Supporting Information

3D confinement regulates stem cell fate

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Supplementary Figures

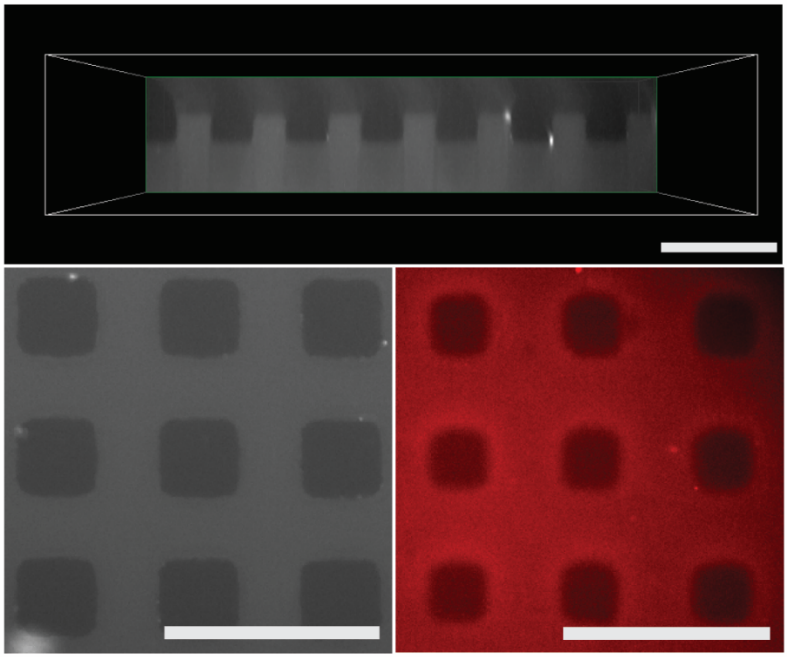


Figure S1. Teflon molds improved pattern transfer to hydrogels as compared with PDMS molds. The patterned hydrogel profile was assessed using fluorescence microscopy. Here the hydrogel pattern was imaged in the x-z (top) and x-y (bottom) planes. The hydrogel was fluorescently labeled with acrylated Rhodamine B. The hydrogel casted using Teflon mold (grey) exhibited improved pattern fidelity as compared with hydrogels formed using PDMS molds (red). Scale bars, 150 μm.

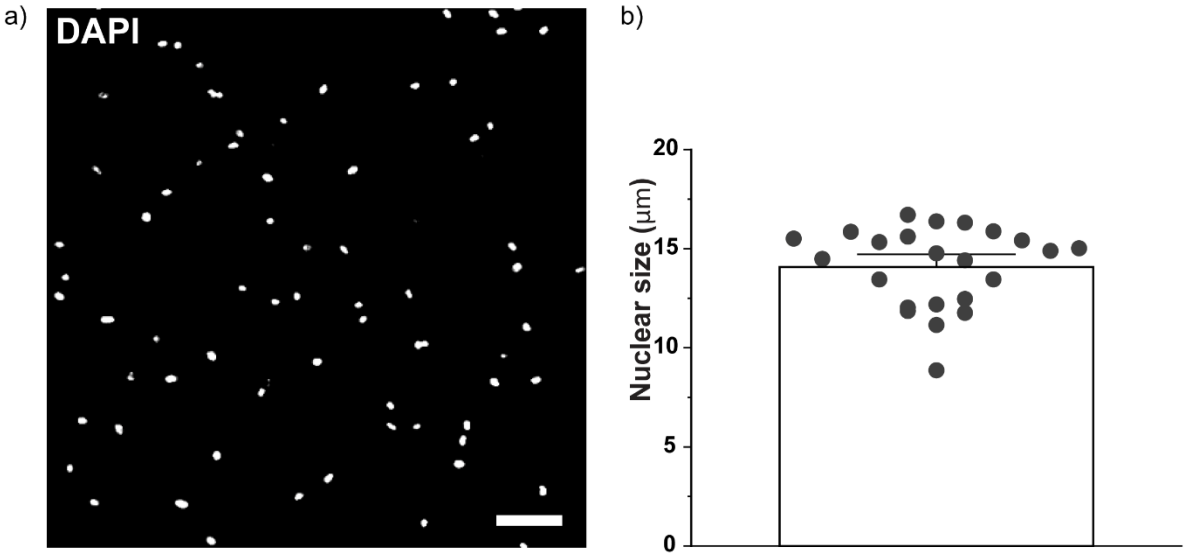


Figure S2. Size of hMSCs nuclei on stiff 2D hydrogels. a) The cell cultured on 2D gels were stained with DAPI. b) Nuclear size is represented as the average major length of the nuclear region of hMSCs at medium stiffness 2D substrates (~16 kPa). The length of the major dimension is measured using ImageJ.

