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Dimensionality and spreading influence MSC YAP/TAZ signaling in hydrogel environments

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Abstract

Improved fundamental understanding of how cells interpret microenvironmental signals is integral to designing better biomaterial therapies. YAP/TAZ are key mediators of mechanosensitive signaling; however, it is not clear how they are regulated by the complex interplay of microenvironmental factors (e.g., stiffness and degradability) and culture dimensionality. Using covalently crosslinked norbornene-functionalized hyaluronic acid (HA) hydrogels with controlled stiffness (via crosslink density) and degradability (via susceptibility of crosslinks to proteolysis), we found that human mesenchymal stem cells (MSCs) displayed increased spreading and YAP/TAZ nuclear localization when cultured atop stiffer hydrogels; however, the opposite trend was observed when MSCs were encapsulated within degradable hydrogels. When stiffness-matched hydrogels of reduced degradability were used, YAP/TAZ nuclear translocation was greater in cells that were able to spread, which was confirmed through pharmacological inhibition of YAP/TAZ and actin polymerization. Together, these data illustrate that YAP/TAZ signaling is responsive to hydrogel stiffness and degradability, but the outcome is dependent on the dimensionality of cell-biomaterial interactions.

Keywords

hydrogel; degradation; stiffness; mechanotransduction; YAP/TAZ; dimensionality

1. Introduction

The ability of stem cells to interact with and interpret their microenvironment drives a myriad of cellular behaviors. Improved fundamental understanding of these interactions is needed to inform the development of therapeutic biomaterials where properties such as stiffness [1] and degradability [2], as well as culture dimension [3] are critical. Hydrogels are attractive biomaterial systems to study cell-microenvironment interactions in a controlled

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manner due to their ability to mimic integral features of the native extracellular matrix (ECM) including high water content, soft tissue mechanics, and cell adhesion [4]. Nearly a decade ago, it was observed that stem cell fate was influenced by the stiffness of the underlying hydrogel substrate [1]. Since then, numerous studies have illustrated the importance of the mechanical environment in regulating cell spreading [5], migration [6], and response to chemotherapeutics [7], as well as stem cell self-renewal [8], differentiation [9], and engraftment [10].

While much of this work has been performed in two-dimensional (2D) culture scenarios (cells plated atop hydrogel films), cells naturally reside in complex three-dimensional (3D) networks that result in altered or even divergent cellular behavior compared to 2D cultures [3]. Hydrogels provide a simplified version of the natural tissue environment to more accurately study cell behavior. Indeed, many cell culture studies have shifted towards 3D where a better understanding of how cell spreading and matrix elasticity influence cell biology is needed. For example, although cells typically spread more in response to increased stiffness in 2D, this is not always the case in 3D. The response is also dependent on the type of hydrogel, as increased stiffness counterintuitively leads to reduced 3D cell spreading and contractility within some covalently crosslinked hydrogels [2], whereas cell spreading is restricted across a wide mechanical range with some physically crosslinked hydrogels [11, 12]. Material viscoelasticity can also influence cell behavior with faster stress relaxation [13] or later onset of stress stiffening [14] promoting preferential mesenchymal stem cell (MSC) osteogenesis. Finally, the degradability of a matrix can influence the ability of cells to interpret this mechanical information from their environment. For example, we recently demonstrated that human MSCs encapsulated in covalently crosslinked hydrogels required a protease-degradable microenvironment to spread and undergo osteogenesis [15].

Due to the large amount of investigation in this area and the implications of hydrogel-based cellular therapies, it is important to better understand the signaling pathways involved and how they relate to these various features in both 2D and 3D environments. YAP/TAZ (Yes-associated protein/ Transcriptional coactivator with PDZ-binding motif) was recently implicated as a master regulator of mechanotransduction, playing a critical role in relaying extracellular mechanical signals to the nucleus to initiate downstream biochemical signaling [16]. While the role of YAP/TAZ as transcriptional co-activators involved in Hippo-related signaling and the (dys)regulation of organ growth during development and disease (e.g., cancer) is well-characterized [17], its role in biomaterial-mediated mechanotransduction is only beginning to be understood. Seminal work from the Piccolo group defined 2D culture conditions, in particular as a function of substrate stiffness, that regulated YAP/TAZ in cell behaviors such as stem cell differentiation [16, 18]. Specifically, elevated substrate stiffness increased YAP/TAZ nuclear translocation.

While our understanding of YAP/TAZ and other mechanosensing pathways in the context of 2D planar systems is improving, determining the critical factors that regulate mechanotransduction in more physiologically and pathologically relevant 3D culture systems has been more complicated. For example, while YAP/TAZ nuclear localization increased with increasing stiffness in 3D Matrigel/collagen matrices [19], no relationship between YAP/TAZ nuclear translocation and stiffness was observed with physically

crosslinked alginate hydrogels [13]. However, these material systems do not represent many of the commonly used hydrogels in the biomedical community; for example, to our knowledge no study to date has investigated how YAP/TAZ signaling is regulated in 3D covalently crosslinked hydrogels where both hydrogel stiffness and degradation can be tailored. Additionally, neither study performed a direct comparison of cells interacting with the same material in 2D and 3D environments. The quantification of YAP/TAZ signaling is also more challenging in 3D environments and these previous studies either subjectively evaluated YAP/TAZ signaling as primarily nuclear or cytoplasmic [19], or quantified YAP/TAZ nuclear to cytoplasmic intensity without correlating it to other cellular metrics such as cell morphology [13].

