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ORIGINAL ARTICLE

Hydrogel Culture Surface Stiffness Modulates Mesenchymal Stromal Cell Secretome and Alters Senescence

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Current cell culture surfaces used for the expansion and production of mesenchymal stromal cells (MSCs) are not optimized for the production of highly secretory and nonsenescent cells. In this study, we used poly (ethylene glycol) hydrogel substrates with tunable mechanical and biochemical properties to screen the effect of culture surfaces on pro-regenerative secretome by multiplex enzyme-linked immunosorbent assay, proliferation by PicoGreen DNA analysis, and senescence by senescence-associated β-galactosidase activity. We demonstrate that MSCs cultured on 30 kPa hydrogels, regardless of biochemical functionalization, broadly enhanced the secretion of immunomodulatory and regenerative factors versus stiffer 100 kPa or tissue culture plastic surfaces, but did not support robust proliferation. In contrast, culture on 100 kPa hydrogel surfaces promoted proliferation at a similar level and did not substantially alter the amount of secreted factors as compared with tissue culture plastic. Culture on integrin-engaging, cadherin-engaging, and hyaluronic acid-containing 30 kPa substrates enhanced MSC-conditioned media (CM) angiogenic activity in a human umbilical vein endothelial cell tube formation assay and human THP-1 monocyte chemoattraction in a transwell assay. However, 30 kPa substrate culture did not impact the myogenic activity of MSC CM in a C2C12 myoblast tube formation assay. Culture on selected 100 kPa surfaces enhanced CM angiogenic activity and monocyte chemotaxis, but not myogenic activity. Serial culture on 100 kPa RGD hydrogel surfaces significantly reduced senescence in MSCs versus tissue culture plastic, while maintaining the capacity of the cells to enhance their secretome in response to 30 kPa surfaces. Thus, hydrogel substrates that exhibit stiffness orders of magnitude lower than standard tissue culture plastic can serve as novel surfaces for the production of MSCs with an improved therapeutic secretory capacity and reduced senescence.

Keywords: mesenchymal stromal cell, hydrogel, secretome

Impact Statement

The success of mesenchymal stromal cell (MSC)-based therapies is dependent on the manufacture of a large number of cells with high therapeutic potency. Among the culture surfaces tested in this study, we demonstrate that substrate stiffness rather than biochemical functionalization predominantly guides changes in MSC proliferation and secretory capacity. We have identified substrate parameters to support MSC proliferation, enhance secretion of paracrine factors, and to reduce replicative senescence. By maximizing secretory capacity and reducing senescence through the choice of hydrogel culture materials, these findings have great potential to improve the large-scale production of therapeutic MSCs.

Introduction

Mesenchymal stromal cells (MSCs) are highly paracrine active cells which are of great clinical interest due to their immunomodulatory and proregenerative properties when transplanted *in vivo*.[1] MSC therapeutic potency is primarily attributed to the release of immunomodulatory, angiogenic, and trophic factors, collectively known as the secretome.[1] MSC proregenerative secretory profile and magnitude *in vitro* is correlated with *in vivo* activity, suggesting that the *in vitro* secretome is related to MSC therapeutic function in some applications.[2] To achieve

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therapeutic cell numbers of millions of cells per kg patient body weight from bone marrow, adipose, or umbilical cord primary cells, most clinical therapy protocols employ several rounds of MSC expansion,[3] primarily in two-dimensional flasks or stacks.[4,5] However, these standard culture expansion protocols progressively decrease MSC secretory potency over population doublings,[6,7] leading to significant changes in MSC therapeutic function *in vivo*.[8] MSCs also undergo a progressive decline in fitness through an increase in senescence and decreased expansion potential during standard *in vitro* culture. This restricts the cell yield from a single source and alters their gene expression, differentiation, and secretory phenotypes.[6,8–11] These issues establish a conundrum by which the primary cell source is limited and must be expanded for clinical scale, but expansion leads to reduced therapeutic efficacy/potency and is further restricted by senescence. Thus, there is a need to identify culture strategies that improve these features of MSC potency and fitness.

To explore material-based strategies to improve the manufacture of highly therapeutic MSCs, we designed a modular hydrogel culture system to examine the role of mechanical and biochemical cues governing MSC secretory capacity and cellular longevity. We assessed mechanical and biochemical properties consistent with native *in vivo* perivascular environment of MSCs, where they are highly secretory and capable of long-term self-renewal.[12,13] The *in vivo* MSC perivascular environment and *in vitro* tissue culture polystyrene (TCP) substrates differ fundamentally in elastic modulus. The basement membrane has a modulus between 1 and 100 kPa, whereas TCP is several orders of magnitude stiffer (GPa range).[14,15] Long-term culture on supraphysiological stiffness guides MSC fate toward an osteoblastic lineage,[16–19] reduces secretion of proregenerative/anti-inflammatory factors,[6,7] and reduces proliferation rate over time,[6] ultimately leading to senescence.[10] Interestingly, MSCs cultured on physiological range stiffness materials suggests that MSCs have enhanced transcription and/or secretion of multiple paracrine factors on softer substrates ranging from 1 to 40 kPa Young's modulus versus TCP.[6,20–22] This study explores the two-dimensional culture of MSCs on materials spanning 30–100 kPa in modulus in conjunction with a variety of biochemical modifications.

In addition to acute cellular changes on differing moduli, MSCs also develop a biological mechanical memory of substrate stiffness that becomes irreversible after extended culture.[6,19,23] Long-term culture on TCP leads to decreased paracrine secretion and proliferation that can be partially rescued by transfer to a soft surface after an intermediate (p5) but not late passage number (p11), indicating that cells gradually lose the ability to respond to surface modulus.[6] Extended serial culture of MSCs on 1 kPa surfaces sustains the secretory properties of MSCs[6]; however, proliferation is markedly reduced, consistent with the known role of focal adhesion signaling in controlling proliferation,[24] making this system nonideal for culture expansion.[6] Thus, we examined the role of serial culture on physiological (100 kPa) or supraphysiological ($\sim$GPa) surfaces on the onset of replicative senescence and the ability to respond to changes in mechanical surface cues after expansion.

While substrate stiffness plays a role in influencing MSC potency and fitness, the role of modulus in the context of differing biomolecular functionalizations is not currently understood. In each of the above referenced culture systems, different matrix proteins or adhesive peptides have been used to support attachment of MSCs to the hydrogels, therefore making the interpretation of the role of mechanical cues in combination with biochemical cues in controlling secretome and proliferation difficult. MSCs experience mechanical forces through several cell/matrix and cell/cell interactions, including stimulation of integrin and cadherin receptors, respectively.[25] Transmembrane signaling through cell/cell interactions of cadherins can enhance MSC secretory capacity,[26,27] making cadherins an attractive adhesive target for stimulating MSC secretome. In biomaterials, the presentation of N-cadherin-engaging peptides, such as HAVDI, in combination with integrin-engaging ligand RGD, reduces MSC proliferation likely through a mechanism involving reduced cellular tension[18]; however, the role of controlled biomaterial presentation of N-cadherin ligands on the MSC secretome is unknown. Therefore, culture materials with integrin- and cadherin-engaging ligands merit exploration in their ability to modulate MSC secretory capacity and proliferation.

MSCs also interact with glycosaminoglycans (GAGs) moieties in the perivascular environment, such as hyaluronic acid (HA) and heparan sulfate. Heparan sulfate sequesters many positively charged autocrine/paracrine trophic factors, chemokines, and cytokines that participate in localized signaling with MSCs.[13,28,29] Material culture surfaces presenting HA improve MSC adhesion through ligation of the MSC surface receptor CD44,[30] whereas heparin-mediated autocrine factor sequestration improves MSC adhesion and trilineage differentiation.[29] Therefore, GAG incorporation into culture surfaces can modulate or enhance adhesion, however, the effect of these GAGs on the secretory capacity of MSCs has not been explored, particularly in the context of differing mechanical properties.

Paracrine signals produced by MSCs stimulate functional changes in multiple downstream cell types involved in endogenous wound healing, including endothelial cells, circulating immune cells, and adult tissue progenitor cells such as myoblasts.[31] Vascular regeneration is necessary to support the supply of blood and nutrients to a healing tissue and MSCs can support this activity through angiogenesis and arteriogenesis.[32,33] MSCs secrete several cytokines that recruit innate immune cells,[34] which is a significant early step in the progression of wound healing, vascular remodeling, and tissue repair.[35–39] MSCs may also contribute to repair in muscle injuries through stimulation of myogenic progenitor cells.[40] Therefore, *in vitro* functional activity assays of endothelial cells, myeloid cells, and myoblasts were utilized in this study to assess the impact of biomaterial substrate culture on differing biological activities of MSCs.

Despite the clear impact of both substrate stiffness and biomolecule presentation on MSC phenotype, no studies have designed these surfaces to assess all these parameters on MSC proliferation, secretome, and therapeutic function in parallel, and therefore the impact of each of these parameters relative to one another is unknown. The objective of this study was to use hydrogel materials with tunable mechanical and biochemical properties as culture surfaces to improve the production of highly secretory MSCs. We hypothesized that culture substrate hydrogels with stiffness orders of magnitude less than TCP (kPa range) incorporating

biochemical moieties such as adhesive ligands (integrin-engaging and N-cadherin-engaging) and/or GAGs, can increase MSC secretory function and reduce replicative senescence compared with conventional culture on TCP. To address this hypothesis, we evaluated the *in vitro* secretome of human MSCs grown on a panel of ten different hydrogel formulations, and the functional effect of the secreted factors on endothelial, immune, and muscle cell activity. Results from these studies will help identify the most critical parameters for the expansion and manufacture of highly potent MSCs.