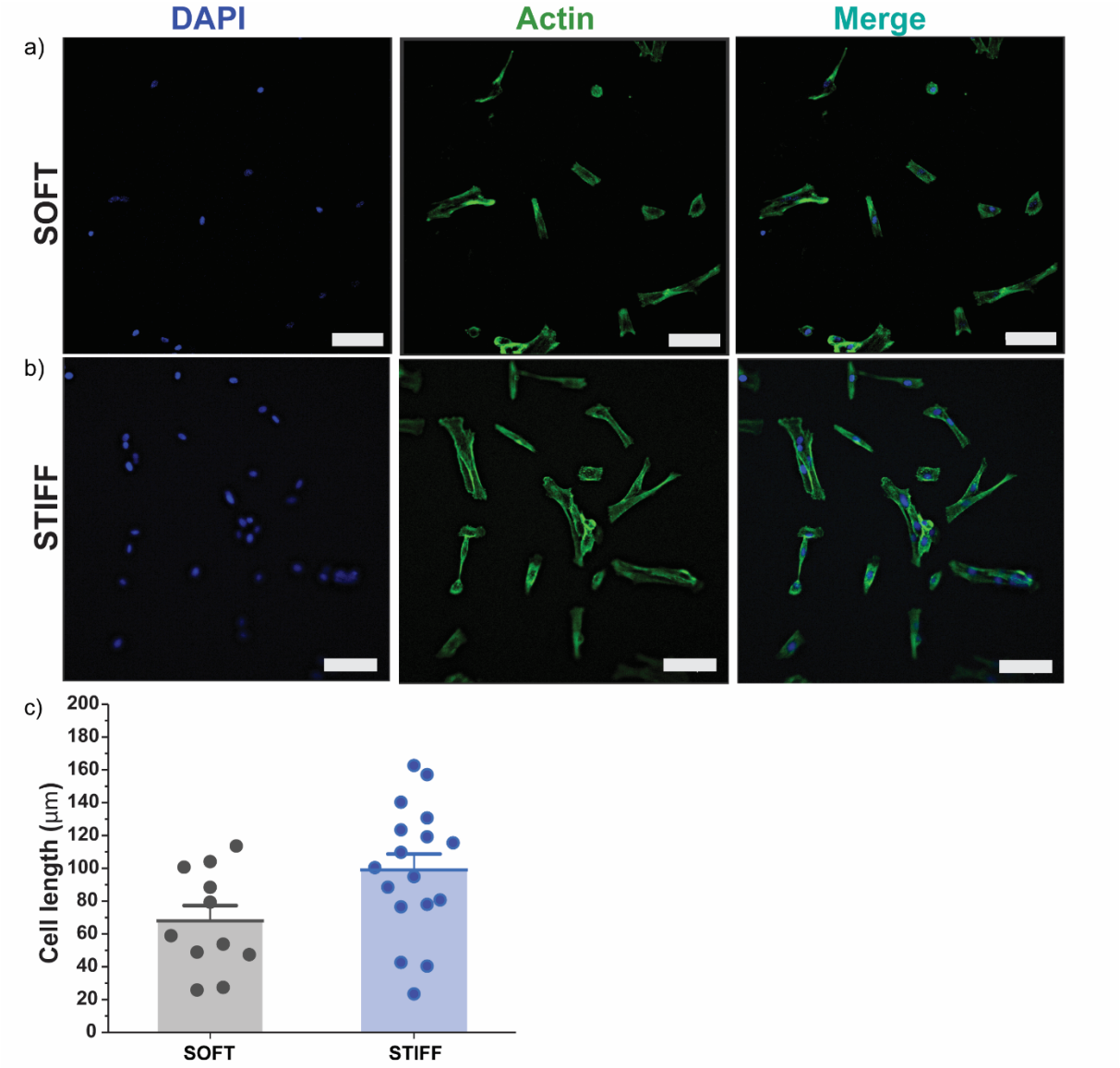


Figure S3. hMSCs obtained different spread morphologies on soft and stiff hydrogels in 2D. hMSCs cultured on soft hydrogels appear to be less elongated than on stiff gels, 1 day after seeding. The length of MSCs was calculated using ImageJ. Scale bar, 100 µm.

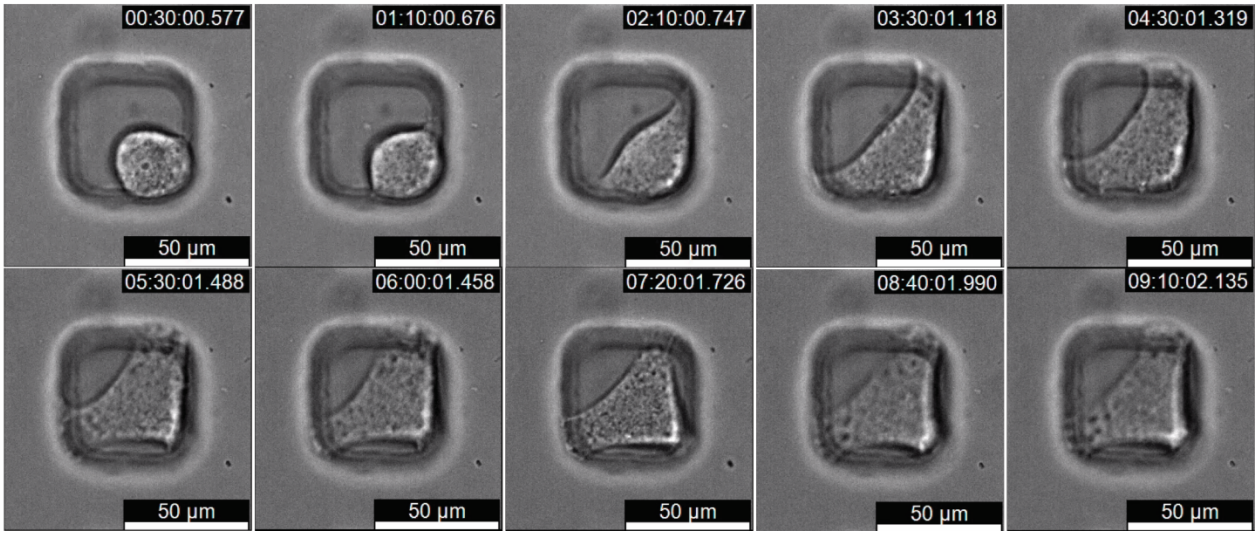


Figure S4. Cells spread within the 3D microniches within hours after encapsulation. Live cell imaging (THUNDER Live Cell Microscope, Leica) of hMSCs in V3 niches revealed that the cells spread rapidly after encapsulation. Cell elongation was observed within 2 h after encapsulation. The cell obtained a spread shape following 4–5 h, which remained stable for the remainder of the time course of the imaging experiment. Total elapsed time, ~9 h.

