Biophysical properties of the cellular microenvironment, including stiffness and geometry, have been shown to influence cell function. Recent findings have implicated 3D confinement as an important regulator of cell behavior. The understanding of how mechanical signals direct cell function is based primarily on 2D studies. To investigate how the extent of 3D confinement affects cell function, a single cell culture platform is fabricated with geometrically defined and fully enclosed microwells and it is applied to investigate how niche volume and stiffness affect human mesenchymal stem cells (hMSC) life and death. The viability and proliferation of hMSCs in confined 3D microniches are compared with unconfined cells in 2D. Confinement biases hMSC viability and proliferation, and this influence depends on the niche volume and stiffness. The rate of cell death increases and proliferation markedly decreases upon 3D confinement. The observed differences in hMSC behavior are correlated to changes in nuclear morphology and YES-associated protein (YAP) localization. In smaller 3D microniches, hMSCs display smaller and more rounded nuclei and primarily cytoplasmic YAP localization, indicating reduced mechanical activation upon confinement. Interestingly, these effects scale with the extent of 3D confinement. These results demonstrate that the extent of confinement in 3D can be an important regulator of cell function.

1. Introduction

Cell function is orchestrated both by the internal machinery of the cell and through reciprocal interactions with the surrounding extracellular matrix (ECM).[1,2] Extracellular regulators of cell function include biochemical cues, such as growth factors and cytokines, and biophysical cues, such as niche elasticity, geometry, and topography.[1–5] The mechanisms by which biophysical signals influence cell function have been elucidated primarily using 2D culture, including standard tissue culture polystyrene and micropatterned substrates. Seminal findings in 2D revealed several mechanisms by which biophysical cues direct cell behavior.[6–11] For example, matrix elasticity induced both short-term and latent effects on stem cell function and differentiation.[12–14] Substrate topography controlled cell morphology, orientation, and proliferation.[15,16] Geometric confinement in 2D directed cell life and death; decreased cell spread area led to decreased proliferation and increased cell death.[17,18] In total, these observations indicate a robust relationship between the physical properties of the microenvironment, cell morphology, and cell function.

While 2D culture has been particularly useful for studying biological processes, it fails to recapitulate critical characteristics of the native 3D cell niche and limits our ability to understand how biophysical properties, such as 3D confinement and stiffness, regulate cell function. Hydrogels have been engineered specifically as ECM mimics to investigate the influence of matrix properties on cell function in 3D.[19–21] Despite the broad utility of 3D platforms, standard encapsulation in bulk materials offers limited control over the geometry and volume of the individual cell niches. To address this limitation, microfabricated platforms have been developed that control previously inaccessible aspects of the 3D cellular environment, including niche geometry and volume.[22–26] Microfabricated hydrogel platforms provided new insight into how 3D niche morphology and volume influence cell shape, contractility, and transcription factor activity. Interestingly, these micropatterned platforms constrained cells to morphologies that deviated from their unconfined 2D cell shape. That is the cells experienced 3D confinement. Throughout
In this manuscript, we use confinement to describe a setting where the morphology that a cell can adopt is restricted relative to an unconfined setting.[27–31] In prior work with microniche, the wells were often similar to the average cell volume (after spreading) and much smaller than the average cell spread length, introducing tight 3D confinement (or compression). Recent studies in viscoelastic and degradable hydrogels suggested that the extent of 3D confinement may impact cell behavior, such as spreading, migration, and proliferation, which require changes in cell volume, shape, or movement.[12–14] For example, viscoelastic gels with slower relaxation times decreased the rates of osteogenic commitment in MSCs (e.g., αvβ1, αvβ3, and αvβ5) and enables cell adhesion and spreading.[37] To create fully confined 3D environments, we sealed the niches with a second, non-patterned hydrogel layer using an enzymatic ligation via Sortase A, which covalently cross-linked substrate peptides present in each of the two hydrogel layers (Figure 1d and Movie S1: Supporting Information).

We fabricated the microniche arrays using traditional microfabrication and soft lithography techniques (Figure 2a). The micropattern was designed in AutoCAD and generated on a silicon wafer via photolithography using SU-8 photoresist (Figure 2b). The silicon wafer was used as a master for the fabrication of intermediate polydimethylsiloxane (PDMS) molds. Teflon molds were cast from the intermediate PDMS patterns to avoid hydrogel patterning directly on PDMS. In our experience, the radical mediated thiol–ene polymerization was inhibited at the interface of PDMS, likely due to oxygen inhibition of primary radicals, limiting pattern transfer fidelity (Figure S1, Supporting Information). We then cast microniche arrays in the PEG-based hydrogels using the Teflon molds and confirmed transfer by visualization of Rhodamine-labeled hydrogels via transmitted light and confocal microscopy (Figure 2c,d). The elastic modulus (and stiffness) of the base hydrogel was controlled by the polymer concentration in the precursor solution. Shear rheometry quantified the mechanical properties of the hydrogels in situ during formation and at equilibrium swelling. We selected hydrogel formulations with three distinct moduli at equilibrium swelling, E = 6, 16, or 30 kPa, referred to as soft, medium, and stiff gels (Figure 2e). These moduli were selected based on prior in vitro investigations of hMSC mechanobiology that demonstrated mechanotransduction over a similar range of stiffness.[5,13,38,39] For example, low moduli PEG-based substrates (E < 10 kPa) mechanically deactivated hMSCs (lower proliferation rates and cytoplasmic YAP localization) while stiff PEG-based substrates (E > 10 kPa) activated hMSCs (increased proliferation rates and nuclear YAP localization).[40]

The microniche arrays were sealed with a hydrogel lid prepared with the same formulation and material properties as the niche environment. This enabled full 3D confinement and ensured that the cells remained confined within the microniche during the culture period, preventing cell escape (Figure S2, Supporting Information). To adhere the non-patterned hydrogel layer to the microniche base, the sealing hydrogel layer was modified with one substrate for Sortase A (GGGG-LERCL-NH₂; 688 µg mL⁻¹, 800 × 10⁻⁶ M) and the micropatterned hydrogel was modified a complementary substrate (Ac-GCRE-DDD-LPMTG-NH₂; 1125 µg mL⁻¹, 800 × 10⁻⁶ M). The work of adhesion between gels was assessed using pull-off tests measuring stable adhesion between hydrogel surfaces in presence of Sortase A enzyme (Figure 2f and Figure S3: Supporting Information). The Sortase-mediated bonding adhered the sealing hydrogel to the base throughout the time course of cell culture and in a manner that did not alter the network structure of the gels at the cell–material interface or interfere with cell function. Further, diffusion experiments demonstrated that the enclosed niche microenvironments allowed sufficient nutrient diffusion and exchange of metabolic products (Figures S4