Materials and Methods

Additional details of methods are available in the Supplementary Data.

Material synthesis and functionalization

Poly(ethylene glycol) diacrylate (PEG-DA, 3.4 kDa) was synthesized according to previously published methods.[36,41] Fully desulfated heparin (Hep^{-}) was prepared as previously described.[42] Hep, Hep^{-}, and HA were each functionalized with methacrylamide.[43] Cell-adhesive peptides, integrin-engaging peptide RGD (GRGDS, Bachem), or N-cadherin-engaging peptide HAVDI (HAVDIGGGC, Genscript), were conjugated to linear acrylate-PEG spacers.[44,45]

Hydrogel fabrication

Hydrogels were prepared with a range of stiffness by varying polymer molecular and final concentration of PEG-DA (Fig. 1B). PEG-DA (3.4 kDa or 600 Da) was dissolved in phosphate-buffered saline (PBS) to create polymer solutions (8–20% wt/v). All hydrogel precursor solutions included 1 mM Acryl-PEG-GRGDS. HAVDI was included at final hydrogel precursor concentration of 1 mM Acryl-PEG-HAVDI and GAG hydrogels (Hep, Hep^{-}, or HA) were formed with 15 mg/mL in its hydrogel precursor solution (Supplementary Tables S1 and S2).

Crosslinking was accomplished by addition of ammonium persulfate (APS; Sigma-Aldrich) and tetramethyl ethylene diamine (TEMED; Bio-Rad). Polymer components were sterile filtered using Spin-X filters (Costar). Hydrogel was formed between glass slides (spacer thickness = 0.5 mm). After 20 min of crosslinking, 12 mm diameter hydrogel disks were formed by biopsy punch, placed in ultra-low-binding 24-well plates, which do not support cell attachment, and incubated in PBS overnight to reach equilibrium swelling and remove leachable products.

Mesenchymal stromal cells culture on hydrogels

Human bone marrow MSCs (Lots 139, 182; RoosterBio, Inc.) were culture expanded for two population doublings after receipt, and frozen in $\sim 5 \times 10^5$ cell aliquots in CryoStor CS10 freezing media (BioLife). Lot 139 was used for all figures in the main text, data from Lot 182 is available in the Supplementary Data. Frozen aliquots were revived on TCP for 3–4 days before seeding test surfaces. MSCs were maintained in Dulbecco's Minimal Essential Medium (DMEM, 1 g/L glucose, sodium pyruvate, L-glutamine; Gibco) supplemented with 10% fetal bovine serum (FBS, lot E16063; Atlanta Biologicals) and 1% antibiotic/antimycotic (Gibco). TrypLE express (Gibco) was used to enzymatically passage MSCs. Hydrogels were washed with PBS and complete media before seeding cells at 15,000 cells per hydrogel disc or TCP well.

For serial culture, cells were maintained on surfaces for 4 days, then passaged to freshly prepared surfaces. For senescence analysis, cells were maintained for three successive passages on 100 kPa hydrogels (12 days). Cell number was determined by collecting MSCs by enzymatic dissociation, freezing cells to lyse, and evaluating DNA content by Quant-it PicoGreen dsDNA assay (Invitrogen).

Generation of conditioned media and secretome analysis

MSCs were incubated on hydrogels or TCP for 4 days to generate conditioned media (CM). CM was centrifuged at

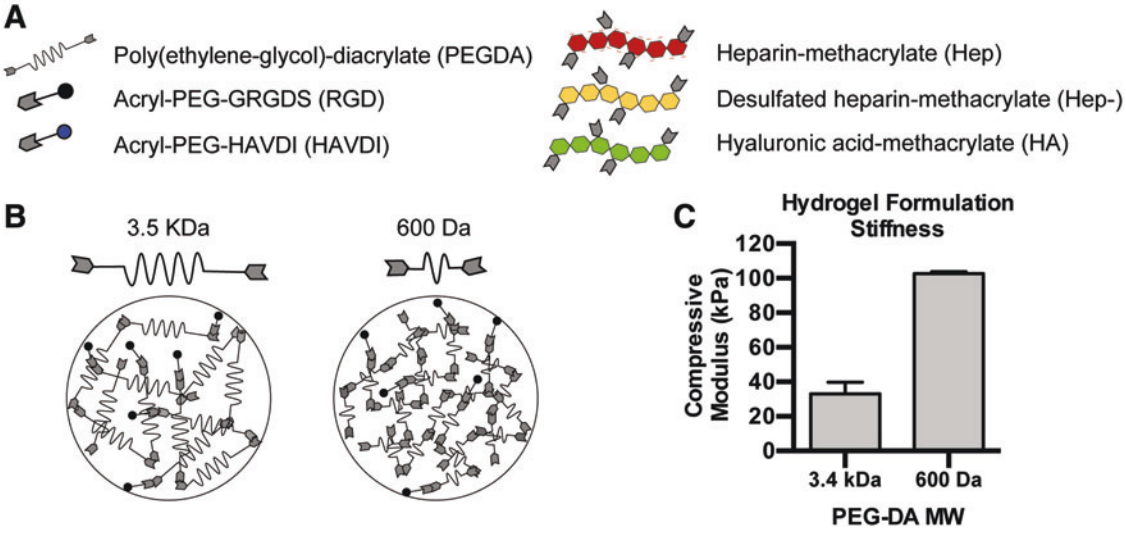

FIG. 1. Hydrogel formulations. **(A)** Hydrogel components. **(B)** Schematic of PEG-DA hydrogels functionalized with RGD using two different molecular weights of PEG-DA to achieve differing mechanical properties. **(C)** Compressive modulus of hydrogel formulations using different PEG-DA molecular weight (mean ± SD). SD, standard deviation. Color images are available online.

1,000 g to remove any debris before freezing the supernatant at −80°C in aliquots. CM was evaluated by Luminex Multiplex enzyme-linked immunosorbent assay for 41 human chemokines, cytokines, and growth factors (HCYTOMAG-60K; EMD Millipore). Integrated Milliplex Analyst software was used for analysis. Complete culture media were used for background subtraction and data were normalized by number of cells per sample.

In vitro *angiogenesis assay*

Human umbilical vein endothelial cells (HUVECs; Lifeline Cell Technology) were expanded on 0.1% gelatin-coated flasks in Endothelial Media (R&D Systems). Matrigel was plated in microwell slides (ibidi) and incubated for 1 h at 37°C. HUVECs suspended in MSC CM (30,000 cells/30 μL) were plated over Matrigel at 15 μL per microwell in duplicate and maintained in a humidified chamber at 37°C for 24 h. Microwells were imaged and analyzed by ImageJ Angiogenesis Analyzer; the ''Total length'' parameter is reported.[46]

In vitro *monocyte chemotaxis assay*

THP-1 monocytes (ATCC TIB202) were maintained in RPMI media with 20% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1% beta-mercaptoethanol. Cells were serum starved in 1% FBS media overnight. Serum-starved THP-1s were seeded into the top chamber of a 96-well 4 μm pore transwell assay in 1% FBS DMEM (10,000 cells/75 μL). Bottom wells were plated with MSC CM diluted 1:1 with 1% FBS DMEM (125 μL total). Negative control for migration was 1% FBS in DMEM, positive control was 10% FBS in DMEM. Cells were allowed to migrate for 8 h at 37°C. Bottom wells were imaged and migrated cells were counted.

In vitro *myogenesis assay*

C2C12 myoblast-like cells (ATTC CRL-1772) were maintained in DMEM (5 g/L glucose) supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% FBS. Once in growth phase, cells were seeded in 48-well plates at 10,000 cells/cm^2 and cultured for 2 days to reach confluence. Growth media were replaced with 1:1 mixture of CM and growth media, and half of the media was replenished on day 3. After 6 days, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X, and blocked in 5% goat serum. Cells were stained with differentiation marker, myosin heavy chain (MHC, MF-20; Developmental Studies Hybridoma Bank) and nuclear counterstain Hoescht 3342. Myofiber length was quantified using ImageJ.[47,48]

Senescence quantification

Senescent cells were identified by senescence-associated β-galactosidase staining following the manufacturer's protocol (No. 9860; Cell Signaling Technology). Briefly, after 4, 8, or 12 days of culture, when cells reached 70–90% confluence on either TCP or RGD 100 kPa hydrogels, cells were washed, fixed, and then incubated with staining solution overnight at 37°C. Staining solution was removed, cells were washed with PBS, and nuclei were stained with Hoechst dye to identify total cell number. Cells were imaged at 20X magnification and number of β-gal+ cells was recorded as a percent of total cells.

Statistical analysis

Samples sizes and replicates are as stated in figure legends, error bars represent mean ± standard deviation. Analysis of differences within a single stiffness of hydrogel used one-way analyses of variance (ANOVAs) with Tukey's *post hoc* comparisons. Analyses across stiffnesses (swelling ratio, compressive moduli) used two-way ANOVA with Tukey's *post hoc* comparisons using Prism software (GraphPad Software). Principal component analysis (PCA) of MSC secretory profiles was performed using SIMCA software (Umetrics).