In this study, we developed a hydrogel system where stiffness and degradability could be tuned across a large parameter range and cells could be cultured either atop or within the hydrogels to investigate how dimensionality alters YAP/TAZ signaling in MSCs. We used a combination of immunostaining and confocal microscopy to accurately quantify YAP/TAZ signaling within cells in 3D hydrogels while also correlating these measurements to metrics of cellular spreading and morphology. We hypothesized that YAP/TAZ activity was not regulated by stiffness alone, but instead through a complex interplay of environmental factors where scenarios generally permissive to cell spreading and the generation of contractility would result in activation of the YAP/TAZ mechanosensing complex.

2. Materials and methods

2.1. NorHA synthesis

HA was modified with norbornene groups as previously described [20]. First, sodium hyaluronate (Lifecore, 75 kDa) was converted to its tetrabutylammonium salt (HA-TBA) using the Dowex 50W proton exchange resin, frozen, and lyophilized. HA-TBA primary hydroxyl groups were then modified with norbornene groups via esterification with 5-norbornene-2-carboxylic acid, 4-(dimethylamino)pyridine (DMAP), and di-tert-butyl dicarbonate (Boc₂O) under nitrogen at 45°C for 20 h. The reaction was quenched with cold water, purified via dialysis (SpectraPor, 6–8 kDa molecular weight cutoff) for 7 d at room temperature, frozen, and lyophilized. The degree of modification was ~ 20% as measured by ¹H NMR (Bruker, Fig. S1).

2.2. Peptide synthesis

Non-degradable (GCHGNSGGSGGNEECG) and protease-degradable (GCNS VPMS MARGSNCG) peptides were synthesized using standard solid state methods as previously described [21]. Peptides were cleaved in trifluoroacetic acid for 2–3 h, precipitated in ether, dialyzed in water at room temperature for 2 d (SpectraPor, 500–1000 Da molecular weight cutoff), lyophilized, and stored at –20°C until use. Successful synthesis was confirmed by MALDI (Fig. S2).

2.3. NorHA hydrogel fabrication

NorHA hydrogels were fabricated via ultraviolet (UV)-light mediated thiol-ene addition reactions [20]. 4 wt% NorHA hydrogels were photopolymerized (2 mW cm^{–2}) in the

presence of 2 mM lithium acylphosphinate (LAP) [22, 23] initiator for either 2 min (2D) or 5 min (3D) in the presence of thiolated RGD (GCGYGRGDS^{SPG}, GenScript) and either the non-degradable or degradable di-thiol peptides at a thiol-norbornene ratio of 0.12, 0.28, or 0.75 corresponding to low, medium, and high relative crosslinking, respectively. 2D hydrogel films were made between untreated and thiolated [20] coverslips (50 μ L, 18 $\times$ 18 mm, $\sim$ 100 μ m thickness) while 3D hydrogel plugs were formed in plastic molds (50 μ L, unswollen dimensions $\sim$ 4.5 mm diameter $\times$ 2.5 mm thickness). Hydrogels were allowed to swell in PBS at 37°C overnight before any subsequent characterization.

2.4. Hydrogel mechanical and swelling characterization

Hydrogel mechanics were assessed using rheology, atomic force microscopy (AFM), and dynamic mechanical analysis (DMA). Rheometer experiments were performed on an AR2000ex (TA Instruments) equipped with a UV light source (Exfo Omnicure S2000) at 0.5% strain and 1 Hz using a cone and plate geometry (1°, 20 mm diameter) at room temperature. 2D hydrogel film mechanics were measured with AFM (Agilent 6000ILM). Force curves (velocity 1 μ m/s, indentation depth analyzed: 500 nm) were taken on at least 3 different areas (2 $\times$ 2 arrays) per hydrogel with a 1 μ m SiO₂ spherical probe (0.06 N/m, Novascan). Elastic moduli were obtained from force curve data using the Sneddon approximation of the Hertz indentation model. 3D hydrogel plug compressive moduli were measured using DMA (TA Instruments). PBS-swollen hydrogel plugs were compressed at a strain rate of 10% per min and the compressive elastic moduli were obtained from the slope of the stress-strain curve between 10–20% strain. Volumetric swelling ratios were calculated from the hydrogel wet weight (after 24 h PBS swelling) and the original hydrogel dry weight.

2.5. Hydrogel degradation quantification

Hydrogel enzymatic degradation in response to collagenase (Type IV, 5 U mL⁻¹, Worthington) or rhMMP-2 (5 nM, R&D Systems) was assessed over the course of 14 days. Hydrogels were incubated in TTC buffer (pH 7.5, 50 mM Tris-HCl, 1 mM CaCl₂, and 0.05% Triton X-100) at 37°C with buffer changes (supplemented with fresh enzyme) performed every 2 days. Buffer samples were stored at –80°C until analysis. HA mass loss was determined by measuring uronic acid content in the buffer samples [24].