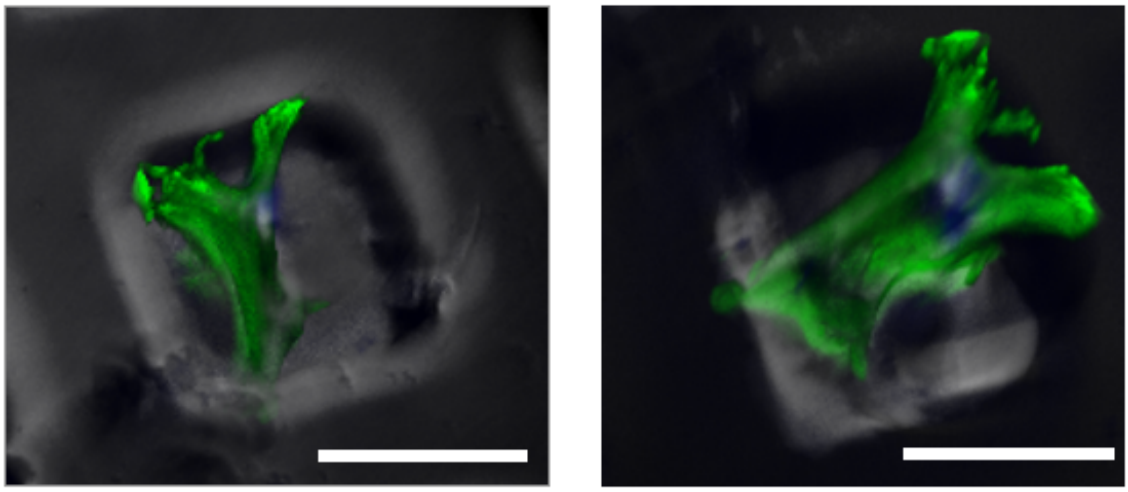


Figure S5. hMSCs adopted 3D spread morphology in the microniches. After 3 days, hMSCs spread in 3D within the microniches, as opposed to spreading along the bottom surface of the niche. The image is generated from a 2D stack on Leica THUNDER microscope. Representative images of cells in V3 niches. Actin was labelled with Phalloidin-iFluor 488 (green) and DNA was labelled with DAPI (blue). Scale bar, 50 μm.

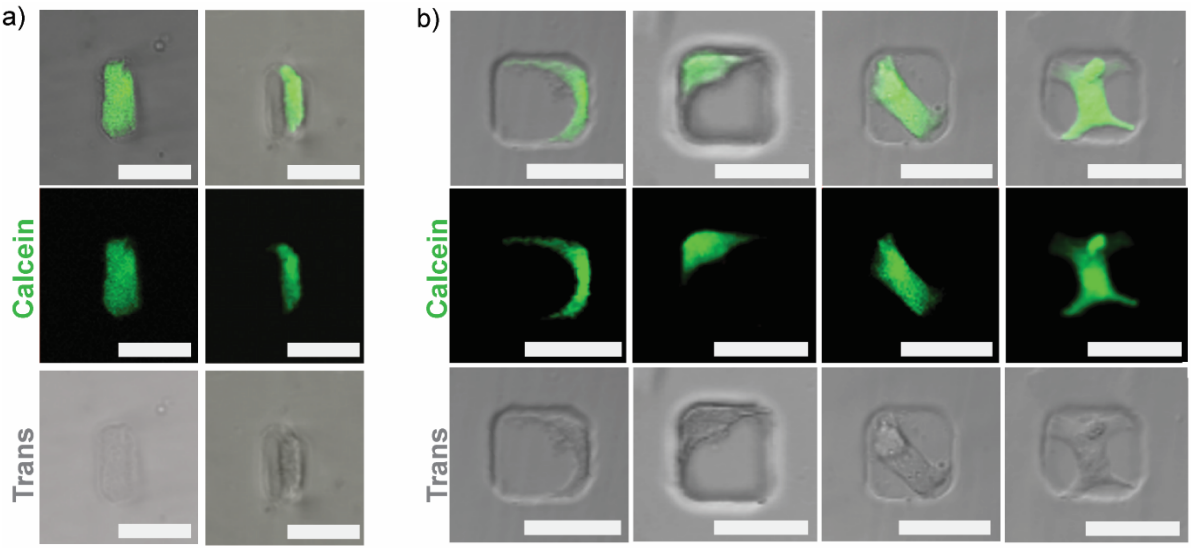


Figure S6. hMSCs spreading within small V1 and large V3 niches. a) In V1 niches, the cells are able to spread and occupy most of the niche area. b) In V3 niches, the cells often inhabit only a fraction of the niche space spreading along to the niche walls, in the corners, or diagonally. The cells were stained with Calcein AM (green). Scale bar, 50 μm.

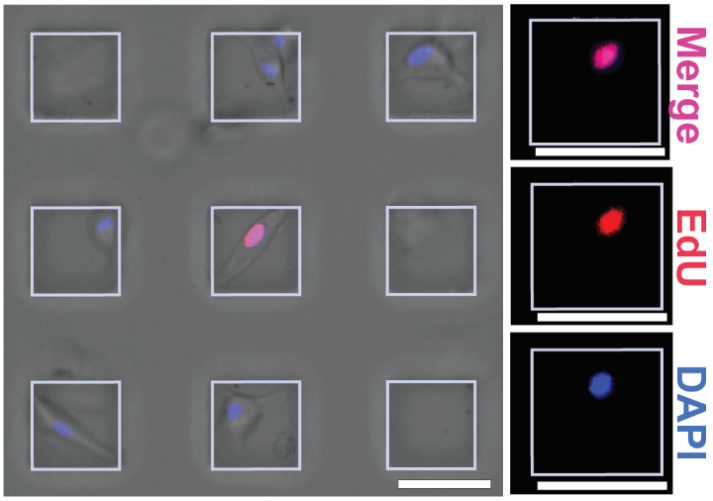


Figure S7. hMSC proliferation was quantified using EdU staining. The proliferation was assessed by calculating the fraction of S-phase cells that stained for 5-ethynyl-2′-deoxyuridine (EdU, red). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, blue). Representative image showing single cells cultured within large, V3 microniches. Scale bars, 50 μm.