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**2. Results**

### 2.1. Single Cell Microniche Arrays to Investigate Physical Regulation of Cell Function in 3D

To investigate the role of 3D confinement and niche elasticity on single stem cell function in 3D, we fabricated arrays of microniche with controlled geometry, volume, and stiffness (Figure 1a,b). We used poly(ethylene glycol) (PEG) hydrogels as the base material for the platform (Figure 1c). To form the gel, 10 kDa eight-arm PEG macromers functionalized with norborne (PEG-NB) were reacted with a dithiol (dithiothreitol; DTT) via a photoinitiated thiol–ene reaction.[36] To enable cell adhesion, the inert PEG hydrogel was functionalized with thiol-containing peptides. Specifically, we included the ECM-derived adhesion peptide CRGDS, which engages integrins (e.g., αvβ1, αvβ3, and αvβ5) and enables cell adhesion and spreading.[37] To create fully confined 3D environments, we sealed the niches with a second, non-patterned hydrogel layer using an enzymatic ligation via Sortase A, which covalently cross-linked substrate peptides present in each of the two hydrogel layers (Figure 1d and Movie S1: Supporting Information).
To investigate the effect of the extent of confinement on cell function, we generated microarray patterns with three distinct niche volumes: 35, 61, and 125 \( \times 10^3 \) \( \mu \)m\(^3\), further denoted as V1, V2, and V3 niches. The dimensions of the single cell niches were large enough to accommodate both the nuclear and cytoplasmic volumes of rounded hMSCs, with the smallest niche dimension (20 \( \mu \)m) exceeding the mean radius of hMSC nuclei (14.1 \( \pm \) 2.0 \( \mu \)m; Figure S6: Supporting Information). The niches were designed in this manner so that the cells and their nuclei would not be deformed upon encapsulation. Single cells were able to spread and fill smaller niches, while having sufficient space to spread, grow, and proliferate in larger niches. However, the largest niche dimension was smaller than the mean hMSC spread length in 2D culture (>50 \( \mu \)m, soft gels; >100 \( \mu \)m, stiff gels) in order to physically confine cells within the 3D micro-environments (Figure S7, Supporting Information). The niches were designed in this manner so that the cells and their nuclei would not be deformed upon encapsulation. Single cells were able to spread and fill smaller niches, while having sufficient space to spread, grow, and proliferate in larger niches. However, the largest niche dimension was smaller than the mean hMSC spread length in 2D culture (>50 \( \mu \)m, soft gels; >100 \( \mu \)m, stiff gels) in order to physically confine cells within the 3D micro-environments (Figure S7, Supporting Information). In 3D, the maximum cell spread length was restricted to the longest dimension of the niche (~64 \( \mu \)m, V1; ~70 \( \mu \)m, V2; ~86 \( \mu \)m, V3). In this manner, the designed niches covered a range of extents of 3D confinement.

After preparing the microarrays, we seeded cells into the niches by applying a suspension of hMSCs on the surface of the patterned hydrogel. To determine a suitable seeding density for single cell occupation, we seeded the cells in the niches at different concentrations (5000–30 000 cells mL\(^{-1}\), 1 mL cm\(^{-2}\)). We allowed the cells to sediment into the microwells under a constant gentle shaking on an orbital shaker (60–70 rpm) for 15 min. Once the cells settled into the microwells, the platform was rinsed with culture medium to remove excess cells from the surface. The niches were then sealed with the non-patterned hydrogel layer. Cell spreading was observed in the niches within 3 h after seeding (Figure 2g and Figure S8, Movie S2: Supporting Information). We found that seeding 20 000 cells cm\(^{-2}\) on patterned surfaces resulted in the highest rate of single cell occupancy (~40%) for all niche geometries (Figure 2h). On day 3, the majority of the cells had spread within the niches attaining 3D morphologies (Figure 2i; Figure S9 and Movie S3: Supporting Information). In V1 niches, cells frequently occupied the full volume of the microniche, whereas cells in V3 niches only spread to occupy a fraction of the niche volume (Figure S10, Supporting Information). On average, the cells in V1 niches occupied 58.0 \( \pm \) 0.6% of the niche volume, 35 \( \pm \) 0.6% in V2 niches, and 26.1 \( \pm \) 0.2% in V3 niches (Figure S11, Supporting Information). The average cell volume expanded with the niche size from 20.3 \( \pm \) 11.3 \( \times 10^3 \) to 32.6 \( \pm \) 19.6 \( \times 10^3 \) \( \mu \)m\(^3\).
with ethidium homodimer (EthD-1) staining, a DNA intercalator and niche stiffness affect cell viability. We monitored cell viability on our platform to investigate how the extent of 3D confinement and matrix stiffness on hMSC viability. Initially, we employed single hMSC culture, we investigated the effects of geometric confinement having established the microarray platform for single hMSC fragments of the AutoCad masks for V1 (20 × 50 × 35 μm³), V2 (35 × 50 × 35 μm³), and V3 (50 × 50 × 50 μm³) niches. The microwells were structured in 10 × 10 arrays to facilitate position identification and rapid image analysis. c) Transmitted light image of the resulting hydrogel pattern showing x-y shape fidelity between the original pattern and the generated microarray. Scale bar, 20 μm. d) Confocal image of a labelled hydrogel demonstrating shape fidelity in the z-dimension. The hydrogel was visualized through the incorporation of a thiolated Rhodamine dye that was covalently attached to the PEG backbone. Scale bar, 50 μm. e) The Young’s modulus of the hydrogel networks was controlled by the polymer fraction yielding soft (3.5 wt%; E = 6 ± 0.6 kPa), medium (5 wt%; E = 16 ± 1.2 kPa) and stiff (6 wt%; E = 30 ± 2.4 kPa) gels. f) The lids adhered to the microarrays following Sortase A linking. The work of adhesion between the two hydrogels was quantified using pull-off experiments with and without the application of the bacterial enzyme Sortase A. The adhesion was sufficient for gels to remain adhered over the time course of the experiment. g) Live imaging showing a representative hMSC spreading within a V3 niche. The cell spread and stabilized its shape over the course of 9 h. Scale bar, 50 μm. h) The highest single cell occupation in the microniche was observed for 20 000 cells cm⁻². i) Cells seeded in V3 niches after 3 days of culture. The actin cytoskeleton was labelled with Phalloidin-iF488 and the nucleus with DAPI. Scale bar, 50 μm.

In addition, the cells within the V2 and V3 niches expressed augmented cytoskeletal organization compared to small size V1 niches, as quantified by increasing F/G-actin ratio with increasing niche volume (Figure S12, Supporting Information). Further, the nuclear volume and morphology varied with niche volume. Nuclear volume increased from 374.7 ± 143.1 and 457.9 ± 199.1 μm³ in V2 and V3 niches while sphericity of the nucleus decreased with increasing niche size (Figure S13, Supporting Information). These data demonstrated that the extent of confinement decreased with increasing niche volume and that the extent of confinement resulted in changes in nuclear volume and morphology.

2.2. 3D Confinement and Matrix Stiffness Affect hMSC Viability

Having established the microarray platform for single hMSC culture, we investigated the effects of geometric confinement and matrix stiffness on hMSC viability. Initially, we employed our platform to investigate how the extent of 3D confinement and niche stiffness affect cell viability. We monitored cell viability with ethidium homodimer (EthD-1) staining, a DNA intercalator that cannot cross the membrane of viable cells. hMSCs that did not stain for EthD-1 (EthD-1⁻) were counted as viable and hMSCs that stained for EthD-1 (EthD-1⁺) were counted as dead. We screened viability across all niche volumes (V1, V2, and V3) in low, medium, and high stiffness gels. On day 1, hMSCs cultured in the 3D microniche exhibited a mean viability ≥80% for all conditions (Figure 3a). On day 3, hMSC viability varied with microniche volume and matrix stiffness. The number of viable cells decreased in low and medium stiffness niches (73.3 ± 12.6% and 79.5 ± 6.8%, respectively) and remained high in high stiffness niches (93.6 ± 5.5%). A weak and non-significant trend in viability was observed for niche volume. On day 3, the V1 niches at low stiffness exhibited heterogeneous results with decreased viability (66.9 ± 17.2%). Whereas the V3 niches with high stiffness exhibited the highest viability observed in 3D (97.5 ± 2.1%; Figure 3b). These data suggested that cells respond to 3D confinement, that niche volume and stiffness influence confinement sensing, and that these effects may be related to mechanical signaling or stiffness sensing.

To further characterize the effect of geometric confinement on cell viability, we compared the viability of hMSCs in 3D microniche (confined) with hMSCs on 2D gels (unconfined) of the...
same stiffness. Cells exhibited high viability (≥90%) in all cases on 2D gels (Figure 3c). hMSC morphology varied with substrate stiffness; cells were less spread and more rounded on low stiffness gels and more spread on high stiffness gels with an average spread length of 57.9 ± 4.1 and 114.1 ± 11.2 µm, respectively (Figure 3d). However, the most notable effect was the overall increase in hMSC viability in 2D as compared with hMSCs in 3D microniche. While the specific niche volume did not have a significant effect on cell viability, 3D confinement dramatically reduced the overall viability of hMSCs. This could have been caused, in part, by mechanical effects during the assembly of the microarrays; however, as cell viability decreased after 3 days of culture in niches, we hypothesized that the cells actively sensed the confinement and that mechanosensitive signaling related this signal to coordinated cell function decisions. Similar mitochondria structure and metabolic activity were observed for 2D and 3D conditions (Figure S14, Supporting Information), suggesting that the decrease in cell viability was not related to changes in nutrient or growth factor transport and supporting the hypothesis that these effects were caused by 3D confinement.