Results

Hydrogel characterization

To screen the combined effect of mechanical properties and biomolecule presentation on MSC-secreted factors and MSC proliferation, 10 PEG-DA-based culture substrates were synthesized with different combinations of adhesive ligands, GAGs, and mechanical properties (Fig. 1A). Hydrogel surfaces made from 8.5 wt% 3.4 kDa PEG-DA exhibited a compressive modulus of 33.0 ± 6.8 kPa and 20 wt% 600 Da PEG-DA formulations exhibited a modulus of 102.4 ± 1.1 kPa, hereafter referred to as 30 and 100 kPa, respectively (Fig. 1B, C). Hydrogel swelling ratios were not significantly different among gels functionalized with adhesive ligands and GAGs in either the 30 or 100 kPa formulations (Supplementary Fig. S1 and Supplementary Table S1). RGD linker (acryl-PEG-NH2) density and GAG density were consistent between 30 and 100 kPa surfaces (Supplementary Fig. S2A, B). Both 30 and 100 kPa formulations were hydrophilic, with contact angles of 29.3 ± 2.6 and 40.2 ± 4.4, respectively (Supplementary Fig. S2C).[49]

Hydrogel stiffness affects MSC proliferation

MSCs adhered to all hydrogel culture surfaces. After 4 days, MSCs cultured on 30 kPa surfaces, regardless of biomolecule presentation, had a lower cell density compared with those on 100 kPa surfaces or TCP (Fig. 2A–C). MSCs cultured on all 100 kPa surfaces exhibited similar morphology to that of MSCs cultured on TCP. Culture on all 30 kPa surfaces resulted in significantly lower cell yield than TCP, with final cell numbers similar to original seeding density (Fig. 2D). All 100 kPa surfaces produced cell numbers similar to that of TCP, however, Hep surfaces were decreased compared with RGD-only hydrogels (Fig. 2E). These results were supported by similar findings in a second MSC donor line (Supplementary Fig. S3).

Hydrogel stiffness dominates modulatory effects on the MSC secretome

MSCs cultured on all 30 kPa gels exhibited an overall increase in abundance of immunomodulatory factor secretion versus TCP and all 100 kPa hydrogels (Fig. 3A). The CM of MSCs cultured on 100 kPa Hep hydrogels exhibited a decrease of immunomodulatory factor secretion versus all surfaces (Fig. 3A). PCA revealed distinct separation

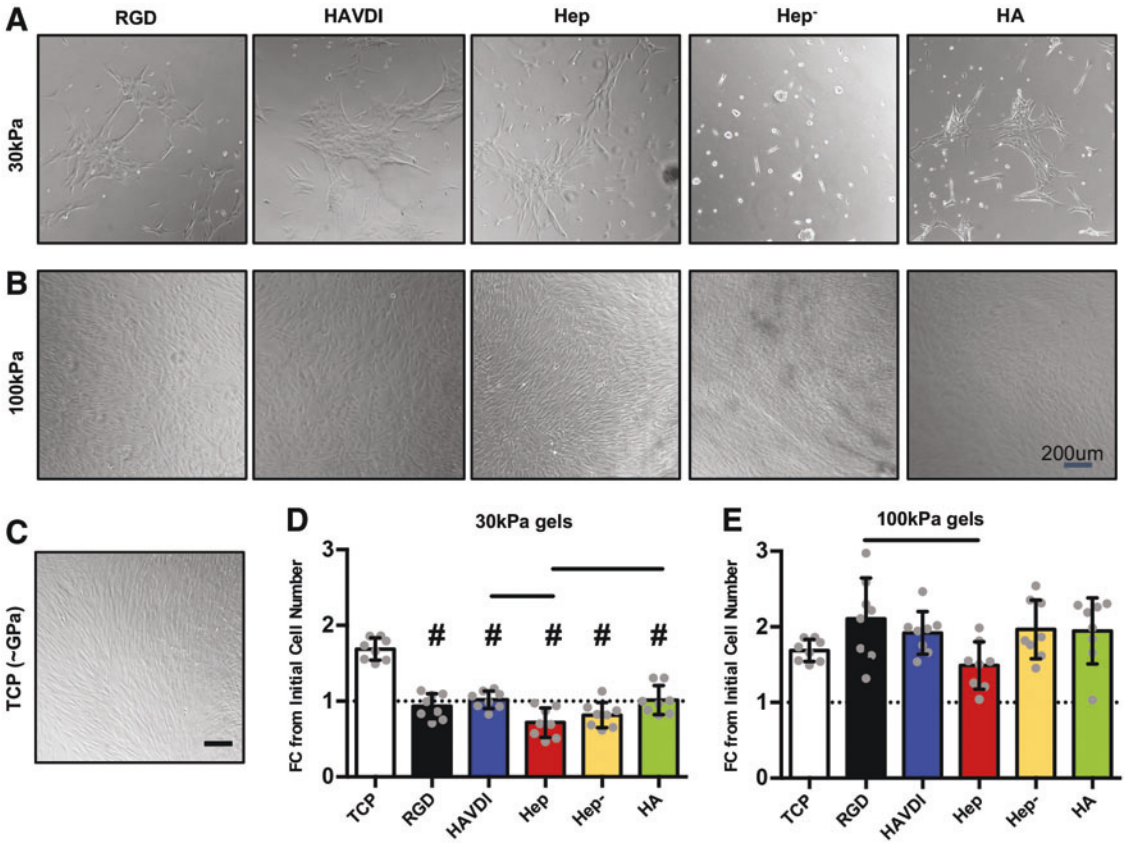

FIG. 2. Increased hydrogel stiffness promotes MSC proliferation. **(A–C)** Representative images of MSCs on **(A)** 30 kPa, **(B)** 100 kPa, or **(C)** TCP after 4 days in culture. **(D)** Fold change (FC) in cell number at 4 days compared with initial cell number on 30 kPa hydrogels or **(E)** 100 kPa gels. *Dotted line* is number of cells seeded (mean ± SD; $^{\#}p < 0.05$ compared with TCP; bars $p < 0.05$ between indicated groups; $n = 7–8$ per group). MSC, mesenchymal stromal cell; TCP, tissue culture polystyrene. Color images are available online.

between the secretome of MSCs cultured on 30 and 100 kPa surfaces along the first principal component (PC1), which accounted for 77% of the variance in the dataset (Fig. 3B, C). All loadings on PC1 are positive and similar in magnitude, which is consistent with the observation that most of the detectable secreted factors are elevated on the 30 kPa hydrogels (Fig. 3C). PCA revealed separation of 100 kPa Hep hydrogel CM from other surfaces along both PC1 and PC2 (Fig. 3B–D). These findings were supported by characterization of MSCs from a second donor, showing broad upregulation in immunomodulatory secretion by cells cultured on all 30 kPa hydrogels, and depletion of secreted factors in the CM of MSCs cultured on 100 kPa Hep gels (Supplementary Fig. S4A). PCA similarly revealed that MSC secretome from the second donor was distinguishable between cells cultured on different stiffness surfaces along PC1, which accounted for 64% of the variance within the dataset (Supplementary Fig. S4B–D).

Quantification of individual immunomodulatory cytokines and chemokines demonstrates increased secretion of several factors, including VEGF, interleukin (IL)-6, IL-8, GRO, and Fractalkine, by MSCs cultured on 30 kPa hydrogels versus those cultured on TCP (Supplementary Fig. S5). Additionally, MSCs cultured on 100 kPa Hep hydrogels exhibited significant decreases in the secretion of all factors versus those cultured on TCP (Supplementary Fig. S4). Interestingly, the secretion of the inflammatory factors, monocyte chemoattractant protein 1 (MCP-1) and IL-6, were downregulated by MSCs cultured on multiple formulations of 100 kPa hydrogels versus TCP (Supplementary Fig. S5).

Hydrogel stiffness and biochemical composition alter angiogenic and immunomodulatory function

In a functional assay for angiogenic activity, CM from MSCs cultured on 30 kPa RGD, HAVDI, and HA-presenting hydrogels increased HUVEC network formation compared with TCP-derived CM (Fig. 4A, B). TCP-derived CM produced network formation similar to standard Endothelial Growth Media (Supplementary Fig. S6). CM from 100 kPa RGD gels improved network formation compared with TCP and all other 100 kPa surfaces, except Hep (Fig. 4C). CM from 30 kPa surfaces, except Hep, enhanced HUVEC network formation compared with 100 kPa surfaces of the same composition (two-way ANOVA, $p < 0.05$).