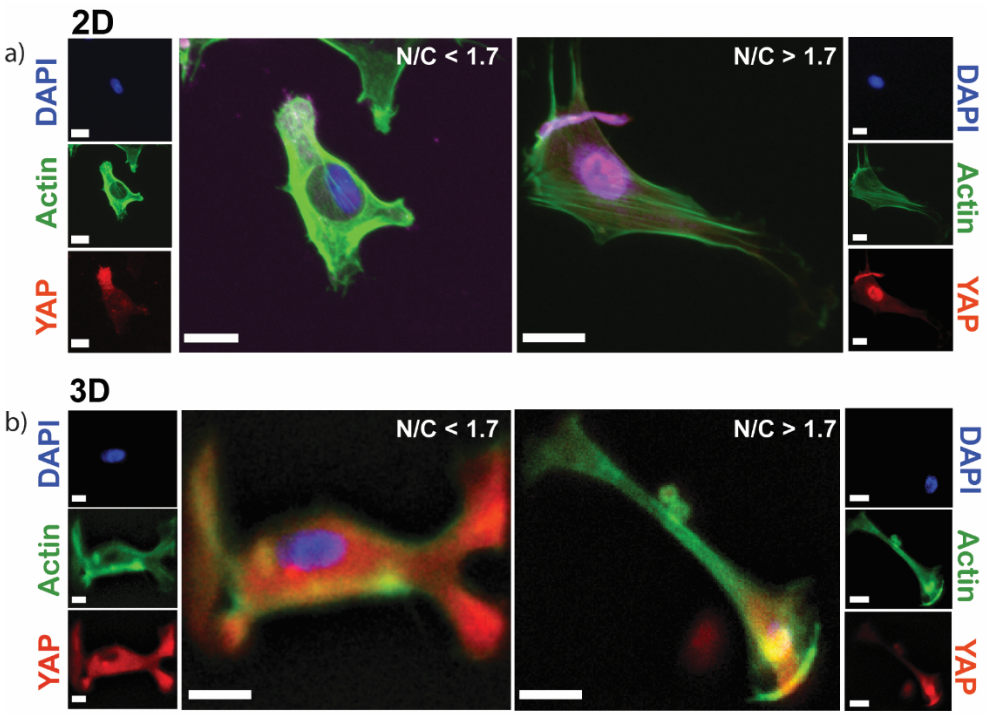


Figure S8. YAP localization in hMSCs. YAP localization was assessed using immunocytochemistry in cells cultured on 2D or in 3D hydrogels and defined as NC ratio. The YAP activation is indicated by NC ratio above 1.6. a) hMSCs cultured on 2D gels expressed both cytoplasmic (left) and nuclear (right) YAP localization. Scale bar, 20 (left) and 30 μm (right). b) hMSCS cultured in large V3 microniches also expressed both cytoplasmic (left) and nuclear YAP localization (right). Scale bar, 10 μm.

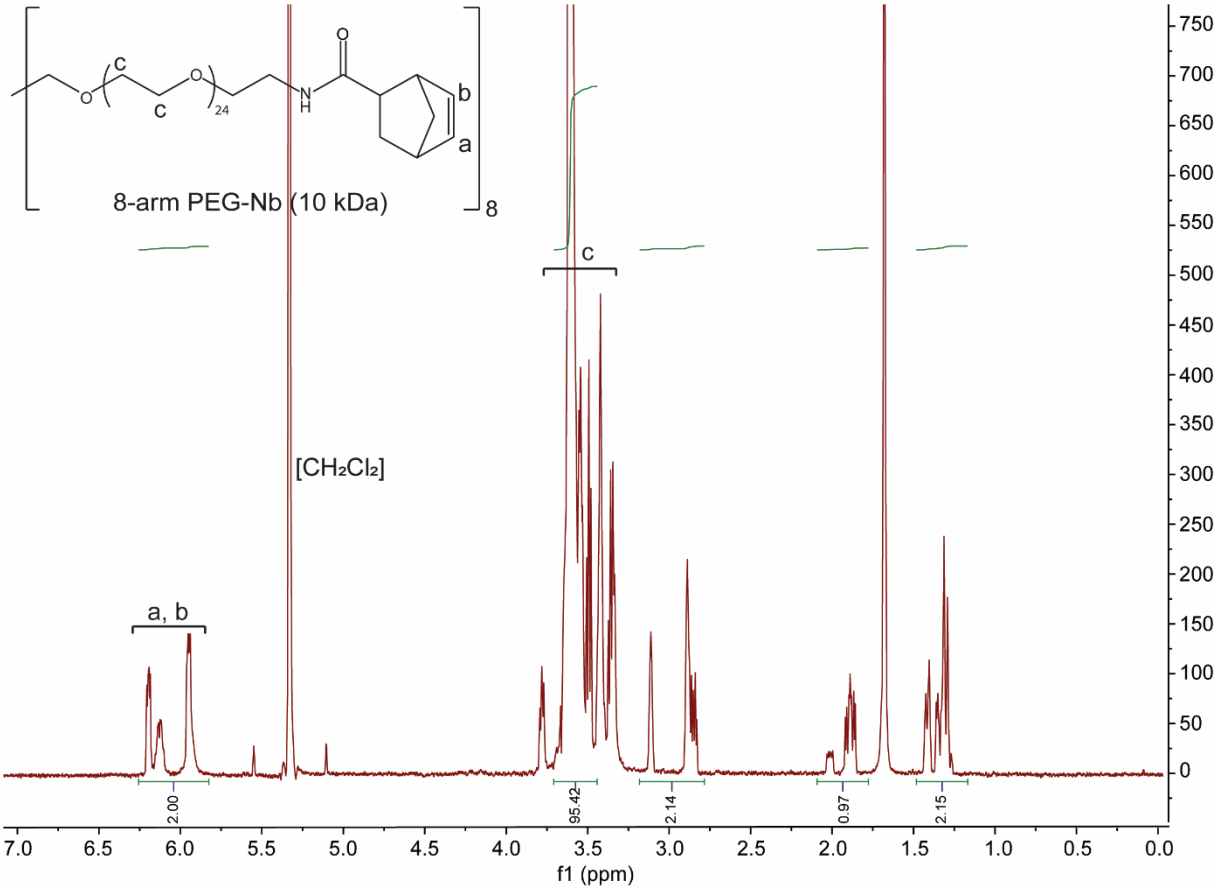
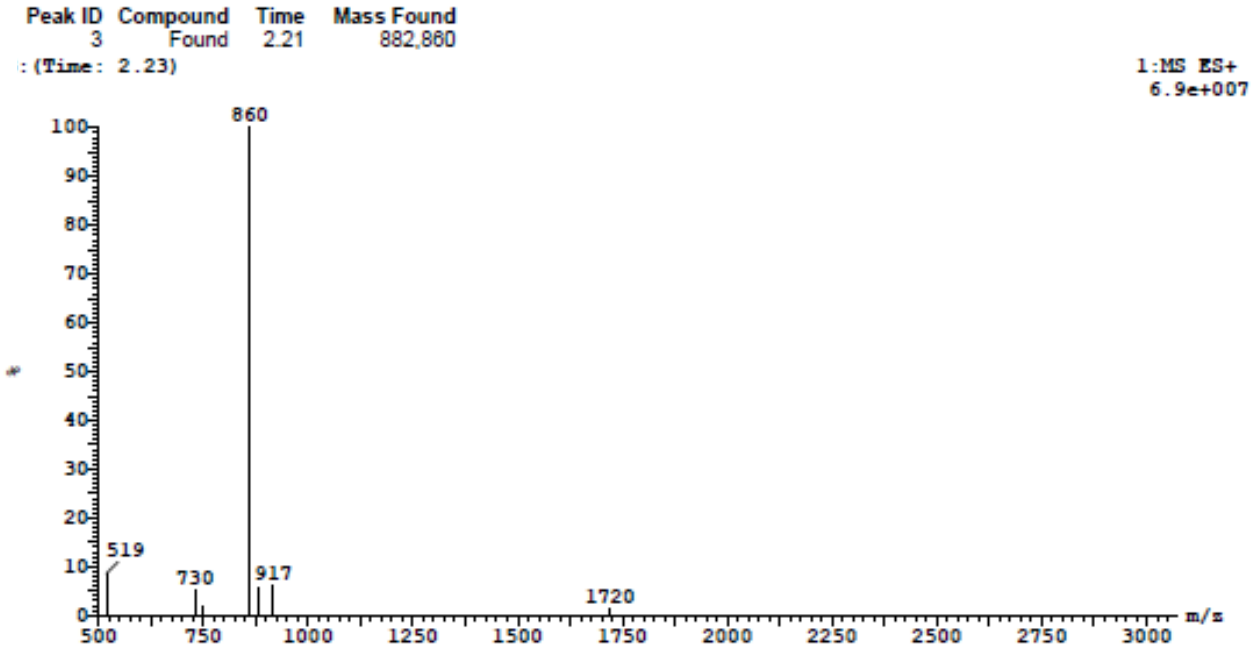


