Cellular Volume and Matrix Stiffness Direct Stem Cell Behavior in a 3D Microniche

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ABSTRACT: The central question addressed in this study is whether cells with different sizes have different responses to matrix stiffness. We used methacrylated hyaluronic acid (MeHA) hydrogels as the matrix to prepare an in vitro 3D microniche in which the single stem cell volume and matrix stiffness can be altered independently from each other. This simple approach enabled us to decouple the effects of matrix stiffness and cell volume in 3D microenvironments. Human mesenchymal stem cells (hMSCs) were cultured in individual 3D microniches with different volumes (2800, 3600, and 6000 μm³) and stiffnesses (5, 12, and 23 kPa). We demonstrated that cell volume affected the cellular response to matrix stiffness. When cells had an optimal volume, they could form clear stress fibers and focal adhesions on soft, intermediate, or stiff matrix. In small cells, stress fiber formation and YAP/TAZ localization were not affected by stiffness. This study highlights the importance of considering cellular volume and substrate stiffness as important cues governing cell–matrix interactions.

KEYWORDS: cell volume, matrix stiffness, hydrogel, 3D cell culture, mechanotransduction, YAP/TAZ

Stem cells reside in a local extracellular microenvironment, or microniche, in which their behavior is tightly regulated by biophysical cues (matrix stiffness, geometry, topography) as well as biochemical signals (ligand density, chemical composition). During the past decade, a range of materials science approaches have been developed to control the various physical and biochemical parameters that govern the interactions between cells and their environment. Of the myriad environmental cues that cells receive, the mechanical properties of the extracellular matrix (ECM) have been shown to play an important role in regulating cell behavior. Differences in matrix stiffness give rise to a range of responses in mesenchymal stem cells (MSCs). For example, it was found that on 2D flat substrates, MSCs will undergo robust osteogenesis on stiff substrates in the range of 40 kPa, whereas soft substrates (~1 kPa) aid adipogenic differentiation. However, in 3D microenvironment, the correlation between matrix stiffness and cell behavior is complicated by the fact that stiff gels tend to reduce cell spreading, and only cells in a degradable (stiff) microenvironments spread and undergo osteogenesis. In addition, cell behavior in a 3D microenvironment is affected by the viscoelastic properties of hydrogels (stress relaxation, stress stiffening).

When cells are embedded within a 3D matrix, it is important to realize that cell volume also directly influences cell fate. Cells exist in a large range of sizes in vivo, which affects many basic cell functions, including cell migration, differentiation, and apoptosis. It has been shown that cell volume regulation impacts not only the mechanical properties of cells, but also gene expression profiles and cell metabolic activity. Decreasing cell volume as a result of water efflux leads to an increase in cell stiffness and ultimately induces stem cells to become prebone cells. Furthermore, cell volume disturbances have been implicated in disease states and it is therefore important to gain a better understanding of how cell volume affects cell function and how cells maintain their optimal size.

Several studies have indicated that mechanical forces can affect cell volume, for example, by opening ion channels. Bush et al. found that cell volume reduced up to 30% under compressive forces. Guo et al. found that stiffer micro-environments resulted in reduced cell volumes through water efflux, impacting on cell fate. However, to the best of our knowledge, there are no in vitro methods that can be used for independently controlling cell volume, independent of substrate stiffness, cell volumes are typically directly correlated to substrate stiffness or mechanical loading. Moreover, most studies probing the importance of cell volume and ECM stiffness are based on 2D substrate, and there is clearly a need for an in vitro 3D model in which cell volume alone, independent of ECM stiffness, can be altered.

Recently, we developed a way to control cell volume and geometry in a 3D microniche and demonstrated that the...
organization of the cytoskeleton is highly sensitive to the precise volume (and to a lesser extent shape). However, in these studies, one crucial parameter was missing, and that is stiffness of the extracellular matrix. Here, we expand upon this work in order to dissect the contribution of ECM stiffness and cell volume on cell behavior, we focus on how different cell volumes enhance or completely abolish the influence of stiffness on cell state (especially formation of actin cytoskeleton and localization of YAP/TAZ).

First, we produced 3D microniches of controlled shape, volume, and stiffness, as shown in Figure 1. We use photopolymerizable methacrylated hyaluronic acid (MeHA) hydrogels to construct artificial single cell 3D microniches. Hyaluronic acid (HA) was selected for several reasons. First, HA can be easily functionalized with proteins such as fibronectin (Fn) to promote cell adhesion, as well as with cationic polymers such as poly(L-lysine)-graft-poly(ethylene glycol) (PLL-b-PEG), to create protein-resistant surfaces to prevent cell adhesion. Second, diffusion of nutrients and oxygen to the cells through the HA hydrogel is rapid enough to support normal cell growth rates, which is essential for 3D cell culture. Fn distribution inside 3D microniches was characterized by confocal fluorescence microscopy after staining with a fluorescent antibody against Fn (Figure S1). Compared with conventional 2D cell culture and microwell systems, our 3D microniche provides a symmetric and nonpolarized environment for cells. The volume of the 3D microniches can be easily adjusted by either changing the surface area of the base of the niches (Figure S2) or their heights (Figure 2b). In this study, the microniche volume was adjusted by changing the height while keeping the lateral dimensions the same. The stiffness of MeHA hydrogels was controlled during the polymerization step, by varying the concentration of HA solution. The stiffness of the hydrogels was measured by colloidal probe atomic force microscopy (AFM) as 5, 12, and 23 kPa, which we will refer to as “soft,” “intermediate,” and “stiff” respectively (Figure 2c). The range of these stiffnesses is large enough to mimic the elasticity of most native tissues. We note that even softer hydrogels (below 5 kPa) were not readily suitable for cell studies because of the difficulty of transferring the complete patterned hydrogels from silicon masters to tissue culture plates.

