Local nascent protein deposition and remodeling guide mesenchymal stromal cell mechanosensing and fate in three-dimensional hydrogels

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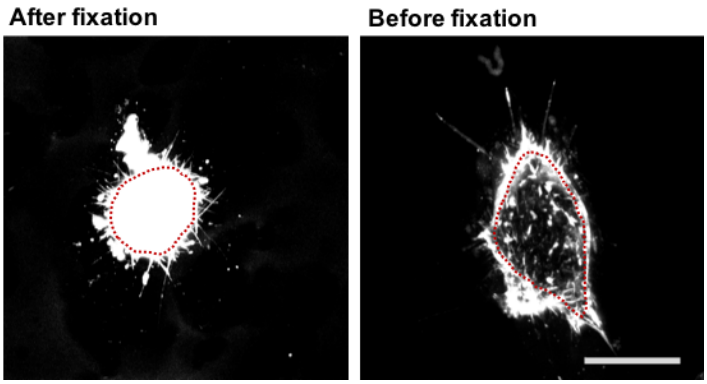
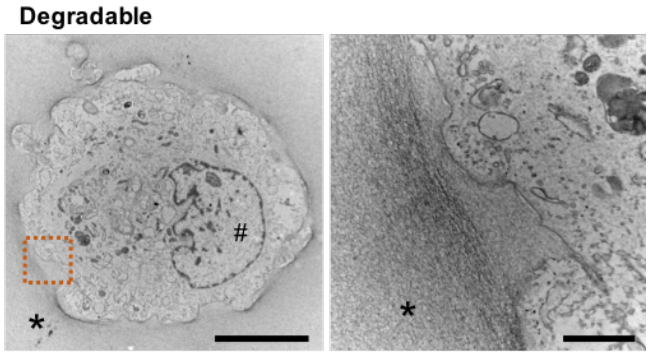
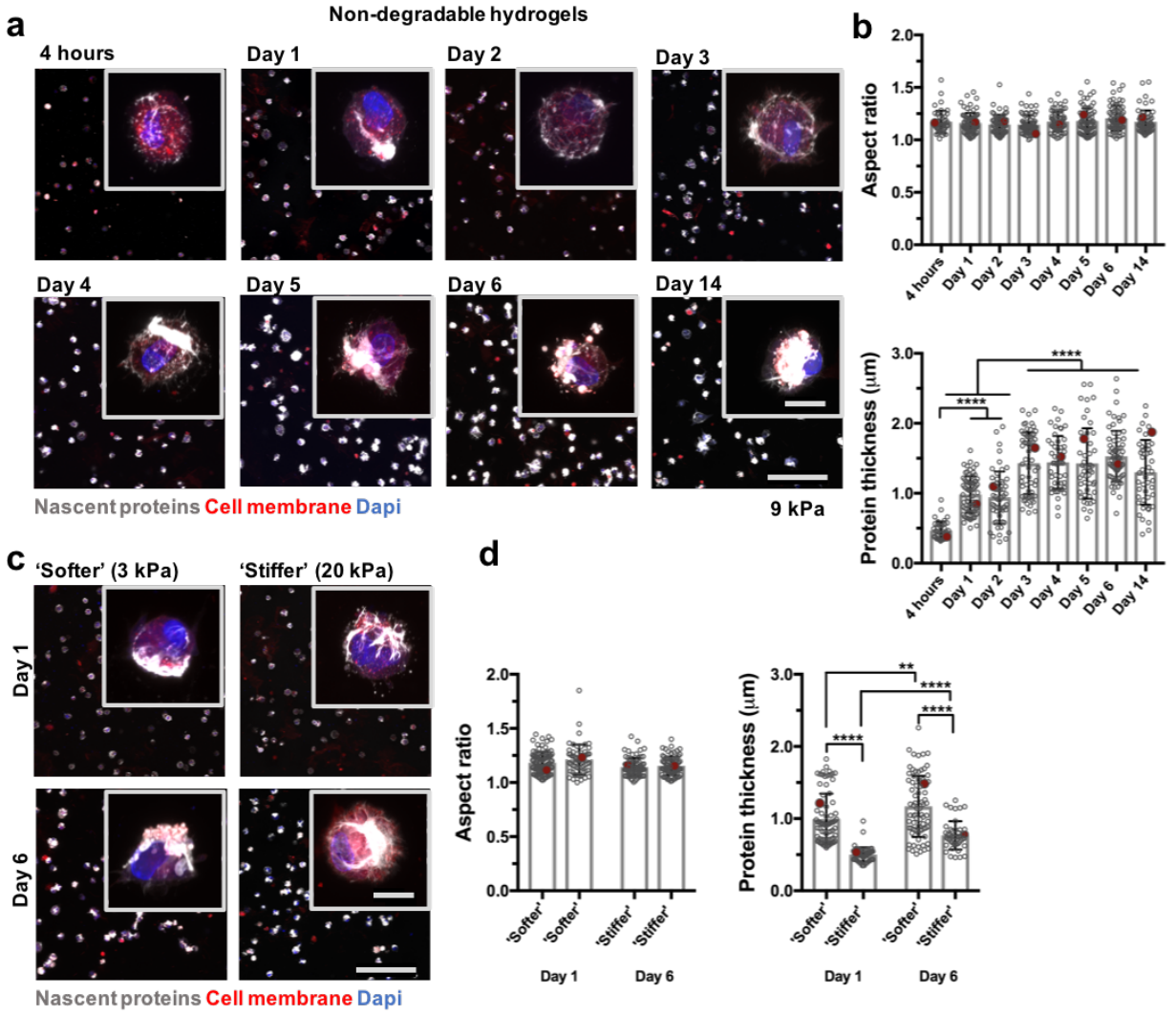
Supplementary Figures



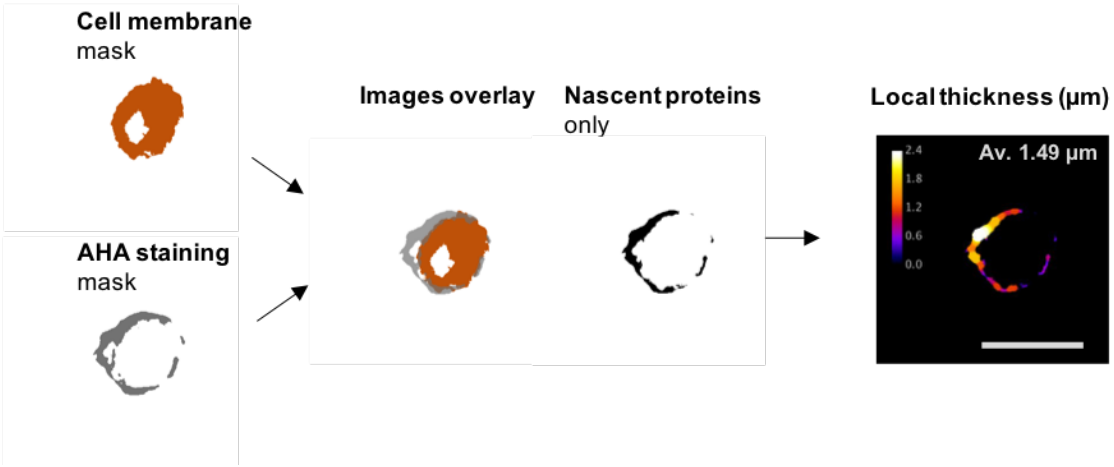
Supplementary Figure 1 Nascent protein labeling prior to cell fixation reduces intra-cellular protein labeling. Representative images of a maximum intensity z-projection of methionine-containing nascent proteins labeled with fluorophore-conjugated cyclooctynes (DBCO-488) after (left) and before (right) adding the fixative. Cells were cultured for 3 days within MMP-degradable hyaluronic acid (HA) hydrogels (media supplemented with azidohomoalanine, AHA). Red lines indicate cell boundaries identified with cell membrane staining (scale bar 20 μm, experiment was repeated three times per condition with similar results).



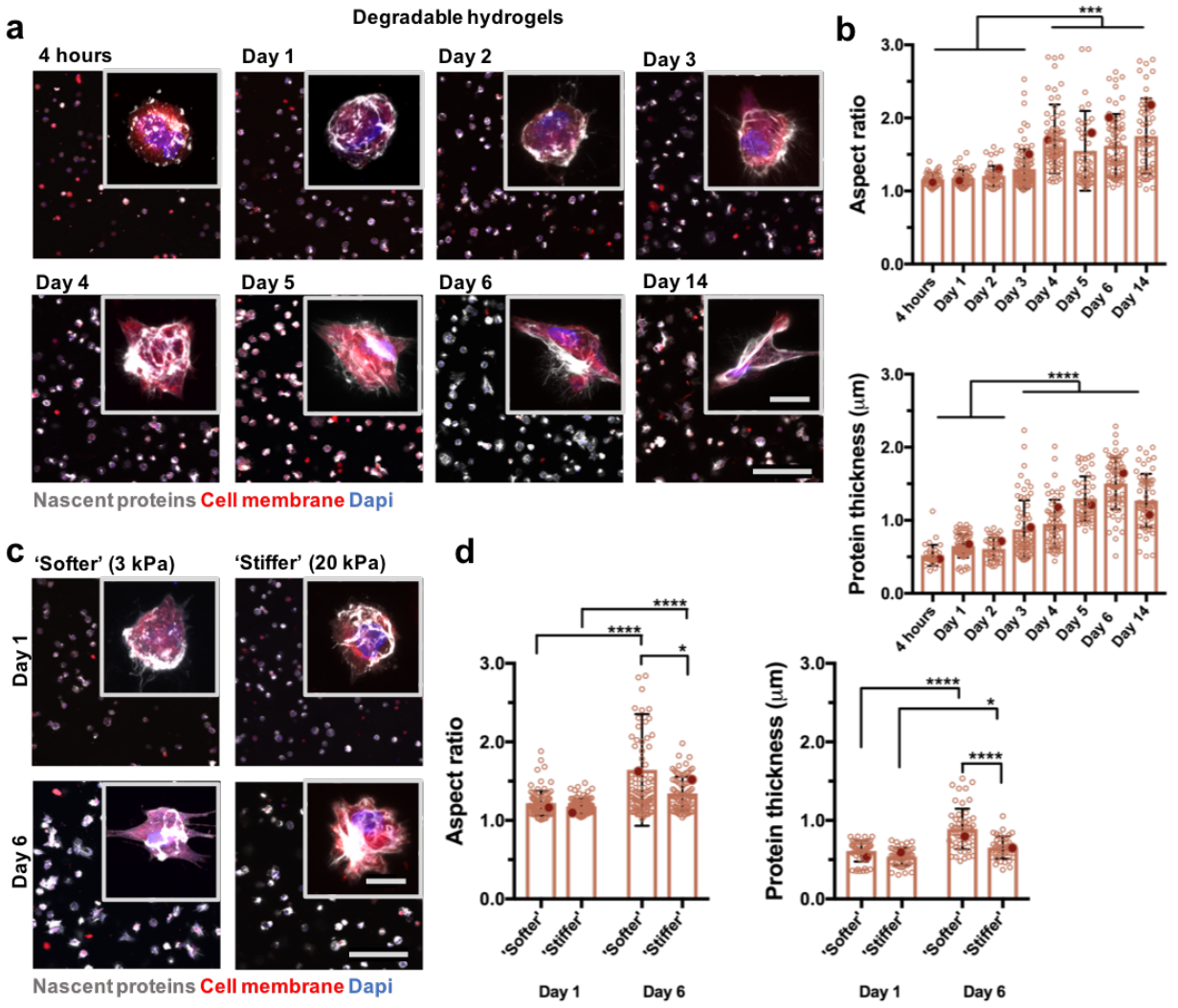
Supplementary Figure 2 TEM images directly after encapsulation. Representative transmission electron microscopy (TEM, * hydrogel, # nucleus) images of hMSCs directly following encapsulation (scale bar 5 μm left, 1 μm right, representative image selected from eight imaged cells). Orange box in left image indicates magnification in right image.