Figure S9. ^{1}H NMR spectrum of PEG$_{10kDa}$-norbornene (500MHz, CD_2Cl_2). Functionalization of the 8-arm PEG with norbornene was determined to be above 95% by comparing the integrated area under the peak for the norbornene vinyl protons (δ = 6.0–6.3, m, 2H) and PEG ether protons (δ = 3.5–3.9, m, 96H).

a)



b)

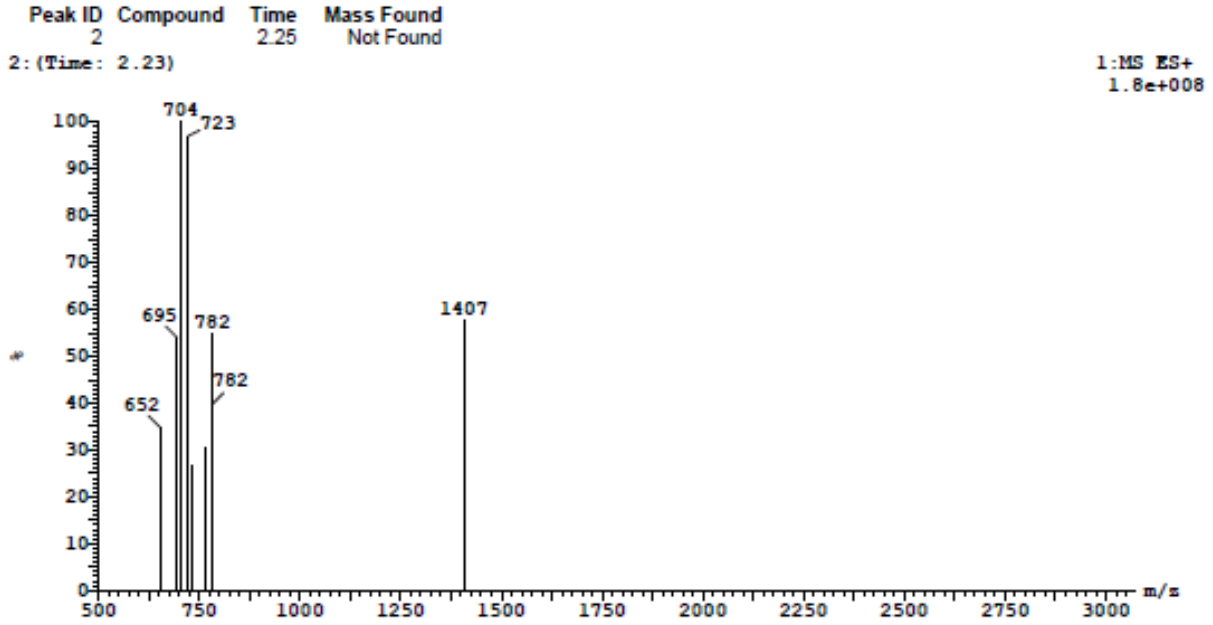


Figure S10. LC-MS (high resolution, positive mode) spectra of Sortase A substrate peptides SAG (GGGGLERCL) and SAT (Ac-GCRE-DDD-LPMTGG). a) SAG: calculated mass: 860.98 g mol^{-1}; measured m/z: 860 $[M]^+$, 1720 $[2M+H]^+$. b) SAT: calculated mass: 1406.49 g mol^{-1}; measured m/z: 704 $[M+2H]^{2+}$, 723 $[M+H+K]^{2+}$, 782 $[M+2K]^{2+}$, 1407 $[M+H]^+$.