Figure 1. Schematic method to encapsulate single cells in 3D microniches. After encapsulating single cells in microwells, a permeable MeHA hydrogel cover coated with fibronectin was directly added on top to compartmentalize single cells in microwells.

Figure 2. (a) Confocal image shows Fn distribution in microwells and 3D microniches. Visualization of Fn by confocal fluorescence microscopy shows an equal distribution of fibronectin on the surface of the microwells. Scale bar 20 μm. (b) Confocal image shows Fn distribution in microwells with different volumes by changing the lateral dimension or height. Scale bar 20 μm. (c) Experimental setup of AFM-indentation based stiffness measurement for MeHA hydrogel. The dot plots show MeHA Hydrogel stiffness with varying macromer concentration. The concentration of MeHA solution for soft, intermediate, and stiff hydrogel is 2, 10, and 15 wt %, respectively. **P < 0.01 (ANOVA using a Tukey post-test).
hMSCs from the same passage were used for all cell experiments in this study. Cells readily spread in 3D microniches with different shapes, stiffness, and volumes (Figure 3a). Three-dimensional images of cells with triangular prism shape that entirely fulfill the 3D microniche are shown in the bottom. (b) β1 integrin staining from top stack to bottom stack for hMSCs in triangular prism shape with and without hydrogel lid. (c) F actin staining and cross-sectional view for cells in 3D microniches with and without hydrogel lid. (d) Percentage of cells that fulfill 3D microniches with different volumes and stiffness.

hMSCs from the same passage were used for all cell experiments in this study. Cells readily spread in 3D microniches with different shapes, stiffnesses, and volumes (Figure 3a). 3D images show that cells completely filled the microniches, and we can thus match cell volume with niche volume (Movie S1). We previously showed that cells with...
Volumetric measurements in our 3D microniche enable us to study the effect of different volumes and mechanical signals. Cells with different volumes were denoted as V1 (2800 μm^3), V2 (3600 μm^3) and V3 (6000 μm^3). All these volumes were bigger than the average starting size of hMSCs (~2100 μm^3), the size of cells in suspension, which means cells were able to spread and expand their volume in the microniches and cell nuclei were not compressed initially in any of the microniches. As the triangular prism shape gave the clearest results on actin organization, we used this shape throughout the study. Figure 3b, c show that after sealing the microwells with a hydrogel lid to create a 3D microenvironment, cells adhere to the lid (coated with Fn) and find localization of β1 integrins and cytoskeleton both on top and bottom of the niches. We manually quantified the percentage of cells that filled 3D microniches with different volumes and stiffness, and found over 75% occupancy rates with fully spread cells (Figure 3d). Thus, unlike previous work, where stiffer surfaces inherently gave rise to smaller cell volumes, our 3D microniche enable us to study the effect of cell volume and hydrogel stiffness independently.

Immuno-staining was performed for visualizing focal adhesions (vinculin staining, green) and filamentous actins (F-actins, red) (Figure 4a, b). To better visualize staining results, we took all confocal images from different z-stacks and then merged into a single stack (Figure S3). In cells of volume V1 (2800 μm^3), focal adhesions were immature and poorly visible, and distributed in a diffuse manner, even for cells cultured in stiff hydrogels, and stiffness had no significant effect on FAs formation (Figure 4c). Mature FAs were observed to be predominately distributed in sharp corners of cells of volume V2 (3600 μm^3), with over 80% of cells forming clear FAs in cells cultured in hydrogels with different stiffnesses (Figure 4c). In cells of volume V3 (6000 μm^3), more cells formed FAs with increasing stiffness; clear FAs structures were observed in over 62% of cells cultured in stiff hydrogels, compared to 38% and 10% of cells that formed FAs in intermediate and soft hydrogels, respectively (Figure 4a, c).