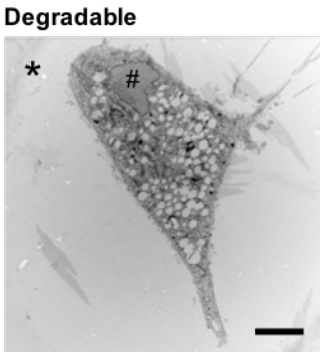
Supplementary Figure 3 Thickness of accumulated nascent proteins in non-degradable hydrogels is dependent on time and initial hydrogel stiffness. a Representative images of nascent proteins (white) of hMSCs encapsulated in non-degradable NorHA hydrogels (9.0 ± 0.7 kPa, mean ± SD, n = 3 independent measurements) and cultured in growth media (supplemented with AHA) for up to 14 days, visualized via fluorescent DBCO labeling (scale bar 200 μm, inset 20 μm). **b** Quantification of hMSC aspect ratio and accumulated protein thickness deposited by hMSCs (n = 40 cells (4 hours), 55 cells (day 2, day 4, day 5, day 14) and 70 cells (day 1, day 3, day 6) from 2 biologically independent experiments), mean ± SD, **** $p \leq 0.0001$ by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **a**). **c** Representative images of nascent proteins (white) of hMSCs encapsulated in 'softer' (3 ± 0.45 kPa, mean ± SD, n = 3 independent measurements) and 'stiffer' (20 ± 1.1 kPa, mean ± SD, n = 3 independent measurements) NorHA hydrogels and cultured for up to 6 days (scale bar 200 μm, inset 20 μm). **d** Quantification of hMSC aspect ratio and accumulated protein thickness deposited by hMSCs (n = 65 cells ('softer' day 1, 6, 'stiffer' day 1) and n = 46 cells ('stiffer', day 6) from 2 biologically independent experiments, mean ± SD, **** $p \leq 0.0001$, ** $p \leq 0.01$ by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **c**).



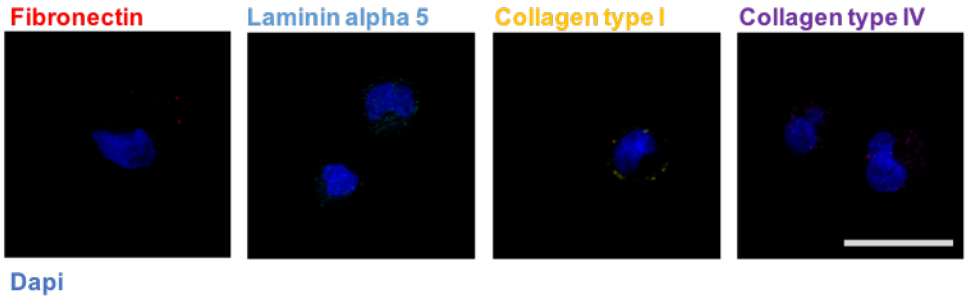
Supplementary Figure 4 Workflow of nascent protein thickness measurements. Schematic illustrating the measurement of average local protein thickness in ImageJ using the plugin 'BoneJ' (scale bar 20 µm).



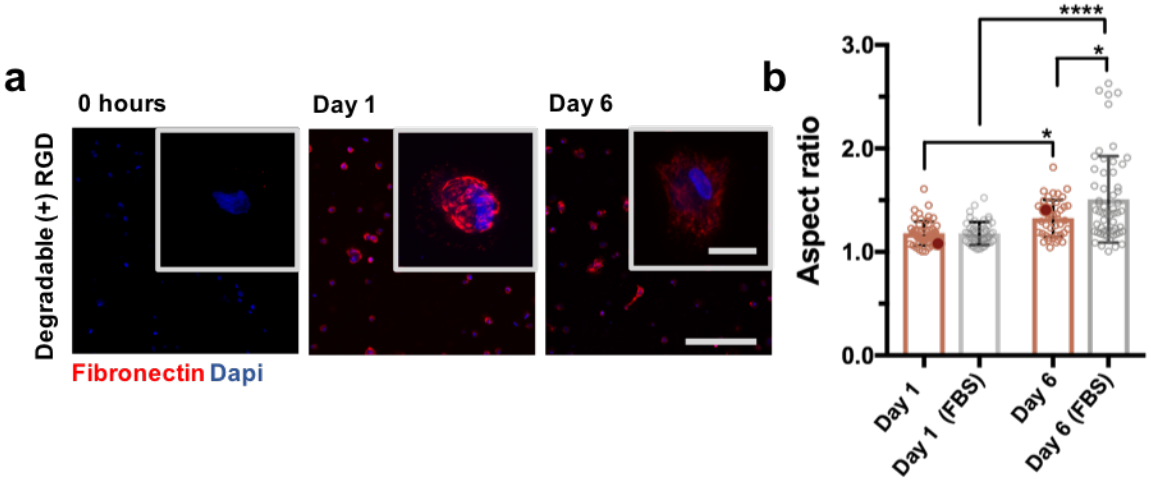
Supplementary Figure 5 Thickness of accumulated nascent proteins in degradable hydrogels is dependent on time and initial hydrogel stiffness. a Representative images of nascent proteins (white) of hMSCs encapsulated in degradable NorHA hydrogels (9.0 ± 0.7 kPa, mean ± SD, n = 3 independent measurements) and cultured in growth media (supplemented with AHA) for up to 14 days, visualized via fluorescent DBCO labeling up to 14 days (scale bar 200 μm, inset 20 μm). **b** Quantification of hMSC aspect ratio and accumulated protein thickness deposited by hMSCs (n = 55 cells per group from 2 biologically independent experiments, mean ± SD, **** $p \leq 0.0001$, *** $p \leq 0.001$ by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **a**). **c** Representative images of the nascent proteins (white) of hMSCs encapsulated in 'softer' (3 ± 0.45 kPa), mean ± SD, n = 3 independent measurements and 'stiffer' (20 ± 1.1 kPa, mean ± SD, n = 3 independent measurements) NorHA hydrogels and cultured for up to 6 days (scale bar 200 μm, inset 20 μm). **d** Quantification of hMSC aspect ratio and accumulated protein thickness deposited by hMSCs (n = 65 cells ('softer' day 1, 6, 'stiffer' day 1) and n = 46 cells ('stiffer', day 6) from 2 biologically independent experiments, mean ± SD, **** $p \leq 0.0001$, * $p \leq 0.05$ by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **c**).



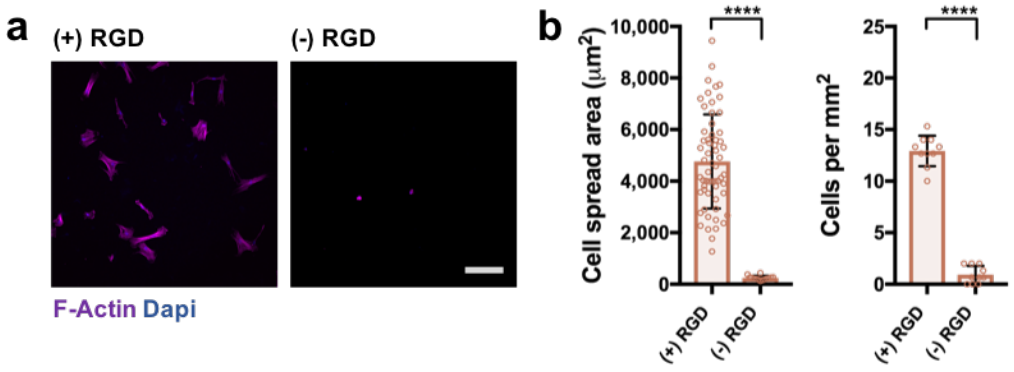
Supplementary Figure 6 TEM images 6 days after encapsulation. Representative transmission electron microscopy (TEM, * hydrogel, # nucleus) image of hMSCs at day 6 (scale bar 5 µm, representative image selected from 6 imaged cells).



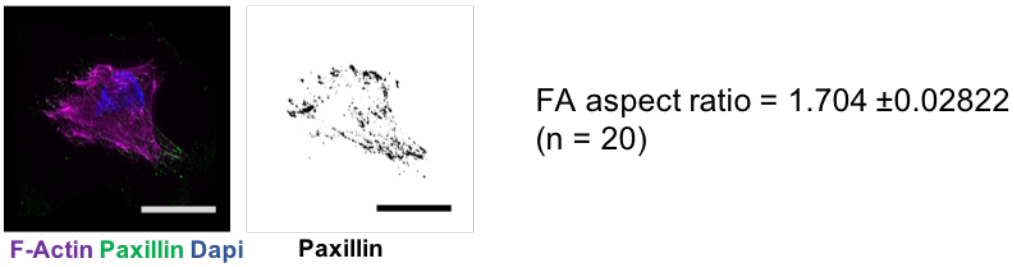
Supplementary Figure 7 Absence of residual ECM proteins on trypsinized hMSCs. Representative images of hMSCs stained for fibronectin, laminin α5 and collagen type 1 and 4 directly following encapsulation in MMP-degradable NorHA hydrogels (scale bar 20 µm, experiments were repeated twice per condition with similar results).



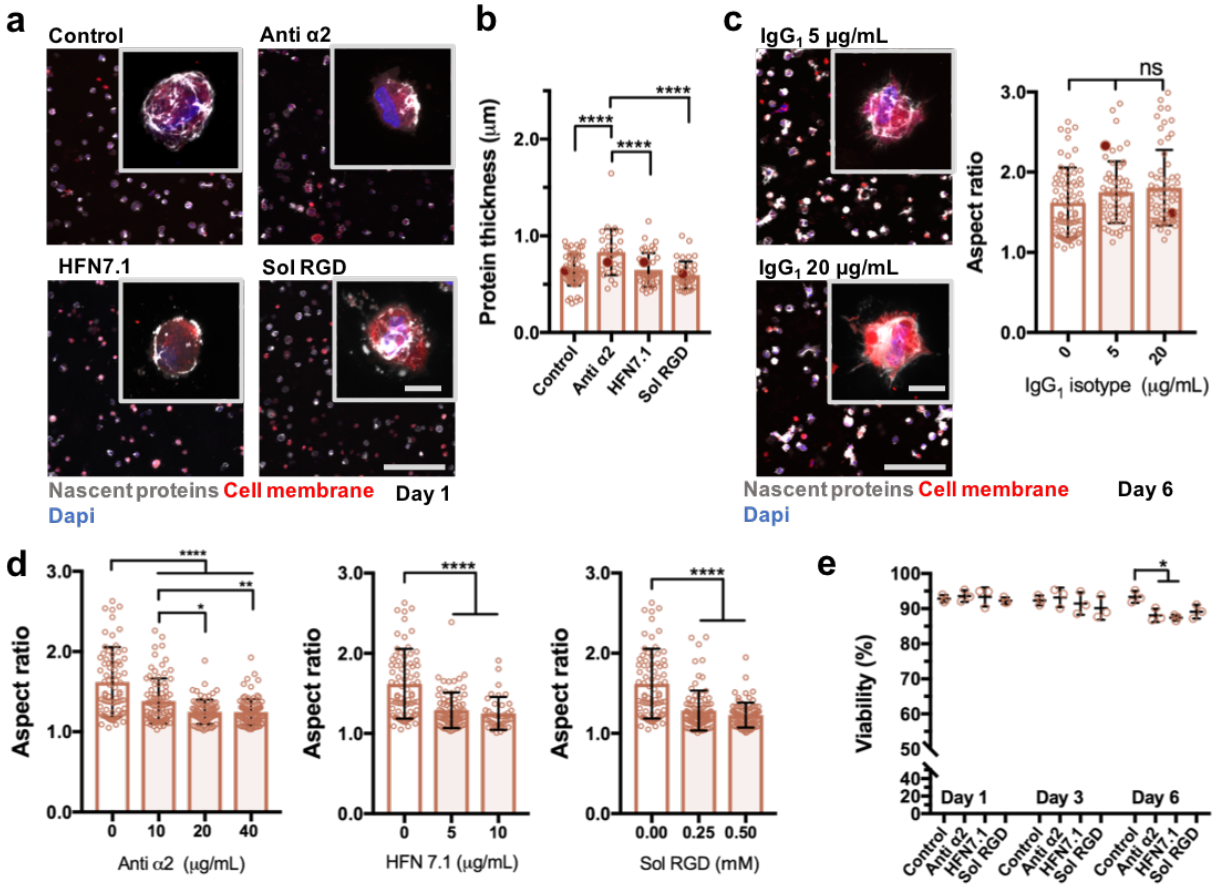
Supplementary Figure 8 hMSC spreading and nascent protein deposition in serum-free, chemically defined media. a Representative images of fibronectin stained hMSCs directly following encapsulation (0 hours) or after culture for 1 or 6 days in serum-free, chemically defined media (scale bar 200 µm, inset 20 µm). **b** Quantification of aspect ratio of hMSCs in degradable NorHA hydrogels (n = 54 (day 1, day 1 (FBS)), n = 58 (day 6 (FBS)) and n = 45 (day 6) from 2 biologically independent experiments, mean ± SD, **** $p \leq 0.0001$, * $p \leq 0.05$, by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **a**).