2.6. Cell culture

Human bone marrow-derived mesenchymal stem cells (MSCs) were purchased from Lonza and used at passage 3 for all experiments. Culture media consisted of α -MEM supplemented with 16.7 v/v% FBS (Gibco), 1 v/v% penicillin streptomycin (Invitrogen), 1 v/v% L-glutamine (Invitrogen), and 1 v/v% fungizone amphotericin B (Invitrogen). All polymer, peptide, and cell culture reagents were either sterile filtered or sterilized via germicidal UV irradiation prior to cell culture. Cells were seeded atop hydrogel thin films (18 $\times$ 18 mm) in 6-well plates at a density of 2 $\times$ 10³ cells/cm² or encapsulated in cylindrical plugs (50 μ L) at 1 million cells/mL. Media was replaced every 3 days except for inhibition studies where media (no inhibitor, 1 μ M verteporfin (VP, Sigma), or 1 μ M cytochalasin D (CytoD, Millipore)) was switched daily. Mitomycin-C-treated cultures were exposed to 10 μ g/mL mitomycin C (Sigma) in serum-free media for 2 h, washed with PBS, and incubated in

complete culture media for at least 1 h prior to seeding. Hydrogel cultures were performed for either 2 days (2D) or 7 days (3D) with the exception of mitomycin C-treated 2D cultures (7 days).

2.7. Live/dead assay

Viability of MSCs encapsulated in hydrogels was assessed using a Live/Dead® cell viability assay (Invitrogen) per the manufacturer's instructions. Viability was quantified as the ratio of calcein-AM-stained living cells to the total cell count.

2.8. Hydrogel staining and imaging

Phase contrast images of MSCs atop 2D hydrogels were acquired on a Zeiss Axiovert 200 inverted microscope (Hitech Instruments, Inc). Cell-hydrogel constructs were prepared for immunocytochemistry by first fixing cells in 10% buffered formalin for 15 min (2D) or overnight (3D, after cutting hydrogel in half), permeabilizing in 0.1% Triton X-100 for 15 min (2D) or 45 min (3D), and blocking non-specific binding sites in 3% BSA in PBS for 1 h (2D, 3D) at room temperature. Constructs were then incubated with primary antibody diluted in blocking buffer against YAP (rabbit polyclonal anti-YAP, Santa Cruz Biotechnology sc-15407, 1:200) overnight at 4°C. Hydrogels were then rinsed thrice with PBS and incubated with secondary antibody (AlexaFluor® 488 goat anti-rabbit, Invitrogen, 1:200) and rhodamine phalloidin to visualize F-actin (Invitrogen, 1:200) for 2 h at room temperature. Hydrogels were then washed twice with PBS, incubated with DAPI for 1 min (1:10000, 2D) or 30 min (1:500, 3D), rinsed again in PBS, and stored at 4°C in the dark until imaging. Fluorescent images were acquired using an Olympus BX51 microscope (B&B Microscopes Limited, 2D) or a Leica SP8 inverted confocal microscope (3D). Using this setup, xy-plane cross-sections were acquired (thickness ~ 150 µm, step-size 0.69 µm).

2.9. Image analysis

All image analysis was performed on a per-cell basis using ImageJ (NIH). For 2D studies, four metrics were acquired: spread area, cell shape index, aspect ratio, and YAP/TAZ nuclear/cytosolic ratio. For each cell, the cellular domains were determined by generating binary masks using Otsu's intensity-based thresholding method from fluorescent actin images. Cellular masks were then used to calculate MSC spread area, and cell shape index (CSI) was calculated using the formula:

$$CSI = \frac{4\pi A}{P^2}$$

where A is the cell area and P is the cell perimeter. With this metric, a line and a circle have CSI values of 0 and 1, respectively. Aspect ratios were calculated as the ratio of the largest and smallest side of a bounding rectangle encompassing the cell. To quantify YAP/TAZ nuclear-to-cytosolic ratio, binary masks of the nuclei were generated using Otsu's intensity-based thresholding method from fluorescent DAPI images and were superimposed with corresponding actin masks to generate masks that encompass the cytosol yet exclude the nucleus. Fluorescent YAP/TAZ images were then superimposed either with the nuclear or cytosol-only masks to isolate YAP/TAZ signal in the nucleus or cytosol, respectively. Total

YAP/TAZ signal intensity was then determined in these domains and their ratio was taken and normalized to the corresponding volumes, as shown in the formula below:

$$\text{Nuclear YAP} = \frac{\frac{\text{Nuclear YAP signal}}{\text{Area of nucleus}}}{\frac{\text{Cytosolic YAP signal}}{\text{Area of cytosol}}}$$

