM) of ECM molecules, ECM molecules can be chemically incorporated into hydrogels in a homogeneous and stable manner, without substantially changing the mechanical stiffness of the hydrogels. We further hypothesize that ECM ligands will interact with matrix stiffness in a nonlinear manner to regulate hADSC chondrogenesis in 3D. Specifically, we incorporated three types of ECM molecules that are commonly found in the cartilage matrix into our hydrogel design, including CS, HA, and HS, at four concentrations (0.5%, 1.25%, 2.5%, and 5% [w/v]).[29] All ECM proteins were modified with methacrylate groups for photocrosslinking to facilitate homogenous incorporation in 3D hydrogels (Fig. 1A). To determine the optimal DOM that allows stable incorporation of ECM molecules without substantially changing the hydrogel network crosslinking density, we synthesized ECM molecules with varying DOM (Fig. 1B). To vary the mechanical stiffness of the hydrogels, poly(ethylene glycol) dimethacrylate (PEGDMA) (4.6 kDa) with varying concentrations (5%, 10%, and 15% [w/v]) was used. Type B gelatin (GelB), a digested form of collagen, was included at a constant concentration to facilitate cell adhesion. A total of 39 combinatorial hydrogels were developed (Table 1) with varying degrees of ECM concentration and matrix stiffness, for supporting chondrogenesis of hADSCs in 3D. Outcomes were analyzed using a fluorescence-based diffusion assay, mechanical testing, gene expression, and immunofluorescence staining.

Materials and Methods

Cell culture

hADSCs were isolated from excised human adipose tissue from informed and consenting patients following procedures as previously described.[30] hADSCs were cultured in a growth medium consisting of Dulbecco's minimal essential medium (Invitrogen) supplemented with 10% (v/v) fetal

Table 1. Compositions of Combinatorial Hydrogels with Varying Biochemical Composition and Mechanical Stiffness

	Biochemical cues												
	CS-MA% (w/v)				*HA-MA% (w/v)*				*HS-MA% (w/v)*				*Control*
Group Number	*0.5*	*1.25*	*2.5*	*5*	*0.5*	*1.25*	*2.5*	*5*	*0.5*	*1.25*	*2.5*	*5*	*0*
PEGDMA (% w/v)													
5	1	2	3	4	5	6	7	8	9	10	11	12	13
10	14	15	16	17	18	19	20	21	22	23	24	25	26
15	27	28	29	30	31	32	33	34	35	36	37	38	39

Each composition is assigned a group number for easy referencing. Biochemical composition was varied by adding different extracellular matrix molecules in varying concentrations, while mechanical stiffness was varied by adding PEGDMA in different concentrations.

PEGDMA, poly(ethylene glycol) dimethacrylate; w/v, weight by volume percentage; CS-MA, chondroitin sulfate-methacrylate; HA-MA, hyaluronic acid-methacrylate; HS-MA, heparan sulfate-methacrylate.

bovine serum (Gibco), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin (Invitrogen), and 10 ng mL^{-1} of basic fibroblast growth factor (PeproTech). hADSCs were subcultured at 90% confluence until passage 4 before use for all experiments.

Synthesizing methacrylated ECM macromolecules

Unless otherwise stated, all reagents are purchased from Sigma. Chondroitin sulfate-methacrylate (CS-MA) was synthesized by modifying a previously reported method.[31] Briefly, CS sodium salt was reacted with N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in an 2-(N-Morpholino) ethanesulfonic acid buffer for 5 min followed by the addition of 2-aminoethyl methacrylate (AEMA). NHS, EDC, and AEMA were mixed at a molar ratio of 1:2:1, stirred for 24 h at room temperature, and then dialyzed against water for 4 days using dialysis tubing (12–14 kDa molecular weight cutoffs [MWCO]). The purified solution was lyophilized and stored at −20°C until use. Heparan sulfate-methacrylate (HS-MA) was synthesized using HS sodium salt following the same protocol.

Hyaluronic acid-methacrylate (HA-MA) was synthesized by modifying previously reported methods.[32,33] Briefly, triethylamine and glycidyl methacrylate were added to 20 kDa MW sodium hyaluronate (Lifecore) and reacted at room temperature for 24 h before acetone precipitation. The precipitate was then dissolved in water and dialyzed against water for 4 days using dialysis tubing (12–14 kDa MWCO). The purified solution was lyophilized and stored at −20°C until use.