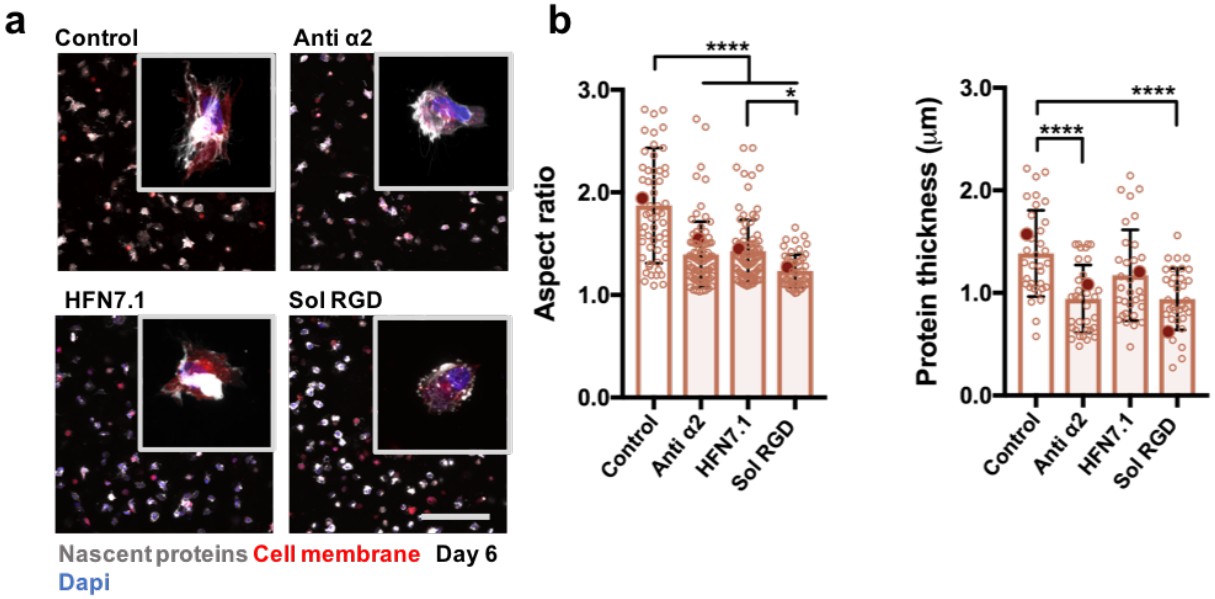
Supplementary Figure 9 Lack of cell attachment and spreading atop unmodified hydrogels. a Representative images of F-Actin immunostaining of hMSCs cultured in growth media (supplemented with AHA) for 18 hours on degradable hydrogels modified with 1 mM RGD ((+) RGD) or unmodified ((-) RGD, scale bar 200 μm). **b** Quantification of cell spread area (representative images in **a**), n = 60 cells ((+) RGD), n = 14 ((-) RGD) from 2 biologically independent experiments) and cell number per mm^2, n = 10 ROIs from 2 biologically independent experiments), mean ± SD, **** $p \leq 0.0001$ by two-tailed Student's *t*-test).



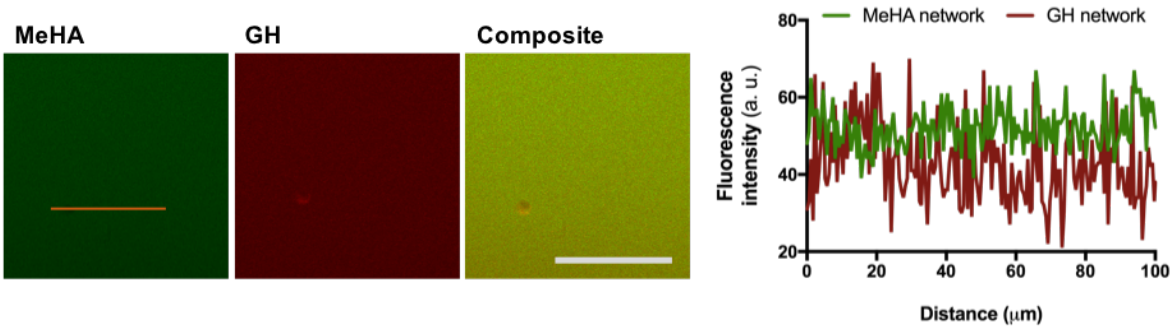
Supplementary Figure 10 Formation of focal adhesions in degradable hydrogels. Representative image of F-Actin and Paxillin immunofluorescence and binary image of Paxillin of an hMSC cultured in an MMP-degradable NorHA hydrogel at day 6 (scale bars 20 μm, n = 20 cells from 2 biologically independent experiments).



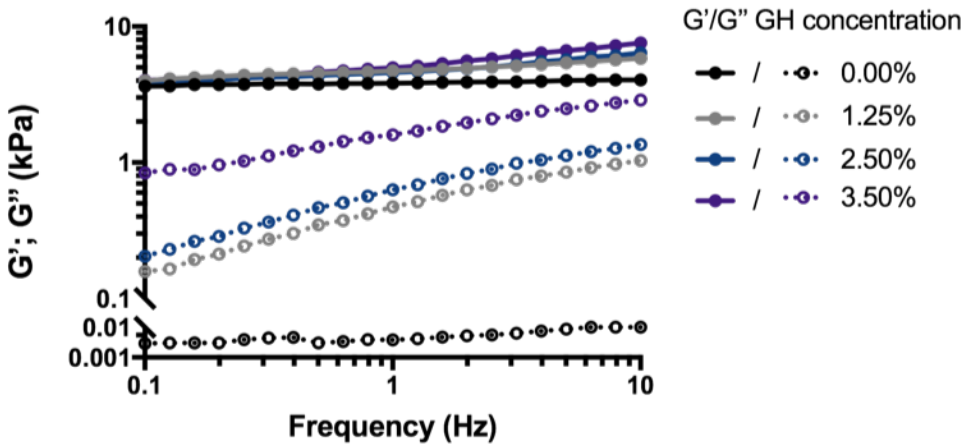
Supplementary Figure 11 Influence of inhibitors of nascent protein adhesion on cell spreading and viability. **a** Representative images and **b** quantification of accumulated nascent proteins (white) of hMSCs encapsulated in degradable hydrogels modified with RGD (1 mM) and treated with anti Integrin α2 (anti α2, 20 μg/mL), human fibronectin (HFN7.1, 5 μg/mL) or soluble RGD (sol RGD, 0.5 mM, scale bar 200 μm, inset 20 μm, (quantifications: n = 70 cells (control), n = 29 (anti α2), n = 41 cells (HFN7.1), n = 30 cells (sol RGD) from 2 biologically independent experiments), mean ± SD, **** $p \leq 0.0001$ by one-way ANOVA with Bonferroni *post hoc,* red dots indicate measurements for representative images in **a**). **c** Representative images and quantification of aspect ratio of hMSCs encapsulated in degradable NorHA hydrogels and treated with IgG1 isotype control antibody (scale bar 200 μm, inset 20 μm, 5, 20 μg/mL, n = 60 cells per group from 2 biologically independent experiments), mean ± SD, with no significant difference by one-way ANOVA with Bonferroni *post hoc*). **d** Quantification of the aspect ratio of hMSCs encapsulated in degradable NorHA hydrogels and treated with anti α2 (10, 20, 40 μg/mL), HFN7.1 (5, 10 μg/mL) or sol RGD (0.25, 0.5 mM, n = 80 cells (anti α2), n = 54 cells (HFN7.1), n = 82 cells (sol RGD) from 2 biologically independent experiments), mean ± SD, **** $p \leq 0.0001$, ** $p \leq 0.01$, * $p \leq 0.05$, by one-way ANOVA with Bonferroni *post hoc*). **e** Viability (analyzed with live-dead staining) of hMSCs treated with anti α2 (20 μg/mL), HFN7.1 (5 μg/mL), sol RGD (0.5 mM, from 2 biologically independent experiments), mean ± SD, * $p \leq 0.05$ by one-way ANOVA with Bonferroni *post hoc*).



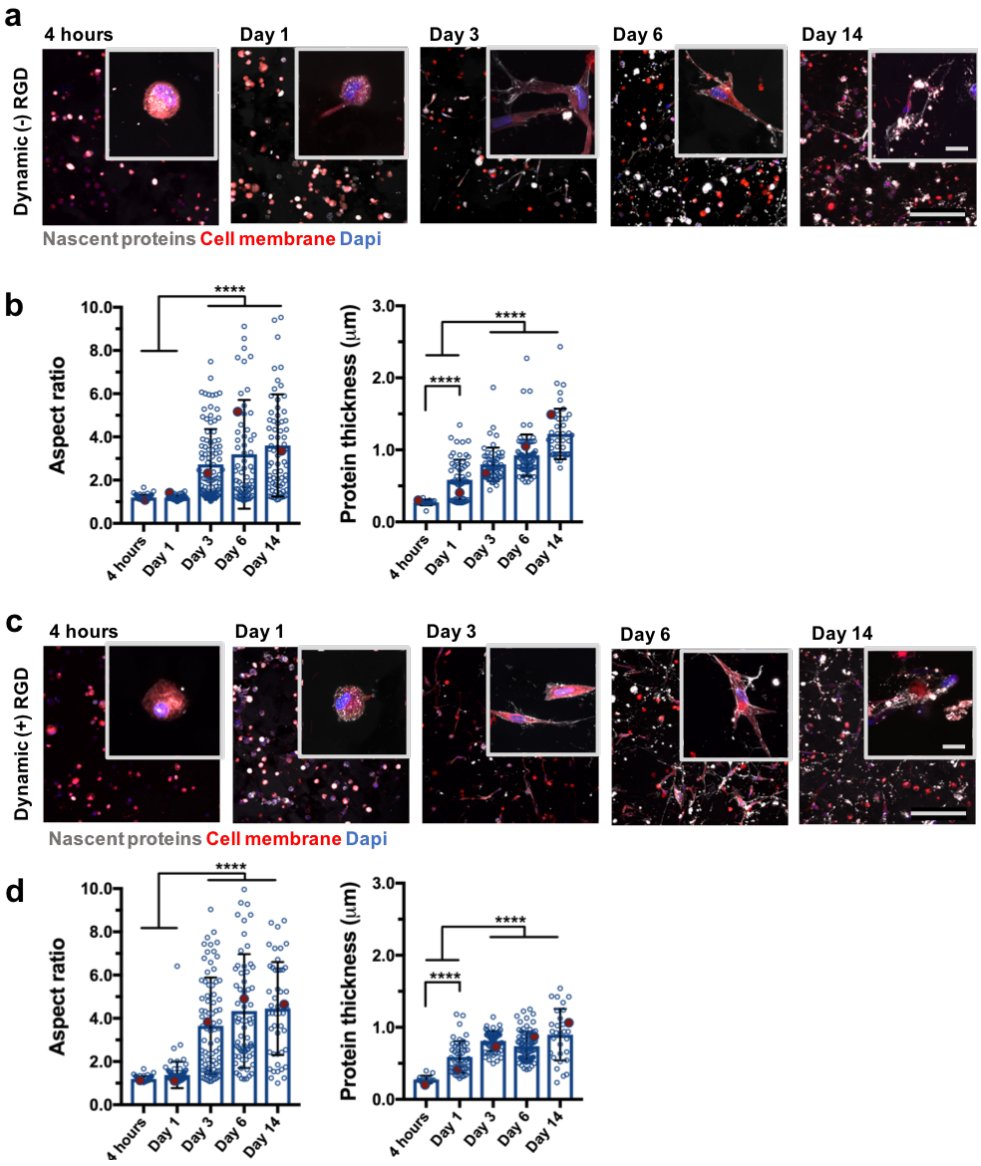
Supplementary Figure 12 Nascent protein deposition and cell spreading in bipotential adipogenic-osteogenic media. Representative images of the accumulated nascent proteins (white) of hMSCs encapsulated in degradable NorHA hydrogels with 1 mM RGD, cultured in adipogenic-osteogenic media (supplemented with AHA) and treated with anti Integrin α2 (anti α2, 20 μg/mL), HFN7.1 (5 μg/mL) or soluble RGD (sol RGD, 0.5 mM), for 6 days (scale bar 200 μm, inset 20 μm). **b** Quantification of aspect ratio and accumulated protein thickness deposited by hMSCs (aspect ratio: n = 57 cells (control, sol RGD), n = 83 cells (anti α2, HFN7.1), n = 68 (day 6, day 14); protein thickness: n = 34 per group from 2 biologically independent experiments), mean ± SD, **** $p \leq 0.001$, * $p \leq 0.05$, by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **a**).