For 3D studies, four metrics were acquired (volume, cell shape index, aspect ratio, and YAP/TAZ nuclear/cytosolic ratio) by following similar procedures done for the 2D images. For each cell, the nuclear and cytosolic domains were determined by generating binary masks of 3-dimensional image stacks of DAPI and actin fluorescent images using Otsu's intensity-based thresholding method. Image J's 3D Objects Counter function was then used to calculate volume (V) and surface area (A_o) for each domain. These values were used to calculate 3D cell shape index using the following formula:

$$CSI = \frac{\pi^{\frac{1}{3}} (6V)^{\frac{2}{3}}}{A_o}$$

With this metric, a line and a sphere have CSI values of 0 and 1, respectively. Aspect ratios were calculated as the ratio of the largest and smallest side of a bounding box encompassing the cell. To quantify YAP/TAZ nuclear/cytosolic ratio, nuclei masks were inverted and superimposed with corresponding actin masks to generate masks that encompass the cytosol yet exclude the nucleus. 3-dimensional YAP/TAZ image stacks of the same cells were then superimposed with these masks, resulting in new image stacks containing cytosolic YAP/TAZ. Similar methods were employed using nuclei masks to obtain nuclear YAP/TAZ image stacks. These image stacks were then subject to Image J's 3D Objects Counter function to calculate total pixel intensity of YAP/TAZ in the nuclear and cytosolic domains. Using this data, the ratio between nuclear and cytosolic YAP/TAZ was calculated and normalized to the volumes of the nuclear and cytosolic domains within the cell, as shown in the formula below:

$$\text{Nuclear YAP} = \frac{\frac{\text{Nuclear YAP signal}}{\text{Volume of nucleus}}}{\frac{\text{Cytosolic YAP signal}}{\text{Volume of cytosol}}}$$

As validation of the methodology outlined above, images taken of MSCs at different depths (up to 150 μm) within the hydrogel showed no significant differences in YAP/TAZ, F-actin (phalloidin), or nuclear (DAPI) signal intensity (Fig. S3).

2.10 Gene expression analysis

PCR analysis was performed for select 3D cultures. MSC-laden NorHA hydrogels were rinsed in PBS and mechanically agitated using a handheld rotor to permit RNA isolation via Trizol (Invitrogen). Three hydrogels were combined for each replicate. RNA was reverse transcribed to cDNA and then PCR was conducted on an Applied Biosystems 7300 Real-Time PCR system. Targets included ankyrin repeat domain 1 (*Ankrd1*) and connective tissue

growth factor (*Ctgf*) with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) used as a housekeeping gene. Gene expression relative to MSCs cultured in medium stiffness degradable hydrogels was determined using the delta-delta Ct method.

2.11. Statistical analysis

All experiments were conducted with at least 3 independent hydrogels and 40 cells per group. One-analysis of variance (ANOVA) followed by Tukey-HSD post-hoc tests were performed on all data sets. Tukey box plots of single cell data contained boxes corresponding to second and third data set quartiles with error bars indicating the maximum and minimum values or 1.5*interquartile range, whichever value was smaller. Error is reported in bar graphs as the standard error of the mean unless otherwise noted. Significance was indicated by *, **, or *** corresponding to $P < 0.05$, 0.01, or 0.001.

3. Results

3.1. NorHA hydrogels enable tuning of substrate stiffness and degradability

We modified hyaluronic acid (HA) with norbornene groups [20] (~ 20% of disaccharide repeat units) to form hydrogels where parameters such as substrate stiffness and network degradability could be readily altered (Fig. 1a). Norbornene-HA (NorHA) was reacted with thiol-containing peptides (through cysteine incorporation) via ultraviolet (UV) light-mediated thiol-ene addition reactions between norbornene groups and thiols. This allowed simultaneous incorporation of RGD peptide (GCGYGRGDSPG, final concentration of 2 mM in hydrogels) to permit cell attachment and crosslinking with either protease-degradable (GCNS VPMSMRGGSNCG) or non-degradable (GCHGNSGGSGGNEECG)[21] peptides (Fig. 1b). NorHA hydrogels (4 wt%) were crosslinked with low, medium, or high concentrations of peptide crosslinkers corresponding to 12, 28, and 75% norbornene consumption, respectively. Identical hydrogels were cast as either thin films for seeding of MSCs atop the hydrogels for 2D studies (Fig. 1c) or as hydrogel plugs for encapsulation of MSCs within hydrogels for 3D studies (Fig. 1d). Hydrogels readily polymerized within 2 min of UV light exposure (2 mW cm^{-2}) (Fig. S4). Resultant mechanics were characterized via atomic force microscopy (AFM, 2D thin films, Fig. 1c) or dynamic mechanical analysis (DMA, 3D plugs, Fig. 1d) and similar elastic moduli were observed for equivalent variants (low: $E \sim 1 \text{ kPa}$, medium: $E \sim 5 \text{ kPa}$, high: $E \sim 20 \text{ kPa}$) in both 2D and 3D assessment. We also performed AFM on 3D free swollen hydrogels (thickness ~ 0.5 mm) of the medium stiffness formulation and obtained similar results to our DMA measurement (AFM: $4.9 \pm 0.3 \text{ kPa}$, DMA: $5.2 \pm 0.5 \text{ kPa}$).