Type B gelatin-methacrylate (GelB-MA) was synthesized as previously reported.[34] Methacrylic anhydride was added to GelB at 50°C and reacted for 3 h, followed by acetone precipitation at 40°C. The precipitate was dissolved in water and dialyzed for 4 days using dialysis tubing (12–14 kDa MWCO). The purified solution was lyophilized and stored at −20°C until use.

ECM molecules with 1/2, 1/4, or 1/8 DOM were synthesized by adding proportionally less methacrylation reagents as compared to the original protocol above. Details of quantities of reagents used for synthesizing CS-MA, HA-MA, HS-MA, and GelB-MA with different DOM are shown in Supplementary Table S1 (Supplementary Data are available online at www.liebertpub.com/tea). Chemical structures for all polymers were verified by NMR analysis (Supplementary Fig. S1) and a schematic of ECM molecules with different DOM is provided (Fig. 1A).

Diffusion assay

To examine the stability of GelB with varying DOM in 3D hydrogels, GelB-MA was chemically labeled with fluorescein molecules (FC) before hydrogel formation and used in the diffusion assay. Acellular hydrogels (4 μL) containing final concentrations of 5% (w/v) PEGDMA and 4% (w/v) GelB-MA-FC at 1/2, ¼, or 1/8 DOM were fabricated, and those containing nonfluorescently labeled GelB-MA were used as controls. To test the stability of cartilage-specific ECM molecules in hydrogels, all ECM molecules were labeled with FC to produce CS-MA-FC, HS-MA-FC, and HA-MA-FC. Acellular hydrogels (4 μL) containing final concentrations of 5% (w/v) PEGDMA, 4% (w/v) GelB-MA, and 1.25%, 2.5%, or 5% (w/v) of fluorescently labeled ECM molecules at 1/2, ¼, or 1/8 DOM were fabricated. Nonfluorescently labeled CS-MA, HS-MA, and HA-MA were used as controls.

To measure the amount of ECM molecules that leach out from 3D hydrogels, 160 μL of phosphate-buffered saline (PBS) was added to the hydrogels and at each time point, 120 μL of supernatant was removed and 120 μL of fresh PBS was added. Fluorescence intensity in the supernatant was measured at 0.5, 1, 2, 4, 6, 8, and 24 h, and 2, 3, 4, 5, 6, and 7 days using a plate reader (Spectramax M2e; Molecular Devices). Measured fluorescence of supernatant was corrected by measured fluorescence of controls and compared to a calibration curve to determine the concentration of ECM molecules that had leached out from 3D hydrogels into the supernatant. ECM molecules without extensive leaching were chosen for further studies.

Combinatorial hydrogels

To examine the effects of interactive biochemical and mechanical niche signaling on the chondrogenesis of hADSCs, we developed 39 combinatorial hydrogel compositions with varying biochemical compositions and mechanical stiffness (Table 1). PEGDMA (MW 4.6 kDa) was dissolved in sterile Dulbecco's PBS to achieve a final concentration of 5%, 10%, and 15% (w/v). To tune the biochemical cues, different types of ECM molecules with optimized DOM (CS-MA, HA-MA,

Table 2. Primer Sequences Used in Reverse Transcription–Polymerase Chain Reaction

Gene name	Forward primer	Reverse primer
GAPDH	5′ CGCTCTCTGCTCCTCCTGTT 3′	5′ CCATGGTGTCTGAGCGATGT 3′
Aggrecan	5′ TGAGGAGGGCTGGAACAAGTACC 3′	5′ GGAGGTGGTAATTGCAGGGAACA 3′
Collagen type II	5′ TCACGTACACTGCCCTGAAG 3′	5′ TTGCAACGGATTGTGTTGTT 3′

and HS-MA) were added to the PEGDMA solution at varying concentrations (0.5%, 1.25%, 2.5%, and 5% [w/v]). GelB-MA was included at 4% (w/v) in all hydrogel compositions to provide adhesion for the hADSCs. All polymer precursor solutions contained 0.05% (w/v) of photoinitiator Igracure D2959 (Ciba Specialty Chemicals). PEGDMA hydrogels with 4% (w/v) GelB-MA, but without any additional cartilage ECM molecules were used as controls.