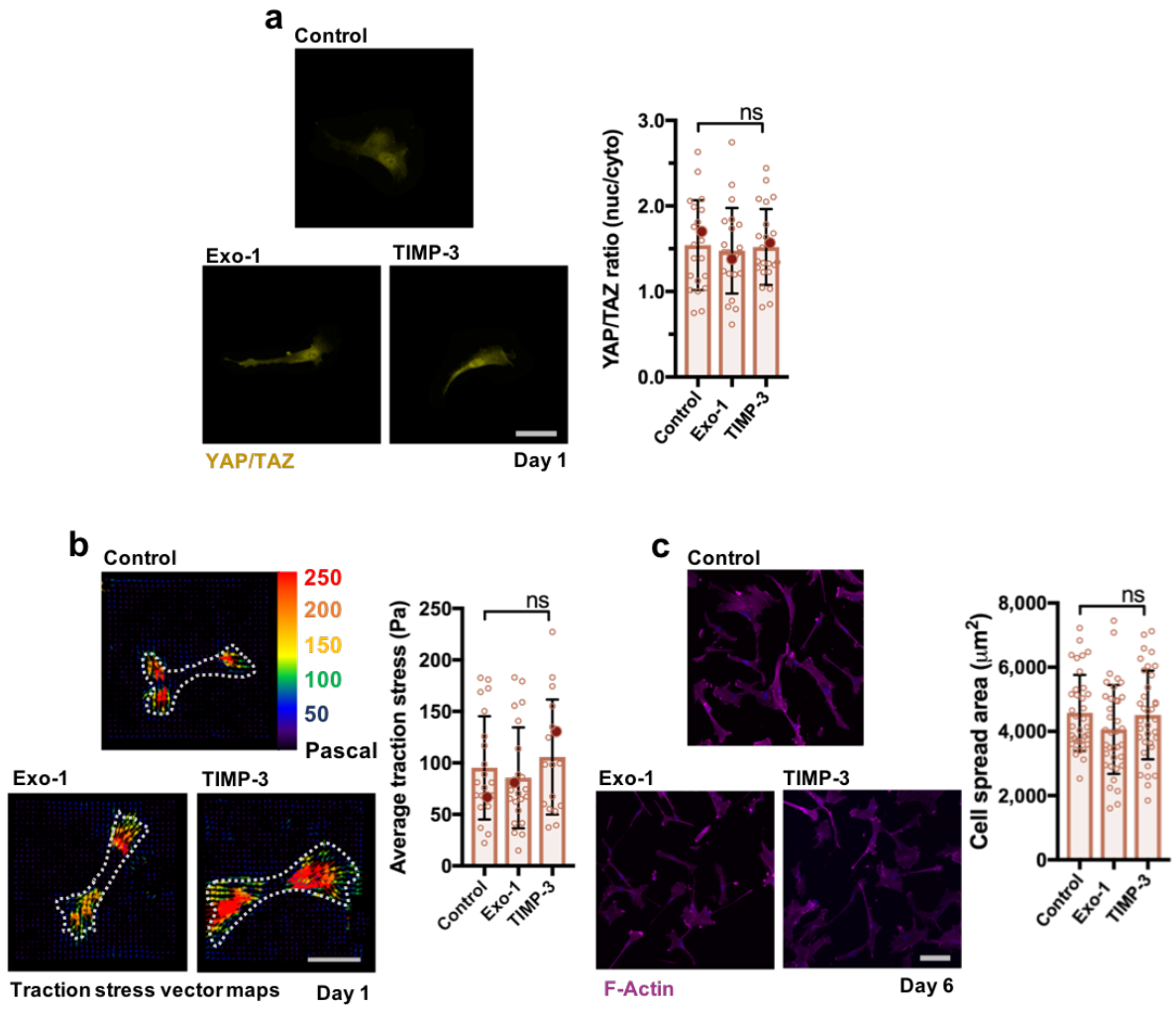
Supplementary Figure 13 Microstructural homogeneity of DN hydrogels. Representative confocal images of double network (DN) hydrogels (guest-host (GH) 2.50%, covalent crosslink ratio 0.3) directly following crosslinking, in which methacrylated hyaluronic acid (MeHA) and GH networks were separately labeled (MeHA with thiolated fluorescein isothiocyanate (FITC, green) and GH with cyclodextrin-658 (red) (scale bar 100 μm, experiments were repeated twice with similar results). Orange line indicates the pixel regions used to generate intensity profiles of MeHA and GH networks.



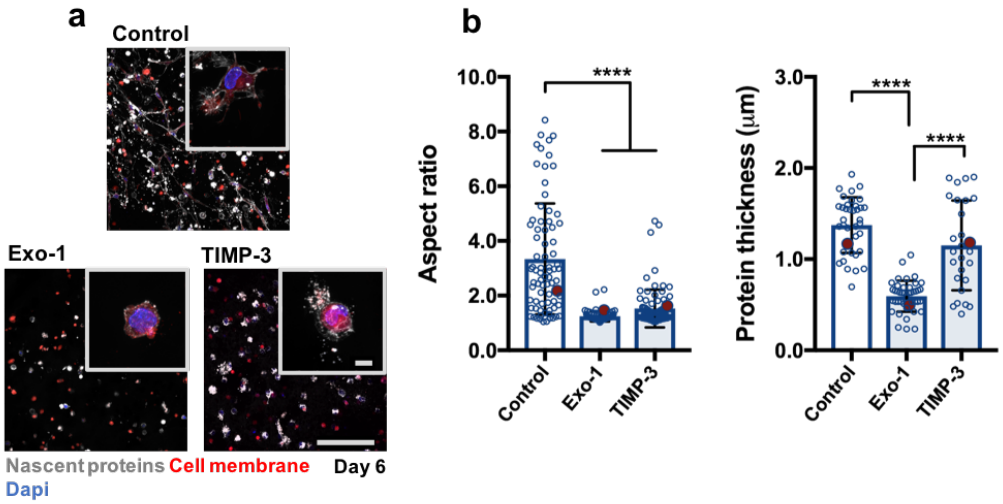
Supplementary Figure 14 Frequency dependence of DN hydrogel properties. Representative oscillatory frequency sweeps of double-network (DN) hydrogels with varied guest-host (GH) concentration (covalent crosslink ratio 0.3, shear elastic moduli (G', continuous lines) and shear viscous moduli (G'', dashed lines). Measurements were taken at 0.5% strain and repeated three times per group with similar results.



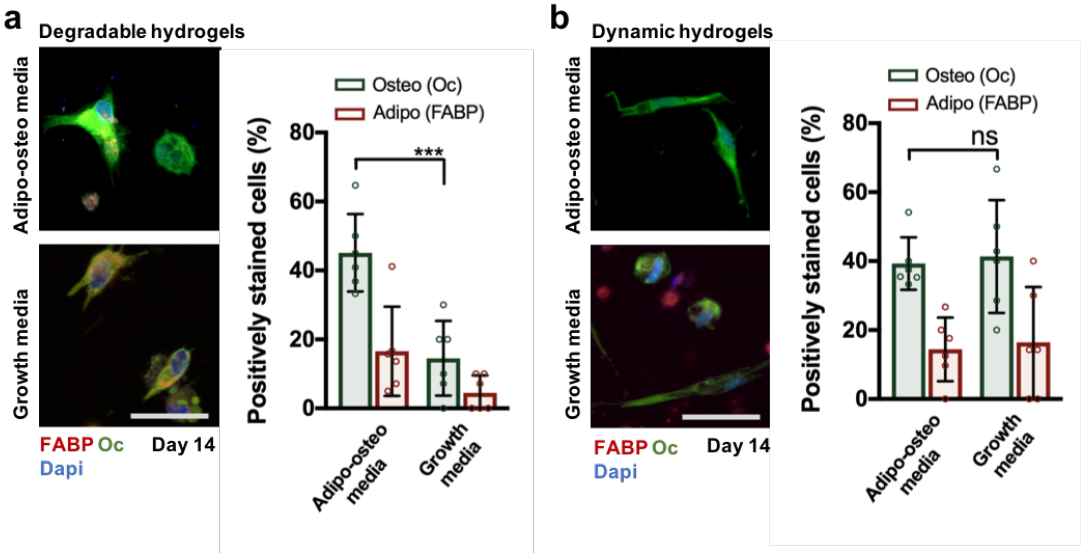
Supplementary Figure 15 Thickness of accumulated nascent proteins in dynamic hydrogels is dependent on time. a Representative images of the accumulated nascent proteins (white) of hMSCs encapsulated in dynamic double-network (DN) hydrogels without RGD modification and cultured in growth media (supplemented with AHA) up to 14 days (scale bar 200 µm, inset 20 µm). **b** Quantification of aspect ratio and accumulated protein thickness deposited by hMSCs (aspect ratio: n = 51 cells (4 hours), n = 85 cells (day 1, day 3), n = 68 (day 6, day 14); protein thickness: n = 70 (4 hours, day 1, day 3, day 6), n = 47 (day 14) from 2 biologically independent experiments), mean ± SD, **** $p \leq 0.0001$ by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **a**). **c** Representative images of the accumulated nascent proteins (white) of hMSCs encapsulated in dynamic double-network (DN) hydrogels with 1 mM RGD and cultured in growth media (supplemented with AHA) up to 14 days (scale bar 200 µm, inset 20 µm). **d** Quantification of aspect ratio and accumulated protein thickness deposited by hMSCs (aspect ratio: n = 51 cells (4 hours), n = 92 cells (day 1), n = 83 (day 3), n = 71 (day 6) and n = 48 (day 14); protein thickness: n = 31 (4 hours), n = 58 (day 1, day 3, day 6), n = 30 (day 14) from 2 biologically independent experiments), mean ± SD, **** $p \leq 0.0001$ by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **c**).



