Supplementary data for:

Dynamic Surfaces For The Study Of Mesenchymal Stem Cell Growth Through Adhesion Regulation.

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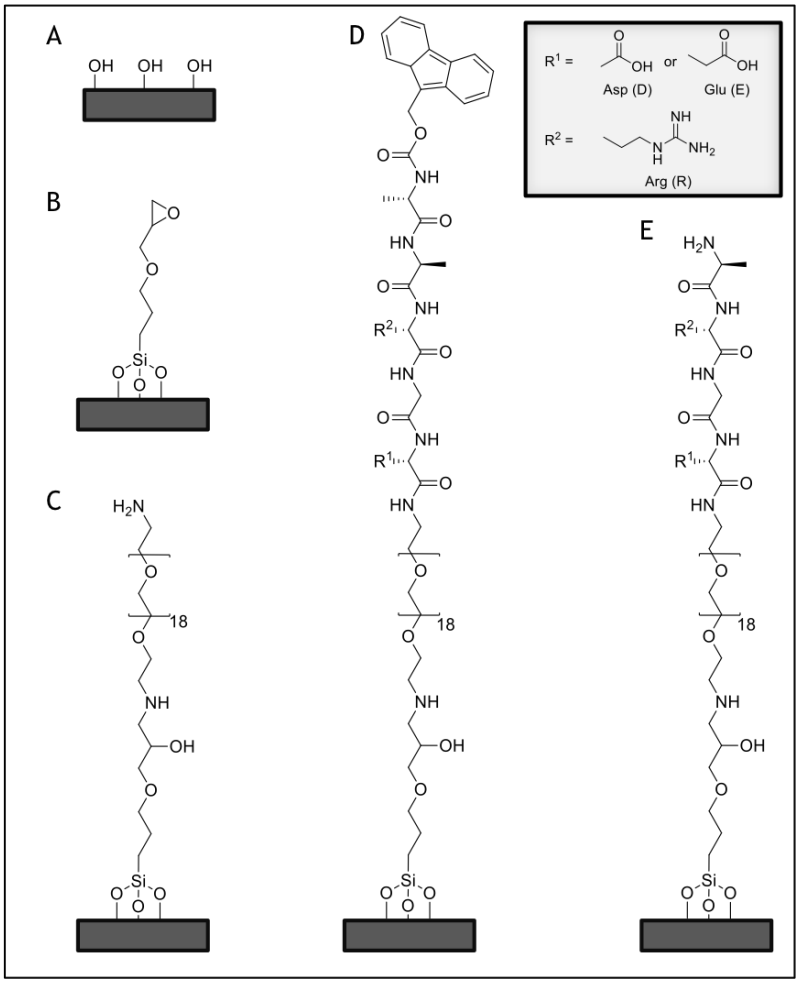
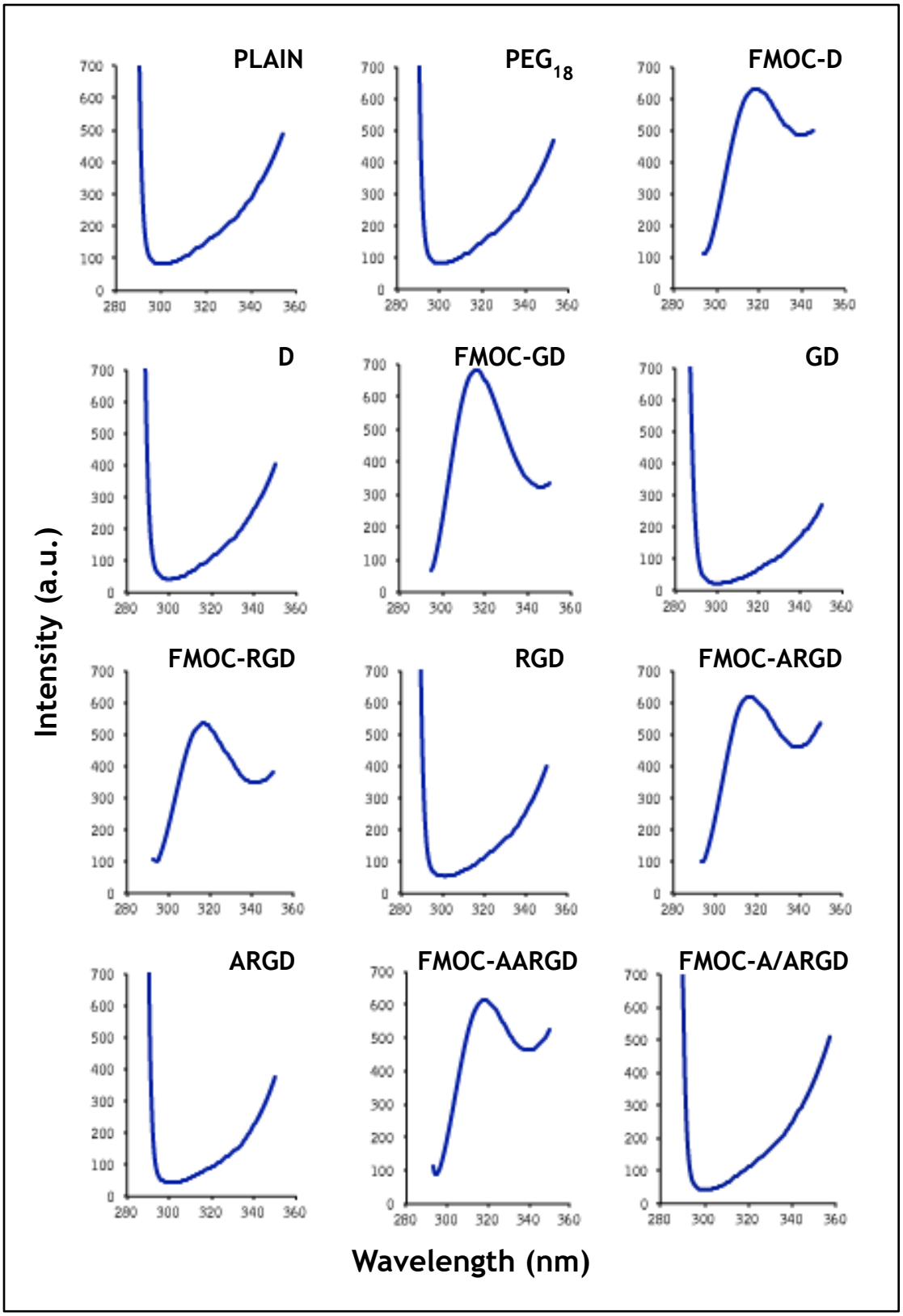
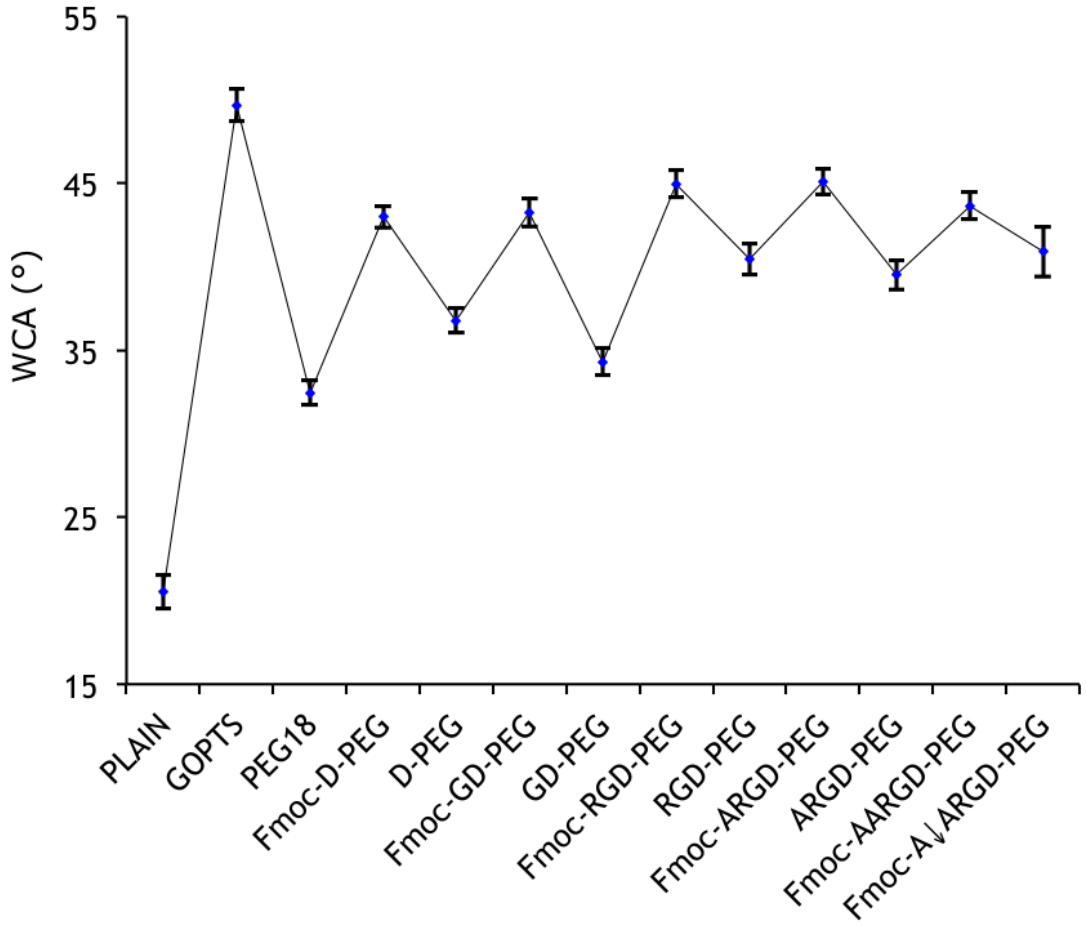


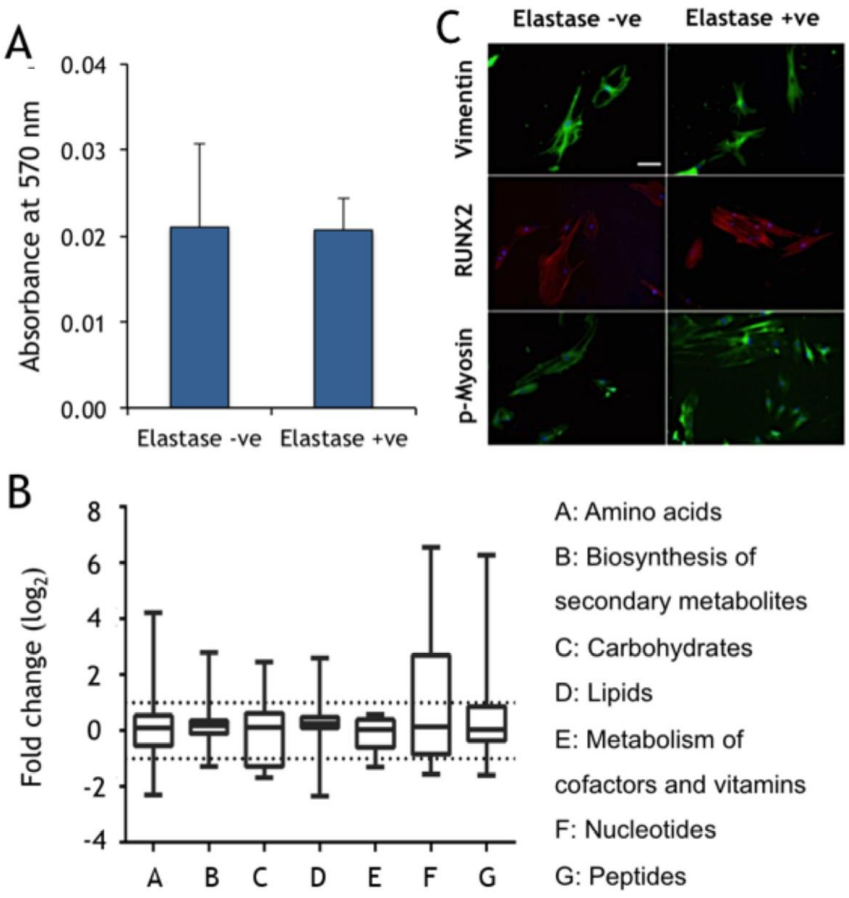
Supplementary figure 1: Preparation of enzyme-responsive FMOC based substrates. Chemically cleaned glass coverslips (A) were modified with GOPTS solution (B). Addition of a PEG diamine provided the surface