Cell encapsulation and in vitro *culture*

Passage 5 hADSCs were homogenously suspended in combinatorial hydrogels at 15 million cells mL^{-1}. The cell–hydrogel mixture was pipetted into a 96-well mold (50 μL per sample) and exposed to UV light (365 nm, 4 mW cm^{-2}) for 5 min to induce crosslinking. Cell viability was assessed 24 h postencapsulation using the LIVE/DEAD Cell Viability Assay Kit (Molecular Probes). All hydrogel samples were cultured at 37°C, 5% CO_2 in 1.5 mL of chondrogenic medium for 21 days, with medium change every other day. Chondrogenic media are composed of high-glucose Dulbecco's modified Eagle's medium (Gibco), 100 nM dexamethasone (Sigma), 50 μg mL^{-1} ascorbic-2-phosphate (Sigma), 40 μg mL^{-1} proline (Sigma), 5 μg mL^{-1} insulin–transferrin–selenium premix (BD Biosciences), 0.001 mg mL^{-1} sodium pyruvate, 10 ng/mL recombinant human transforming growth factor beta 3 (TGF-β3) (Peprotech), 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (Invitrogen).

Mechanical testing

Unconfined compression tests were conducted using an Instron 5944 materials testing system (Instron Corporation) fitted with a 10 N load cell (Interface, Inc.). The test setup consisted of custom-made aluminum compression platens lined with polytetrafluoroethylene to minimize friction. Acellular hydrogels were fabricated and immersed in the PBS solution for 24 h at room temperature to reach equilibrium before mechanical testing. All tests were conducted in the PBS solution at room temperature. Before each test, a preload of ∼2 mN was applied, and the upper plate was lowered at a rate of 1% strain s^{-1} to a maximum strain of 30%. The compressive modulus was determined from the curve fit equation for strain between 10% and 20% strain.

RNA extraction and reverse transcription–polymerase chain reaction

At the end of the 21-day culture, RNA was extracted from 3D combinatorial hydrogels ($n = 3$/group) to quantify the gene expression of chondrogenic markers, including aggrecan (*Agg*) and collagen type II (*Col II*). Total RNA was extracted using a Trizol method as we previously reported[27] and cDNA was synthesized by reverse transcription using the Superscript First Strand Synthesis System (Invitrogen). Reverse transcription–polymerase chain reaction (RT-PCR) was performed using the Power® SYBR Green Kit (BD Biosciences) following the manufacturer's protocol. All samples were run for 40 PCR cycles and analyzed on the Applied Biosystems 7900 Real-Time PCR System (Invitrogen). The human primer sequence is listed in Table 2. Gene expression levels were normalized using the ΔΔCt method, in which target gene expression was first normalized to an endogenous gene, GAPDH, and then normalized by the gene expression level in the control group composed of 5% (w/v) PEGDMA without additional ECM molecules.

Histological analyses

To visualize the ECM distribution inside 3D hydrogels, immunofluorescence staining of *Col II* and *Col X* was performed in selected groups. Samples were harvested after 21 days of culture and fixed in 4% paraformaldehyde overnight at 4°C. Fixed hydrogels were then transferred to 30% sucrose solution (Sigma) overnight at 4°C before snap freezing following standard cryopreservation protocols. For immunostaining, sections were incubated for 15 min at 37°C for enzymatic antigen retrieval, and then incubated with a blocking buffer containing 2% goat serum and 3% bovine serum albumin. The rabbit polyclonal antibody for *Col II* and *Col X* (Abcam) was diluted at 1:75 and added to the samples separately and incubated overnight at 4°C. The secondary antibody was diluted at 1:200 (Alexa Fluor 488 goat anti-rabbit) with Hoechst (4 μg/mL), and sections were incubated with the secondary antibody for 1 h at room temperature. Samples were mounted with a mounting medium (Vectashield) and images were taken with a Zeiss fluorescence microscope.

Statistical test

Statistical analysis was performed using one- or two-way analysis of variance and Tukey's *post hoc* test was used to compare between individual groups. All data are presented as mean ± standard deviation ($n = 3$/group). GraphPad Prism (Graphpad Software) was used to perform statistical analysis.