Figure 3. 3D confinement decreased cell viability. a) hMSCs were viable (>80%) in all conditions in 3D microniche culture after 1 day (n = 3; at least 500 cells per condition; comparisons between stiffness and size groups were made using two-way ANOVA with Tukey’s test for post-hoc analysis). b) After 3 days, the high stiffness niches displayed the highest numbers of viable (EthD-1−) cells (n = 3; at least 500 cells per condition; comparisons between stiffness and size groups were made using two-way ANOVA with Tukey’s test for post-hoc analysis). c) On 2D gels, cell viability was close to 100% for all conditions after 3 days in culture (n = 3; at least 2000 cells per condition; comparisons between stiffness groups were made using one-way ANOVA, with Tukey’s test for post-hoc analysis). d) In unconfined 2D culture, hMSCs were more elongated on stiff gels as compared with soft gels (n = 3; 30 cells per condition; comparison of means between groups was made using a Two-Sample t-Test). e) In both conditions, hMSC spreading was confined in the 3D microniche relative to unconfined 2D gels. In 2D, cell spreading increased with gel stiffness and, in all conditions, cells adopted spread morphologies with mean cell lengths greater than the maximum available distance in the V1, V2, and V3 niches. In 3D, cell spreading was constrained by the geometry of the microniche, providing geometric confinement of the cells. Representative images of hMSC morphology on day 3 in 2D and 3D culture. Cell cytoplasm was imaged with Calcein AM. Scale bars, 200 µm. For a–c) plots represent mean ± s.e.m., for d) bar plots represent mean ± s.e.m.
2.3. hMSC Proliferation Decreases upon 3D Confinement

Having shown that the rates of cell death increased upon confinement, we then investigated the proliferative capacity of hMSCs in the confined microniches as a function of niche stiffness and volume. Seminal results demonstrated that cell proliferation rates decreased with decreasing cell adhesive area on 2D adhesive islands (75–3000 µm²).[42] Other findings demonstrated that proliferation in 3D—porous ECM-derived and synthetic bulk hydrogels—is reduced with respect to 2D culture.[43,44] Therefore, we hypothesized that proliferation rates would decrease upon confinement in 3D niches relative to 2D and that the rate of proliferation would correlate positively with niche volume. Further, we hypothesized that increased matrix stiffness would promote hMSC proliferation similar to observations in vivo or in 2D culture.[45] Increased tissue stiffness has been associated with increased cell proliferation and invasion in different types of cancers.[46,47] Similarly, stiffness has been shown to activate proliferation in vitro on 2D substrates and in porous ECM-derived hydrogels in 3D.[45,48] However, conflicting effects have been observed in bulk 3D biomaterials showing a decrease in cell proliferation with increasing stiffness.[43] This may be caused by the inherent increase in cell confinement at high stiffnesses in traditional bulk hydrogels.

To further study how confinement affects proliferation, we cultured hMSCs in our 3D microneiches for 1 and 3 days and assessed proliferation by calculating the fraction of cells in S-phase via 5-ethynyl-2´-deoxyuridine (EdU) staining (Figure S15, Supporting Information). Strikingly, the mean percentage of proliferating cells on day 1 in all 3D conditions was below 10%, which is much lower than hMSC proliferation observed in standard 2D culture (57 ± 3.0%; P7 hMSCs on TCPS) (Figure 4a).[49] The mean value of EdU+ hMSCs in the 3D niches varied from 4.6 ± 2.3% to 8.1 ± 1.4%. At this early time-point, the percent of proliferating cells increased with niche volume but did not exhibit an obvious trend with niche stiffness. On day 3, the proliferation rate settled below 10% and was lowest for the smallest (V1) niches (Figure 4b). The percent of EdU+ cells remained at ≈7–8% in the V3 niches but dropped to 3.6 ± 0.4% in the V1 low stiffness niches. Overall, hMSCs exhibited the highest proliferation rates in V2 and V3 niches at medium and high stiffnesses (7.7 ± 0.9% and 7.9 ± 0.7% in high

![Figure 4. 3D confinement downregulated hMSC proliferation.](Image)

In 3D, cells displayed mean proliferation rates below 10% after day 1 in all niche conditions. (n = 3; at least 500 cells per condition; comparisons between stiffness and volume groups were made using a two-way ANOVA with Tukey’s test for post-hoc analysis). After 3 days, proliferation in the 3D niches remained low, further decreasing for V1 niches. (n = 3; at least 500 cells per condition; comparisons between stiffness and volume groups were made using a two-way ANOVA with Tukey’s test for post-hoc analysis). On day 1 in 2D culture, the number of EdU+ cells varied from <20 to 36%; the proliferation rate increased with substrate stiffness. After 3 days, 2D proliferation increased further to above 25% for soft and 40% for stiff gels (n = 3; comparisons between stiffness groups were made using a one-way ANOVA with Tukey’s test for post-hoc analysis). After 1 and 3 days of culture, hMSC proliferation rates in the confined 3D microneiches were substantially lower than values for unconfined hMSCs in 2D culture. For a-d) plots represent mean ± s.e.m. For e) bar plots represent mean ± s.e.m.
stiffness niches, respectively). Niche stiffness had a significant effect on proliferation rates resulting in the lowest number of EdU* cells in low stiffness arrays, and proliferation rates increased with stiffness for all niche sizes. The effect of stiffness was most pronounced in V1 and V2 niches.

To relate these observations to geometric confinement, we compared proliferation values for hMSCs in 3D microniches with unconfined hMSCs on 2D gels of the same mechanical properties. After 24 h in culture, the percent of EdU* cells on 2D substrates varied from 19.8 ± 8.1% to 36.0 ± 5.5%; proliferation rates increased with substrate stiffness (Figure 4c). On day 3, 2D proliferation increased further to 25.9 ± 0.8% for soft and 40.1 ± 1.6% for stiff gels (Figure 4c). Overall proliferation rates were markedly higher for unconfined hMSCs on 2D gels than for confined hMSCs in 3D micropatterns for all stiffnesses tested (Figure 4d). These results indicated that 3D geometric confinement and matrix stiffness affect stem cell life and death.

2.4. Geometric Confinement and Niche Stiffness Regulate YAP Localization in 3D

Our observations demonstrated that stem cell viability and proliferation were influenced by geometric confinement in 3D—proliferation rates decreased upon mechanical confinement and rates of cell death increased, and both of these effects were coupled to niche volume and stiffness. Since the cells on 2D displayed average spread lengths that exceeded the longest dimension of the niche, we hypothesized that physical restriction in confined micropatterns may result in decreased mechanotransduction by inhibiting cell elongation and cytoskeleton maturation. Of the many effects involved in mechanosensing, we opted to investigate YAP, which shares homology with transcriptional coactivator with PDZ-binding motif (TAZ), as a potential mediator of the response to 3D confinement. YAP/TAZ nuclear activity is governed, in part, by mechanical cues that are transduced through the cytoskeleton and YAP/TAZ localization depends on internal cell tension. Previously, YAP/TAZ activation has been shown to depend on the available adhesive area on confined 2D micropatterned fibronectin islands; YAP/TAZ was activated in cells on large islands and inactivated on smaller islands.

To investigate if the effects of mechanical confinement on cell function were indeed mediated by YAP signaling, we characterized YAP localization (nuclear or cytoplasmic) in hMSCs cultured in confined 3D micropatterns and on unconfined 2D gels. The nuclear-cytoplasmic distribution of YAP is not a binary state but rather a time capture of continuous nuclear and cytoplasmic shuttling dynamics. We quantified YAP distribution by calculating nuclear and cytoplasmic mean fluorescent intensities, further defined as the nuclear/cytoplasmic ratio (N/C); we defined YAP nuclear localization at N/C values above 1.7 (Figure 5a and Figure S16, Supporting Information). YAP localization was assessed after 1 and 3 days in culture. First, we quantified YAP localization for hMSCs in 2D. As expected, the N/C scaled with the stiffness of the 2D substrates (Figure 5b). On day 3, the mean N/C on soft gels was low (1.2 ± 0.5) indicating mechanical deactivation. Increased mean N/C values were observed on medium and stiff gels (1.8 ± 0.8 for medium and 1.7 ± 0.7 for stiff niches).