Monocyte chemotaxis toward MSC CM was enhanced by MSC culture on HAVDI- and HA-presenting surfaces of both stiffnesses compared with CM from TCP culture (Fig. 5). Assay controls showed that THP-1 cells migrate dose dependently toward higher serum concentrations (Supplementary Fig. S6B). TCP CM supported migration

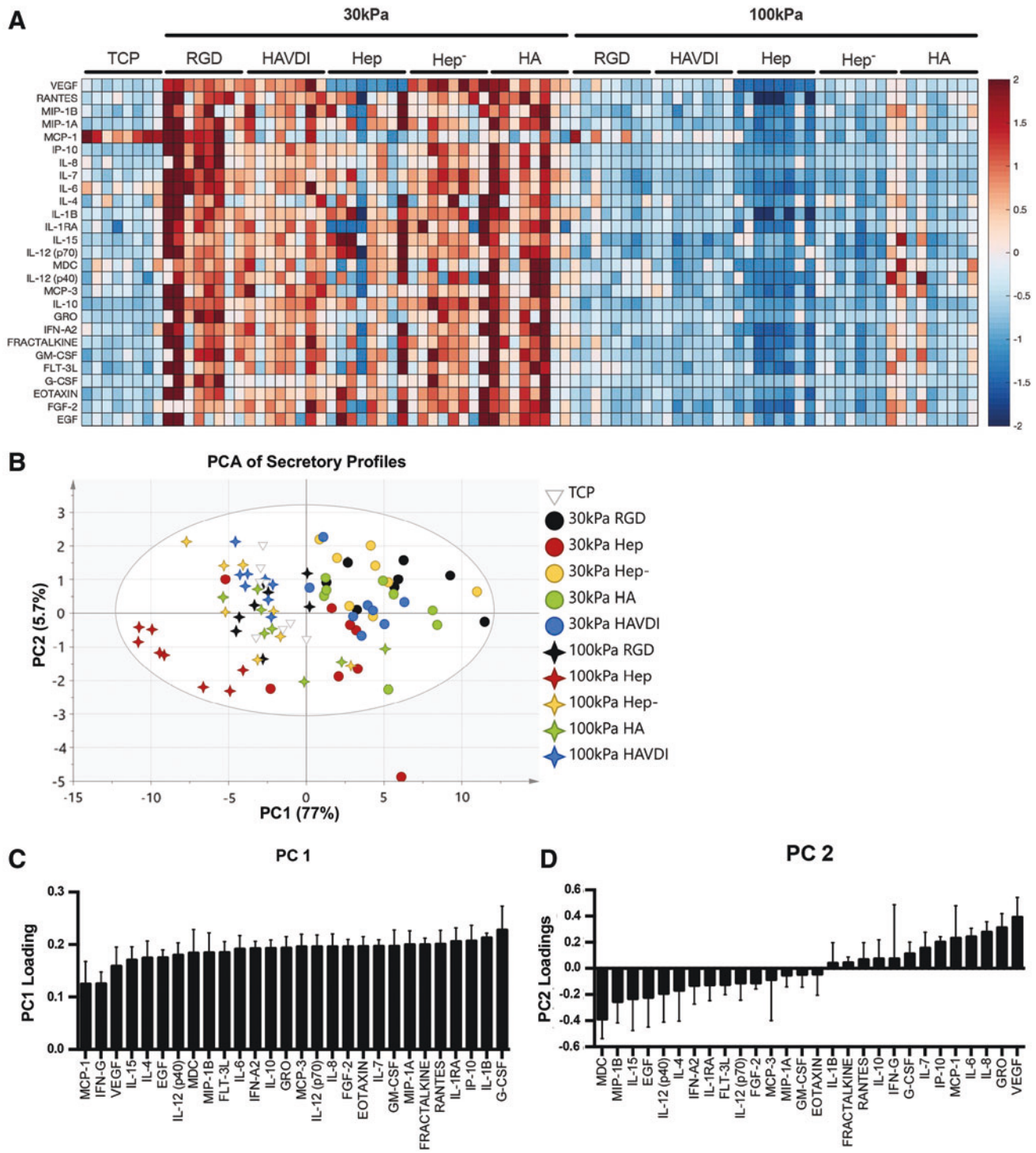

FIG. 3. Hydrogel stiffness dominates modulatory effects on MSC secretome. **(A)** Relative abundance of secreted factors from MSCs cultured on each surface, colors based on z-score. **(B)** PCA of MSC secretome on different surfaces shows separation of 30 and 100 kPa surfaces along PC1 and separation of Hep surfaces from other groups along PC2. Secretory factor loading along PC1 **(C)** and PC2 **(D)**. ($n = 7$–8 per group.) PCA, principal component analysis. Color images are available online.

greater than the assay-negative control of 1% FBS, but not different from unconditioned media with equivalent FBS concentration of 5% (Supplementary Fig. S6B). No significant differences in monocyte chemotaxis were found between CM from 30 and 100 kPa surfaces.

Myoblast-like cells produced MHC+ myotubes when cultured in CM of MSCs cultured on all surfaces (Fig. 6A). Myotube length was not enhanced by hydrogel-stimulated CM compared with standard TCP-derived CM. TCP-derived CM supported myotube formation greater than the

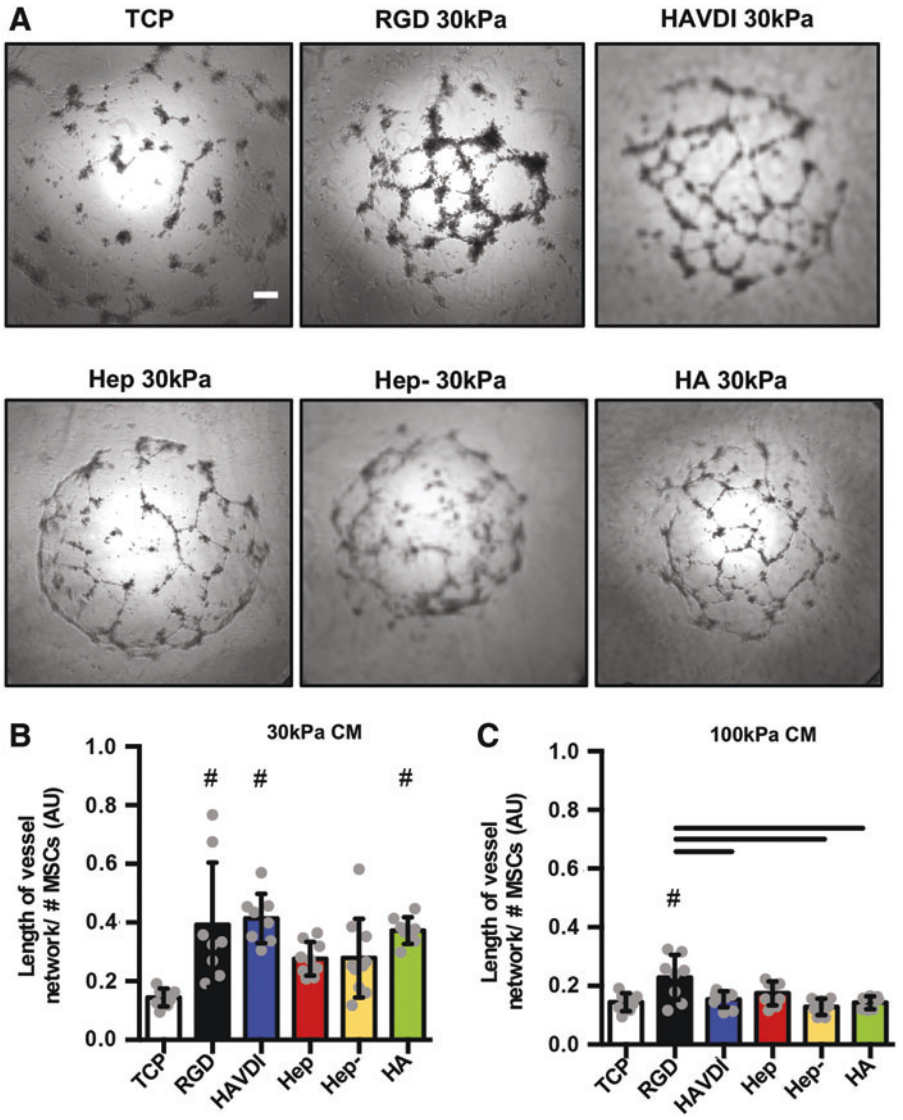

FIG. 4. Hydrogel culture enhances MSC *in vitro* paracrine angiogenic activity. **(A)** Representative images of HUVEC network formation on Matrigel with MSC CM (24 h, scale bar 200 μm). **(B–C)** Quantification of HUVEC network length in MSC-CM from 30 kPa hydrogels **(B)** or 100 kPa hydrogels **(C)**. (Mean ± SD; $^{\#}p < 0.05$ compared with TCP; bars $p < 0.05$ between indicated groups; $n = 4$–6 per group.) CM, conditioned media; HUVEC, human umbilical vein endothelial cells. Color images are available online.

negative control of basal MSC media, but less than the positive control of C2C12 differentiation media (Supplementary Fig. S6C). There were no significant differences in C2C12 myotube formation between CM from 30 and 100 kPa hydrogels. CM produced on 100 kPa Hep surfaces decreased myotube formation versus CM from TCP, 100 kPa Hep^{-}, and HA cultures (Fig. 6).