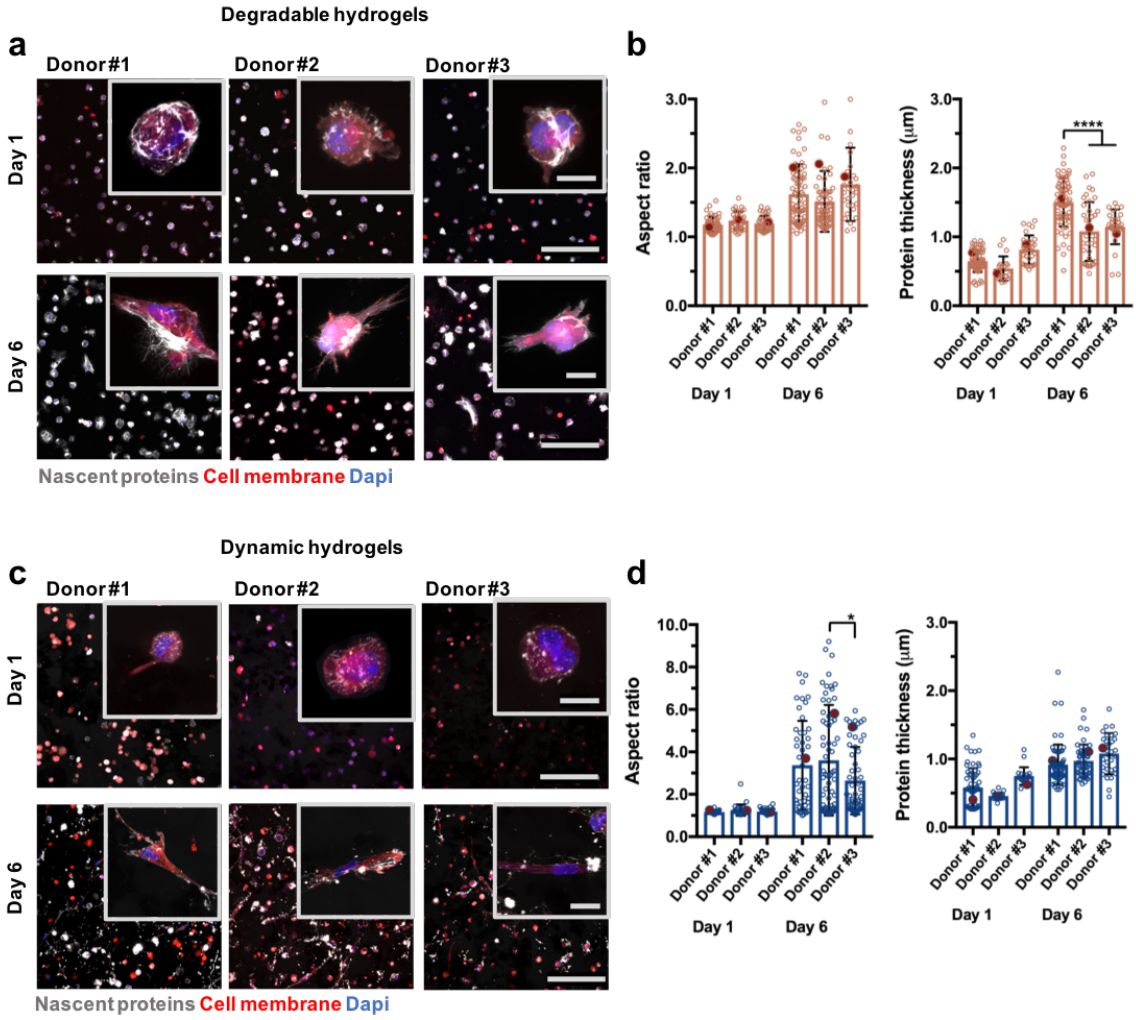
Supplementary Figure 16 Treatment with Exo-1 or TIMP-3 does not attenuate hMSC spreading or contractility atop HA hydrogels. a Representative images of YAP/TAZ immunostaining and quantification of nuclear to cytoplasmic (nuc/cyto) ratio of hMSCs cultured on top of non-degradable NorHA hydrogels (9 kPa, (+) RGD) for 24 h (day 1) in "AHA growth media" supplemented with DMSO only, 5 nM TIMP-3 or 120 nM Exo-1 (scale bar 50 μm, n = 22 cells per group from 2 biologically independent experiments, mean ± SD with no significant difference by one-way ANOVA with Bonferroni *post hoc*). **b** Representative traction stress vector maps and quantification of average traction stress of hMSCs at day 1 (scale bar 50 μm, n = 23 cells per group from 2 biologically independent experiments, mean ± SD with no significant difference by one-way ANOVA with Bonferroni *post hoc*). **c** Representative images of F-Actin and quantification of cell spread area after 6 days (scale bar 50 μm, n = 36 cells per group from 2 biologically independent experiments, mean ± SD with no significant difference by one-way ANOVA with Bonferroni *post hoc*).



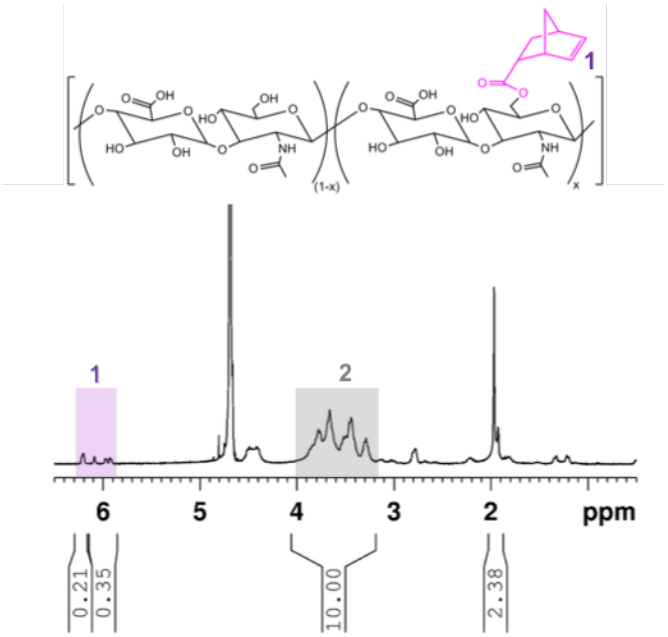
Supplementary Figure 17 Nascent protein deposition and cell spreading in bipotential adipogenic-osteogenic media. Representative images of accumulated nascent proteins (white) of hMSCs encapsulated in dynamic DN hydrogels without RGD modification and cultured in adipogenic-osteogenic media (supplemented with AHA) for 6 days (scale bar 200 μm, inset 20 μm). **b** Quantification of aspect ratio and accumulated protein thickness deposited by hMSCs (aspect ratio: n = 79 cells (control, Exo-1), n = 88 cells (TIMP-3); protein thickness: n = 42 (control, Exo-1), n = 28 (TIMP-3) from 2 biologically independent experiments), mean ± SD, **** $p \leq 0.0001$ by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **a**).



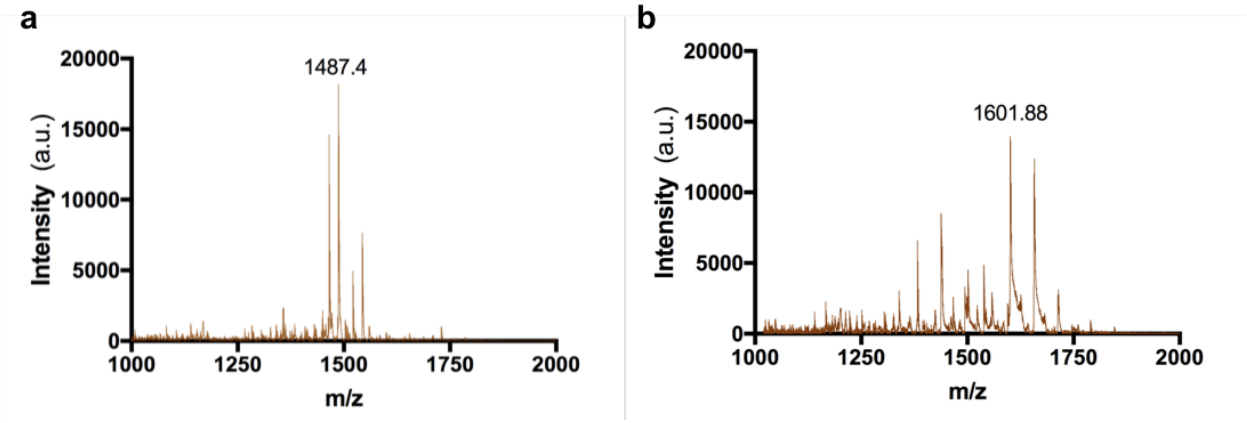
Supplementary Figure 18 Comparison of hMSC differentiation in growth and adipogenic-osteogenic media. Immunostaining for fatty-acid binding protein (FABP, adipogenic marker) and osteocalcin (Oc, osteogenic marker) and quantification of positively stained cells (percentage, %) towards osteogenesis (positive for Oc) and adipogenesis (positive for FABP) after 14 days in adipogenic-osteogenic media or hMSC growth media in **a** degradable NorHA and **b** dynamic DN hydrogels (scale bar 50 μm, quantification: n = 6 hydrogels from 2 biologically independent experiments), mean ± SD,*** $p \leq 0.001$ and no significant difference (ns) by one-way ANOVA with Bonferroni *post hoc*).



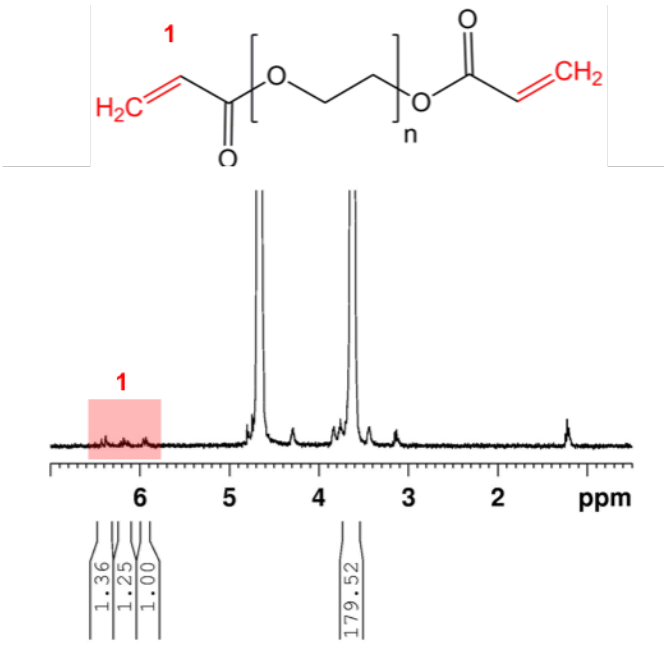
Supplementary Figure 19 hMSC donor dependence on spreading and accumulation of nascent proteins in degradable and dynamic hydrogels. a Representative images of nascent proteins (white) for 3 different hMSC donors (#1 male 23 years, #2 female 18 years, #3 male 22 years) encapsulated in degradable hydrogels modified with RGD (1 mM) and cultured in growth media (supplemented with AHA) up to 6 days (scale bar 200 µm, inset 20 µm). **b** Quantification of the aspect ratio and accumulated nascent protein thickness of hMSCs encapsulated in degradable hydrogels (aspect ratio: n = 51 cells (Donor #1), n = 75 (Donor #2), n = 60 (Donor #3); protein thickness: n = 79 cells (Donor #1), n = 41 (Donor #2), n = 30 (Donor #3) from 2 biologically independent experiments; mean ± SD, **** $p \leq 0.0001$ by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **a**). **c** Representative images of nascent proteins (white) for 3 different hMSC donors encapsulated in dynamic DN hydrogels without RGD and cultured in growth media (supplemented with AHA) for up to 6 days (scale bar 200 µm, inset 20 µm). **d** Quantification of the aspect ratio and accumulated nascent proteins of hMSCs (aspect ratio: n = 79 cells (Donor #1), n = 63 (Donor #2), n = 30 (Donor #3); protein thickness: n = 75 cells (Donor #1), n = 54 (Donor #2), n = 32 (Donor #3) from 2 biologically independent experiments; mean ± SD, * $p \leq 0.05$ by one-way ANOVA with Bonferroni *post hoc*, red dots indicate measurements for representative images in **c**).