In confined 3D micropatterns, hMSCs displayed mean YAP N/C values below the activation limit of 1.7 for all niche conditions on days 1 and 3. This suggested a general mechanical deactivation for confined cells as compared with unconfined cells in 2D culture. The mean percentage of YAP activation varied from 8.2 ± 5.6% in soft V2 niches to 23.9 ± 8.3% for stiff V3 niches (Figure 5c). On day 1, mean YAP N/C did not correlate with niche volume and exhibited a weak trend with stiffness (Figure 5d). On day 3, the cells showed increased nuclear YAP localization in larger niches for soft and medium gels (Figure 5e). Interestingly, stiffness had a dominant effect on YAP N/C: activation and N/C values were highest in stiff niches compared with other 3D micropatterned conditions, and N/C was independent of niche volume at this stiffness. Overall, suppressed YAP activation (mechanical deactivation, decreased YAP N/C) in 3D niches correlated with our viability and proliferation measurements. It has been shown previously that proliferation correlates with mechanical activation of cells, which can be regulated through YAP signaling. Furthermore, YAP activation is a known suppressor of cell death. This indicates that the changes in cell function within confined 3D environments may be in part regulated through YAP-mediated mechanotransduction.

3. Discussion

When culturing cells outside of the body, the context matters and functional differences are observed between 2D and 3D culture. In 2D, cell death rates are generally low and proliferation rates are often supraphysiologic. In 3D, proliferation rates often decrease and cell death rates increase. These differences in cell function are often attributed to changes in cell polarization, adhesion, and uptake of soluble factors. Recently, an additional focus has been placed on physical confinement of cells in 3D as a factor that contributes to the context-dependent differences in cell function. Seminal work on micropatterned 2D substrates demonstrated the role of confinement in directing cell behavior. Further studies corroborated the link between confinement and function suggesting that these effects are, in part, regulated by mechanotransduction. Studying the effects of confinement on cell function in 3D is more challenging due to the general lack of control over geometric parameters of the cell microenvironment. Micropatterned 3D platforms have been developed to culture individual cells in defined microwells with fully controllable geometrical parameters to investigate 3D confinement. For example, the Huck and Pruitt groups introduced sophisticated platforms to culture single cells in micropatterns of a similar volume as the cell. These platforms demonstrated how tight 3D confinement influences stem cell fate and cytoskeletal architecture. However, the question of if and how does the extent of 3D confinement influence cell function remained unexplored.

To investigate this question, we developed a platform to culture and monitor single hMSCs in microenvironments with tunable confinement to investigate systematically how the extent of 3D confinement affects cell viability and proliferation.
This platform facilitated full 3D confinement of single cells within the niche environment through adhesion between two gels via covalent ligation by bacterial enzyme Sortase A. Niche sealing avoided cells escaping the niches and ensuring that the cells experienced 3D confinement during the whole culture duration. We assessed morphological parameters of the cell, cell function, and mechanical activation based on the extent of confinement in 3D niches. To our knowledge this is the first study that assesses cell function under precisely controlled 3D confinement that span dimensions from the cell volume to several times the cell volume.

We observed that culturing cells in confined 3D microneiches resulted in substantial differences in cell morphology and function. We detected adaptation of cell volume to the dimensions of the niche via increased spreading and volume expansion at increasing niche size. Correspondingly, the ratio of F- to G-actin increased with the niche volume. Nuclear volume increased and nuclear sphericity decreased in larger niches. These data indicated that cells sensed the extent of confinement and that increased 3D confinement (smaller niches) constrained nuclear volume and maintained nuclear sphericity, as a potential mechanism of mechanosensing of the extent of 3D confinement.

Further, we studied cell viability and proliferation in unconfined 2D culture and confined 3D culture across a range of niche volumes and stiffnesses. In 3D, cell death increased with confinement and was significantly elevated relative to 2D culture. In addition, we observed a marked decrease in cell proliferation in 3D microneiches as compared with 2D culture across all niche volumes and stiffnesses. Even though the largest volume niches exceeded the average hMSC volume, proliferation in 3D was significantly lower than in 2D. The observed proliferation rates in 3D were similar to in vivo proliferation rates in soft tissue, which typically do not exceed 10% of S-phase cells except during tissue development, regeneration, or tumor growth.[64–66] These data indicate that cells sensed confinement in their microenvironment even when the niche volume exceeded that of an individual cell and that confinement affected the rates of cell life and death.

We hypothesized that the observed effects of physical confinement may be mediated by mechanosensitive signaling pathways, based on the changes in nuclear volume and sphericity.[64,67] We investigated YAP localization as a function of confinement and stiffness. YAP activity is, in part, governed by mechanical loading transduced through cytoskeleton, and reduced YAP activation in hMSCs has been correlated with...
reduced viability and proliferation in 2D culture.\textsuperscript{[68–70]} Furthermore, YAP activation has been shown to induce cell spreading and expansion and to modify nuclear shape in 2D and 3D culture.\textsuperscript{[11,20]} Consistent with our proliferation results, YAP activation in 3D was reduced relative to 2D, indicating that cells experienced lower mechanical stresses upon confinement. Traction dynamics of actin stress fibers are related to their length and in 2D cells balance external forces by adjusting the stiffness and organization of cytoskeleton, including its length, to sub-strate stiffness.\textsuperscript{[48,72,73]} 2D confinement of cells to smaller spread areas has been shown to suppress stress fiber formation.\textsuperscript{[67,74]} 3D niches impose similar physical restrictions on cell elongation, length, and organization of actin stress fibers. Therefore, 3D confinement may act similarly to 2D confinement hindering actin network formation resulting in dampened cell contractility and stiffness sensing.\textsuperscript{[75,63]} This was supported by the observation that the F/G actin ratio decreased with increasing extents of confinement (Figure S12, Supporting Information). That is, hMSCs cultured in smaller (V1) niches expressed reduced elongation and cytoskeletal organization, indicated by the lower average F/G actin ratio, which was consistent with actin cytoskeleton changes observed by Shivashankar and co-workers in response to lateral confinement.\textsuperscript{[76]} Correspondingly, both proliferation and YAP activation in 3D niches scaled positively with niche volume—larger niches allowed for increased cell elongation. Related studies that assessed proliferation with respect to the diameter of scaffold porosity have shown similar results—larger pore sizes stimulated cell proliferation.\textsuperscript{[77,78]}

In a recent study, Lee et al. observed that non-isometric volume expansion increased with decreased confinement in fast relaxing viscoelastic hydrogels, resulting in increased YAP activation.\textsuperscript{[79]} This resulted in increased YAP activation; however, YAP activation was not directly coupled to volume expansion when it was induced by hypoosmotic conditions. Furthermore, Major et al. have shown that cell volume correlated to nuclear volume and YAP localization.\textsuperscript{[80]} Bao et al. found that YAP activation increased in cells in microniches with higher anisotropy or sharp angle containing geometries that allowed best spreading, highest presence of stress fibers, and FA localization.\textsuperscript{[25,26]} Furthermore, YAP activation was maximal in niches where the z height was set to 9 µm, which could affect nuclear morphology imposing shearing forces on the nuclei. These studies indicate that YAP activation is tightly bound to changes in nuclear morphology induced by volume expansion through integrin connections. Previously, it was demonstrated that nuclear flattening increases nuclear pore diameter on the cytosol side and reduces it on the nucleoplasmic side, lowering the export rate of YAP/TAZ in comparison to the import rate and favoring its nuclear accumulation.\textsuperscript{[84]} Similarly, in our microwells, hMSCs spreading resulted in larger nuclear sizes and lower nuclear sphericity in V2 and V3 niches that correlated with YAP activation. Furthermore, decreasing nuclear size at increasing confinement (smaller niches) is known to correlate with an increasing molecular crowding, which could obstruct nuclear import of YAP.\textsuperscript{[85]}

In this manner, altered nuclear size and morphology could explain differences in cell function between 2D and 3D substrates. 3D cell culture results in less polarized cell spreading as compared with 2D; cell polarity has been shown to alter mechanotransduction.\textsuperscript{[83,86]} Namely, cell polarization on 2D substrates has been shown to induce stretching of the nuclear envelope.\textsuperscript{[46]} In our niches, hMSCs spread in 3D given the isotropic distribution of adhesive cues. We hypothesize that the 3D cell morphology and limited cell elongation in our confined niches resulted in a less polarized nuclear conformation, reducing YAP activation at high stiffness as compared with 2D culture. Chaudhuri and co-workers have shown that, in contrast to 2D substrates, 3D conditions allow for non-polarized adhe-sion, imposing reduced stretching forces on the nucleus and resulting in lower nuclear cross-sectional area.\textsuperscript{[85]} Our results corroborated these findings and demonstrated that 3D confinement with dimensions larger than the cell volume also restrict cell elongation and nuclear size and morphology. In addition, our results indicated that these effects scale with the extent of 3D confinement.