Extended culture on hydrogel substrates reduces MSC senescence

MSCs that were previously culture expanded on TCP (~PDL 16.8) were passaged to either TCP or 100 kPa RGD hydrogels (Fig. 7A). MSCs serially cultured on TCP exhibited increasing levels of senescence over three passages

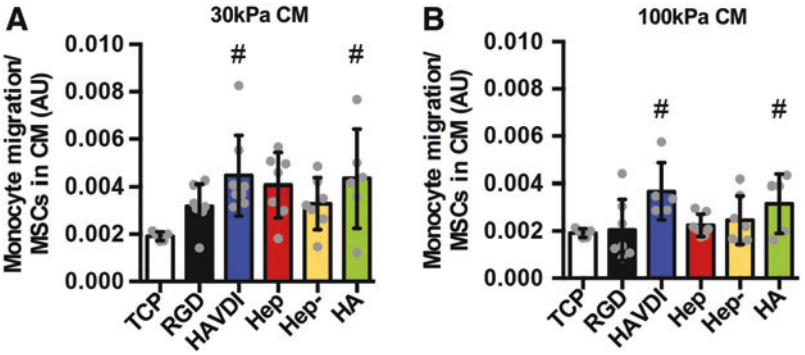

FIG. 5. MSCs cultured on HAVDI or HA hydrogels enhance monocyte chemotaxis. Migration of THP-1 cells toward CM from MSCs cultured on **(A)** 30 kPa or **(B)** 100 kPa hydrogels. (Mean ± SD; $^{\#}p < 0.05$ compared with TCP; $n = 5$–8 per group.) HA, hyaluronic acid. Color images are available online.

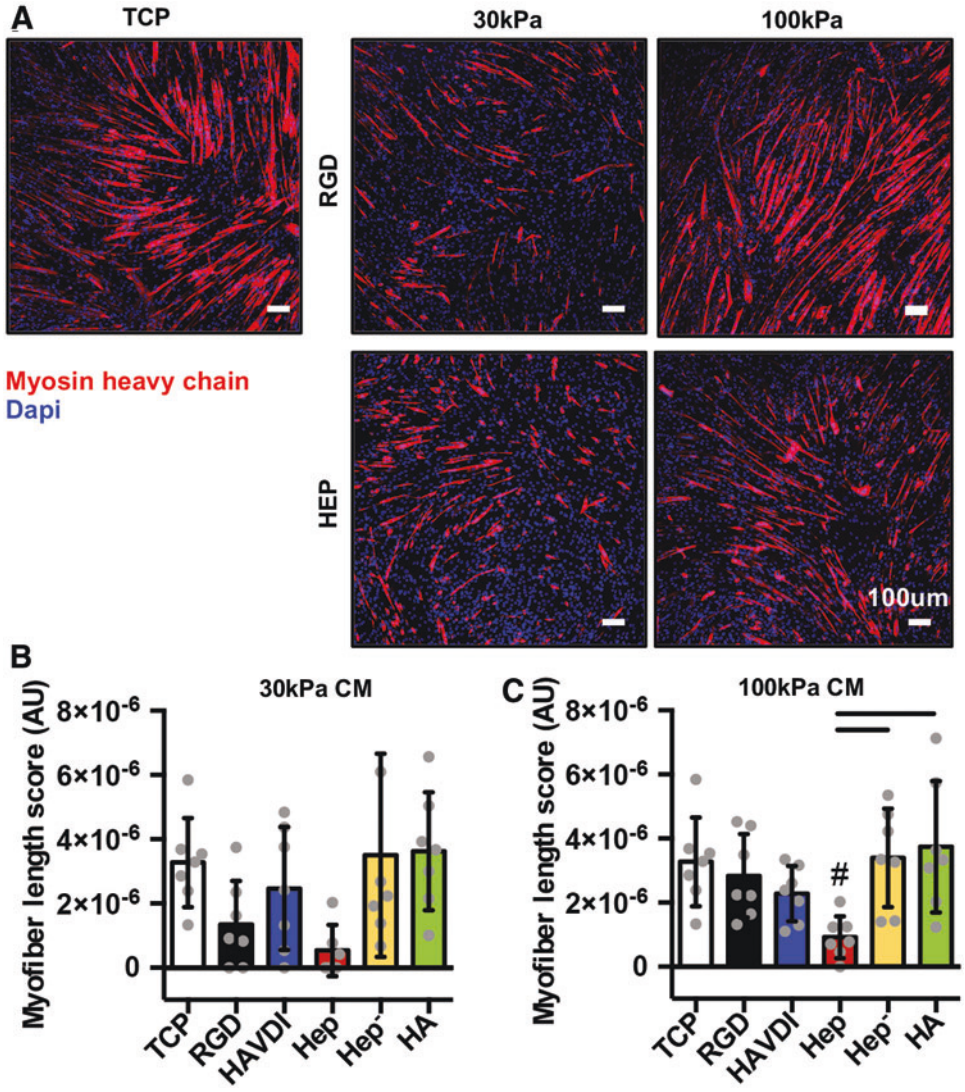

FIG. 6. MSC CM does not enhance *in vitro* myogenic activity. **(A)** Representative images of C2C12 myoblast-like cells cultured with MSC CM from indicated conditions (scale bar 100 μm). **(B, C)** Quantification of the fraction of myofibers over 400 μm relative to the number of MSCs in the CM on **(B)** 30 kPa hydrogels or **(C)** 100 kPa hydrogels. (Mean ± SD; $^{\#}p < 0.05$ compared with TCP; bars $p < 0.05$ between indicated groups; $n = 7–9$ per group.) Color images are available online.

(''p1''–''p3'') as indicated by increased percentage of cells staining positive for senescence-associated β-gal (p1: 5.27% ± 3.04%, p2:13.67% ± 4.02%, p3: 25.06% ± 6.60%) (Fig. 7B, C). MSCs cultured on 100 kPa RGD hydrogels exhibited significantly lower levels of β-gal^{+} cells over the course of the successive passages (Fig. 7B, C). These findings were supported by similar results in a second MSC donor (Supplementary Fig. S7) demonstrating that MSCs cultured on 100 kPa RGD hydrogels undergo less senescence than on TCP.

MSCs retain the ability to respond to different stiffness surfaces after sustained culture on hydrogel

MSCs serially passaged on 100 kPa RGD hydrogels retained the ability to increase secretion of many growth factors, cytokines, and chemokines when transferred to 30 kPa hydrogels compared with TCP. The highest secreted factors, VEGF, IL-8, and GRO, are shown in Figure 7D.

Discussion

This work used hydrogel culture surfaces that differed in both substrate stiffness and biochemical composition to explore the relationship between culture surface parameters, MSC secretory activity, proliferation, and senescence. Overall, we found that mechanical properties of hydrogels dominate the effect on MSC secretome. Softer 30 kPa hydrogels, regardless of biochemical functionalization, broadly enhanced secretion of detected immunomodulatory and regenerative factors versus stiffer surfaces, but did not support robust proliferation. Hydrogel surfaces of 100 kPa supported proliferation, and altered secretion of selected factors compared with TCP. Extended culture on 100 kPa RGD hydrogels significantly reduced MSC senescence-associated β-gal activity versus TCP, while maintaining the MSC's capacity to enhance secretory capacity in response to 30 kPa hydrogels. It should be noted that these results were generally consistent in both cell donors that were used in this study to address potential effects of donor heterogeneity. Taken together, these studies identify hydrogel culture parameters that can be utilized either to stimulate a heightened therapeutic secretome in cultured MSCs, or to expand healthy (nonsenescent) MSCs that retain the ability to respond to changes in their mechanical environment.

Of the multiple culture surface parameters screened, substrate stiffness, which was consistent across different