3.2. MSCs show differential spreading trends with stiffness based on dimensionality

Following characterization of the NorHA hydrogel variants, MSCs were cultured either atop of (2D) or within (3D) hydrogels with low, medium, or high relative crosslinking (Fig. 2). MSCs readily adhered to 2D hydrogel culture substrates and showed over 80% viability when encapsulated within 3D hydrogels containing protease-degradable crosslinks, as indicated by live/dead staining (Fig. S5). MSCs increased in spread area with a reduced cell shape index (CSI) on 2D hydrogel substrates of increasing stiffness (Fig. 2a). F-actin staining demonstrated greater organization of stress fibers for MSCs on stiffer substrates,

especially for the high stiffness group (Fig. 2b). MSC volumes in 3D showed more complex trends with predominantly elongated MSCs observed in low stiffness variants, spread MSCs displaying protrusions in the medium group, and smaller rounded cells in highly crosslinked hydrogels (Fig. 2c,d). The MSC shape index increased with increasing hydrogel stiffness, indicative of more rounded cells (Fig. 2c). Additionally, although MSC aspect ratio did not change as a function of stiffness in 2D, MSCs encapsulated in low stiffness hydrogels were significantly ($P < 0.001$) more elongated than in hydrogels of higher crosslink density (Fig. S6).

3.3. MSCs display opposite YAP/TAZ nuclear localization trends with stiffness in 2D versus 3D cultures

Fluorescence imaging was used to examine YAP/TAZ nuclear to cytoplasmic intensity ratios in order to understand relationships between hydrogel stiffness and dimensionality with YAP/TAZ signaling (Fig. 3, S7). YAP/TAZ relative nuclear intensity was progressively higher for MSCs cultured atop hydrogels of increasing stiffness (Fig. 3a, b). When YAP/TAZ was evaluated qualitatively with binary outcomes of either cytosolic or nuclear localization ~ 35% of MSCs on medium stiffness hydrogels showed nuclear localization compared to ~ 95% in the high stiffness group (Fig. S8). However, since both methods showed similar trends we believe the more quantitative evaluation is more appropriate and have therefore reported nuclear YAP/TAZ ratios throughout this work. Single cell scatter plots showing YAP/TAZ nuclear localization as functions of three cell morphology metrics (spread area, CSI, and aspect ratio) revealed complex trends between groups. For 2D cultures, there was a trend correlating greater YAP/TAZ nuclear localization with greater spreading with distinct populations evident for the low, medium, and high stiffness groups (Fig. 3a). There was a more subtle population separation between the experimental groups for the other two morphology metrics, although a population predominantly made up of MSCs with low YAP/TAZ, high CSI was evident for the low stiffness group.

The relationship between morphology metrics and YAP/TAZ signaling was quite different for 3D encapsulation studies. YAP/TAZ nuclear translocation was significantly higher ($P < 0.001$) in the low and medium stiffness groups when compared to the higher stiffness group (Fig. 3c,d). Scatter plots of single cells revealed a weaker correlation between YAP/TAZ signaling and cell volume compared to YAP/TAZ versus cell area in 2D. Instead, a distinct population of low YAP/TAZ, high CSI cells for the high stiffness group was observed separate from higher YAP/TAZ, lower CSI cells for the low and medium groups (Fig. 3c). Although the low and medium 3D groups exhibited similar YAP/TAZ nuclear intensity ratios, MSC morphology was different between the two groups, as stated above. While 2D experiments were performed for only 2 days to prevent cell overgrowth as opposed to 7 days in 3D, quantification of spreading and YAP/TAZ metrics after 7 days for mitomycin C-treated cultures on 2D medium hydrogels showed that extended culture time did not substantially alter the observed trends for 2 day cultures (Fig. S9). We also performed a control that exposed MSCs on 2D medium hydrogels to 5 min of UV light (same light exposure used for 3D MSC encapsulation experiments) and showed that there were no resulting differences in spread area, CSI, aspect ratio, or nuclear YAP/TAZ (Fig. S10).

3.4. Degradable hydrogel environments significantly enhance YAP/TAZ nuclear translocation in 3D

While it was clear that 3D degradable environments supported MSC spreading and activation of YAP/TAZ signaling (especially at lower stiffnesses), we wanted to ascertain the specific role of hydrogel degradation in mediating YAP/TAZ signaling. To do this, we assessed MSC spreading and YAP/TAZ localization in medium stiffness hydrogels crosslinked with either protease-degradable or non-degradable peptides. These two variants showed similar gelation kinetics, swelling ratio, stiffness, and cell viability after 7 days (Fig. S11). In addition, degradable hydrogels eroded in response to collagenase or matrix metalloproteinase-2 (MMP-2) exposure, while non-degradable variants showed minimal differences in mass loss compared to controls with no enzyme (Fig. S12). MSCs within degradable hydrogels were significantly larger, less rounded, and had significantly higher YAP/TAZ nuclear localization when compared to MSCs in stiffness-matched non-degradable hydrogels (Fig. 4, S13). MSCs separated into distinct populations for the non-degradable (low YAP/TAZ and high CSI) and degradable (high YAP/TAZ and low CSI) groups (Fig. 4h). We also observed elevated expression of YAP target genes *Ankrd1* and *Ctgf* in the degradable hydrogels (Fig. S14).