Results

Effects of DOM on ECM diffusion

To determine the lowest DOM of GelB-MA needed for stable ECM incorporation, we performed a diffusion assay by measuring the amount of fluorescently labeled GelB-MA with varying DOM (1/2, 1/4, or 1/8) that leached out from hydrogels containing 5% (w/v) PEGDMA. GelB-MA with 1/4 DOM resulted in the lowest amount of leaching (less than 10% in total) and reached stabilization within 10 h (Supplementary Fig. S2A), and thus was chosen for further

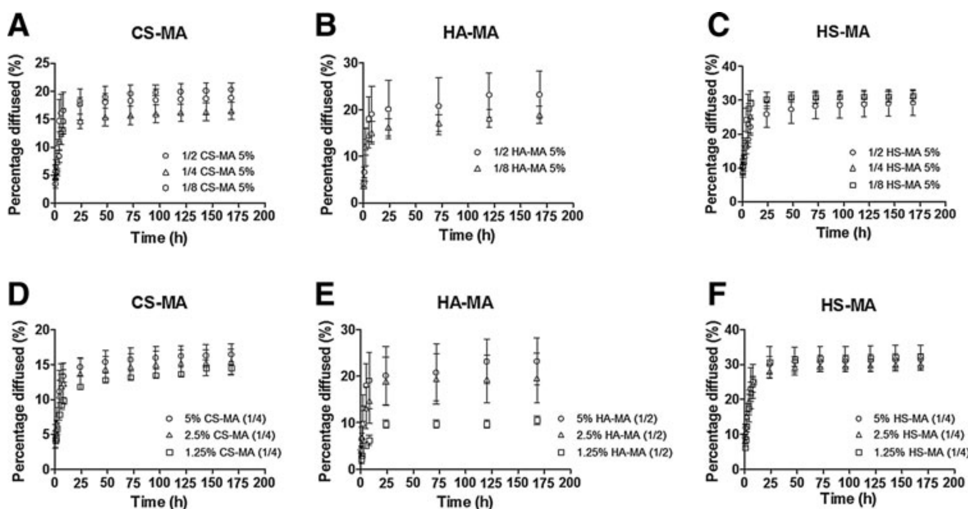

FIG. 2. **(A–C)** Effects of varying DOM on the amount of ECM molecules diffused out of hydrogels as measured by a fluorescence-based diffusion assay over 7 days. **(D–F)** Effects of varying ECM concentration (1.25–5% [w/v]) on the cumulative diffusion of ECM molecules over 7 days. All hydrogels contained 5% (w/v) poly(ethylene glycol) dimethacrylate (PEGDMA) and 4% (w/v) type B gelatin-methacrylate (GelB-MA) in addition to varying ECM molecule concentrations.

studies. Similarly, the leaching of three other ECM molecules, CS-MA, HA-MA, and HS-MA, all reached stabilization within 10 h (Supplementary Fig. S2B–D). Next, we examined the effects of varying DOM and concentrations of CS-MA, HA-MA, and HS-MA on ECM diffusion over 7 days (Fig. 2). For CS-MA and HA-MA, varying DOM exhibited a diffusion of 15–20% of total crosslinked fluorescently labeled ECM molecules within the first 24 h of hydrogel formation, while hydrogels containing HS-MA-FC exhibited diffusion of up to 30% total crosslinked fluorescently labeled HS-MA-FC within 24 h (Fig. 2A–C). For all ECM containing hydrogels, the diffusion of fluorescently labeled ECM molecules reached a plateau after 10 h of hydrogel formation. For all three types of ECM molecules examined (CS-MA, HA-MA, and HS-MA), increasing DOM generally led to a slight decrease in leaching, but the difference was not statistically significant (Fig. 2A–C). Meanwhile, increasing the concentration of ECM molecules in the hydrogels also had negligible effects on leaching in CS-MA- and HS-MA-containing hydrogels, whereas increasing HA-MA from 1.25% to 5% resulted in an increase in the amount of ECM leaching (Fig. 2D–F). All leaching tapered off after 10 h, and is generally limited to 15–30% of the total encapsulated ECM amount.