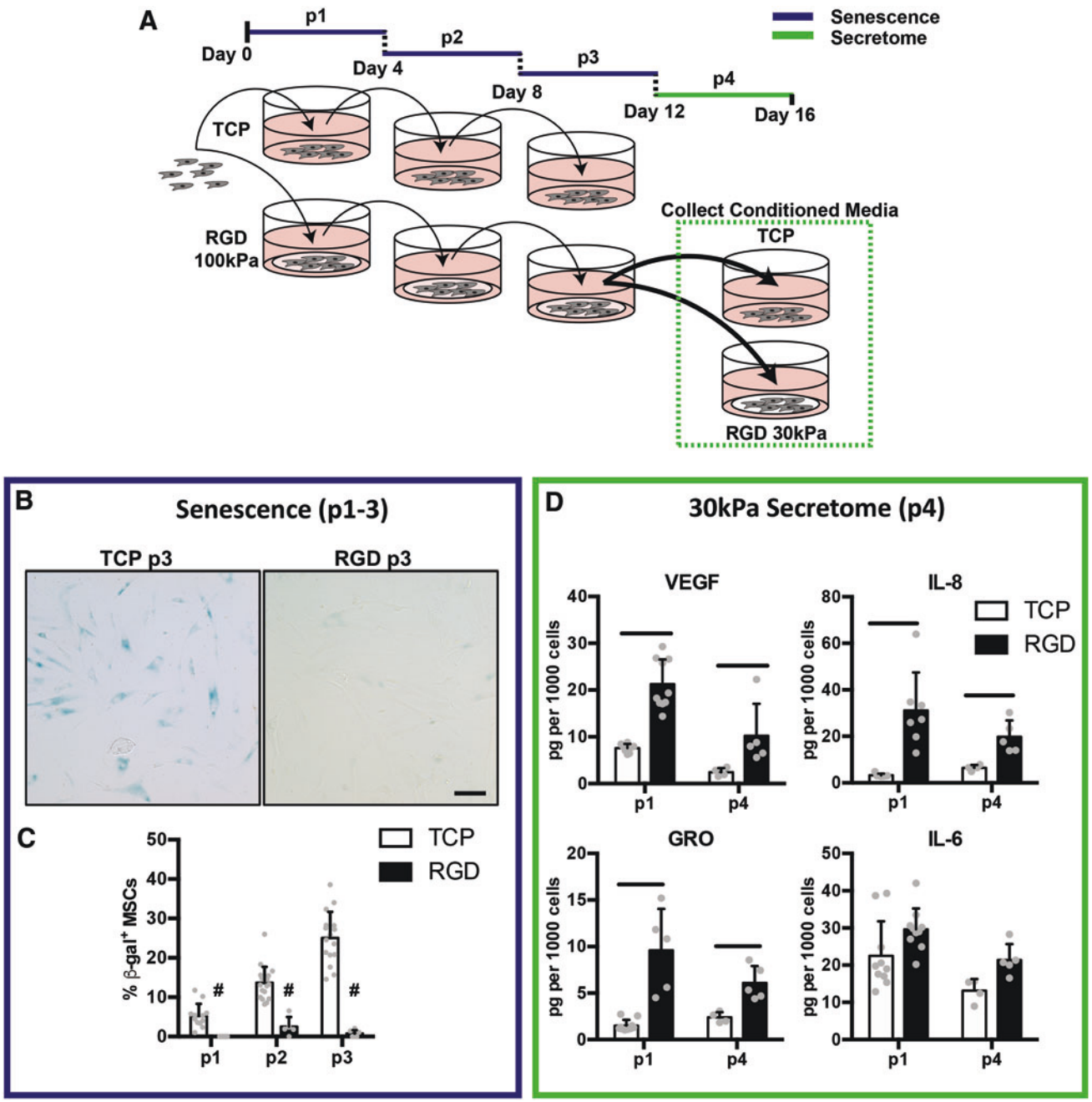

FIG. 7. MSCs serially passaged on 100 kPa RGD hydrogel surfaces have reduced senescence. **(A)** MSCs that were previously culture expanded on TCP (~PDL 16.8) were passaged to either TCP or 100 kPa RGD hydrogels. Cells were maintained on either TCP or RGD 100 kPa hydrogels for three successive passages (p1–p3; 4 days each passage). On day 12, cells from the 100 kPa RGD hydrogels were passaged to either TCP or 30 kPa RGD hydrogels for analysis of the secretome. **(B)** β-gal staining for senescence at day 12 of culture on TCP or RGD. **(C)** Quantification of β-gal staining over three passages (two-way ANOVA, $^{\#}p < 0.05$ vs TCP). **(D)** Individual factors (highest secreted factors from initial screen) exhibited differences in secretion by MSCs replated onto either TCP or 30 kPa hydrogels after serial culture on 100 kPa RGD hydrogels ($n = 4$, two-way ANOVA, $p < 0.05$). ANOVA, analysis of variance. Color images are available online.

hydrogel functionalizations (Supplementary Fig. S1), had a much greater impact on MSC proliferation than did biochemical composition. Modification of plastic or glass substrates with adhesive peptides and proteins have previously been shown to improve MSC adhesion and proliferation[50,51]; however, the impact of adhesive ligands in comparison to substrate stiffness has not been directly compared. MSCs cultured on softer 30 kPa hydrogels, regardless of functionalization, exhibited reduced cell yield (Fig. 2) versus their 100 kPa counterparts (Fig. 2). Ligand density, characterized by incorporation of PEG with terminal amines into the hydrogel network, was consistent between 30 and 100 kPa formulations (Supplementary Fig. S2); therefore, stiffness is the primary variable contributing to this

difference. Although changes in proliferation were only assessed using a single adhesive ligand density (1 mM RGD), these findings support previous work demonstrating that decreased culture substrate stiffness is related to decreased proliferation.[52–54] Altogether, these findings suggest that substrate stiffness causes a greater impact on MSC proliferation compared with type of biomolecule presented, within the range of biomolecule types tested in this study.

Multivariate analysis of MSC secretome indicated that substrate stiffness had a greater impact on MSC secretory profiles than biochemical composition. Hydrogels of 30 kPa stiffness broadly enhanced the level of immunomodulatory factors versus either 100 kPa hydrogels or TCP (Fig. 3). This stiffness-related amplification of secretion is supported by previous findings of increases in several individual paracrine factors on substrate's orders of magnitude lower stiffness than TCP (1–10 kPa vs. ~GPa).[20,21,55] On some 30 kPa substrates, cell aggregation and clustering were observed, a phenomenon that can broadly enhance the secretory capacity of MSCs.[56–58] MSCs cultured on 100 kPa gels did not exhibit a distinct secretory profile from those on TCP, however, some differences were observed in individual factors, including MCP-1 and IL-6 (Supplementary Fig. S5). Although the relationship between MSC secretion and proliferation mediated by culture substrate is not fully understood, MSCs induced into quiescent phenotypes using soluble or surface-based techniques, have been shown to experience high metabolic activity and the secretion of various proteins.[59,60] Thus, substrate stiffness may simultaneously modulate MSC proliferative and secretory activity.

Some biomolecular functionalization impacted detection of cell-secreted factors. Hep-functionalized 100 kPa surfaces had decreased detection of many factors in CM (Fig. 3), consistent with the known strong interaction of heparan sulfate with many positively charged proteins, which may have pulled down these proteins from the CM.[61,62] Previous studies with heparin-functionalized materials have demonstrated the ability of fully sulfated heparin materials to sequester proteins secreted by both human MSCs[63] and murine chondrocytes.[64] When the charged sulfate groups are removed to make Hep^{-}, higher levels of secreted proteins are found in the CM, suggesting that protein sequestration requires the charge associated with fully sulfated Hep. Further studies would be necessary to confirm this pull down and determine whether retention of MSC paracrine factors at the culture surface impacts cellular behavior.

Because MSC paracrine activity impacts several biological functions of wound healing,[31] several *in vitro* assays were employed to assess downstream biologic effects of the hydrogel-stimulated MSC secretome. Angiogenesis, innate immune cell recruitment, and myotube formation were explored as these are each critical elements of the musculoskeletal repair process. These functional effects were assessed at a single dose of CM to assess changes when MSCs were cultured on different surfaces. MSC CM from 30 kPa substrates, with the exception of Hep, exhibited increased angiogenic potency versus their 100 kPa counterparts (Fig. 4). This finding supports previous results showing surfaces that promote MSC aggregation and surfaces around 40 kPa enhance VEGF secretion and improve proangiogenic signaling.[21,58]

Monocyte chemotaxis, which is a critical first step in the innate immune injury response,[65] was enhanced only by CM from MSCs cultured on HAVDI- and HA-functionalized surfaces, regardless of hydrogel stiffness, compared with TCP control (Fig. 5). This finding was surprising as multivariate analysis of MSCs cultured on 100 kPa hydrogels functionalized with HAVDI or HA did not show overall secretory profiles distinct from those on TCP (Fig. 3); however, other factors not included in our secretome panel may also be modulated by culture surfaces, and contribute to recruitment of monocytes. Many of the surfaces in addition to HAVDI or HA, modulated the secretion of monocyte chemotactic factors, fractalkine and MCP-1, known to recruit anti-inflammatory and proinflammatory monocytes, respectively.[38,39] Since these monocytes were not polarized to either pro- or anti-inflammatory phenotype, it is unclear whether this finding would be beneficial for a wound healing or regenerative response. Future assessment of the polarization state of recruited monocytes may reveal other functional effects of culture surface priming, in terms of the type of inflammatory response MSCs cultured on these surfaces may stimulate.

Myogenic activity was not impacted by substrate stiffness or biochemical composition, except for the effects of potential protein sequestration observed in Hep-functionalized hydrogel surfaces (Fig. 6). MSCs cultured on 100 kPa Hep-functionalized hydrogels reduced overall myotube length compared with TCP, suggesting that MSC-derived factors promote myotube development, however, the effect is not enhanced by hydrogel culture. Although MSCs have been shown to exhibit myogenic function *in vivo*,[66,67] this *in vitro* CM assay, along with other previous studies, suggest that bidirectional signaling, myogenic cytokine supplementation, or additional cell types, may be required to evaluate the impact of conditioning methods on MSC myogenic function.[68–70] Taken together, these *in vitro* functional assays suggest that enhancing the secretome of MSCs by culture on tailored substrates can improve their angiogenic and immunomodulatory activity, which may have a functional impact on subsequent regenerative processes.

While certain hydrogel formulations impacted MSC secretome, others improved long-term MSC culture. RGD 100 kPa hydrogels maintained similar cell expansion to TCP, however, reduced replicative senescence during serial cell expansion (Fig. 7A–C). Previous strategies to abrogate senescence include media additives, such as reactive oxygen species-scavenging molecules or intracellular signaling pathway inhibitors.[71,72] We are the first to report a culture surface that alone reduces early markers of senescence. MSC senescence induced by long-term culture on TCP alters cell morphology, downregulates cytoskeletal organization pathways, and focal adhesion organization.[10,73,74] Therefore, material substrates softer than TCP may reduce deleterious cytoskeleton reorganization, thereby reducing MSC senescence. The cytoskeletal changes MSCs may undergo on 100 kPa hydrogels require further exploration, however, these substrates offer a novel strategy to support production of clinical-scale nonsenescent MSCs.