3.5. MSC spreading in 3D degradable environments is YAP/TAZ and actin-dependent

After exploring how MSC YAP/TAZ nuclear localization was regulated by stiffness and degradability in the context of cell spreading and dimensionality, we next used pharmacological inhibition to evaluate how blocking YAP-mediated transcription as well as actin polymerization alters MSC response to hydrogel environments (Fig. 5, S15, S16). We cultured MSCs in 3D medium degradable hydrogels either with no inhibitor as a control, the small molecule inhibitor verteporfin (VP), which blocks YAP-mediated transcription by disrupting the TEAD-YAP complex [25], or the small molecule inhibitor cytochalasin D (CytoD), which blocks actin polymerization [26]. MSC viability after 7 days of culture in VP-supplemented media remained > 80% (Fig. S17). Both VP and CytoD treatment in degradable hydrogels led to similar MSC behaviors to those in non-degradable hydrogels with significantly lower cell volume, significantly higher CSI, and significantly lower YAP/TAZ nuclear localization (Fig. 5, S15). VP treatment also led to the down-regulation of YAP target genes including *Ctgf* (Fig. S18). Single cell scatter plots revealed an inhibited MSC profile of low YAP/TAZ nuclear intensity coupled with lower cell volume, higher CSI, and lower aspect ratio compared to non-inhibited cultures (Fig. 5g,h,i). VP and CytoD-treated MSCs cultured atop 2D medium stiffness hydrogels showed a similar response to 3D cultures with reduced MSC spread area and YAP/TAZ nuclear localization observed (Fig. S15, S16).

4. Discussion

Hydrogels are being increasingly developed as not only biomedical therapies, but also as *in vitro* models of cell-matrix interactions; thus, it is important to understand how cells respond to various hydrogel properties. Considering the stark differences observed in cell behavior as a function of stiffness between 2D and 3D studies, we aimed to develop a hydrogel system amenable to both 2D and 3D culture where stiffness and degradability could be

independently tuned. We used norbornene-modified hyaluronic acid (NorHA) to do this. Norbornenes react with thiols in a light-mediated thiol-ene addition reaction. Unlike other commonly used photocrosslinking moieties (e.g., (meth)acrylates), norbornenes do not react with each other to form non-degradable kinetic chains but instead preferentially react with thiol radicals [20] provided in our system by either thiolated RGD adhesion peptides or di-thiol peptide crosslinkers. We took advantage of the high number of functional groups on NorHA to synthesize a suite of hydrogels with identical polymer concentration but variable crosslinking density to span a physiologically relevant stiffness range (~ 1–20 kPa, Fig. 1). We were also able to vary the degradability of this system at a set crosslink density by simply tuning the sequence of the peptide crosslinker [21] (Fig. S12).

Despite the noted differences in cellular outcomes between 2D and 3D environments, to our knowledge no study has examined differences in spreading and cell behavior in mechanically and biochemically equivalent 2D and 3D hydrogel systems across wide mechanical ranges. Using our NorHA platform, we performed this comparison using hydrogels with low (~ 1 kPa modulus), medium (~ 5 kPa modulus), or high (~ 20 kPa modulus) relative crosslinking and a uniform RGD concentration of 2mM (Fig. 2, 3). MSCs cultured atop hydrogels of increasing stiffness displayed increased spreading and YAP/TAZ nuclear localization; however, we observed more complex, and even opposing, trends in 3D cultures (Fig. 6a). Our 2D findings are not surprising based on a plethora of previous studies on polyacrylamide and other classic hydrogel substrates that converged on the idea that cells cultured atop unrestrained planar substrates display increased spreading and contractility.

Despite our increased understanding of cell behavior in 2D planar systems, the critical factors regulating mechanotransduction in more physiologically and pathologically relevant 3D systems are more undefined. Work from the Mooney group showed that stem cell differentiation in physically crosslinked 3D hydrogels was stiffness dependent based on ligand mobility and integrin clustering with an optimum stiffness of ~ 20 kPa preferentially guiding osteogenesis [11]. More recent work from the same group has also demonstrated the importance of stress relaxation in this response, with quicker relaxation times leading to increased osteogenesis [13]. In contrast to 2D systems, cell response in physically crosslinked hydrogels does not seem to correlate directly with spreading. However, cells behave much differently in covalently crosslinked networks derived from commonly used polymers in the biomedical field such as poly(ethylene glycol) and HA, where cells encapsulated in hydrogels of increasing stiffness commonly spread less than in more loosely crosslinked hydrogels [2]. In this study and in contrast to our 2D results, MSCs encapsulated in hydrogels with protease-degradable crosslinks were able to spread more easily and promote YAP/TAZ nuclear localization in lower stiffness hydrogels. This finding is contrary to previous (and largely 2D) studies of YAP/TAZ signaling and implicates culture dimensionality as a critical factor regulating YAP/TAZ activity.

Recent studies suggest that in order for cells to spread in covalently crosslinked environments they must be able to degrade their local environment [15, 27]. Covalently crosslinked environments with high crosslink density present more crosslinks that cells must degrade, restricting their ability to pull on the surrounding matrix and cluster integrin-ligand complexes, and perhaps explaining the divergent behaviors observed in 3D. Interestingly, we

observed similar levels of YAP/TAZ nuclear localization in low and medium 3D groups despite stark differences in cell morphology. Although similar CSI was observed between the two groups, MSCs in low stiffness hydrogels were smaller with highly elongated, high aspect ratio shapes, while MSCs in medium hydrogels were larger with many projections. This could be due to differences in crosslink density between the two systems, as cells need to degrade fewer crosslinks at the lower crosslink density in order to generate space to spread, perhaps leading to the elongated morphology. MSC heterogeneity could also contribute to sub-populations identified within experimental groups, especially in 3D systems where cells are encapsulated within the hydrogels.

