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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.

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 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

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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 ditert-butyl dicarbonate (Boc_2O) 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 days at room temperature, frozen, and lyophilized. The degree of modification was ~20% as measured by ^{1}H NMR (Bruker, Fig. S1).

2.2. Peptide synthesis

Non-degradable (G**C**HGNSGGSGGNEE**C**G) and protease-degradable (G**C**NS*VPMS*↓*MRGG*SN**C**G) 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 days (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 (GCGYG*RGD*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 μL, 18 × 18 mm, ~100 μm thickness) while 3D hydrogel plugs were formed in plastic molds (50 μL, unswollen dimensions ~ 4.5 mm diameter × 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 μm/s, indentation depth analyzed: 500 nm) were taken on at least 3 different areas (2 × 2 arrays) per hydrogel with a 1 μm SiO_2 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 and 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 recombinant human matrix metalloproteinase-2 (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 $CaCl_2$, and 0.05% Triton X-100) at 37 °C with buffer changes (supplemented with fresh