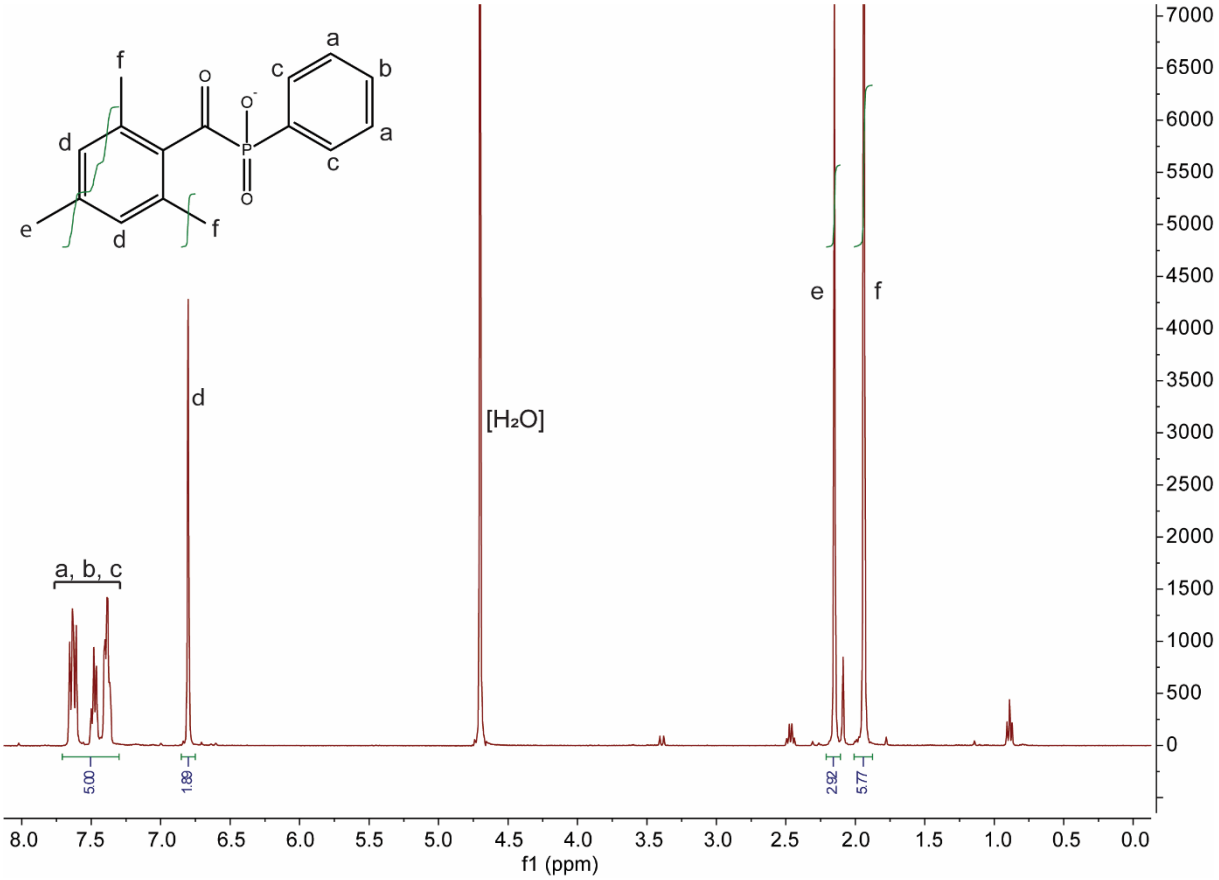


Figure S11. ^{1}H NMR spectrum of LAP (500MHz, D_2O). δ 7.75 (m, 2H), 7.59 (m, 1H), 7.49 (m, 2H), 6.91 (s, 2H), 2.26 (s, 3H), 2.05 (s, 6H).

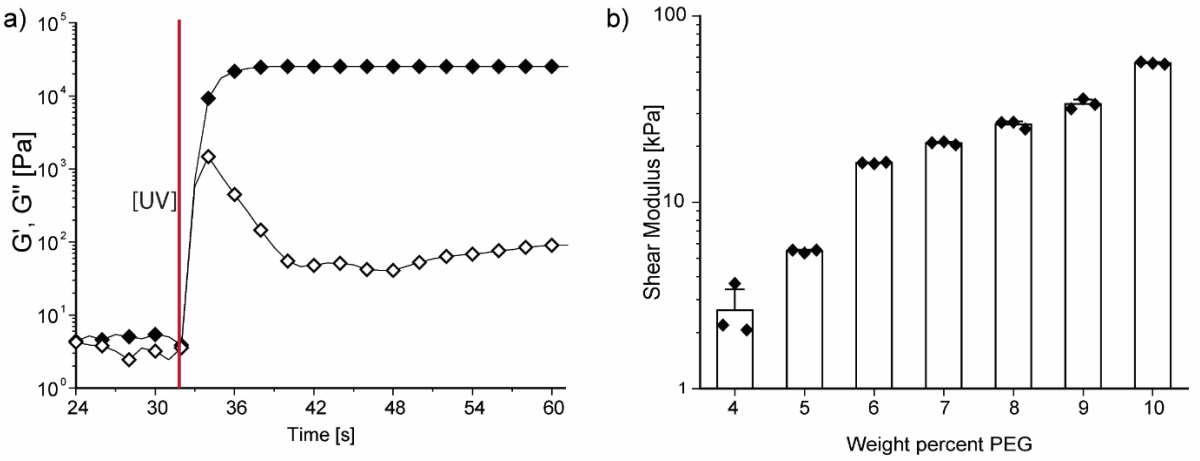


Figure S12. Mechanical properties of PEG hydrogels. a) Dynamic time sweep rheology of 8-arm PEG-NB reacted with DTT upon UV light exposure (λ = 365 nm, I = 20 mW cm^{-2}) demonstrated that gelation occurred within seconds after UV exposure, indicated by the vertical red line. Gelation was complete within 30 s after UV exposure, indicated by G′ reaching a plateau value. b) The equilibrium swollen shear modulus scaled with the PEG wt%, provided a handle to tune the stiffness of the PEG-based hydrogels. Bar plots represent mean + s.e.m.

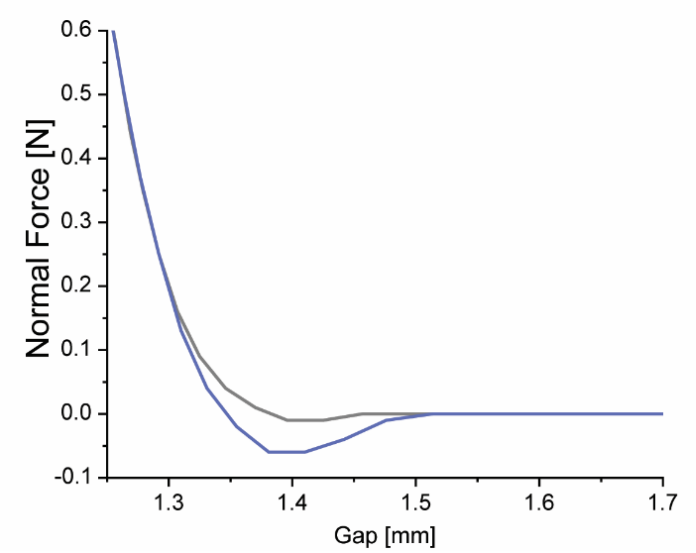


Figure S13. Normal force (F_n)–displacement curves for measuring adhesion between two gels adhered via covalent bond formation between two substrate peptides catalyzed by bacterial enzyme Sortase A. The pull-off test were performed on the rheometer in extensional mode. The geometry was raised with a velocity of 0.01 mm s^{-1} and F_n upon retraction was measured and F_n–displacement curves were recorded. 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.