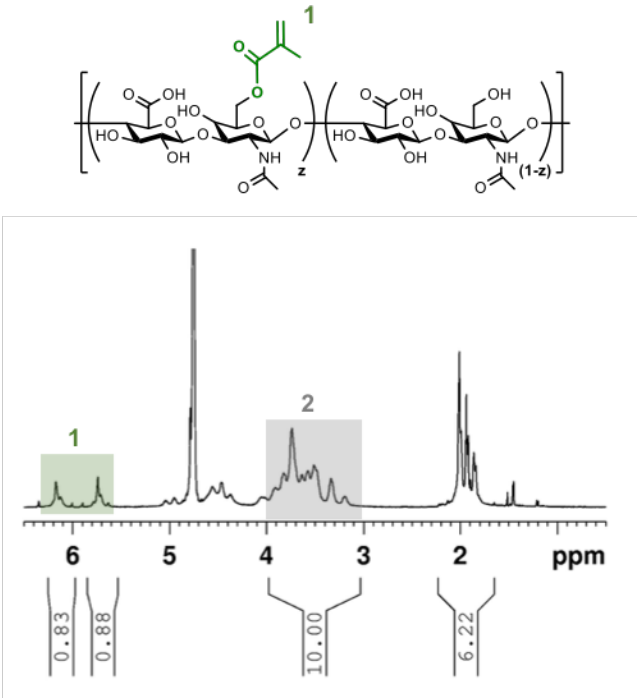
Supplementary Figure 20 ^{1}H NMR spectrum of norbornene-functionalized hyaluronic acid (NorHA) in D_2O. Modification of HA with norbornene (23%) determined by integration of vinyl protons (2H, shaded violet) relative to the sugar ring of HA (10H, shaded grey), analysis was done on each batch used to ensure consistency of modification.



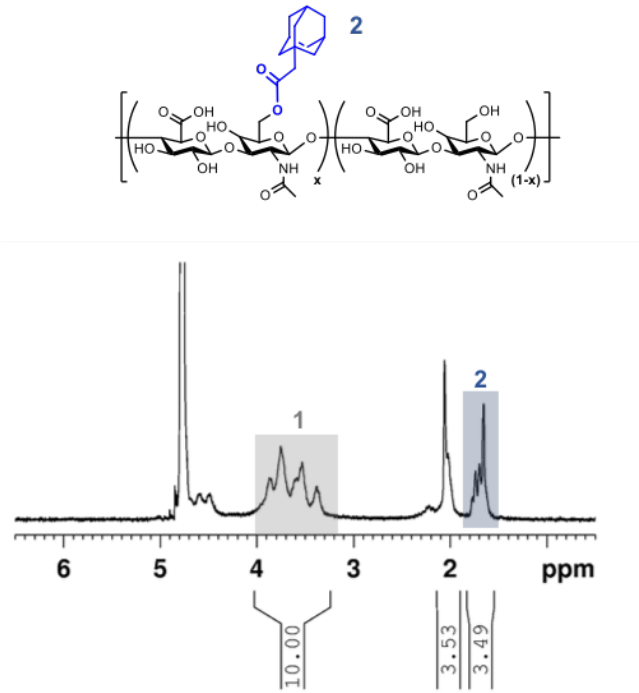
Supplementary Figure 21 MALDI spectra of dithiol peptide crosslinkers. Matrix Assisted Laser Desorption/Ionization (MALDI) spectra for **a** non-degradable dithiol peptide (sequence GCHGNSGGSGGNEECG) with an expected mass (plus alcohol from resin) of 1462 g/mol and actual mass of 1487 g/mol, and **b** degradable dithiol peptide (sequence GCNSVPMSMRGGSNCG) with an expected mass (plus alcohol from resin) of 1597 g/mol and actual mass of 1602 g/mol, all studies were performed with the same batch of material.



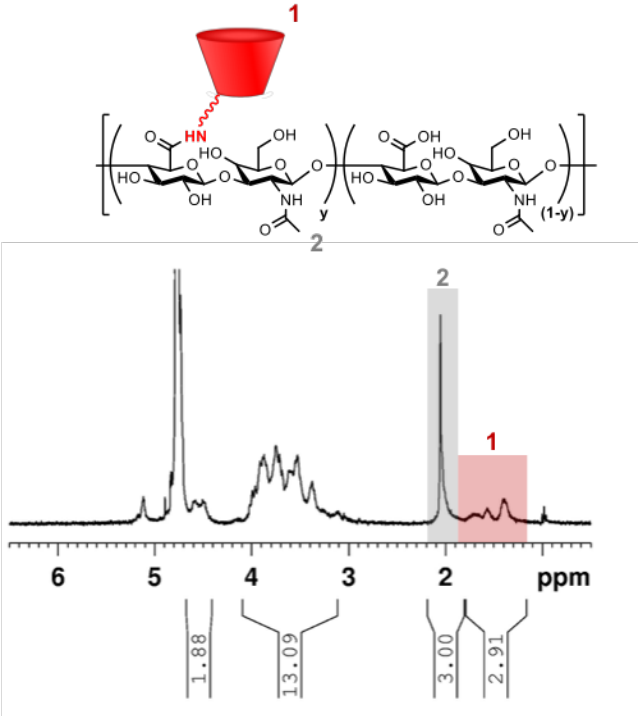
Supplementary Figure 22 ^{1}H NMR spectrum of poly(ethylene glycol)-diacrylate (PEG-DA) in D_2O. Modification of PEG (10 kDa) with acrylates on both ends determined by integration of the acrylate end-groups (each 1H, shaded red), all studies were performed with the same batch of material.



Supplementary Figure 23 ^{1}H NMR spectrum of methacrylated hyaluronic acid (MeHA) in D_2O. Methacrylate modification (86%) determined by integration of the vinyl protons (1H each, shaded green) relative to the sugar ring of HA (10H, shaded grey), all studies were performed with the same batch of material.



Supplementary Figure 24 ^{1}H NMR spectrum of adamantane-functionalizated hyaluronic acid (Ad-HA) in D_2O. Modification of HA with Ad (29%) determined by integration of the ethyl multiplet (12H, shaded blue) relative to the sugar ring of HA (10H, shaded grey), all studies were performed with the same batch of material.



Supplementary Figure 25 ^{1}H NMR spectrum of β-cyclodextrin-functionalized hyaluronic acid (CD-HA) in D_2O. Modification of HA with CD (24.3%) determined by integration of the hexane linkers (12H, shaded red) relative to the N-acetyl singlet of HA (3H, shaded grey), all studies were performed with the same batch of material.

Supplementary Table

Figure	Panel	Statistical Test	Comparison	P-value	F-value/t-value	Degrees of freedom
1	e	ANOVA Bonferroni *post-hoc* test	4 hours vs. Day 2	<0.0001	6.671	207
			4 hours vs. Day 4	<0.0001	13.5	207
			4 hours vs. Day 6	<0.0001	15.73	207
			Day 2 vs. Day 4	<0.0001	7.526	207
			Day 2 vs. Day 6	<0.0001	9.565	207
			Day 4 vs. Day 6	>0.9999	1.38	207
2	c (left)	ANOVA Bonferroni *post-hoc* test	Day 1 vs. Day 2	>0.9999	0.3515	253
			Day 1 vs. Day 4	<0.0001	8.104	253
			Day 1 vs. Day 6	<0.0001	6.666	253
			Day 2 vs. Day 4	<0.0001	7.715	253
			Day 2 vs. Day 6	<0.0001	6.278	253
			Day 4 vs. Day 6	0.6522	1.61	253
	c (right)	ANOVA Bonferroni *post-hoc* test	Day 1 vs. Day 2	>0.9999	0.7499	234
			Day 1 vs. Day 4	<0.0001	6.228	234
			Day 1 vs. Day 6	<0.0001	18.31	234
			Day 2 vs. Day 4	<0.0001	6.238	234
			Day 2 vs. Day 6	<0.0001	16.79	234
			Day 4 vs. Day 6	<0.0001	11.42	234
3	b (left)	ANOVA Bonferroni *post-hoc* test	Control vs. Sol RGD	<0.0001	11.42	554
			Control vs. Anti α2	<0.0001	9.218	554
			Control vs. HFN7.1	<0.0001	8.128	554
			Sol RGD vs. Anti α2	0.0306	2.812	554
			Sol RGD vs. HFN7.1	0.001	3.828	554
			Anti α2 vs. HFN7.1	>0.9999	1.093	554
	b (right)	ANOVA Bonferroni *post-hoc* test	Control vs. Sol RGD	0.0258	2.884	233
			Control vs. Anti α2	0.0545	2.631	233
			Control vs. HFN7.1	>0.9999	0.3944	233
			Sol RGD vs. Anti α2	<0.0001	5.299	233
			Sol RGD vs. HFN7.1	0.009	2.982	233
			Anti α2 vs. HFN7.1	0.2721	2.012	233
	d	ANOVA Bonferroni *post-hoc* test	Control vs. Sol RGD	<0.0001	9.047	185
			Control vs. Anti α2	<0.0001	7.09	185
			Control vs. HFN7.1	<0.0001	9.735	185
			Sol RGD vs. Anti α2	0.103	2.405	185
			Sol RGD vs. HFN7.1	0.0572	0.5631	185
			Anti α2 vs. HFN7.1	0.0172	3.022	185

	f	Two-way ANOVA Bonferroni *post-hoc* test	Osteo: Control vs. Sol RGD	0.0008	4.243	40
			Osteo: Control vs. Anti α2	<0.0001	7.36	40
			Osteo: Control vs. HFN7.1	<0.0001	7.119	40
			Osteo: Sol RGD vs. Anti α2	0.0202	3.118	40
			Osteo: Sol RGD vs. HFN7.1	0.0385	2.877	40
			Osteo: Anti α2 vs. HFN7.1	>0.9999	0.2409	40
			Adipo: Control vs. Sol RGD	0.076	2.611	40
			Adipo: Control vs. Anti α2	0.0026	3.83	40
			Adipo: Control vs. HFN7.1	0.0003	4.533	40
			Adipo: Sol RGD vs. Anti α2	>0.9999	1.22	40
			Adipo: Sol RGD vs. HFN7.1	0.3703	1.922	40
			Adipo: Anti α2 vs. HFN7.1	>0.9999	0.7024	40
4	d	ANOVA Bonferroni *post-hoc* test	0.00% vs. 1.25%	>0.9999	0.1836	207
			0.00% vs. 2.50%	<0.0001	6.478	207
			0.00% vs. 3.50%	<0.0001	7.268	207
			1.25% vs. 2.50%	<0.0001	7.392	207
			1.25% vs. 3.50%	<0.0001	8.311	207
			2.50% vs. 3.50%	>0.9999	0.9471	207
	e	ANOVA Bonferroni *post-hoc* test	0.0 vs. 0.2	0.2144	2.119	151
			0.0 vs. 0.3	0.1298	2.321	151
			0.0 vs. 0.4	>0.9999	1.008	151
			0.2 vs. 0.3	>0.9999	1.01	151
			0.2 vs. 0.4	<0.0001	5.475	151
			0.3 vs. 0.4	<0.0001	6.843	151
5	b (left)	ANOVA Bonferroni *post-hoc* test	Control vs. TIMP-3	<0.0001	11.96	432
			Control vs. Exo-1	<0.0001	11.9	432
			TIMP-3 vs. Exo-1	>0.9999	0.5994	432
	b (right)	ANOVA Bonferroni *post-hoc* test	Control vs. TIMP-3	<0.0001	6.71	147
			Control vs. Exo-1	<0.0001	4.451	147
			TIMP-3 vs. Exo-1	<0.0001	9.886	147
	d	ANOVA Bonferroni *post-hoc* test	Control vs. TIMP-3	<0.0001	7.313	134
			Control vs. Exo-1	<0.0001	11.43	134
			TIMP-3 vs. Exo-1	0.0013	3.619	134
	f	Two-way ANOVA Bonferroni	Osteo: Control vs. Exo-1	<0.0001	5.215	30
			Osteo: Control vs. TIMP-3	0.0008	4.111	30