For F-actin staining, less than 18% of V1 cells had clear and parallel fibrous actin, whereas in over 80% of cells actin staining showed monomeric or spot-like structure (Figure 4b), independent of stiffness. It should be noted that overall F-actin concentration (intensity per stack) was comparable in cells on different stiffness (Figure 4c). Clear and well-organized actin cytoskeletons were observed in most cells with V2 (2800 μm^3) volume, where the percentage of cells with polymerized actin slightly increased with increasing hydrogel stiffness, from 67% on soft hydrogel to 78 and 82% on intermediate and stiff hydrogels, respectively. F-actin concentrations were similar in cells with V1 volume in hydrogels with different stiffnesses (Figure 4c). In contrast, in cells with the largest volume (V3, 6000 μm^3), a strong relationship between stress fiber formation and hydrogel stiffness was observed: in 5 kPa gels, stress fibers were barely seen; in 12 kPa hydrogel, around 10% of cells formed moderate stress fibers; around 55% of cells with clear cytoskeleton organization could be observed in stiff gels. Compared with cells in soft hydrogels, F-actin concentration increased 1.8-fold between V3 cells in soft or stiff hydrogels (Figure 4c). These results are in contrast to previous findings on 2D substrates, where stiff substrates always yielded more stress fibers in cells, but these cells also always showed smaller volumes. In our study, we show that cells of volume V2 appear to be in some “optimal” state, always forming stable FAs and stress fibers, irrespective of the stiffness of the hydrogels. V1 cells did not form stable FAs and stress fibers, regardless of stiffness of hydrogels, indicating that in these studies cell volume overrides the effect of stiffness in affecting cell behavior. In contrast, in the largest cells, stiffness appeared to be the major determinant for FA and stress fiber formation.

Finally, we investigated how cell volume and hydrogel stiffness affect YAP/TAZ localization in single hMSC. YAP/TAZ is considered a key regulator in cell mechanotransduction, with nuclear localization typically associated with stiff substrates. However, no study has investigated how YAP/TAZ is affected by cell volume.

Figure 5. (a) Images that show the effect of different stiffness and volume on YAP/TAZ localization. (b) Quantification of nuclear YAP/TAZ localization in cells with different volumes and hydrogel stiffness. Data are shown as mean ± SD, ANOVA one-way analysis followed by Tukey post hoc test shows significance levels of *p < 0.05, **p < 0.01, and N.S.: p > 0.05. (c) Schematic image shows cell volume regulation and matrix stiffness direct stem cell behavior in a 3D microniche.
TAZ activity can be controlled in 3D hydrogels where both stiffness and cell volume can be independently tailored. Representative images of YAP/TAZ staining for hMSCs with different volumes and hydrogel stiffness are shown in Figure S5a. Quantification of nuclear YAP/TAZ localization was performed manually. Cells were considered to have nuclear YAP/TAZ localization when the level of fluorescence of YAP/TAZ in the nucleus was higher than the level in the cytoplasmic region (Figure S5b). We found that in low volume ($V_1$) cells, YAP/TAZ was predominately localized in the cytoplasm, with no effect on stiffness. When cell volume reached $V_2$, more than 60% of cells exhibited nuclear YAP/TAZ localization, even in soft gels. Typically, we would expect that increasing stiffness would increase nuclear YAP/TAZ localization, but we found no significant differences between soft and stiff substrates for cell volume $V_2$. In contrast, for cells with largest ($V_3$) volumes, the percentage of cells with YAP/TAZ nuclear localization increased significantly from 10% in soft microniches, to 35% in intermediate stiffness niches, and to 70% in the stiffest hydrogels. Our work is different from previous findings on 2D substrates, where people have shown that increased cell spreading area and stiffer substrates lead to increased actin formation, focal adhesion size and nuclear YAP/TAZ formation.\(^{30}\) Cell volume and matrix stiffness cannot be controlled and decoupled in 2D, and has not been previously studied as an independent parameter. We demonstrated, for the first time, that cell volume can affect the stiffness sensing in a 3D microenvironment. Small niche volumes might lead to cells filling the niche prior to the development of the actin-cytoskeleton structure, whereas the largest volumes lead to dilute intracellular macromolecule concentrations, possibly requiring a stiffer environment to provide a positive feedback to form actin cytoskeleton. Overall, these results demonstrate that YAP/TAZ activity in a 3D microenvironment is strongly impacted by cell volume, regardless of hydrogel stiffness.

In conclusion, we have shown here how 3D microniches allowed us to probe the effects of cell volume and matrix stiffness in a decoupled way. We demonstrated, for the first time, that focal adhesion formation, stress fiber organization, and YAP/TAZ activity of hMSCs in 3D hydrogels is not merely regulated by substrate stiffness but is sensitive to cell volume (Figure 5c). Interestingly, cell volume always impacts cell behavior, whereas matrix stiffness showed only a strong influence for the largest cells. Our study illustrates that the interplay of cell size (and shape) and matrix stiffness must be considered when studying cell mechanotransduction and designing new biomaterials. We believe that these results add to our understanding of mechanotransduction and opens up new routes to regulate YAP/TAZ signaling, which could be particularly relevant for tissue engineering applications, cell biology studies, or organoid development.

### ASSOCIATED CONTENT

**2 Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b19396.

Experimental details and supplementary figures (PDF)

Movie S1, 3D construction of F-actin and DAPI staining in microniches (AVI)

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**REFERENCES**