To further explore biophysical factors regulating 3D spreading and YAP/TAZ behavior, we then investigated degradation as a regulator of such signaling. We previously observed that in contrast to the seminal literature for 2D systems, hydrogels with non-degradable covalent crosslinks suppressed MSC spreading and promoted adipogenesis, even at high (15–90 kPa) stiffnesses previously shown to favor osteogenesis in 2D or in 3D alginate systems. It was determined that these non-degradable crosslinks were restrictive to cell spreading and contractility, limiting osteogenesis [15]. However, the signaling mechanisms behind this finding were unclear. Based on our previous work and the aforementioned results we hypothesized that MSC spreading in 3D covalently crosslinked hydrogels required a degradable environment that promoted increased YAP/TAZ nuclear translocation. Taking advantage of our tunable NorHA system, we fabricated two stiffness- matched medium hydrogel groups with similar biophysical properties but crosslinked with either protease-degradable or non-degradable peptide crosslinkers. Using this system we demonstrated that MSCs encapsulated in non-degradable hydrogels were significantly smaller, more rounded, and displayed lower nuclear YAP/TAZ (Fig. 4). Critically this finding demonstrated for the first time that YAP/TAZ signaling in 3D biomimetic hydrogels is regulated by degradation, independent of bulk hydrogel stiffness, although further investigation of how local mechanical changes [27, 28] in response to degradation and cell-mediated ECM production are needed (Fig 6b).

While clearly observing that hydrogel stiffness and degradability could regulate MSC YAP/TAZ signaling in a dimensionality dependent manner, we investigated the dependence of MSC spreading on YAP/TAZ and actin polymerization. YAP and TAZ do not directly bind to DNA but instead exert transcriptional control by interacting with numerous transcription factors, most notably the TEAD family [29]. We blocked YAP-mediated transcription using VP, which disrupts the TEAD-YAP complex by selectively binding YAP [25], and also blocked actin polymerization with CytoD [26]. We then evaluated MSC morphology and YAP/TAZ nuclear translocation in both 2D and 3D cultures (Fig. 5, S15, S16). Previous work with 2D substrates showed that YAP/TAZ silencing led to decreased cell spreading, focal adhesion size, and collagen I gene expression [30] and also demonstrated that VP inhibition reduced cell contractility and expression of the YAP target gene *Ctgf* [31]. Indeed, we observed reduced MSC spreading and nuclear YAP/TAZ in both 2D and 3D cultures treated with either VP or CytoD. Although VP does not inhibit YAP/TAZ nuclear translocation, we likely observed reduced nuclear YAP/TAZ due to resulting alterations in MSC contractility as suggested by previous studies [30, 31]. Additionally, while YAP/TAZ inhibition has been shown to influence cell proliferation [30],

we do not believe that this significantly influenced cell spreading since all cultures were performed at low cell densities to prevent confounding factors such as cell-cell contacts. Previous work also correlated YAP/TAZ silencing [32] and lowered YAP/TAZ nuclear localization [33] with reduction in MMP activity. Since crosslink degradation is dependent on MMP activity and a precursor to spreading in our hydrogels, this could also explain the reduction in MSC spreading in 3D environments observed in response to VP treatment. Taken together, these data indicate that inhibiting YAP/TAZ or actin polymerization likely alters MSC response to hydrogel properties such as degradability by decreasing spreading (Fig. 6c).

5. Conclusions

Our data strongly suggest that YAP/TAZ signaling in engineered hydrogels is not merely regulated by substrate stiffness, but rather is sensitive to other parameters such as dimensionality and degradability. Additionally, our results suggest that cellular microenvironments generally permissive to MSC spreading promote active YAP/TAZ signal transduction in both 2D and 3D environments. This study illustrates how the interplay of these factors must be considered when designing therapeutic biomaterials to either encourage (or suppress) YAP/TAZ nuclear translocation and downstream signaling events. This may be particularly relevant for tissue engineering applications where MSC environments permissive or restrictive to YAP/TAZ signaling could alter differentiation and tissue repair. Approaches to manipulate biomaterial-mediated YAP/TAZ signaling may also have applications in studies of cell biology, organ development, and disease progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. NorHA hydrogels enable independent tuning of substrate stiffness, degradability, and dimensionality

(a) Hyaluronic acid modified with norbornenes (~ 20 wt% of disaccharide repeat units) that permit (b) UV light-mediated thiol-ene addition reactions between thiolated RGD to permit cell attachment (2 mM final concentration) and with dithiol peptide crosslinkers (protease-degradable: GCNS VPMS MRGGS NCG, non-degradable: GCHGNSGGSGGNEECG) in the presence of the photoinitiator lithium acylphosphinate (LAP). Hydrogels (4 wt%) were fabricated as either thin films for seeding of MSCs atop for 2D culture or as plugs for MSC

encapsulation within for 3D studies, across a range of crosslink densities (Low, Medium, High). AFM and DMA were used to quantify the elastic moduli of (c) 2D hydrogel thin films and (d) 3D hydrogel plugs, respectively ($n = 3$ hydrogels per group, error bars represent s.e.m.). **: $P < 0.01$, ***: $P < 0.001$.