		post-hoc test	Osteo: Exo-1 vs. TIMP-3	0.8346	1.104	30
			Adipo: Control vs. Exo-1	<0.0001	6.709	30
			Adipo: Control vs. TIMP-3	<0.0001	6.058	30
			Adipo: Exo-1 vs. TIMP-3	>0.9999	0.6507	30
S3	b (upper)	ANOVA Bonferroni *post-hoc* test	4 hours vs. Day 1	>0.9999	0.6305	515
			4 hours vs. Day 2	0.9983	1.274	515
			4 hours vs. Day 3	0.997	1.32	515
			4 hours vs. Day 4	>0.9999	0.1364	515
			4 hours vs. Day 5	>0.9999	0.3815	515
			4 hours vs. Day 6	0.907	1.746	515
			4 hours vs. Day 14	>0.9999	0.003466	515
			Day 1 vs. Day 2	>0.9999	0.7503	515
			Day 1 vs. Day 3	>0.9999	0.7921	515
			Day 1 vs. Day 4	>0.9999	0.8634	515
			Day 1 vs. Day 5	0.9998	1.136	515
			Day 1 vs. Day 6	0.1885	2.688	515
			Day 1 vs. Day 14	>0.9999	0.6747	515
			Day 2 vs. Day 3	>0.9999	0.01958	515
			Day 2 vs. Day 4	0.9701	1.567	515
			Day 2 vs. Day 5	0.8644	1.823	515
			Day 2 vs. Day 6	0.0276	3.31	515
			Day 2 vs. Day 14	0.9957	1.353	515
			Day 3 vs. Day 4	0.9533	1.63	515
			Day 3 vs. Day 5	0.8175	1.893	515
			Day 3 vs. Day 6	0.0187	3.421	515
			Day 3 vs. Day 14	0.9925	1.406	515
			Day 4 vs. Day 5	>0.9999	0.2751	515
			Day 4 vs. Day 6	0.8765	1.803	515
			Day 4 vs. Day 14	>0.9999	0.1411	515
			Day 5 vs. Day 6	0.9798	1.516	515
			Day 5 vs. Day 14	>0.9999	0.4013	515
			Day 6 vs. Day 14	0.8465	1.851	515
	b (lower)	ANOVA Bonferroni *post-hoc* test	4 hours vs. Day 1	<0.0001	6.839	427
			4 hours vs. Day 2	<0.0001	5.955	427
			4 hours vs. Day 3	<0.0001	12.43	427
			4 hours vs. Day 4	<0.0001	12.05	427
			4 hours vs. Day 5	<0.0001	11.8	427
			4 hours vs. Day 6	<0.0001	14.04	427
			4 hours vs. Day 14	<0.0001	10.14	427
			Day 1 vs. Day 2	>0.9999	0.6257	427
			Day 1 vs. Day 3	<0.0001	6.716	427

			Day 1 vs. Day 4	<0.0001	6.507	427
			Day 1 vs. Day 5	<0.0001	6.25	427
			Day 1 vs. Day 6	<0.0001	8.474	427
			Day 1 vs. Day 14	0.0004	4.403	427
			Day 2 vs. Day 3	<0.0001	6.903	427
			Day 2 vs. Day 4	<0.0001	6.718	427
			Day 2 vs. Day 5	<0.0001	6.476	427
			Day 2 vs. Day 6	<0.0001	8.538	427
			Day 2 vs. Day 14	<0.0001	4.73	427
			Day 3 vs. Day 4	>0.9999	0.1583	427
			Day 3 vs. Day 5	>0.9999	0.05304	427
			Day 3 vs. Day 6	>0.9999	1.475	427
			Day 3 vs. Day 14	>0.9999	1.765	427
			Day 4 vs. Day 5	>0.9999	0.2007	427
			Day 4 vs. Day 6	>0.9999	1.232	427
			Day 4 vs. Day 14	>0.9999	1.833	427
			Day 5 vs. Day 6	>0.9999	1.441	427
			Day 5 vs. Day 14	>0.9999	1.626	427
			Day 6 vs. Day 14	0.0442	3.18	427
	d (left)	ANOVA Bonferroni *post-hoc* test	'D1 Softer' vs. 'D1 Stiffer'	0.0478	2.671	302
			'D1 Softer' vs. 'D6 Softer'	0.5343	1.706	302
			'D1 Softer' vs. 'D6 Stiffer'	0.3627	1.885	302
			'D1 Stiffer' vs. 'D6 Softer'	0.0005	3.984	302
			'D1 Stiffer' vs. 'D6 Stiffer'	>0.9999	0.767	302
			'D6 Softer' vs. 'D6 Stiffer'	0.0066	3.297	302
	d (right)	ANOVA Bonferroni *post-hoc* test	'D1 Softer' vs. 'D1 Stiffer'	<0.0001	9.332	258
			'D1 Softer' vs. 'D6 Softer'	0.0035	3.481	258
			'D1 Softer' vs. 'D6 Stiffer'	0.0008	3.885	258
			'D1 Stiffer' vs. 'D6 Softer'	<0.0001	12.59	258
			'D1 Stiffer' vs. 'D6 Stiffer'	<0.0001	4.42	258
			'D6 Softer' vs. 'D6 Stiffer'	<0.0001	6.882	258
S5	b (upper)	ANOVA Bonferroni *post-hoc* test	4 hours vs. Day 1	>0.9999	0.2462	511
			4 hours vs. Day 2	>0.9999	0.5732	511
			4 hours vs. Day 3	0.6834	2.056	511
			4 hours vs. Day 4	<0.0001	7.766	511
			4 hours vs. Day 5	<0.0001	5.125	511
			4 hours vs. Day 6	<0.0001	6.436	511
			4 hours vs. Day 14	<0.0001	7.938	511
			Day 1 vs. Day 2	>0.9999	0.336	511
			Day 1 vs. Day 3	0.8538	1.84	511
			Day 1 vs. Day 4	<0.0001	7.746	511

		Day 1 vs. Day 5	<0.0001	5.014	511
		Day 1 vs. Day 6	<0.0001	6.371	511
		Day 1 vs. Day 14	<0.0001	7.913	511
		Day 2 vs. Day 3	0.9882	1.454	511
		Day 2 vs. Day 4	<0.0001	7.374	511
		Day 2 vs. Day 5	0.0001	4.667	511
		Day 2 vs. Day 6	<0.0001	6	511
		Day 2 vs. Day 14	<0.0001	7.558	511
		Day 3 vs. Day 4	<0.0001	7.123	511
		Day 3 vs. Day 5	0.003	3.902	511
		Day 3 vs. Day 6	<0.0001	5.483	511
		Day 3 vs. Day 14	<0.0001	7.283	511
		Day 4 vs. Day 5	0.3272	2.464	511
		Day 4 vs. Day 6	0.9759	1.539	511
		Day 4 vs. Day 14	>0.9999	0.5862	511
		Day 5 vs. Day 6	>0.9999	1.048	511
		Day 5 vs. Day 14	0.1083	2.884	511
		Day 6 vs. Day 14	0.703	2.034	511
b (lower)	ANOVA Bonferroni *post-hoc* test	4 hours vs. Day 1	0.7242	2.011	441
		4 hours vs. Day 2	0.9987	1.253	441
		4 hours vs. Day 3	<0.0001	5.606	441
		4 hours vs. Day 4	<0.0001	6.644	441
		4 hours vs. Day 5	<0.0001	11.57	441
		4 hours vs. Day 6	<0.0001	15.6	441
		4 hours vs. Day 14	<0.0001	11.58	441
		Day 1 vs. Day 2	>0.9999	0.6733	441
		Day 1 vs. Day 3	0.0005	4.341	441
		Day 1 vs. Day 4	<0.0001	5.591	441
		Day 1 vs. Day 5	<0.0001	11.4	441
		Day 1 vs. Day 6	<0.0001	16.44	441
		Day 1 vs. Day 14	<0.0001	11.5	441
		Day 2 vs. Day 3	0.0003	4.485	441
		Day 2 vs. Day 4	<0.0001	5.601	441
		Day 2 vs. Day 5	<0.0001	10.81	441
		Day 2 vs. Day 6	<0.0001	15.07	441
		Day 2 vs. Day 14	<0.0001	10.82	441
		Day 3 vs. Day 4	0.9948	1.37	441
		Day 3 vs. Day 5	<0.0001	7.35	441
		Day 3 vs. Day 6	<0.0001	11.99	441
		Day 3 vs. Day 14	<0.0001	7.24	441
		Day 4 vs. Day 5	<0.0001	5.892	441
		Day 4 vs. Day 6	<0.0001	10.25	441

			Day 4 vs. Day 14	<0.0001	5.706	441
			Day 5 vs. Day 6	0.0077	3.665	441
			Day 5 vs. Day 14	>0.9999	0.4519	441
			Day 6 vs. Day 14	0.0005	4.349	441
	d (left)	ANOVA Bonferroni *post-hoc* test	'D1 Softer' vs. 'D1 Stiffer'	>0.9999	0.7192	353
			'D1 Softer' vs. 'D6 Softer'	<0.0001	7.427	353
			'D1 Softer' vs. 'D6 Stiffer'	0.2127	2.111	353
			'D1 Stiffer' vs. 'D6 Softer'	<0.0001	8.36	353
			'D1 Stiffer' vs. 'D6 Stiffer'	0.0266	2.864	353
			'D6 Softer' vs. 'D6 Stiffer'	<0.0001	5.091	353
	d (right)	ANOVA Bonferroni *post-hoc* test	'D1 Softer' vs. 'D1 Stiffer'	0.2851	1.995	188
			'D1 Softer' vs. 'D6 Softer'	<0.0001	8.045	188
			'D1 Softer' vs. 'D6 Stiffer'	>0.9999	1.15	188
			'D1 Stiffer' vs. 'D6 Softer'	<0.0001	10.25	188
			'D1 Stiffer' vs. 'D6 Stiffer'	0.0145	3.076	188
			'D6 Softer' vs. 'D6 Stiffer'	<0.0001	6.5	188
S8	b	ANOVA Bonferroni *post-hoc* test	0 hours vs. Day 1	>0.9999	0.4748	244
			0 hours vs. Day 6	0.1349	2.489	244
			0 hours vs. Day 6 (FBS)	<0.0001	6.705	244
			0 hours vs. Day 1 (FBS)	>0.9999	0.4833	244
			Day 1 vs. Day 6	0.0258	3.045	244
			Day 1 vs. Day 6 (FBS)	<0.0001	7.499	244
			Day 1 vs. Day 1 (FBS)	>0.9999	0.01772	244
			Day 6 vs. Day 6 (FBS)	0.0011	3.923	244
			Day 6 vs. Day 1 (FBS)	0.0289	3.009	244
			Day 6 (FBS) vs. Day 1 (FBS)	<0.0001	7.368	244
S9	b (left)	*t*-test, *two-tailed*	(+) RGD vs. (-) RGD	<0.0001	9.212	72
	b (right)	*t*-test, *two-tailed*	(+) RGD vs. (-) RGD	<0.0001	22.27	18
S11	b	ANOVA Bonferroni *post-hoc* test	Control vs. Sol RGD	0.811	1.501	172
			Control vs. Anti α2	<0.0001	4.743	172
			Control vs. HFN7.1	>0.9999	0.007385	172
			Sol RGD vs. Anti α2	<0.0001	5.533	172
			Sol RGD vs. HFN7.1	>0.9999	1.358	172
			Anti α2 vs. HFN7.1	0.0001	4.339	172
	c	ANOVA Bonferroni *post-hoc* test	IgG_1 (μg/mL): 20 vs. 5	>0.9999	0.7079	196
			IgG_1 (μg/mL): 20 vs. 0	0.0578	2.018	196
			IgG_1 (μg/mL): 5 vs. 0	0.2415	1.757	196