Mechanical testing

The unconfined compression test was performed to measure the Young's modulus of combinatorial hydrogels. Our data showed 3 almost horizontal lines, indicating that the mechanical stiffness of the hydrogels was largely dominated by the concentration of PEGDMA, and varying ECM molecules up to 5% (w/v) had negligible effects on hydrogel stiffness (Fig. 3). By increasing the PEGDMA concentration (5, 10, and 15% [w/v]), we obtained hydrogels at three stiffness ranging around 3, 30, and 90 kPa (Fig. 3). The stiffness of control hydrogels without additional ECM molecules is shown as dashed lines.

Chondrogenic gene expressions

To determine the effects of varying biochemical and mechanical niche cues on the chondrogenic differentiation of hADSCs in 3D, we quantified gene expressions of two cartilage markers, *Agg* and *Col II*, after culturing hADSCs in 39 combinatorial hydrogels for 21 days (Fig. 4 and Supplementary Fig. S3). Our results show that the effect of matrix stiffness on chondrogenesis is dependent on the biochemical composition of hydrogels, suggesting that matrix stiffness and biochemical cues interact in a nonlinear manner to regulate chondrogenesis of hADSCs in 3D. In control groups without additional ECMs, increasing the hydrogel stiffness by increasing the PEGDMA concentration from 5% (w/v) to 15% (w/v) resulted in an 11-fold increase in *Agg* gene expression. Similarly, in hydrogels containing lower concentrations of HA (0.5% to 2.5% [w/v]), increasing hydrogel stiffness led to an increase in *Agg* expression. However, a reverse trend was observed in hydrogels containing higher concentrations of HA (5% w/v), in which increasing hydrogel stiffness led to a decrease in *Agg* expression. *Col II* gene expression in combinatorial hydrogels also showed that the effects of matrix stiffness on hADSC chondrogenesis are dependent upon the types and concentration of biochemical cues. In groups containing

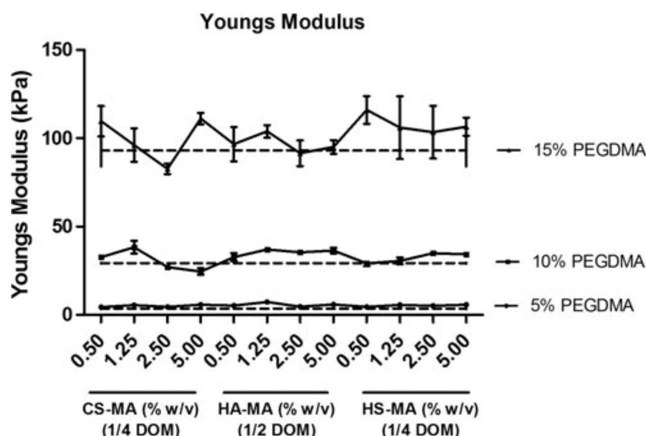

FIG. 3. Young's modulus of combinatorial hydrogels with varying types and concentrations of ECM molecules. Dotted lines represent stiffness of control hydrogels (5–15% [w/v] PEGDMA) without any ECM molecules.

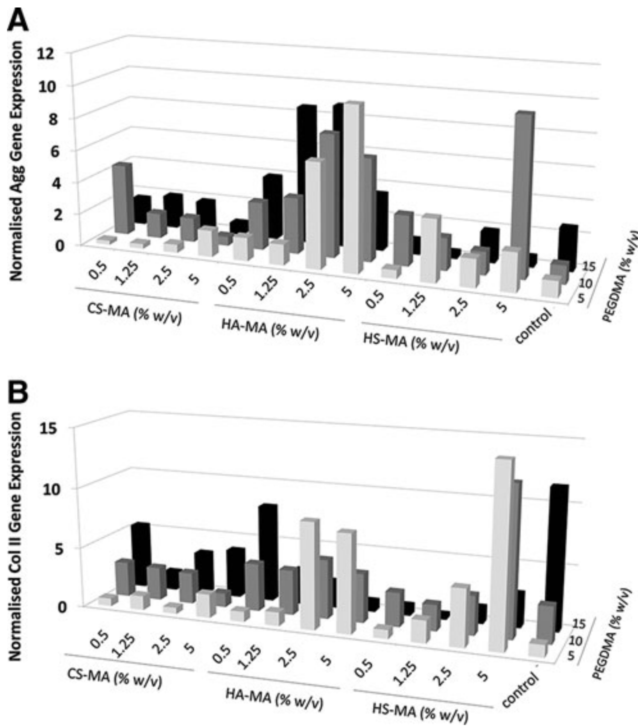

FIG. 4. Quantitative gene expression of aggrecan (*Agg*) **(A)** and collagen type II (*Col II*) **(B)** by human adipose-derived stromal cells (hADSCs) in 39 combinatorial hydrogels after 21 days of *in vitro* culture. Results were normalized by the gene expression in the control group containing 5% (w/v) PEGDMA without any ECM molecules.

