Combinatorial hydrogels for deciphering the role of cell-hydrogel interactions on MSC chondrogenesis

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Introduction: During cartilage development mesenchymal cells reside in a 3D environment that presents them with both cell-ECM (e.g., fibronectin) and cell-cell (e.g., cadherins) signals[1]. Hydrogels functionalized with peptides of the RGD sequence from fibronectin[2] or the HAV sequence from N-cadherin[3] enhance the viability and chondrogenic differentiation of encapsulated mesenchymal stem cells (MSCs). To further explore the role that these signals play in engineered hydrogel environments, the screening of a wide range of these biochemical cues alone and in combination would aid in guiding future material design. To address this, hydrogels were created using hyaluronic acid (HA) macromers functionalized with norbornene groups (NorHA) for both crosslinking and patterning of peptide gradients for screening of viability and differentiation[4].

Methods: *Base hydrogel formation:* hydrogels were prepared by mixing NorHA macromer, di-thiol crosslinker, and 0.05 wt% photoinitiator (I2959), pipetting into PDMS molds (5x5x1 mm^3 squares), and photopolymerizing with UV light (10 mW/cm^2) for 10 min (Fig. 1a). *Biochemical gradient formation:* base hydrogels were incubated in either a 5 mM solution of mono-thiolated rhodamine-labeled **RGD** peptide (GCGYG**RGD**SPG) or a 2 mM solution of fluorescein-labeled **HAV** peptide (**HAV**DIGGGC) and 0.05 wt% I2959 in PBS, and exposed to UV light for different times using an opaque sliding mask (Fig. 1b). *Imaging:* using a Leica TCS SP8 confocal microscope with a motorized x-y stage, high-resolution 3D tile scan images of the top 200 µm of hydrogels were acquired. *Biochemical gradient characterization:* ImageJ and hydrogel confocal images were used to obtain a horizontal profile of RGD or Ncad fluorescence (e.g., yellow rectangles in Fig. 1d-e). *Cell viability:* MSCs (5E6 cells/ml, Lonza) were encapsulated in base hydrogels, exposed to biochemical gradients, and cultured for 7 days in chemically-defined media supplemented with TGF-β3 (10 ng/ml). Viability was assessed using a Live/Dead (Invitrogen) assay (live, green; dead, red). *Cellular characterization:* MSC-laden hydrogels were stained with Sox9 primary (abcam, 1:200, 4ºC, 16 h) and secondary (AlexaFluor 488, 1:200, 2 h) antibodies. *Single cell analysis:* hydrogel confocal images of 1-day Sox9 stained samples were divided into 10 regions in the horizontal direction (e.g., yellow rectangles in Fig. 1f-g), and total nuclei intensity was obtained and normalized to nuclear volume (Fig. 1c).

Results: NorHA hydrogels created by the fabrication scheme presented in Fig. 1a-b feature an RGD (Fig. 1d) or an HAV (Fig. 1e) gradient, as confirmed by confocal microscopy. MSCs encapsulated in hydrogels with spatially-controlled RGD and HAV were highly viable across the hydrogels for at least 7 days in 3D culture (RGD: 93.0% ± 2.6%; HAV: 91.8% ± 1.7%). Rapid confocal imaging of hydrogels stained with Sox9 after 1-day in culture were acquired and used to quantify nuclear Sox9 on a per-cell basis in 10 regions across the hydrogel. MSCs in an RGD hydrogel stained for viability and nuclear Sox9 are shown in Fig. 1f-g. Scatter plots of nuclear Sox9 show concentration-dependent changes in chondrogenic readouts resulting from RGD (Fig. 1h) and HAV (Fig. 1i), where Sox9 generally increased and decreased with higher HAV and RGD peptide concentrations, respectively.

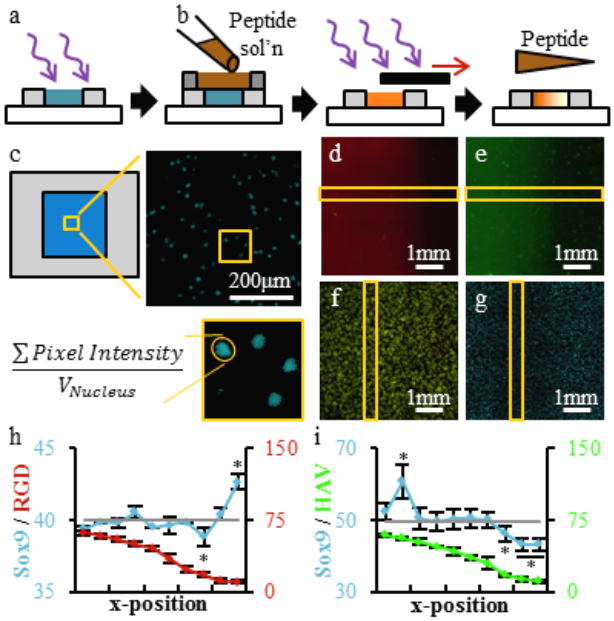

Fig 1. Combinatorial hydrogel fabrication scheme (a-b). To analyze transcription factor Sox9, (c) whole hydrogels were imaged and nuclear Sox9 fluorescence of individual cells was normalized to nuclear volume. Confocal images of (d) RGD and (e) HAV biochemical gradients and (f) viability and (g) nuclear Sox9 fluorescence. Plots of nuclear Sox9 fluorescence and (h) RGD and (i) HAV gradients across hydrogels. * denotes $p < 0.01$ compared to global mean (gray horizontal line).

Conclusions: We developed combinatorial hydrogels in tandem with rapid single cell imaging to identify how hydrogel modification impacts early chondrogenic signaling. The concentration of both RGD and HAV affected early MSC transcriptional activity towards chondrogenesis. Ongoing work includes modifying NorHA hydrogels with orthogonal gradients of both RGD and HAV peptides via sequential incubation with mono-thiolated peptides to investigate their synergy across a range of chondrogenic readouts (e.g., aggrecan, type II collagen).

References: [1]DeLise AM. Osteoarth and Cart. 2000;8:309-334. [2]Salinas CN. Biomat. 2008;29:2370-2377. [3]Bian L. PNAS. 2013;110:10117-10122. [4]Gramlich WM. Biomat. 2013;34:9803-9811.