with amine functionality to enable subsequent coupling of amino acids (C). The complete surface structure was built up through stepwise coupling of amino acids and FMOC deprotection stages. As the amino acids were FMOC-protected the amino acid sequence naturally terminated in the FMOC blocking group (D). The structure also contains an Ala-Ala dipeptide that formed the designated enzyme-cleavable site. Enzymatic digestion resulted in the removal of the FMOC capping group and one of the alanine residues (E). R groups are (R^1) CH_2COOH for aspartic acid or CH_2CH_2COOH for glutamic acid, and (R^2) is $(CH_2)_3NHC(NH)(NH_2)$ for arginine. This method was used for PEG-blocked substrates where the terminal FMOC group was removed with piperidine and replaced with PEG.


PLAIN
PEG18
FMOC-D
D
FMOC-GD
GD
FMOC-RGD
RGD
FMOC-ARGD
ARGD
FMOC-AARGD
FMOC-A/ARGD
Intensity (a.u.)
Wavelength (nm)
0
100
200
300
400
500
600
700
280
300
320
340
360

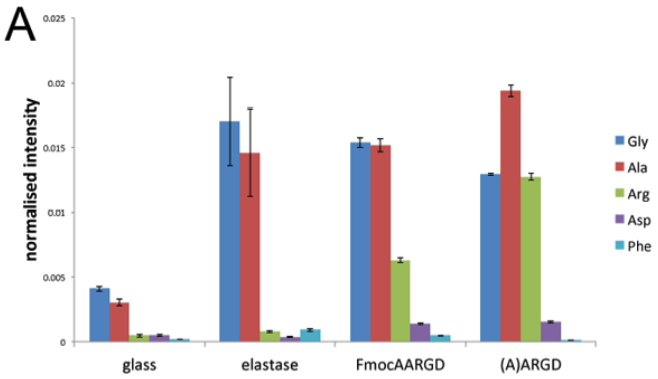
Supplementary figure 2: Stepwise monitoring of peptide synthesis using solid-state fluorescence spectroscopy. The emission spectra show the stepwise synthesis of the peptide chain from FMOC-D to FMOC-AARGD and FMOC-A↓ARGD (surface after enzymatic removal of FMOC-A). The presence of surface-tethered FMOC groups is identified by the 315 nm emission peak (when excited at 270 nm) indicating successful amino acid coupling. The absence of this peak after FMOC-deprotection stages and enzymatic digestion indicates successful removal of the FMOC groups. Plain and PEG_{18} references show that the 315 nm peak is intrinsic to the FMOC group.