	d (left)	ANOVA Bonferroni *post-hoc* test	Anti α2 (μg/mL): 0 vs. 10	<0.0001	5.388	328
			Anti α2 (μg/mL): 0 vs. 20	<0.0001	8.285	328
			Anti α2 (μg/mL): 0 vs. 40	<0.0001	8.828	328
			Anti α2 (μg/mL): 10 vs. 20	0.0144	3.06	328
			Anti α2 (μg/mL): 10 vs. 40	0.006	3.323	328
			Anti α2 (μg/mL): 20 vs. 40	>0.9999	0.08731	328
	d (middle)	ANOVA Bonferroni *post-hoc* test	HFN7.1 (μg/mL): 0 vs. 5	<0.0001	6.353	190
			HFN7.1 (μg/mL): 0 vs. 10	<0.0001	5.76	190
			HFN7.1 (μg/mL): 5 vs. 10	>0.9999	0.6006	190
	d (right)	ANOVA Bonferroni *post-hoc* test	RGD (mM): 0.00 vs. 0.25	<0.0001	7.124	241
			RGD (mM): 0.00 vs. 0.50	<0.0001	8.234	241
			RGD (mM): 0.25 vs. 0.50	0.6728	1.218	241
	e	ANOVA Bonferroni *post-hoc* test	Control vs. Sol RGD	0.0885	3.096	8
			Control vs. Anti ⟨2	0.0289	3.86	8
			Control vs. HFN7.1	0.0145	4.36	8
			Sol RGD vs. Anti ⟨2	>0.9999	0.7635	8
			Sol RGD vs. HFN7.1	>0.9999	1.263	8
			Anti α2 vs. HFN7.1	>0.9999	0.5	8
S12	b (left)	ANOVA Bonferroni *post-hoc* test	Control vs. Sol RGD	<0.0001	9.321	278
			Control vs. Anti α2	<0.0001	7.753	278
			Control vs. HFN7.1	<0.0001	7.32	278
			Sol RGD vs. Anti α2	0.0716	2.531	278
			Sol RGD vs. HFN7.1	0.013	3.094	278
			Anti α2 vs. HFN7.1	>0.9999	0.6023	278
	b (right)	ANOVA Bonferroni *post-hoc* test	Control vs. Sol RGD	<0.0001	4.858	130
			Control vs. Anti ⟨2	<0.0001	4.832	130
			Control vs. HFN7.1	0.1453	2.28	130
			Sol RGD vs. Anti ⟨2	>0.9999	0.02607	130
			Sol RGD vs. HFN7.1	0.0812	2.503	130
			Anti ⟨2 vs. HFN7.1	0.087	2.478	130
S15	b (left)	ANOVA Bonferroni *post-hoc* test	4 hours vs. Day 1	>0.9999	0.05008	364
			4 hours vs. Day 3	<0.0001	5.143	364
			4 hours vs. Day 6	<0.0001	6.267	364
			4 hours vs. Day 14	<0.0001	7.693	364
			Day 1 vs. Day 3	<0.0001	5.982	364
			Day 1 vs. Day 6	<0.0001	7.126	364
			Day 1 vs. Day 14	<0.0001	8.788	364
			Day 3 vs. Day 6	0.8915	1.704	364

			Day 3 vs. Day 14	0.0117	3.272	364
			Day 6 vs. Day 14	>0.9999	1.396	364
	b (right)	ANOVA Bonferroni *post-hoc* test	4 hours vs. Day 1	0.0013	3.899	238
			4 hours vs. Day 3	<0.0001	6.507	238
			4 hours vs. Day 6	<0.0001	8.238	238
			4 hours vs. Day 14	<0.0001	11.39	238
			Day 1 vs. Day 3	0.0005	4.125	238
			Day 1 vs. Day 6	<0.0001	6.919	238
			Day 1 vs. Day 14	<0.0001	11.49	238
			Day 3 vs. Day 6	0.132	2.497	238
			Day 3 vs. Day 14	<0.0001	7.473	238
			Day 6 vs. Day 14	<0.0001	5.495	238
	d (left)	ANOVA Bonferroni *post-hoc* test	4 hours vs. Day 1	>0.9999	0.5877	337
			4 hours vs. Day 3	<0.0001	7.518	337
			4 hours vs. Day 6	<0.0001	9.312	337
			4 hours vs. Day 14	<0.0001	8.835	337
			Day 1 vs. Day 3	<0.0001	8.13	337
			Day 1 vs. Day 6	<0.0001	10.14	337
			Day 1 vs. Day 14	<0.0001	9.383	337
			Day 3 vs. Day 6	0.2231	2.296	337
			Day 3 vs. Day 14	0.1699	2.399	337
			Day 6 vs. Day 14	>0.9999	0.3329	337
	d (right)	ANOVA Bonferroni *post-hoc* test	4 hours vs. Day 1	<0.0001	4.852	227
			4 hours vs. Day 3	<0.0001	8.443	227
			4 hours vs. Day 6	<0.0001	7.523	227
			4 hours vs. Day 14	<0.0001	9.037	227
			Day 1 vs. Day 3	<0.0001	5.331	227
			Day 1 vs. Day 6	0.0015	3.864	227
			Day 1 vs. Day 14	<0.0001	6.236	227
			Day 3 vs. Day 6	0.533	1.943	227
			Day 3 vs. Day 14	0.6537	1.852	227
			Day 6 vs. Day 14	0.0056	3.498	227
S16	a	ANOVA Bonferroni *post-hoc* test	Control vs. Exo-1	>0.9999	0.4375	63
			Control vs. TIMP-3	>0.9999	0.15	63
			Exo-1 vs. TIMP-3	>0.9999	0.2958	63
	b	ANOVA Bonferroni *post-hoc* test	Control vs. Exo-1	>0.9999	0.623	57
			Control vs. TIMP-3	>0.9999	0.6255	57
			Exo-1 vs. TIMP-3	0.6818	1.221	57

	c	ANOVA Bonferroni *post-hoc* test	Control vs. TIMP-3	>0.9999	0.1962	103
			Control vs. Exo-1	0.3217	1.625	103
			TIMP-3 vs. Exo-1	0.4695	1.427	103
S17	b (left)	ANOVA Bonferroni *post-hoc* test	Exo-1 vs. Control	<0.0001	10.7	239
			Exo-1 vs. TIMP-3	0.399	1.508	239
			Control vs. TIMP-3	<0.0001	9.484	239
	b (right)	ANOVA Bonferroni *post-hoc* test	Exo-1 vs. Control	<0.0001	10.75	104
			Exo-1 vs. TIMP-3	<0.0001	7.016	104
			Control vs. TIMP-3	0.0231	2.718	104
S18	a	*t*-test, two-tailed	Adipo-osteo vs. Growth	0.0007	4.804	10
	b	*t*-test, two-tailed	Adipo-osteo vs. Growth	0.7863	0.2785	10
S19	b (left)	*ANOVA Bonferroni post-hoc test*	Day 6 Donor #1 vs. Day 6 Donor #2	>0.9999	1.719	285
			Day 6 Donor #1 vs. Day 6 Donor #3	0.9746	1.853	285
			Day 6 Donor #1 vs. Day 1 Donor #1	<0.0001	6.786	285
			Day 6 Donor #1 vs. Day 1 Donor #2	<0.0001	5.147	285
			Day 6 Donor #1 vs. Day 1 Donor #3	<0.0001	5.965	285
			Day 6 Donor #2 vs. Day 6 Donor #3	0.0312	3.107	285
			Day 6 Donor #2 vs. Day 1 Donor #1	<0.0001	4.953	285
			Day 6 Donor #2 vs. Day 1 Donor #2	0.0053	3.618	285
			Day 6 Donor #2 vs. Day 1 Donor #3	0.0003	4.343	285
			Day 6 Donor #3 vs. Day 1 Donor #1	<0.0001	7.047	285
			Day 6 Donor #3 vs. Day 1 Donor #2	<0.0001	5.815	285
			Day 6 Donor #3 vs. Day 1 Donor #3	<0.0001	6.467	285
			Day 1 Donor #1 vs. Day 1 Donor #2	>0.9999	0.7161	285
			Day 1 Donor #1 vs. Day 1 Donor #3	>0.9999	0.178	285
			Day 1 Donor #2 vs. Day 1 Donor #3	>0.9999	0.5095	285
	b (right)	*ANOVA Bonferroni post-hoc test*	Day 6 Donor #1 vs. Day 6 Donor #2	<0.0001	7.282	245
			Day 6 Donor #1 vs. Day 6 Donor #3	<0.0001	5.545	245
			Day 6 Donor #1 vs. Day 1 Donor #1	<0.0001	17.39	245
			Day 6 Donor #1 vs. Day 1 Donor #2	<0.0001	12.94	245
			Day 6 Donor #1 vs. Day 1 Donor #3	<0.0001	11.01	245
			Day 6 Donor #2 vs. Day 6 Donor #3	>0.9999	0.9597	245
			Day 6 Donor #2 vs. Day 1 Donor #1	<0.0001	7.287	245

		Day 6 Donor #2 vs. Day 1 Donor #2	<0.0001	6.62	245
		Day 6 Donor #2 vs. Day 1 Donor #3	0.0033	3.75	245
		Day 6 Donor #3 vs. Day 1 Donor #1	<0.0001	7.701	245
		Day 6 Donor #3 vs. Day 1 Donor #2	<0.0001	7.094	245
		Day 6 Donor #3 vs. Day 1 Donor #3	0.0002	4.421	245
		Day 1 Donor #1 vs. Day 1 Donor #2	>0.9999	1.445	245
		Day 1 Donor #1 vs. Day 1 Donor #3	0.1481	2.601	245
		Day 1 Donor #2 vs. Day 1 Donor #3	0.0211	3.23	245
d (left)	*ANOVA Bonferroni post-hoc test*	Day 1 Donor #1 vs. Day 1 Donor #2	>0.9999	0.2096	274
		Day 1 Donor #1 vs. Day 1 Donor #3	>0.9999	0.05963	274
		Day 1 Donor #2 vs. Day 1 Donor #3	>0.9999	0.1619	274
		Day 6 Donor #1 vs. Day 6 Donor #2	>0.9999	0.7079	274
		Day 6 Donor #1 vs. Day 6 Donor #3	0.5342	2.112	274
		Day 6 Donor #1 vs. Day 1 Donor #1	<0.0001	5.343	274
		Day 6 Donor #1 vs. Day 1 Donor #2	<0.0001	5.112	274
		Day 6 Donor #1 vs. Day 1 Donor #3	<0.0001	5.657	274
		Day 6 Donor #2 vs. Day 6 Donor #3	0.0286	3.135	274
		Day 6 Donor #2 vs. Day 1 Donor #1	<0.0001	6.39	274
		Day 6 Donor #2 vs. Day 1 Donor #2	<0.0001	6.139	274
		Day 6 Donor #2 vs. Day 1 Donor #3	<0.0001	6.859	274
		Day 6 Donor #3 vs. Day 1 Donor #1	0.0033	3.745	274
		Day 6 Donor #3 vs. Day 1 Donor #2	0.0081	3.503	274
		Day 6 Donor #3 vs. Day 1 Donor #3	0.0014	3.969	274
d (right)	*ANOVA Bonferroni post-hoc test*	Day 1 Donor #1 vs. Day 1 Donor #2	>0.9999	1.1	240
		Day 1 Donor #1 vs. Day 1 Donor #3	0.0771	2.824	240
		Day 1 Donor #2 vs. Day 1 Donor #3	<0.0001	7.225	240
		Day 6 Donor #1 vs. Day 6 Donor #2	<0.0001	6.955	240
		Day 6 Donor #1 vs. Day 6 Donor #3	0.177	2.537	240
		Day 6 Donor #1 vs. Day 1 Donor #1	>0.9999	1.8	240
		Day 6 Donor #1 vs. Day 1 Donor #2	<0.0001	7.807	240
		Day 6 Donor #1 vs. Day 1 Donor #3	<0.0001	7.489	240
		Day 6 Donor #2 vs. Day 6 Donor #3	0.0214	3.227	240

	Day 6 Donor #2 vs. Day 1 Donor #1	<0.0001	8.545	240
	Day 6 Donor #2 vs. Day 1 Donor #2	<0.0001	8.303	240
	Day 6 Donor #2 vs. Day 1 Donor #3	0.0002	4.398	240
	Day 6 Donor #3 vs. Day 1 Donor #1	0.9123	1.884	240
	Day 6 Donor #3 vs. Day 1 Donor #2	0.2057	2.483	240
	Day 6 Donor #3 vs. Day 1 Donor #3	0.0066	3.565	240