Lastly, increased cell death was most apparent in soft and medium stiffness niches, while, in stiff niches, cells maintained high viability close to 2D levels. Across all conditions, proliferation increased with niche stiffness. In 2D, increased stiffness stimulates cell survival and proliferation due to elevated cell tension transduced through the cytoskeleton and contractile machinery of the cell.\textsuperscript{[59,86]} Although stiffness sensing in 3D niches might be reduced due to inhibited spreading, mech-anotransduction is also regulated by the size and stability of focal adhesions, myosin contractility, and abundance of stress fibers, which are typically downregulated on low stiffness sub- strates.\textsuperscript{[87]} Thus, mechanotransduction may have been upregulated in stiff niches independent of cell elongation, resulting in the relative increase in cell viability and proliferation.

4. Conclusion

This study improves our understanding of how the physical milieu regulates stem cell function, with a specific emphasis on how the extent of 3D confinement affects cell viability and proliferation. By culturing hMSCs in microengineered niches, we demonstrated the role of 3D confinement and niche stiffness on hMSC viability and proliferation. Importantly, we observed significantly increased cell death rates and decreased rates of proliferation in hMSCs cultured within confined 3D microniche cultures compared with cells on unconfined 2D gels. This effect increased with increased extents of 3D confinement (smaller niche volumes). We related the observed effects of geometric confinement and niche stiffness on cell behavior to changes in nuclear volume and morphology and YAP localization, indicating that mechanotransduction pathways mediate these effects. Further investigation is needed to quantitatively describe the effects of confinement on the mechanical activation of cells and how it relates to cytoskeletal organization, focal adhesion formation, and generation of traction forces. A general understanding of how cell behavior is affected by 3D confinement will be an important step toward mapping a complete regulation profile of in vivo cell behavior. While we focused here on the role of confinement on single cell function, 3D confinement is likely to be involved also in the regulation of multicellular structures influencing growth, development, and homeostatic profiles of tissues.
5. Experimental Section

Hydrogel Platform Design—Synthesis of Norbornene-Functionalized PEG: 8-arm PEG amine (Mn ~10 kDa; 4 g, 0.4 mmol PEG, 3.2 mmol NH2), 1 eq. NH2; JenKem USA) was dissolved in anhydrous dimethylformamide (DMF; 5 mL; Sigma-Aldrich) and purged with argon. N,N-Disopropylethylamine (DIPEA; 2.23 mL, 12.8 mmol, 4 eq.; Sigma Aldrich) was added to the PEG solution followed by the addition of 1-bis(dimethylamino)methylene)-1H-1,2,3-triazolol-5,6-bipyridine 3-oxide hexafluorophosphate] (HATU; 2.43 g, 6.4 mmol, 2 eq.; Sigma-Aldrich). Dimethylformamide (DMF; 5 mL; Sigma-Aldrich) and purged with argon was added dropwise over stirring to the protein solution until reaching 20% glycerol (Sigma-Aldrich) in DMF at RT. Deprotection was performed with 20% piperidine in DMF treatment twice for 1 min at 50 °C, followed by three DMF washes.

Coupling was performed twice (thrice for first coupling) for 5 min at 50 °C, using a reaction mix in DMF containing protected amino-acids 0.2 m stock, HBTU 0.4 m stock, and N-methylmorpholine 0.4 m stock, in 40/20/40 proportions, for a total of 1 mmol (2 equivalents) amino acid per coupling. The resin was then washed three times with DMF before removing the deprotection/coupling cycle. For N-terminus acetylation, the resin was treated in situ with 20% acetic anhydride in DMF for 10 min at RT. Finally, the resin was washed with dichloromethane (DCM; Sigma-Aldrich), dried, with nitrogen for 60 min, and cleared for 2 h at RT. The cleaving solution for SAG (optimized to avoid side products from reaction with amine linker in addition to side reactions with cleaved protecting groups and cysteine oxidation) contained TFA/H3O/TIPS/EDDT in proportions 85/7.5/5/2.5. The cleaving solution for SAT (further optimized to avoid methionine oxidation) contained TFA/TIPS/EDDT/thiaoisole/anisole in proportions 83.75/3.75/6.25/2.5, where TIPS (Sigma-Aldrich) is trisopropylsilane and EDDT is 2,2’-ethylenedioxy diethanethiol (Sigma-Aldrich). The crude peptides were precipitated in ice cold diethyl ether (Et2O; Sigma-Aldrich), collected by centrifugation, further washed with ice cold Et2O, dried under nitrogen flow, and resuspended in a minimal amount of acetonitrile/water/TFA, followed by purification by preparative reverse-phase high-performance liquid chromatography (HPLC; Agilent 1260 infinity) on a 55 mm diameter C18-capped silica column (Agilent), using a gradient from 10% to 90% acetonitrile in water over 40 min in the presence of 0.1% TFA. LC-MS (high resolution, positive mode, Figure S18; Supporting Information): SAG, calculated mass: 860 g mol⁻¹; measured m/z: 860 [M⁺], 1720 [2M+H⁺]; SAT, calculated mass: 1406.49 g mol⁻¹; measured m/z: 704 [M+2H⁺], 1407 [M+H⁺].

Hydrogel Platform Design—Synthesis of Lithium Phenyl-2,4,6-trimethylbenzophosphininate: Lithium phenyl-2,4,6-trimethylbenzophosphininate (LAP) was synthesized as described previously,[8] with 2,4,6-trimethylbenzoylchloride (3.2 g, 18 mmol) of was added dropwise to an equimolar amount of continuously stirred dimethyl phenylphosphonitride (3.1 g; 18 mmol) at RT and under argon. The reaction was stirred overnight under argon atmosphere. Lithium bromide (6.1 g; 72 mmol) was dissolved in 100 mL of 2-butanol and added to the reaction mixture. The reaction was heated to 50 °C to induce product precipitation. After 10 min, a solid precipitate formed. The reaction was cooled to RT over 4 h and then filtered to recover the precipitate. The precipitate was washed 3 times with 2-butanol (50 mL) to remove unreacted lithium bromide and dried under vacuum. The product was recovered in near quantitative yield. 1H NMR in D2O: 7.57 (m, 2H), 7.42 (m, 2H), 7.33 (m, 2H), 6.74 (s, 2H), 2.09 (s, 3H), 1.88 (s, 6H) (Figure S19, Supporting Information).

Hydrogel Platform Design—Rheological Characterization: The crosslinking kinetics and mechanical properties of the PEG hydrogels (3–10 wt%) were quantified using a strain-controlled shear rheometer (MCR 502; Anton Paar). The hydrogel precursor solution was loaded between an 8-arm PEG with norbornene was determined to be above 95% via 1H NMR in CD2Cl2, as shown in the integrated areas under the peaks of the norbornene vinyl protons (δ = 6.0–6.3, m, 2H) and the PEG ether protons (δ = 3.5–3.9, m, 9H; Figure S17, Supporting Information).
ethanol, and acetone. After air drying, the substrate was placed in a Petri dish (silicon mat) or in a glass staining container (glass slides) and submerged in Sigmacote (Sigma-Aldrich) for 5 min. The container was sealed with a lid to avoid evaporation of Sigmacote. Subsequently, Sigmacote was removed and the surface was washed again twice with dH2O and air dried. In addition, the substrate was dried in oven at 100 °C for 30 min to produce a durable coating. Used Sigmacote was stored in a glass container and reused.