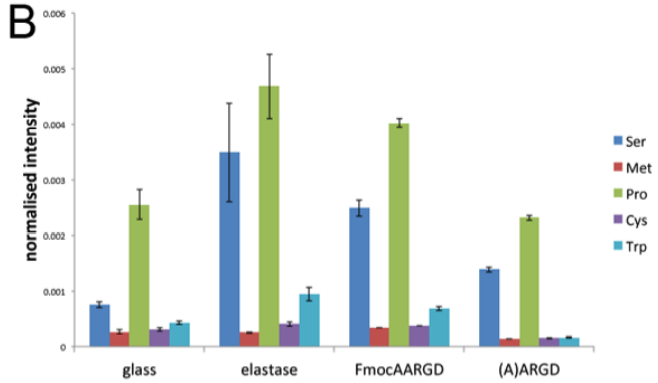


Supplementary figure 3: Water contact angle (WCA) measurements were used to follow the stepwise synthesis of peptide surfaces. There was a significant increase in contact angle after silanisation of the substrates (Plain to GOPTS surfaces), which then dropped after introducing the PEG_{18} monolayer. Contact angle was seen to be greater when hydrophobic FMOC protecting groups were in place compared with surfaces that were deprotected; n=3.



Supplementary figure 4: Analysis of 0.1 mg/ml elastase on cell responses. (A) MTT assay for metabolic activity shows no difference between control samples and samples incubated with elastase. Results are mean±SD, n=3, statistics by t-test. **(B)** Metabolomics showed little change across main metabolite groupings. Of 591 identified metabolites (IDEOM confidence score of 5 or above) analysed using t-test with Bonferroni's correction, just 27 identified metabolites were significantly different. **(C)** Vimentin, RUNX2 and phosphomyosin (p-myosin) were analysed and no effects on stress fibre formation were observed in samples incubated with elastase. No RUNX2 was noted in samples showing elastase alone did not activate osteogenesis. Red = actin, green = vimentin/p-myosin/RUNX2 (non shown) and blue = nuclei.