Supplementary Tables

Statistical significance was determined for p-values less than 0.05.

Cell viability

Cell viability 3D Day 1 (Figure 3a)

After 1 day of culture in 3D niches, hMSCs exhibited high viability without variation in mean viability values between niche volumes or stiffnesses.

Table S1. Two-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
Stiffness	2	69.15756	34.57878	0.559	5.81E-01
Volume	2	42.62572	21.31286	0.34455	0.71311
Interaction	4	482.7966	120.6992	1.95123	0.14552
Model	8	594.5799	74.32249	1.2015	0.35178
Error	18	1113.443	61.85794	--	--

Table S2. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium–Soft	3.54487	3.70759	1.35215	0.61297	0.05	0	-5.91744	13.00718
Stiff–Soft	3.22215	3.70759	1.22905	0.66605	0.05	0	-6.24016	12.68446
Stiff–Medium	-0.32272	3.70759	0.1231	0.99583	0.05	0	-9.78503	9.13959

Table S3. Tukey's comparison of means (volume)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
V2–V1	2.7291	3.70759	1.04098	0.74564	0.05	0	-6.73321	12.19141
V3–V1	2.59674	3.70759	0.9905	0.76631	0.05	0	-6.86557	12.05905
V3–V2	-0.13235	3.70759	0.05048	0.9993	0.05	0	-9.59466	9.32996

Viability 3D Day 3 (Figure 3b)

After 3 days of culture in 3D niches, the mean hMSC viability in high stiffness niches was higher than in low and medium stiffness niches.

Table S4. Two-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
Stiffness	2	2389.653	1194.827	22.48079	7.65E-06
Volume	2	287.7307	143.8654	2.70684	0.09112
Interaction	4	915.791	228.9477	4.30768	0.01126
Model	8	3593.011	449.1264	8.45036	5.48E-05
Error	20	1062.976	53.14879	--	--
Corrected Total	28	4655.987	--	--	--

Table 5. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium–Soft	7.19691	3.34967	3.0385	0.10529	0.05	0	-1.27763	15.67145
Stiff–Soft	21.28969	3.26033	9.2347	6.59E-06	0.05	1	13.04118	29.53821
Stiff–Medium	14.09278	3.34967	5.9499	0.00121	0.05	1	5.61824	22.56732

Table S6. Tukey's comparison of means (volume)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
V2–V1	7.35488	3.34967	3.10519	0.09638	0.05	0	-1.11966	15.82942
V3–V1	6.77329	3.34967	2.85965	0.13275	0.05	0	-1.70125	15.24784
V3–V2	-0.58159	3.26033	0.25227	0.98263	0.05	0	-8.8301	7.66693

Cell viability 2D Day 3 (Figure 3c)

After 3 days of culture on 2D substrates, hMSC viability was close to 100% for all stiffness conditions without significant variation between means.

Table S7. One-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	2	2.44186	1.22093	0.31901	0.73848
Error	6	22.96363	3.82727		
Total	8	25.40548			

Table S8. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium Low	-1.24383	1.59735	1.10123	0.7287	0.05	0	-6.14505	3.65738
High Low	-0.86806	1.59735	0.76854	0.85348	0.05	0	-5.76927	4.03315
High Medium	0.37577	1.59735	0.33269	0.9701	0.05	0	-4.52544	5.27698

Proliferation

Proliferation 3D Day 1 (Figure 4a)

After 1 day in 3D culture, hMSC proliferation was low and was not significantly affected by niche stiffness or volume.

Table S9. Two-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
Stiffness	2	22.06112	11.03056	2.4523	0.11433
Volume	2	0.35833	0.17916	0.03983	0.96104
Interaction	4	5.38363	1.34591	0.29922	0.87461
Model	8	27.80308	3.47539	0.77264	0.63132
Error	18	80.96499	4.49806	--	--
Corrected Total	26	108.7681	--	--	--

Table S10. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium–Soft	-2.17097	0.99978	3.07088	0.10359	0.05	0	-4.72256	0.38063
Stiff–Soft	-0.70862	0.99978	1.00236	0.76148	0.05	0	-3.26022	1.84297
Stiff–Medium	1.46235	0.99978	2.06852	0.33159	0.05	0	-1.08925	4.01394

Table S11. Tukey's comparison of means (volume)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
V2–V1	0.23025	0.99978	0.32569	0.97123	0.05	0	-2.32135	2.78184
V3–V1	0.25641	0.99978	0.36269	0.96446	0.05	0	-2.29519	2.808
V3–V2	0.02616	0.99978	0.03701	0.99962	0.05	0	-2.52543	2.57776

Proliferation 3D Day 3 (Figure 4b)

After 3 days in 3D niches, the proliferation rate settled below 10% and mean proliferation was significantly affected by both stiffness and volume.

Table S12. Two-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
Stiffness	2	31.09483	15.54742	6.03209	0.00989
Volume	2	34.54075	17.27038	6.70056	0.00668
Interaction	4	11.18792	2.79698	1.08517	0.39328
Model	8	76.8235	9.60294	3.72575	0.00975
Error	18	46.39415	2.57745	--	--
Corrected Total	26	123.2177	--	--	--

Table S13. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL

Medium–Soft	2.01741	0.75681	3.76982	0.03984	0.05	1	0.08591	3.94891
Stiff–Soft	2.46816	0.75681	4.61211	0.01149	0.05	1	0.53666	4.39966
Stiff–Medium	0.45075	0.75681	0.84229	0.82422	0.05	0	-1.48075	2.38225

Table S14. Tukey's comparison of means (volume)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
V2–V1	1.85184	0.75681	3.46042	0.06149	0.05	0	-0.07966	3.78334
V3–V1	2.71052	0.75681	5.065	0.00575	0.05	1	0.77902	4.64202
V3–V2	0.85869	0.75681	1.60458	0.50594	0.05	0	-1.07281	2.79019

Proliferation 2D Day 1 (Figure 4c)

The mean proliferation of hMSCs increased with stiffness of the 2D substrates, the variation between mean proliferation values of hMSCs on low and high stiffness substrates was significant.