0.5% (w/v) of CS-MA or HA-MA as biochemical cues, *Col II* gene expression increased 8.5-folds and 9.5-folds, respectively, when the PEGDMA concentration increased from 5% (w/v) to 15% (w/v). In contrast, hydrogels containing a high level of HA and HS (5% [w/v]) showed a reverse trend, and increasing the PEGDMA concentration from 5% (w/v) to 15% (w/v) resulted in up to 90% decrease in *Col II* gene expression. ECM molecules also conferred a dose-dependent effect on *Col II* gene expression. For HA-containing hydrogels, increasing biochemical cue concentrations resulted in a dose-dependent increase in *Col II* expression in soft hydrogels (5%), whereas a reverse trend was observed in stiff hydrogels (15% [w/v]) (Supplementary Fig. S3D–F).

Immunostaining

Given the robust effects of HA-MA in regulating chondrogenic gene expression, six groups containing HA-MA at two doses (0.5% and 5% [w/v]) and three matrix stiffness (5%, 10%, or 15% [w/v] PEGDMA) were stained for cartilage ECM. In softer hydrogels (5% [w/v] PEGDMA), increasing the HA-MA concentration led to a more intense deposition of Col II (Fig. 5A, D). This trend was reversed at higher mechanical stiffness (15% PEGDMA), in which an increase in the HA-MA concentration led to a decrease in the amount of pericellular Col II produced (Fig. 5C, F). Varying HA-MA concentrations resulted in negligible changes in Col II staining for hydrogels with intermediate stiffness (Fig. 5B, E). Col X secretion appeared to be low in hydrogels with low and moderate mechanical stiffness (5% and 10% [w/v]), and increased in stiff hydrogels (15% [w/v]), regardless of HA-MA concentrations (Supplementary Fig. S4).

Discussion

In this study, we report the development of an ECM-containing hydrogel platform with independently tunable mechanical stiffness and biochemical composition for promoting chondrogenesis of hADSCs in 3D. To tune the matrix stiffness, we have chosen PEGDMA, a bioinert polymer to provide a blank slate without any biochemical cues. By modulating the DOM, three cartilage ECM molecules (CS, HA, and HS) were chemically incorporated into 3D hydrogels. A lower DOM allows ECM molecules to be incorporated with less influence on the hydrogel cross-linking density, whereas a higher DOM allows more stable incorporation (Fig. 1B). To identify the optimal DOM that allows interlocking of ECM molecules without substantially changing hydrogel stiffness, we used fluorescently-labeled