A
normalised intensity
0.025
0.02
0.015
0.01
0.005
0
glass
elastase
FmocAARGD
(A)ARGD
Gly
Ala
Arg
Asp
Phe


B
normalised intensity
0.006
0.005
0.004
0.003
0.002
0.001
0
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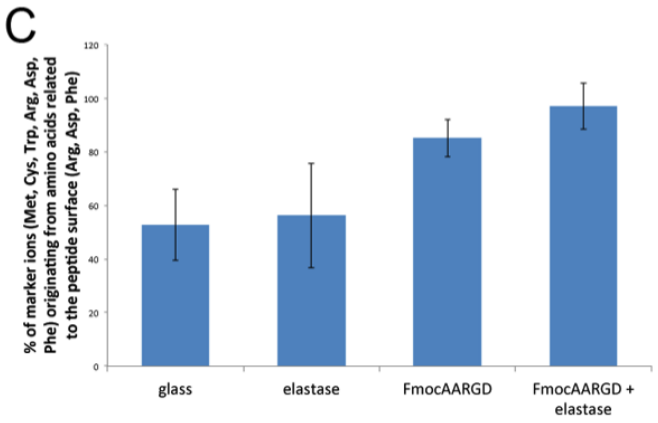
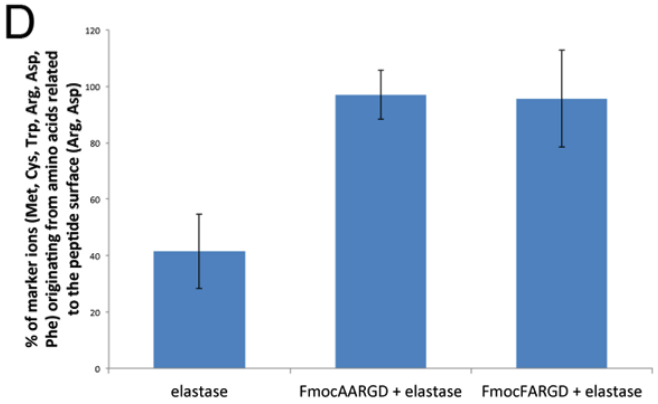
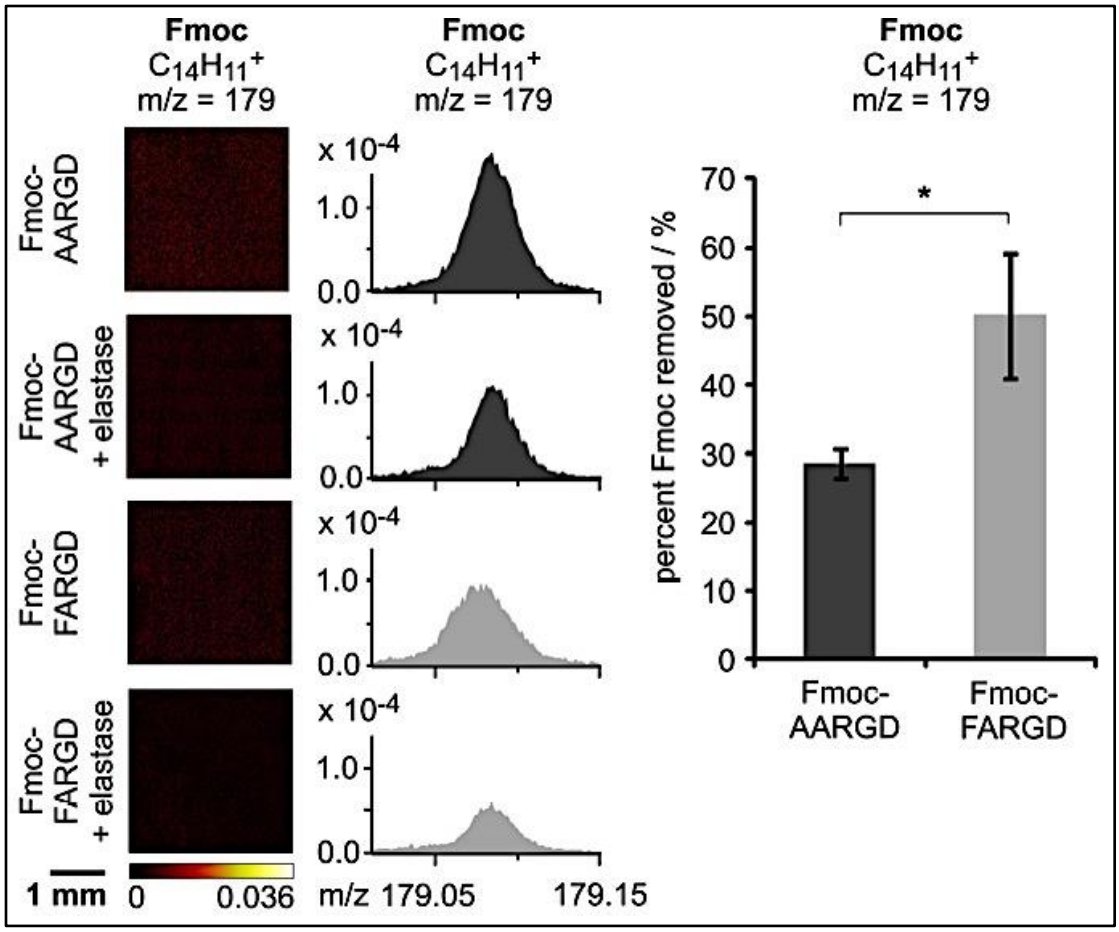
elastase
FmocAARGD
(A)ARGD
Ser
Met
Pro
Cys
Trp

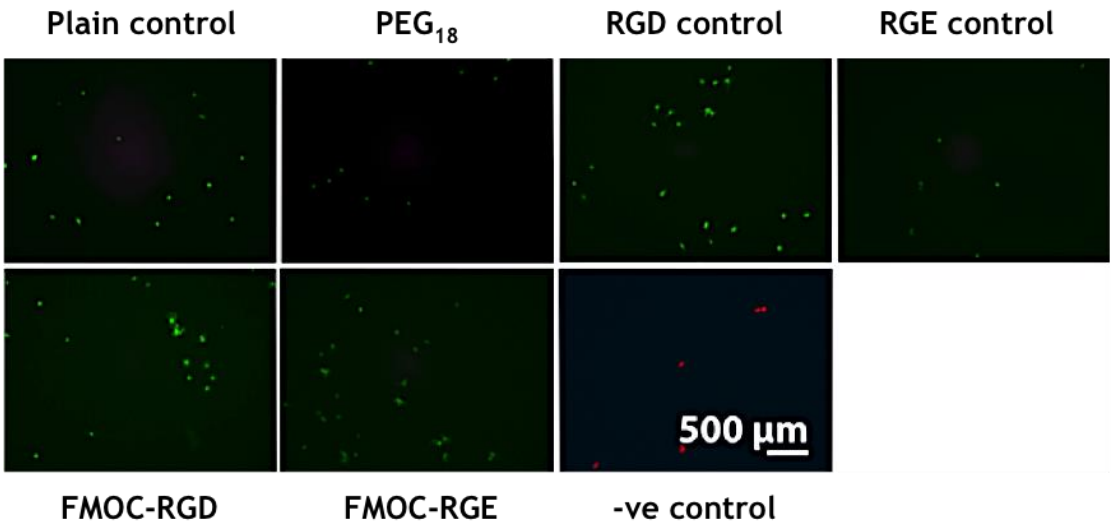

C
% of marker ions (Met, Cys, Trp, Arg, Asp, Phe) originating from amino acids related to the peptide surface (Arg, Asp, Phe)
120
100
80
60
40
20
0
glass
elastase
FmocAARGD
FmocAARGD + elastase


D
% of marker ions (Met, Cys, Trp, Arg, Asp, Phe) originating from amino acids related to the peptide surface (Arg, Asp)
120
100
80
60
40
20
0
elastase
FmocAARGD + elastase
FmocFARGD + elastase

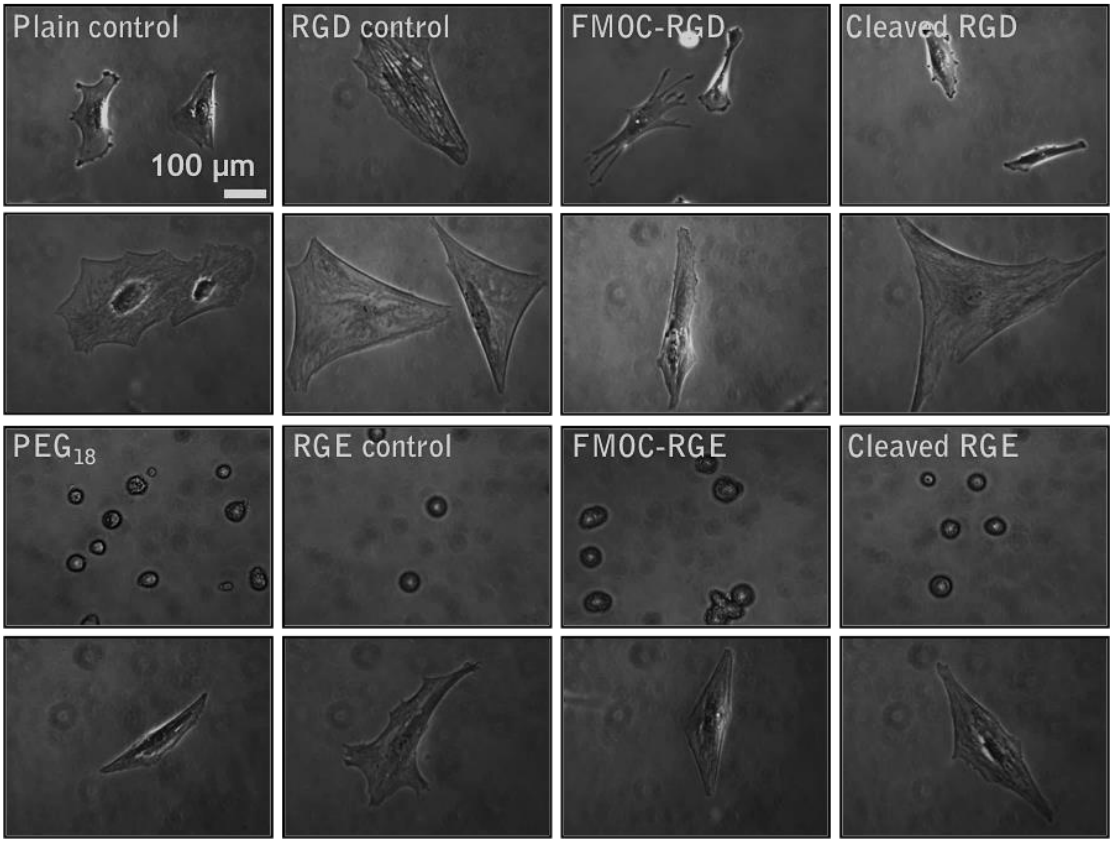
Supplementary Figure 5. TOF-SIMS analysis to confirm RGD is left on the surface after elastase cleavage. To determine if RGD is still present on the surfaces after exposure to elastase, ToF-SIMS mass spectra from enzyme exposed Fmoc-AARGD surfaces were compared to mass spectra from a glass control