Hydrogel Platform Design—Silanization with (3-mercaptopropyl) trimethoxysilane: 3-(Trimethoxysilyl)propyl methacrylate (TMPMA; Sigma Aldrich) has been used to covalently link thiol-containing gels to glass slides. Hydrogels cast on methacrylated slides did not lift from the glass when immersed in medium during cell culture and remain attached during staining procedures. To prepare the glass slides for silanization, the glass slides were cleaned with SDS, rinsed thoroughly with water, washed with ethanol and acetone, and dried in an oven at 80 °C for an hour. 1 mL of TMPMA was diluted in 200 mL of ethanol and 6 mL of dilute acetic acid (1:10 glacial acetic acid:dH2O) was added to the mixture directly prior use. The glass slides were placed in a glass staining container and submerged in the activated TMPMA solution allowing full contact of the glass surface with the silane solution. The solution was allowed to react for 5 min. The excess solution was poured off and the glass slides were rinsed with ethanol to remove the residual reagent and dried in air at RT.

Hydrogel Platform Design—Hydrogel Adhesion via Enzymatic Ligation: Two hydrogel slabs (0.1 and 0.5 mm) were prepared at the same polymer wt% including complementary Sortase A peptide substrates (SAG or SAT, 800 × 10⁻⁶ m). Two glass slides were treated with Sigmacote (Sigma-Aldrich) and separated with a 0.1 or 0.5 mm silicone rubber spacer. The hydrogel precursor solution was injected between the glass slides and polymerized upon exposure to UV light (λ = 365 nm, t = 20 mW cm⁻²). The surface of the hydrogel slab containing SAT peptide was dried in air and treated with Sortase A solution (20 µL; 86 mg mL⁻¹, 4 × 10⁻⁶ m). Directly after, the second hydrogel layer containing SAG peptide was placed on top of the thicker slab substrate and pressed gently. The adhered hydrogels were submerged in a minimal amount of DPBS buffer containing Ca²⁺ and stored in the incubator at 37 °C for half an hour. Adhesion was assessed visually upon mechanical agitation (Movie S1, Supporting Information).

The work of adhesion following enzymatic cross-linking of two PEG hydrogel layers was assessed using pull-off tests performed using the normal force transducer of the rheometer (MCR-502; Anton Paar). Preswollen gels formulated with SAG or SAT (as described above) were attached to the Petter plate or the 8 mm parallel plate geometry (PP-08) of the rheometer using cyanacrylate glue. The surface of the hydrogel adhered to the Petter plate, at a speed of 0.01 mm s⁻¹ during which the normal force (F_n) upon retraction was measured and F₁–displacement curves were recorded (Figure S3, Supporting Information). In order to calculate the work of adhesion (J m⁻²) from the recorded curves, the retraction force was integrated as a function of the displacement, followed by dividing the resulting adhesion energy by the known contact area (surface area of geometry) at the interface.

Hydrogel Platform Design—BSA-FITC Diffusion within 2D and 3D Hydrogels: The diffusion experiments were performed in bulk hydrogels and assembled hydrogel platform (3D microriches). The surface of the hydrogel was dried with a Kimwipe tissue (Kimberly-Clark Professional) to remove the excess liquid and solution of bovine serum albumin covalently labeled with fluorescein isothiocyanate (2 mg mL⁻¹; BSA-FITC, Sigma Aldrich) was applied to the surface of the hydrogel. The gel adhered to a coverslip was mounted on confocal microscope. The z-stacks images were taken every 5 min over a period of 1 h to visualize the extent of BSA-FITC diffusion through the hydrogel bulk and into niches (Figures S4 and S5, Supporting Information).

Microfabrication—Microfabrication of Silicon Master: The microniche arrays were designed in AutoCAD (Autodesk). The obtained designs were used to fabricate polyethylene terephthalate (PET) based transparency masks with soft photographic negative emulsion film, right reading emulsion up (JP-Photodata). The resulting mask was used to transfer the design to a silicon wafer via photolithography in a clean room facility. A layer of photoresist SU-8 50 (MicroChem Inc.) was spin-coated onto a plasma-cleaned silicon wafer. Manufacturer recommendations were followed for prebaking, lithography, postbaking, and photoresist development. The resulting silicon wafer contained a micropost pattern. The pattern profile was assessed using white light interferometry (WLI), confirming that the dimensions of the microposts matched the dimensions of the desired microniche (V1 (20 × 50 × 35 µm³), V2 (35 × 50 × 35 µm³), and V3 (50 × 50 × 50 µm³)). The masters with correct dimensions were used for the fabrication of the intermediate molds.

Microfabrication—PDMS Mold Fabrication: The silicon master fabricated in the previous step was used for casting intermediate PDMS molds. In short, Sylgard 184 (Dow Inc.) was mixed with Sylgard curing agent (Dow Inc.) at a 10:1 mixing ratio by weight. The mixture was placed under vacuum in a desiccator for 30 min to remove trapped air bubbles. The silicon master was placed in a Petri dish and the PDMS mixture was gently poured onto the silicon master avoiding bubble formation. The Petri dish containing both the master with PDMS prepolymer was placed in an oven at 70 °C and cured for 6 h. The generated PDMS with a microwell pattern was separated from the silicon wafer and used in the following steps.

Microfabrication—Teflon Mold Fabrication: The PDMS mold obtained in the previous step was used to fabricate a Teflon mold. The patterned PDMS mold was placed in the glass Petri dish and the surface was covered with Teflon beads. The Petri dish containing both the master with PDMS prepolymer was placed in an oven at 200 °C for 2 h. After cooling to RT and detachment of the two materials, the generated Teflon mold with microposts was released and used for hydrogel patterning to produce the microniche arrays.

Microfabrication—Hydrogel Patterning: The hydrogel precursor solution was prepared as described in the procedure for the preparation of 2D hydrogels. The silicon spacer (0.5 mm) was placed on the TMPMA treated glass slide and the Teflon mold was placed on top of the spacer. The hydrogel precursor solution was carefully injected between the glass slide and the Teflon mold until the whole interstitial volume was filled, avoiding the formation of air bubbles on the surface of the mold. The precursor solution was placed under UV light (λ = 365 nm, t = 20 mW cm⁻²) for 1 to 3 min depending on the hydrogel formulation. Teflon mold was carefully removed from the hydrogel using a spatula as a lever while minimizing shear forces on the hydrogel. The patterned hydrogel adhered to the TMPMA-treated glass slide was immersed in PBS until further use.

Microfabrication—Hydrogel Pattern Assessment: The hydrogel profile was analyzed using fluorescence confocal microscopy. In order to visualize the hydrogel profile, the hydrogel was labeled with acryloxyethyl thiocarbamoyl Rhodamine B (Sigma-Aldrich; Figure S1: Supporting Information). The acrylated Rhodamine B was dissolved in dH₂O at a concentration of 1 mg mL⁻¹. From this stock solution 50 µL was added to 1 mL of hydrogel precursor solution (0.005 wt%). The hydrogel was polymerized under each Teflon mold and immersed in PBS for 30 min to swell out all unreacted Rhodamine dye. Subsequently, the pattern was imaged via confocal microscopy (LSM 780, Zeiss). 3D images were reconstructed from Z-stacks and dimensions were analyzed using ZEN software (Zeiss).

2D and 3D Cell Culture—Cell Culture: Human bone marrow-derived stromal cells (hMSCs) were isolated from bone marrow aspirates of healthy donors obtained during orthopaedic procedures with informed consent and in accordance with the local ethical committee (University Hospital Basel; Prof. Kummer; approval date 26/03/2007, Ref. Number 78/07). Cells were cultured at 37 °C in a humidified atmosphere at 5% CO₂ in minimal essential medium with alpha modification and...
nucleosides (MEMoR, Sigma-Aldrich) supplemented with fetal bovine serum (FBS; 10%; Gibco), penicillin/streptomycin (P/S; 100 U mL⁻¹; Gibco), and fibroblast growth factor 2 (FGF-2; 5 ng mL⁻¹; PeproTech). Cells were passaged before reaching 90% confluency and the medium was changed every 2–3 days.