Substrate-dependent modulation of MSC cytoskeletal and morphological behaviors suggests potential mechanisms for the decrease in senescence observed. Mechanically induced changes in MSC morphology and nuclear translocation

of the mechanical rheostat transcriptional activator Yes-associate protein (YAP) are reversible in shorter term cultures, however, long exposure to stiff surfaces causes cells to develop an irreversible mechanical memory that may be epigenetically mediated.[6,23] Previous work showed that MSCs cultured over 11 passages on TCP were not able to recover secretory responsiveness to softer surfaces.[6]

Extended culture on our 100 kPa RGD hydrogels not only reduces senescence, but also, allows MSCs to retain responsiveness to the difference between 30 kPa hydrogel and TCP as evidenced by changes in their secretome (Fig. 7). After 12 days of culture on 100 kPa RGD hydrogels, a timeframe shown to induce irreversible epigenetic changes,[23] MSCs significantly increased expression of pro-regenerative and immunomodulatory factors (VEGF, IL-8, GRO) without enhancing inflammatory factors such as IL-6 (Fig. 7D). These specific factors were chosen as representative factors since they were the secreted in levels $\sim$10-fold higher than other analytes in initial secretome. Given the observation of reduced senescence in serial hydrogel culture, these cells may retain a more healthy secretory phenotype compared with previous studies, where cells were not able to respond to soft surfaces after sustained culture.[6,23] This exciting finding indicates that our materials screen identified substrates that could be used to stimulate or prime MSCs for regenerative activity after expansion on other substrates designed to improve MSC fitness by reducing senescence. Further investigation is needed to verify the impact of these substrates on regenerative activity and senescence using three-dimensional culture or other higher-yield cell expansion systems, such as suspension culture in bioreactors, as well as its applicability to the expansion of other adherent cell types.

Conclusion

The goal of this study was to assess the impact of substrate stiffness as well as adhesive ligand and GAG presentation on MSC secretome, proliferation, and replicative senescence relative to standard culture surfaces (TCP). Culture on 30 kPa hydrogels halted MSC proliferation, however, broadly enhanced secretion of immunomodulatory factors, resulting in improved MSC angiogenic potency. MSCs cultured on 100 kPa hydrogel substrates promoted MSC proliferation at levels similar to that of TCP, but did not exhibit any amplification in secretome. However, extended culture on 100 kPa hydrogels reduced replicative senescence in MSCs compared with those cultured on TCP, while still maintaining mechanosensitivity to enhance secretory activity when passaged onto softer substrates. The success of MSC-based therapies is dependent on the manufacture of large number of cells with high levels of therapeutic potency. Thus, maximizing secretory capacity and reducing replicative senescence through the choice of hydrogel culture materials tailored for each goal has great potential to improve the large-scale production of therapeutic MSCs.

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Supplementary Material

Supplementary Data
Supplementary Table S1
Supplementary Table S2
Supplementary Figure S1
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References

1. Spees, J.L., Lee, R.H., and Gregory, C.A. Mechanisms of mesenchymal stem/stromal cell function. Stem Cell Res Ther **7,** 125, 2016.
2. Kim, H.Y., Kim, H., Oh, K.W., *et al.* Biological markers of mesenchymal stromal cells as predictors of response to autologous stem cell transplantation in patients with amyotrophic lateral sclerosis: an investigator-initiated trial and in vivo study. Stem Cells **32,** 2724, 2014.
3. Galipeau, J. and Sensebe, L. Mesenchymal stromal cells: clinical challenges and therapeutic opportunities. Cell Stem Cell **22,** 824, 2018.
4. Jossen, V., van den Bos, C., Eibl, R., and Eibl, D. Manufacturing human mesenchymal stem cells at clinical scale: process and regulatory challenges. Appl Microbiol Biotechnol **102,** 3981, 2018.
5. Mizukami, A., Pereira Chilima, T., Orellana, M., *et al.* Technologies for large-scale umbilical cord-derived MSC expansion: experimental performance and cost of goods analysis. Biochem Eng J **135,** 36, 2018.
6. Rao, V.V., Vu, M.K., Ma, H., Killaars, A.R., and Anseth, K.S. Rescuing mesenchymal stem cell regenerative properties on hydrogel substrates post serial expansion. Bioeng Transl Med **4,** 51, 2019.
7. Choi, M.R., Kim, H.Y., Park, J.Y., *et al.* Selection of optimal passage of bone marrow-derived mesenchymal stem cells for stem cell therapy in patients with amyotrophic lateral sclerosis. Neurosci Lett **472,** 94, 2010.
8. von Bahr, L., Sundberg, B., Lonnies, L., *et al.* Long-term complications, immunologic effects, and role of passage for outcome in mesenchymal stromal cell therapy. Biol Blood Marrow Transplant **18,** 557, 2012.
9. Banfi, A., Muraglia, A., Dozin, B., Mastrogiacomo, M., Cancedda, R., and Quarto, R. Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: implications for their use in cell therapy. Exp Hematol **28,** 707, 2000.
10. Wagner, W., Horn, P., Castoldi, M., *et al.* Replicative senescence of mesenchymal stem cells: a continuous and organized process. PLoS One **3,** e2213, 2008.

11. Shibata, K.R., Aoyama, T., Shima, Y., *et al.* Expression of the p16INK4A gene is associated closely with senescence of human mesenchymal stem cells and is potentially silenced by DNA methylation during in vitro expansion. Stem Cells **25,** 2371, 2007.
12. Crisan, M., Yap, S., Casteilla, L., *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell **3,** 301, 2008.
13. Caplan, A.I. New MSC: MSCs as pericytes are sentinels and gatekeepers. J Orthop Res **35,** 1151, 2017.
14. Last, J.A., Liliensiek, S.J., Nealey, P.F., and Murphy, C.J. Determining the mechanical properties of human corneal basement membranes with atomic force microscopy. J Struct Biol **167,** 19, 2009.
15. Miller, R.T. Mechanical properties of basement membrane in health and disease. Matrix Biol **57,** 366, 2017.
16. Engler, A.J., Sen, S., Sweeney, H.L., and Discher, D.E. Matrix elasticity directs stem cell lineage specification. Cell **126,** 677, 2006.
17. Wen, J.H., Vincent, L.G., Fuhrmann, A., *et al.* Interplay of matrix stiffness and protein tethering in stem cell differentiation. Nat Mater **13,** 979, 2014.
18. Cosgrove, B.D., Mui, K.L., Driscoll, T.P., *et al.* N-cadherin adhesive interactions modulate matrix mechanosensing and fate commitment of mesenchymal stem cells. Nat Mater **15,** 1297, 2016.
19. Yang, C., Tibbitt, M.W., Basta, L., and Anseth, K.S. Mechanical memory and dosing influence stem cell fate. Nat Mater **13,** 645, 2014.
20. Liu, F.D., Pishesha, N., Poon, Z., Kaushik, T., and Van Vliet, K.J. Material viscoelastic properties modulate the mesenchymal stem cell secretome for applications in hematopoietic recovery. ACS Biomat **3,** 3292, 2017.
21. Abdeen, A.A., Weiss, J.B., Lee, J., and Kilian, K.A. Matrix composition and mechanics direct proangiogenic signaling from mesenchymal stem cells. Tissue Eng Part A **20,** 2737, 2014.
22. Darnell, M., O'Neil, A., Mao, A., Gu, L., Rubin, L.L., and Mooney, D.J. Material microenvironmental properties couple to induce distinct transcriptional programs in mammalian stem cells. Proc Natl Acad Sci U S A **115,** E8368, 2018.
23. Killaars, A.R., Grim, J.C., Walker, C.J., Hushka, E.A., Brown, T.E., and Anseth, K.S. Extended exposure to stiff microenvironments leads to persistent chromatin remodeling in human mesenchymal stem cells. Adv Sci (Weinh) **6,** 1801483, 2019.
24. Provenzano, P.P., and Keely, P.J. Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling. J Cell Sci **124,** 1195, 2011.
25. Docheva, D., Popov, C., Mutschler, W., and Schieker, M. Human mesenchymal stem cells in contact with their environment: surface characteristics and the integrin system. J Cell Mol Med **11,** 21, 2007.
26. Qazi, T.H., Mooney, D.J., Duda, G.N., and Geissler, S. Biomaterials that promote cell-cell interactions enhance the paracrine function of MSCs. Biomaterials **140,** 103, 2017.
27. Lee, E.J., Choi, E.K., Kang, S.K., *et al.* N-cadherin determines individual variations in the therapeutic efficacy of human umbilical cord blood-derived mesenchymal stem cells in a rat model of myocardial infarction. Mol Ther **20,** 155, 2012.
28. Kalluri, R. Basement membranes: structure, assembly and role in tumour angiogenesis. Nat Rev Cancer **3,** 422, 2003.
29. Kim, M., Kim, Y.H., and Tae, G. Human mesenchymal stem cell culture on heparin-based hydrogels and the modulation of interactions by gel elasticity and heparin amount. Acta Biomater **9,** 7833, 2013.
30. Corradetti, B., Taraballi, F., Martinez, J.O., *et al.* Hyaluronic acid coatings as a simple and efficient approach to improve MSC homing toward the site of inflammation. Sci Rep **7,** 7991, 2017.
31. Caplan, A.I., and Correa, D. The MSC: an injury drugstore. Cell Stem Cell **9,** 11, 2011.
32. Kinnaird, T., Stabile, E., Burnett, M.S., *et al.* Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res **94,** 678, 2004.
33. Kehl, D., Generali, M., Mallone, A., *et al.* Proteomic analysis of human mesenchymal stromal cell secretomes: a systematic comparison of the angiogenic potential. NPJ Regen Med **4,** 8, 2019.
34. Saldana, L., Valles, G., Bensiamar, F., Mancebo, F.J., Garcia-Rey, E., and Vilaboa, N. Paracrine interactions between mesenchymal stem cells and macrophages are regulated by 1,25-dihydroxyvitamin D3. Sci Rep **7,** 14618, 2017.
35. Awojoodu, A.O., Ogle, M.E., Sefcik, L.S., *et al.* Sphingosine 1-phosphate receptor 3 regulates recruitment of anti-inflammatory monocytes to microvessels during implant arteriogenesis. Proc Natl Acad Sci U S A **110,** 13785, 2013.
36. Krieger, J.R., Ogle, M.E., McFaline-Figueroa, J., Segar, C.E., Temenoff, J.S., and Botchwey, E.A. Spatially localized recruitment of anti-inflammatory monocytes by SDF-1alpha-releasing hydrogels enhances microvascular network remodeling. Biomaterials **77,** 280, 2016.
37. Olingy, C.E., San Emeterio, C.L., Ogle, M.E., *et al.* Non-classical monocytes are biased progenitors of wound healing macrophages during soft tissue injury. Sci Rep **7,** 447, 2017.
38. Mojumdar, K., Liang, F., Giordano, C., *et al.* Inflammatory monocytes promote progression of Duchenne muscular dystrophy and can be therapeutically targeted via CCR2. EMBO Mol Med **6,** 1476, 2014.
39. Miteva, K., Pappritz, K., El-Shafeey, M., *et al.* Mesenchymal stromal cells modulate monocytes trafficking in coxsackievirus B3-induced myocarditis. Stem Cells Transl Med **6,** 1249, 2017.
40. De Lisio, M., Jensen, T., Sukiennik, R.A., Huntsman, H.D., and Boppart, M.D. Substrate and strain alter the muscle-derived mesenchymal stem cell secretome to promote myogenesis. Stem Cell Res Ther **5,** 74, 2014.
41. Hahn, M.S., Taite, L.J., Moon, J.J., Rowland, M.C., Ruffino, K.A., and West, J.L. Photolithographic patterning of polyethylene glycol hydrogels. Biomaterials **27,** 2519, 2006.
42. Tellier, L.E., Miller, T., McDevitt, T.C., and Temenoff, J.S. Hydrolysis and sulfation pattern effects on release of bioactive bone morphogenetic protein-2 from heparin-based microparticles. J Mater Chem B **3,** 8001, 2015.
43. Seto, S.P., Casas, M.E., and Temenoff, J.S. Differentiation of mesenchymal stem cells in heparin-containing hydrogels via coculture with osteoblasts. Cell Tissue Res **347,** 589, 2012.
44. Moon, J.J., Saik, J.E., Poche, R.A., *et al.* Biomimetic hydrogels with pro-angiogenic properties. Biomaterials **31,** 3840, 2010.