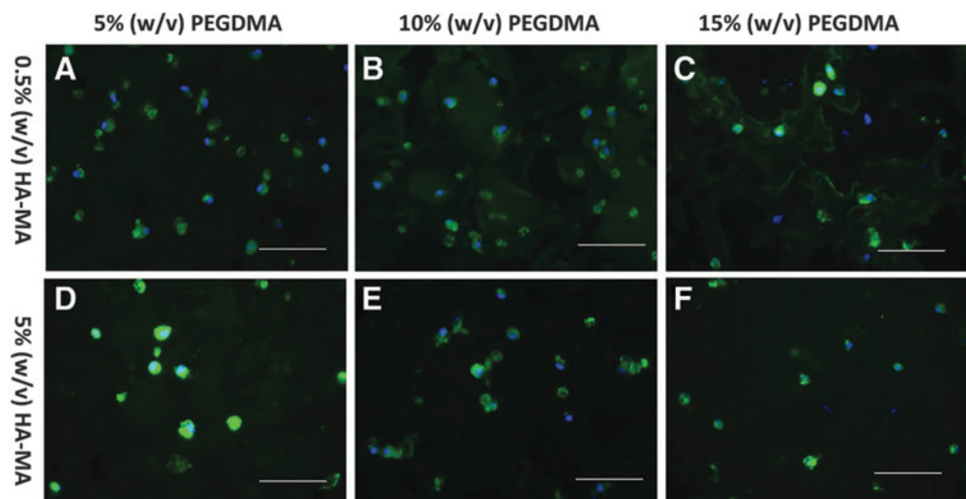

FIG. 5. Effect of varying hydrogel stiffness and HA concentration on Col II deposition by ADSCs in three-dimensional hydrogels, as shown by immunostaining. **(A–F)** Three PEGDMA concentrations (5%, 10%, and 15% [w/v]) and two hyaluronic acid-methacrylate (HA-MA) concentrations (0.5% and 5%) were examined. Green, Col II; blue, nuclei. Scale bar = 100 μm. Color images available online at www.liebertpub.com/tea

ECM molecules modified with varying DOM, and measured unbound ECM molecules that diffuse out from hydrogels over time. For all ECM molecules tested, we observed only an initial burst release of ECM molecules within the first 2 h, which quickly stabilized afterward. It should be noted that the diffusion assay was performed with frequent changes of supernatant to create a sink condition; therefore, the results would be an overestimate of the actual amount of leached out ECM molecules in the 50 μL hydrogels. Our diffusion results confirmed that ECM molecules with low DOM allowed stable incorporation of these biochemical cues into the hydrogels across the range of concentrations (up to 5% [w/v]) examined in our study. Based on the results of the leaching assay, we have chosen 1/4 DOM CS-MA, 1/2 DOM HA-MA, and 1/4 DOM HS-MA (henceforth be called CS-MA, HA-MA, and HS-MA) for constructing combinatorial hydrogels to further explore the interactive signaling of matrix stiffness and ECM biochemical cues on the chondrogenesis of hADSCs in 3D culture.

Previous studies have explored various cartilage ECM-derived macromolecules as scaffolds for guiding stem cell chondrogenesis, including CS,[2,18,20,26] HA,[21,35,36] and HS.[37–39] Whereas these studies demonstrated the potential benefit of these ECM macromolecules for promoting cartilage repair, few studies have compared the effects of different types of cartilage-derived ECM molecules and dose effects on chondrogenesis in 3D. Our study addresses this unmet need by decoupling the biochemical cues from mechanical cues, and directly compared the efficacy of three cartilage ECM-derived molecules in promoting chondrogenesis across a broad range of concentrations. Among the three ECM molecules examined in our study, CS-MA showed the most modest dose effects on chondrogenesis, whereas HA-MA demonstrated the highest potency at upregulating chondrogenic gene expression across a range of hydrogel stiffness tested in this study (Fig. 4). Such effects are the most apparent in soft hydrogels ($\sim$3 kPa), in which increasing HA-MA resulted in substantial upregulation of *Agg* and *Col II* expression in a dose-dependent manner (Supplementary Fig. S3). Similar to HA-MA, HS-MA also showed a dose-dependent stimulating effect on chondrogenesis in hydrogels across all stiffness range, with the most robust upregulation observed in hydrogels with soft to intermediate stiffness (3–30 kPa). HS is known to serve as a growth factor reservoir in the ECM, which can bind to growth factors to prolong its bioavailability, and release growth factors as the matrix undergoes remodeling.[40] TGF-β3 is a potent growth factor for chondrogenesis and supplemented in the chondrogenic medium in our study. We speculate that HS may bind to TGF-β3, thereby increasing the local TGF-β3 concentration and promoting chondrogenesis.[41]