**2D and 3D Cell Culture—Fabrication of Hydrogels for 2D Cell Culture and Cell Seeding:** For 2D cell culture experiments, PEG hydrogels (3.5, 5, and 6 wt%) were prepared with E = 6, 16, and 30 kPa, respectively. For 3.5 wt% hydrogels, 8-arm PEG-NB (35 mg; 28 × 10⁻³ M NB) was mixed with freshly prepared DTT (2.0 mg; 28 × 10⁻³ M SH), lithium phenyl-2,4,6-trimethylbenzophosphinate (LAP) photoinitiator (2.5 mg; 0.25 × 10⁻³ M), CRGDS (1 × 10⁻¹ M), and dH₂O. For 5 wt% hydrogels, 8-arm PEG-NB (50 mg; 40 × 10⁻³ M NB) was mixed with freshly prepared DTT (2.84 mg; 40 × 10⁻³ M SH), LAP (2.5 mg, 0.25 × 10⁻³ M), CRGDS (1 × 10⁻¹ M), and dH₂O. For 6 wt% hydrogels, 8-arm PEG-NB (50 mg; 48 × 10⁻³ M NB) was mixed with freshly prepared DTT (3.4 mg, 48 × 10⁻³ M), LAP photoinitiator (2.5 mg, 0.25 × 10⁻³ M), CRGDS (1 × 10⁻¹ M), and dH₂O.

Two glass slides were separated by a silicone spacer (0.5 mm). One of the glass slides was treated with TMPMA to facilitate adhesion of the hydrogel to the glass surface. The other glass slide was treated with Sigmacote to provide a hydrophobic surface that prevented hydrogel adhesion and enabled hydrogel detachment without damaging the surface. The hydrogel precursor solution was injected between the two glass slides and subsequently polymerized under exposure to UV light (λ = 365 nm; I = 20 mW cm⁻²) for 1 to 3 min depending on the gelation time of the hydrogel determined during rheological testing. After careful removal of the Sigmacote treated glass slide, 4- or 8-well chamber separators (SPL Cell culture slides, SPL Life Sciences Co.) were placed on the hydrogel adhered to the MPTMS treated glass slide. The gel was washed with PBS 3x for 5 min to diffuse out residual LAP, stored in PBS, and sterilized under UV light (λ = 20 mW cm⁻²) for 1 to 3 min depending on the gelation time of the hydrogel determined during rheological testing. After careful removal of the Sigmacote treated glass slide, 4- or 8-well chamber separators (SPL Cell culture slides, SPL Life Sciences Co.) were placed on the hydrogel adhered to the MPTMS treated glass slide. The gel was washed with PBS 3x for 5 min to diffuse out residual LAP, stored in PBS, and sterilized under UV light (λ = 20 mW cm⁻²) for 1 to 3 min depending on the gelation time of the hydrogel determined during rheological testing.

**Cell Function Assessment—Live/Dead Assay:** Membrane integrity was assessed by staining with Hoechst 33342 (1 μg mL⁻¹) and propidium iodide (PI; 2 μg mL⁻¹) for 30 min. The cells were washed 3x for 10 min with PBS and resuspended in fresh medium, and imaged.

**Cell Function Assessment—Characterization of Mitochondria Structure using Mitotracker Green FM:** The culture medium was changed every 2 days by substituting half of the old medium with fresh medium.

**Cell Function Assessment—EdU Assay:** The fraction of proliferating cells was determined by incorporating 5-ethyl-2′-deoxyuridine (EdU) in the culture medium for a 12 h pulse on the first and third day of culture for both 2D and 3D samples. Incorporation of EdU was visualized by Alexa Fluor 647 azide staining using EdU DetectPro Imaging Kit (BCK: EdUPro-iM647, BaseClick) according to the manufacturer’s protocol.

In brief, hMSCs were seeded on 2D hydrogels and treated with EdU (10 × 10⁻⁶ M) in culture medium 12 h prior to the end of the experiment. After 12 h of EdU treatment, the typical cell cycle period for hMSCs, samples were fixed by treatment with 4% PFA for 15 min and a subsequent treatment with 4% PFA for an additional 15 min. The 2D samples were washed with PBS 3x for 10 min and with PBS supplemented with 5% bovine serum albumin (BSA, Sigma-Aldrich) 2x for 5 min. All samples were permeabilized with TritonX-100 (0.1% in PBS) for 20 min at RT and washed 2x for 5 min with PBS supplemented with 5% BSA.

The 3D samples were treated the same way as 2D samples. The incubation time with DetectPro reaction cocktail containing an azide functionalized Alexa Fluor 657 was extended to 3 h in the dark at RT. The samples were then washed with PBS 3x for 30 min and with PBS supplemented with 5% BSA 2x for 1 h.

In both 2D and 3D samples, the cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 300 × 10⁻⁶ M; Sigma-Aldrich) in PBS.
anti-mouse Alexa Fluor-647 conjugated secondary antibody (1:400, goat; ab150115, Abcam) was applied for 2 h at RT. Nuclei were labelled with DAPI and F-actin was labelled with Phalloidin-iFluor 488 (1:1000, ab176753; Abcam). After the staining, the sample was washed 3x for 10 min with PBST. The sample was resuspended in PBST with P/S and stored in the fridge until imaging.

Cell Function Assessment—F- and G-Actin Staining: hMSCs culture on 2D hydrogels or within 3D microniches were fixed with 2% PFA for 15 min and a subsequent treatment with 4% PFA for an additional 15 min. The cells were washed and permeabilized with Triton X-100 (0.1%, PBST). The samples were blocked with 5% BSA in PBS for 1 h and incubated with Phalloidin-iFluor 488 (1:1000, ab176753; Abcam) to visualize F-actin, and DNaseI-AF594 (Invitrogen, D12372, 1:200) to visualize G-actin for 2 h at RT. After the staining, the sample was washed 3x for 5 min with PBST and co-stained with (DAPI) for 1 h at room temperature. After co-staining, the sample was again washed 3x for 5 min PBST.

Imaging and Image Analysis—Imaging: For assessment of BSA-FITC diffusion through the hydrogels and hydrogel niches, fluorescence microscopy was performed using an inverted confocal laser scanning microscope (LSM 780, Axio Observer; Zeiss) equipped with an Airyscan detector. The samples were imaged using an EC Plan-Neofluar 10x/0.30 Ph1 M27 objective. Each confocal image (1024 × 1024 pixel resolution) was obtained in z-stack (30 µm stack depth, 10 µm step size). The pixel size was 1.384×1.384 µm. Niches were illuminated at excitation wavelengths of 488 nm, and the emission signal was detected at 500–525 nm.

For quantification of F- to G-actin ratio, fixed cells stained with DAPI, Phalloidin-iFluor-488, and DNaseI-AF594 were illuminated at excitation wavelengths of 405, 488, and 594 nm and detected at 450–480, 500–550, and 610–650 nm, respectively. Each confocal image (1024 × 1024 pixel resolution) was obtained in z-stack (8 µm stack depth, 0.2 µm distance between slices). The samples were imaged using an EC Plan-Neofluor 20x/0.50 Ph2 M27 objective. The pixel size was set to 0.41 µm.

For assessment of nuclear volume and morphology, fixed hMSCs stained with DAPI were illuminated at excitation wavelength of 405 nm and detected at 450–480 nm. The samples were imaged using an EC Plan-Neofluor 10x/0.30 Ph1 M27 objective. Each confocal image (1024 × 1024 pixel resolution) was obtained in z-stack (30 µm stack thickness, 2 µm distance between slices). The pixel size was set to 0.923×0.923 µm.