Fig. 2. MSC spreading is dependent on substrate stiffness and dimensionality

(a) MSC spread area and cell shape index when seeded atop hydrogels of varying stiffness (Low (1 kPa), Medium (5 kPa), High (20 kPa)). (b) Representative F-actin (*red*) and nuclear (*blue*) staining of cells cultured atop hydrogels (2D). (c) MSC volume and cell shape index when encapsulated within hydrogels of varying stiffness (Low (1 kPa), Medium (5 kPa), High (20 kPa)). (d) Representative F-actin (*red*) and nuclear (*blue*) average projections of cells encapsulated within hydrogels (3D). $n > 40$ cells per group. Scale bars: 50 μm . ***: $P < 0.001$.

Fig. 3. MSC YAP/TAZ nuclear localization is differentially regulated by hydrogel stiffness in 2D and 3D cultures

(a) YAP/TAZ nuclear localization for MSCs cultured atop hydrogels of varying stiffness (Low (1 kPa), Medium (5 kPa), High (20 kPa)) reported as mean values, as well as single cell scatter plots of YAP/TAZ nuclear intensity ratio as a function of spread area, cell shape index, and aspect ratio (b) Representative YAP/TAZ staining (*green*) of cells cultured atop of hydrogels (2D). *Dashed white lines*: nuclear outlines. (c) YAP/TAZ nuclear localization for MSCs encapsulated within hydrogels of varying stiffness (Low (1 kPa), Medium (5 kPa), High (20 kPa)) reported as mean values, as well as single cell scatter plots of YAP/TAZ nuclear intensity ratio as a function of volume, cell shape index, and aspect ratio. (d) Representative YAP/TAZ (*green*) average projections of cells encapsulated within hydrogels

(3D). *Dashed white lines*: nuclear outlines. $n > 40$ cells per group. Scale bars: 50 μm . ***; $P < 0.001$.

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Fig. 4. Degradable hydrogel environments significantly enhance YAP/TAZ nuclear translocation in 3D cultures

MSC (a) volume, (b) shape index, and (c) aspect ratio when cultured in stiffness-matched (Medium (5 kPa) formulation) non-degradable and degradable hydrogels after 7 days. (d) Representative F-actin (*red*) and nuclear (*blue*) average projections of cells encapsulated within stiffness-matched non-degradable and degradable hydrogels. MSC YAP/TAZ nuclear localization (e) quantification and (f) staining (*green*) in stiffness-matched (Medium (5 kPa) formulation) non-degradable and degradable hydrogels after 7 days. *Dashed white lines*: nuclear outlines. Single cell scatter plots of YAP/TAZ nuclear intensity ratio as a function of (g) volume, (h) cell shape index, and (i) aspect ratio. Medium degradable data reproduced from Fig. 2, 3 for comparison. $n > 40$ cells per group. Scale bars: 50 μm . ***: $P < 0.001$.

Fig. 5. MSC spreading in 3D degradable environments is YAP/TAZ and actin-dependent MSC (a) volume, (b) shape index, and (c) aspect ratio when cultured in Medium (5 kPa) degradable hydrogels after 7 days with no inhibitor, YAP/TAZ inhibitor verteporfin (VP (+)), or actin polymerization inhibitor cytochalasin D (CytoD (+)). (d) Representative F-actin (red) and nuclear (blue) average projections of cells encapsulated in Medium (5 kPa) degradable hydrogels cultured in media with no inhibitor, VP (+), or CytoD (+). MSC YAP/TAZ nuclear localization (e) quantification and (f) staining (green) in degradable hydrogels after 7 days with no inhibitor, VP (+), or CytoD (+). *Dashed white lines*: nuclear outlines. Single cell scatter plots of YAP/TAZ nuclear intensity ratio as a function of (g)

volume, **(h)** cell shape index, and **(i)** aspect ratio. Medium degradable data reproduced from Fig. 2, 3 for comparison. $n > 40$ cells per group. Scale bars: 50 μm . ***: $P < 0.001$.

Fig. 6. Summary of relationships between hydrogel stiffness and degradation with respect to YAP/TAZ signaling of MSCs in both 2D and 3D

(a) Increased hydrogel stiffness promotes increased spreading and YAP/TAZ nuclear localization for MSCs cultured atop hydrogels 2D cultures; however, increased crosslinking results in decreased spreading and YAP/TAZ signaling for MSCs encapsulated within hydrogels for 3D cultures. The increased crosslinking in 3D hydrogels presents a greater number of crosslinks that need to be cleaved for spreading. (b) A degradable hydrogel environment is necessary for cell spreading and YAP/TAZ nuclear translocation in 3D

cultures, since stiffness-matched non-degradable hydrogels limited both spreading and YAP/TAZ signaling. (c) Small molecule inhibition of YAP/TAZ or actin polymerization alters how MSCs interact with hydrogel environments by reducing spreading.

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