Recent studies have highlighted the importance of matrix stiffness on influencing stem cell fate.[23,24,42,43] Most work so far has been performed on 2D culture, and studies for elucidating how the cell–matrix interactions regulate cell differentiation in 3D are emerging.[23,44] A recent study has examined the effects of varying matrix stiffness on osteogenesis in 3D using alginate-based hydrogels with stiffness ranging from 5 to 110 kPa, and showed that osteogenesis peaked in hydrogels with intermediate stiffness of 22 kPa in 3D hydrogels.[23] However, how matrix stiffness influences chondrogenesis in 3D remains largely unknown. A recent study using HA hydrogels suggested that chondrogenesis in 3D was upregulated as matrix stiffness decreased.[45] Given the complex interactions among the multifactorial niche signaling, we have designed combinatorial hydrogels to help elucidate how ECM cues interact with matrix stiffness to regulate hADSC chondrogenesis in 3D. Previous combinatorial studies on cell–material interactions in 2D and 3D have shown that matrix biochemical cues and stiffness interact in a nonlinear manner that cannot be predicted from sequential optimizations alone.[27,46] In our studies, we obtained hydrogels with tunable stiffness across a broad range from 3 to 90 kPa by varying the concentration of PEGDMA, and increasing methacrylated ECM molecules up 5% (w/v) did not markedly influence the matrix stiffness (Fig. 4). Among the three ECM molecules examined, varying matrix stiffness showed the highest impact on hydrogels containing CS-MA. In soft hydrogels made of 5% (w/v) PEGDMA, CS-MA across different dosages generally resulted in down-regulation of both *Agg* and *Col II* gene expression (Fig. 4). Increasing the PEGDMA concentration from 5% to 10%, which corresponds to an increase in hydrogel stiffness from 3 to 30 kPa, resulted in an increase in chondrogenic expression in most CS-MA-containing groups. For HA-MA-containing hydrogels, HA-MA increased chondrogenesis in a dose-dependent manner in hydrogels with low and intermediate stiffness (3–30 kPa), but a reverse trend was observed in stiff hydrogels (90 kPa). We further observed a decrease in *Col II* gene expression and protein deposition in stiffer hydrogels containing 15% (w/v) PEGDMA. In addition, *Agg* gene expression was also decreased in stiff hydrogels with high concentrations (5% [w/v]) of ECM molecules. It is possible that stiffer hydrogels limit cell–cell contact, which has been shown to promote chondrogenesis of mesenchymal stem cells.[47,48]

Despite the high chondrogenic gene expression, the amount of Col II deposition in our study appeared to be limited mostly to the pericellular regions. This might be caused by the limited degradation of the chosen PEGDMA hydrogel network, or the presence of gelatin in all formulations. A recent study has suggested that gelatin could inhibit chondrogenesis in 3D by modulating the cell shape and spreading.[49] Future studies will examine the effects of the removal of gelatin on chondrogenesis. To further facilitate cell proliferation and ECM deposition, hydrolytically degradable or enzymatically degradable sequences can be incorporated into the hydrogel network.[50,51] Alternatively, we have shown that dynamic hydrogels containing stimulus-responsive porogens may also be used to enhance cell proliferation and uniform ECM deposition.[52] Last, the native cartilage matrix contains multiple types of ECM molecules, and the platform reported herein may also be applied to study the interactive signaling of ECM biochemical cues on stem cell chondrogenesis in different combinations and ratios in 3D.

Conclusions

In this study, we report the development of combinatorial ECM-containing hydrogels with decoupled matrix stiffness and biochemical compositions for promoting hADSC chondrogenesis in 3D culture. By modulating the DOM on

cartilage-derived ECM molecules, these ECM molecules can be incorporated into hydrogels across a broad range of concentration with minimal influence on hydrogel stiffness. Using fluorescence-labeled ECM molecules and a diffusion assay, we verified that ECM molecules with optimal DOM can be stably incorporated within the hydrogels. Among the three ECM molecules examined in our study, HA-MA demonstrated the highest potency in enhancing chondrogenic gene expression across all stiffness ranges. Such effects were most apparent in soft hydrogels ($\sim$3 kPa), in which increasing HA-MA resulted in substantial upregulation of *Agg* and *Col II* expression in a dose-dependent manner. This trend was reversed in HA-MA-containing hydrogels with higher stiffness (90 kPa). Our results showed that ECM biochemical cues and matrix stiffness interact in a nonlinear manner that cannot be predicted from sequential optimizations alone, and highlighted the need of such combinatorial platforms for probing stem cell–niche interactions. The ECM-containing hydrogels reported herein could provide a useful platform for elucidating how ECM biochemical cues and matrix stiffness interact together to regulate stem cell fate, and for rapidly optimizing scaffold compositions to support stem cell differentiation for tissue regeneration applications.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Fan Yang, PhD
Department of Orthopaedic Surgery and Bioengineering
Stanford University
300 Pasteur Drive
Stanford, CA 94305

E-mail: fanyang@stanford.edu

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