For assessment of cell volume, live hMSCs were stained with Calcein AM and illuminated at excitation wavelengths of 494 nm, and detected at 500–525, 600–640, and 655–700 nm. For assessment of YAP localization, fixed hMSCs, stained with DAPI, Phalloidin-iFluor 488, and Alexa Fluor-647 conjugated secondary antibody, were illuminated at excitation wavelengths of 405 and 650 nm and detected at 450–480, 500–550, and 655–700 nm, respectively.

For live time-lapse imaging was performed on an inverted wide field fluorescence microscope (THUNDER Live Cell; Leica). The samples were imaged using an HC PL Fluorotar 10x/0.32 PH1 objective. Transmitted light channel was recorded over the course of 12 h at specified positions, the movie was reconstructed from recorded images (Movie S1, Supporting Information). All live cell measurements were performed at 37 °C in an atmosphere containing 5% CO2.

For assessment of 3D spreading morphology fluorescence microscopy was performed using a THUNDER Live Cell microscope. The samples were imaged using an HC PL APO 40x/0.95 dry objective. Images were obtained in z-stack (stack depth was chosen according to the niche dimensions and step size was set automatically to optimize ICC). The pictures were processed using THUNDER Large Volume Computational Clearing settings, Feature Scale (nm): 5000, Strength (%): 92, Adaptive Deconvolution set with a refractive index of the aqueous mounting medium of 1.33. The 3D images were reconstructed from z-stacks and rendered using LAS X 3D software (Leica).

Imaging and Image Analysis—Image Processing: The images were exported in batch in .srm or .czi format using ZEN 3.1 blue edition using the z-stack alignment module. The z-stack .srm or .czi images were exported as TIF RGB images (Nuclear volume and shape: z-stack with DAPI signal; Cell volume: z-stack with Calcein signal; Live/Dead: maximum intensity projection (MIP); EdU: MIP; YAP: single plane at highest DAPI signal; Actin: single plane with Phalloidin and AF594 signal) in batch process using a Fiji macro, an open-source freeware (available at https://imagej.net/software/fijij/). All 3D cell images were overlaid with the AutoCAD masks with the corresponding well pattern to enhance the niche outlines to facilitate the following analyses. The overlay was performed in MATLAB using 2D cross-correlation between the binary mask of the well pattern and the binarized bright field image of the wells.

Imaging and Image Analysis—Nuclear Volume: Image-stacks with DAPI signal were pre-processed in FIJI. The contrast was adjusted to remove background noise and all images were smoothed subsequently with the built-in “Image→smooth” function. Afterward, images were imported as 3D images in CellProfiler, an open-source freeware (available at https://cellprofiler.org/). Noise correction was performed removing features with less than 5 pixels diameter using the built-in function. The image slices were thresholded using Otsu threshold algorithm with 2 classes, resulting in a binary image: background and object. Subsequently, image stacks were converted into 3D objects and their size and surface area was calculated by counting voxel number. Resulting data was corrected by applying the voxel dimensions in x, y, and z-direction to the volume and surface area respectively. The correction of axial distortion in voxel volume was performed using correction values obtained from imaging fluorescent microbeads (microParticles GmbH PS-FluoGrün-Fi226, diameter = 10.23 ± 0.13 µm) at the same microscope settings and the same hydrogel conditions as the image stacks. Overall, four images were analyzed per condition, featuring 62 cells for V1, 59 cells for V2, and 65 cells for V3 niches. Dethatched dead cells and cells that were not within a niche were not considered. To further quantify nuclear shape, dependent on the cell’s microenvironment, sphericity was calculated using the method described by Lee et al.[79]

\[
\text{Sphericity} = \left( 6V_C / \pi A_C \right)^{1/3}
\]

(1)

Imaging and Image Analysis—Cell Volume: Images with live Calcein staining were pre-processed in FIJI adjusting contrast and smoothness. The same CellProfiler pipeline as for the calculation of nuclear volume was be used for further analysis. The correction of axial distortion in voxel volume was performed using correction values obtained from imaging fluorescent microbeads (microParticles GmbH PS-FluoGrün-Fi226, diameter = 10.23 ± 0.13 µm) at the same microscope settings and the
same hydrogel conditions as the image stacks. A total of six images was analyzed per condition, featuring 99, 85, and 76 cells for V1, V2, and V3 niches respectively. Rounded, detached cells and cells outside of niches were not considered for this analysis.

**Imaging and Image Analysis—Viability Analysis:** Images were analyzed using Cell Profiler. Separate pipelines were constructed for 2D and 3D experiments. For 2D, the calcein AM positive cells and EthD-1 positive nuclei were identified using separate IdentifyPrimaryObject modules applying appropriate size and intensity thresholding. The identified objects were related via the RelateObjects module and the fraction of viable cells was reported as the fraction of cells that stained negative for EthD-1.

For 3D, additionally the wells were identified using the IdentifyPrimaryObject module. The objects were related via the RelateObjects module and the wells containing multiple cells or nuclei were excluded from the analysis (only the wells containing single cells were analyzed). The fraction of viable cells was reported as the fraction of encapsulated cells staining negative for EthD-1.

**Imaging and Image Analysis—Proliferation Analysis:** Images were analyzed using Cell Profiler. For 2D, the nuclear regions were identified using the IdentifyPrimaryObject module. The fraction of proliferating cells was reported as the fraction of total nuclei that stained positive for EdU divided by the total number of nuclei, indicated by DAPI staining.

For 3D, the wells were identified using the IdentifyPrimaryObject module. The wells with multiple cells were excluded from the analysis (only the wells containing single cells were analyzed).

**Imaging and Image Analysis—YAP N/C Ratio:** Images with DAPI and YAP signal were analyzed using Cell Profiler. For 2D, nuclei (DAPI) and YAP cells were identified using separate IdentifyPrimaryObject modules with appropriate size and intensity thresholds. The nuclear region was identified as a region that stained positive for DAPI. The area outside of the nuclear region that stained positively for YAP was identified as a cytoplasm using the IdentifyTertiaryObject module. YAP mean fluorescence intensities within nuclear and the cytoplasmic regions were calculated using the MeasureObjectIntensity module. The ratio of mean fluorescence intensities within the nucleus and cytoplasm was quantified and reported as the YAP N/C ratio.

For 3D, the wells were identified using the IdentifyPrimaryObject module. The objects were related to each other and only the wells containing single cells were analyzed.

**Imaging and Image Analysis—Quantification of F- to G-Actin Ratio:** For quantification of F- to G-actin ratio the images with DAPI, iF488, and AF594 signal were analyzed with Cell Profiler. The nuclear and cytoplasmic regions were identified using separate IdentifyPrimaryObject modules. The nuclear region was identified as a region that stained positive for DAPI. The area outside of the nuclear region that stained positively for iF488 (F-actin) or AF594 (G-actin) was identified as a cytoplasm using the IdentifyTertiaryObject module. Integrated fluorescence intensities of F- and G-actin within the cytoplasmic regions were calculated using the MeasureObjectIntensity module. The ratio of mean fluorescence intensities of F- to G-actin within the cytoplasm was quantified and reported as the F/G ratio.

**Statistical Analysis:** All experiments were performed with at least three biological replicates. Statistical analyses were performed with OriginPro 2019. Quantitative data for viability, proliferation, and YAP N/C ratio were presented as mean ± standard error or the mean (s.e.m.). Differences between experimental conditions were assessed using one-way or two-way analysis of variance (ANOVA) with Tukey post-hoc test. The significance levels were set at p = 0.05 (*).

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**Conflict of Interest**

The authors declare no conflict of interest.

**Author Contributions**

The project was conceived of and designed by O.D. and M.W.T. The experiments were carried out by O.D., A.B., S.J., M.A.B., N.B., and M.L.A.B., F.H., G.B., and N.B. provided assistance in developing image analysis tools or developing the strategy for enzymatic ligation and contributed to writing the supporting information. The manuscript was written by O.D. and M.W.T. and all authors have approved the final version of the manuscript.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request. All Cell Profiler pipelines have been uploaded on the GitHub repository: https://github.com/MEL-ETH/Single_Cell_ECM.

**Keywords**

3D niche, confinement, human mesenchymal stem cells, proliferation, single cell culture

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**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.
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