45. Yang, P.J., Levenston, M.E., and Temenoff, J.S. Modulation of mesenchymal stem cell shape in enzyme-sensitive hydrogels is decoupled from upregulation of fibroblast markers under cyclic tension. Tissue Eng Part A **18,** 2365, 2012.
46. Carpentier, G. ImageJ contribution: angiogenesis analyzer. ImageJ News, 2012.
47. San Emeterio, C.L., Olingy, C.E., Chu, Y., and Botchwey, E.A. Selective recruitment of non-classical monocytes promotes skeletal muscle repair. Biomaterials **117,** 32, 2017.
48. Hayashi, S., and Yonekura, S. Thermal stimulation at 39 degrees C facilitates the fusion and elongation of C2C12 myoblasts. Anim Sci J **90,** 1008, 2019.
49. Munoz-Pinto, D.J., Grigoryan, B., Long, J., Grunlan, M., and Hahn, M.S. An approach for assessing hydrogel hydrophobicity. J Biomed Mater Res A **100,** 2855, 2012.
50. Qian, L., and Saltzman, W.M. Improving the expansion and neuronal differentiation of mesenchymal stem cells through culture surface modification. Biomaterials **25,** 1331, 2004.
51. Cimino, M., Goncalves, R.M., Barrias, C.C., and Martins, M.C.L. Xeno-free strategies for safe human mesenchymal stem/stromal cell expansion: supplements and coatings. Stem Cells Int **2017,** 6597815, 2017.
52. Winer, J.P., Janmey, P.A., McCormick, M.E., and Funaki, M. Bone marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli. Tissue Eng Part A **15,** 147, 2009.
53. Mao, A.S., Shin, J.W., and Mooney, D.J. Effects of substrate stiffness and cell-cell contact on mesenchymal stem cell differentiation. Biomaterials **98,** 184, 2016.
54. Sun, M., Chi, G., Li, P., *et al.* Effects of matrix stiffness on the morphology, adhesion, proliferation and osteogenic differentiation of mesenchymal stem cells. Int J Med Sci **15,** 257, 2018.
55. Marklein, R., Soranno, D., and Burdick, J. Magnitude and presentation of mechanical signals influence adult stem cell behavior in 3-dimensional macroporous hydrogels. Soft Matter **8,** 8113, 2012.
56. Cha, J.M., Shin, E.K., Sung, J.H., *et al.* Efficient scalable production of therapeutic microvesicles derived from human mesenchymal stem cells. Sci Rep **8,** 1171, 2018.
57. Zimmermann, J.A., and McDevitt, T.C. Pre-conditioning mesenchymal stromal cell spheroids for immunomodulatory paracrine factor secretion. Cytotherapy **16,** 331, 2014.
58. Lee, E.J., Park, S.J., Kang, S.K., *et al.* Spherical bullet formation via E-cadherin promotes therapeutic potency of mesenchymal stem cells derived from human umbilical cord blood for myocardial infarction. Mol Ther **20,** 1424, 2012.
59. Lemons, J.M., Feng, X.J., Bennett, B.D., *et al.* Quiescent fibroblasts exhibit high metabolic activity. PLoS Biol **8,** e1000514, 2010.
60. Rumman, M., Majumder, A., Harkness, L., *et al.* Induction of quiescence (G0) in bone marrow stromal stem cells enhances their stem cell characteristics. Stem Cell Res **30,** 69, 2018.
61. Muñoz, E.M., and Linhardt, R.J. Heparin-binding domains in vascular biology. Arterioscler Thromb Vasc Biol **24,** 1549, 2004.
62. Krilleke, D., Ng, Y.S., and Shima, D.T. The heparin-binding domain confers diverse functions of VEGF-A in development and disease: a structure-function study. Biochem Soc Trans **37,** 1201, 2009.
63. Hudalla, G.A., Kouris, N.A., Koepsel, J.T., Ogle, B.M., and Murphy, W.L. Harnessing endogenous growth factor activity modulates stem cell behavior. Integr Biol (Camb) **3,** 832, 2011.
64. Rinker, T.E., Philbrick, B.D., Hettiaratchi, M.H., Smalley, D.M., McDevitt, T.C., and Temenoff, J.S. Microparticle-mediated sequestration of cell-secreted proteins to modulate chondrocytic differentiation. Acta Biomater **68,** 125, 2018.
65. Ogle, M.E., Segar, C.E., Sridhar, S., and Botchwey, E.A. Monocytes and macrophages in tissue repair: implications for immunoregenerative biomaterial design. Exp Biol Med (Maywood) **241,** 1084, 2016.
66. Sassoli, C., Zecchi-Orlandini, S., and Formigli, L. Trophic actions of bone marrow-derived mesenchymal stromal cells for muscle repair/regeneration. Cells **1,** 832, 2012.
67. Ruehle, M.A., Stevens, H.Y., Beedle, A.M., Guldberg, R.E., and Call, J.A. Aggregate mesenchymal stem cell delivery ameliorates the regenerative niche for muscle repair. J Tissue Eng Regen Med **12,** 1867, 2018.
68. Di Rocco, G., Iachininoto, M.G., Tritarelli, A., *et al.* Myogenic potential of adipose-tissue-derived cells. J Cell Sci **119,** 2945, 2006.
69. Kulesza, A., Burdzinska, A., Szczepanska, I., *et al.* The mutual interactions between mesenchymal stem cells and myoblasts in an autologous co-culture model. PLoS One **11,** e0161693, 2016.
70. Qazi, T.H., Mooney, D.J., Duda, G.N., and Geissler, S. Niche-mimicking interactions in peptide-functionalized 3D hydrogels amplify mesenchymal stromal cell paracrine effects. Biomaterials **230,** 119639, 2020.
71. Gharibi, B., Farzadi, S., Ghuman, M., and Hughes, F.J. Inhibition of Akt/mTOR attenuates age-related changes in mesenchymal stem cells. Stem Cells **32,** 2256, 2014.
72. Baker, N., Boyette, L.B., and Tuan, R.S. Characterization of bone marrow-derived mesenchymal stem cells in aging. Bone **70,** 37, 2015.
73. Geissler, S., Textor, M., Kuhnisch, J., *et al.* Functional comparison of chronological and in vitro aging: differential role of the cytoskeleton and mitochondria in mesenchymal stromal cells. PLoS One **7,** e52700, 2012.
74. Kasper, G., Mao, L., Geissler, S., *et al.* Insights into mesenchymal stem cell aging: involvement of antioxidant defense and actin cytoskeleton. Stem Cells **27,** 1288, 2009.

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