Table S15. One-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	2	411.2374	205.6187	5.14752	0.04992
Error	6	239.671	39.94517		
Total	8	650.9084			

Table S16. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium–Low	5.46792	5.16044	1.49848	0.5704	0.05	0	-10.3661	21.30194
High– Low	16.26892	5.16044	4.45848	0.04509	0.05	1	0.4349	32.10294
High–Medium	10.801	5.16044	2.96	0.17151	0.05	0	-5.03302	26.63502

Proliferation 2D Day 3 (Figure 4d)

After 3 days on 2D gels, mean hMSC proliferation increased with stiffness. The differences in mean proliferation values of hMSCs between low and high and medium and high stiffness substrates were significant.

Table S17. One-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	2	621.3567	310.6784	8.24585	0.01444
Error	7	263.7386	37.67694		
Total	9	885.0953			

Table S18. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium–Low	1.0108	1.23004	1.16215	0.7044	0.05	0	-2.76339	4.78499

High– Low	14.27127	1.23004	16.4081	6.09E-05	0.05	1	10.49708	18.04546
High– Medium	13.26047	1.23004	15.24595	9.29E-05	0.05	1	9.48627	17.03466

YAP localization

YAP N/C 2D Day 3 (Figure 5b)

After 3 days on 2D culture, the N/C ratio increased with substrate stiffness. The mean N/C ratio values between medium and low stiffness substrates were significantly different.

Table S19. One-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	Prob>F
Model	2	0.45691	0.22845	8.88094	0.0161
Error	6	0.15434	0.02572		
Total	8	0.61125			

Table S20. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium– Low	0.55179	0.13096	5.95892	0.01324	0.05	1	0.14998	0.95361
High– Low	0.28574	0.13096	3.08581	0.15316	0.05	0	-0.11607	0.68756
High– Medium	-0.26605	0.13096	2.87311	0.1854	0.05	0	-0.66786	0.13577

YAP percent activation Day 3 (Figure 5c)

After 3 days in 3D niches, mean YAP activation (defined as N/C > 1.7) did not correlate with niche volume. A weak dependence with stiffness was observed, with highest variation between high and medium stiffnesses.

Table S21. Two-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
Stiffness	2	0.06332	0.03166	3.5171	0.05136
Volume	2	0.03133	0.01566	1.74	0.20377
Interaction	4	0.01868	0.00467	0.51881	0.72301
Model	8	0.11333	0.01417	1.57368	0.20156
Error	18	0.16203	0.009	--	--
Corrected Total	26	0.27536	--	--	--

Table S22. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium– Low	-0.02303	0.04473	0.72829	0.86507	0.05	0	-0.13718	0.09111
High–Low	0.08926	0.04473	2.82231	0.14196	0.05	0	-0.02489	0.2034
High– Medium	0.11229	0.04473	3.5506	0.05427	0.05	0	-0.00186	0.22644

Table S23. Tukey's comparison of means (volume)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
V2–V1	0.00866	0.04473	0.27394	0.97955	0.05	0	-0.10548	0.12281
V3–V1	0.0762	0.04473	2.40935	0.23101	0.05	0	-0.03795	0.19034
V3–V2	0.06753	0.04473	2.13541	0.30987	0.05	0	-0.04661	0.18168

YAP N/C ratio 3D Day 1 (Figure 5d)

On day 1 in 3D niches, mean YAP N/C ratios did not correlate with niche volume and exhibited a weak trend with stiffness, specifically N/C values between low and high stiffness niches were significantly different.

Table S24. Two-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
Stiffness	2	0.67774	0.33887	5.35206	0.01197
Volume	2	0.02955	0.01478	0.23338	0.79363
Model	4	0.69831	0.17458	2.75727	0.05113
Error	24	1.51957	0.06332	--	--
Corrected Total	28	2.21788	--	--	--

Table S25. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium–Low	0.12437	0.11862	1.48286	0.55434	0.05	0	-0.17184	0.42059
High–Low	0.35842	0.1131	4.48183	0.01112	0.05	1	0.07599	0.64085
High–Medium	0.23405	0.1131	2.9266	0.11765	0.05	0	-0.04839	0.51648

Table S26. Tukey's comparison of means (volume)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
V2 –V1	-0.00194	0.11561	0.02374	0.99984	0.05	0	-0.29066	0.28678
V3 –V1	-0.05693	0.11253	0.71552	0.86916	0.05	0	-0.33795	0.22408
V3 –V2	-0.05499	0.11561	0.67269	0.88335	0.05	0	-0.34371	0.23373

YAP N/C ratio 3D Day 3 (Figure 5e)

After 3 days in 3D niches, mean YAP N/C ratios exhibited a weak correlation with stiffness and volume but were not significantly different.

Table S27. Two-way ANOVA

	DF	Sum of Squares	Mean Square	F Value	P Value
Stiffness	2	0.15858	0.07929	2.42836	0.11145
Volume	2	0.1548	0.0774	2.37056	0.11686
Model	4	0.31338	0.07835	2.39946	0.08092
Error	22	0.71833	0.03265	--	--

Corrected Total	26	1.03172	--	--	--

Table S28. Tukey's comparison of means (stiffness)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
Medium Low	0.06709	0.08518	1.11387	0.71437	0.05	0	-0.14689	0.28107
High Low	0.18538	0.08518	3.07776	0.09774	0.05	0	-0.0286	0.39936
High Medium	0.11829	0.08518	1.96389	0.36383	0.05	0	-0.09569	0.33227

Table S29. Tukey's comparison of means (volume)

	MeanDiff	SEM	q Value	Prob	Alpha	Sig	LCL	UCL
V2 V1	0.04899	0.08518	0.81329	0.83468	0.05	0	-0.16499	0.26297
V3 V1	0.17942	0.08518	2.97873	0.11161	0.05	0	-0.03456	0.3934
V3 V2	0.13043	0.08518	2.16543	0.2962	0.05	0	-0.08355	0.34441

Supplementary Movies

Supplementary Movie 1. Sortase A sealing. Demonstrates the adhesion between two hydrogel slabs following enzymatic ligation between two substrate peptides of bacterial enzyme Sortase A. The adhesion between two gels holds under agitation.

Supplementary Movie 2. Live cells. This movie follows a single hMSC spreading in large V3 niche for 9 h post seeding.

Supplementary Movie 3. 3D Cell Spreading. A short video of a single hMSC spread in 3D within large V3 niche (**Figure S5**) showing the cell from different perspectives.