surface that was subjected to the same enzyme exposure and washing protocols as the peptide surfaces. A peak search was performed on the calibrated mass spectra to identify signals associated with the amino acids of interest[1]. Mass fragments for all amino acids that are expected to be on the peptide surfaces (Ala, Phe, Arg, Gly, Asp) (A) as well as other amino acids such as Met, Pro, Trp, Ser and Cys (B) were found on all three surfaces. As the latter amino acids are not expected to be present on the peptide surfaces, this indicates that the enzyme has not been completely removed by the washing steps and also contributes to the intensities of the ions expected on the peptide surface (Ala, Phe, Arg, Gly, Asp). To account for the enzyme's contribution to the amino acid signals on the peptide surface, we did not directly compare ion intensities but determined the relative amount of peptide surface related amino acids to that of amino acids that mark the presence of elastase (C). This procedure builds on the assumption that the amino acid composition of elastase as detected by ToF-SIMS is similar on the glass control and the peptide surfaces. The amino acid ion fragments selected as markers for elastase were Met, Cys and Trp (enzyme markers) because they are more specific higher mass ions with low variability (low standard deviations). Arg and Asp (surface markers) were chosen as markers for the peptide surfaces and hence as indicators for the presence of RGD on the surface for the same reasons (D). To obtain more robust data for each set, we summed up the intensities of the selected amino acid ion fragments in each set of markers (enzyme marker and surface marker). We hypothesised that if the amino acid signals on the peptide surfaces originate only from adsorbed enzymes, the relative intensities of the two marker sets should remain unchanged and thus comparable to that of enzymes adsorbed on glass. The relative amount of surface markers compared to the sum of all five amino acid marker fragments (enzyme + surface markers) was calculated and plotted. The results show that surface markers only make up 42 ± 13% of the sum of intensities whereas on the Fmoc-AARGD peptide surface the surface markers are close to 100% (97 ± 9%). This significant difference indicates that most ion intensities for Arg and Asp originate from the peptide surface and thus suggests that the RGD sequence is still present on the surface after exposure to elastase.



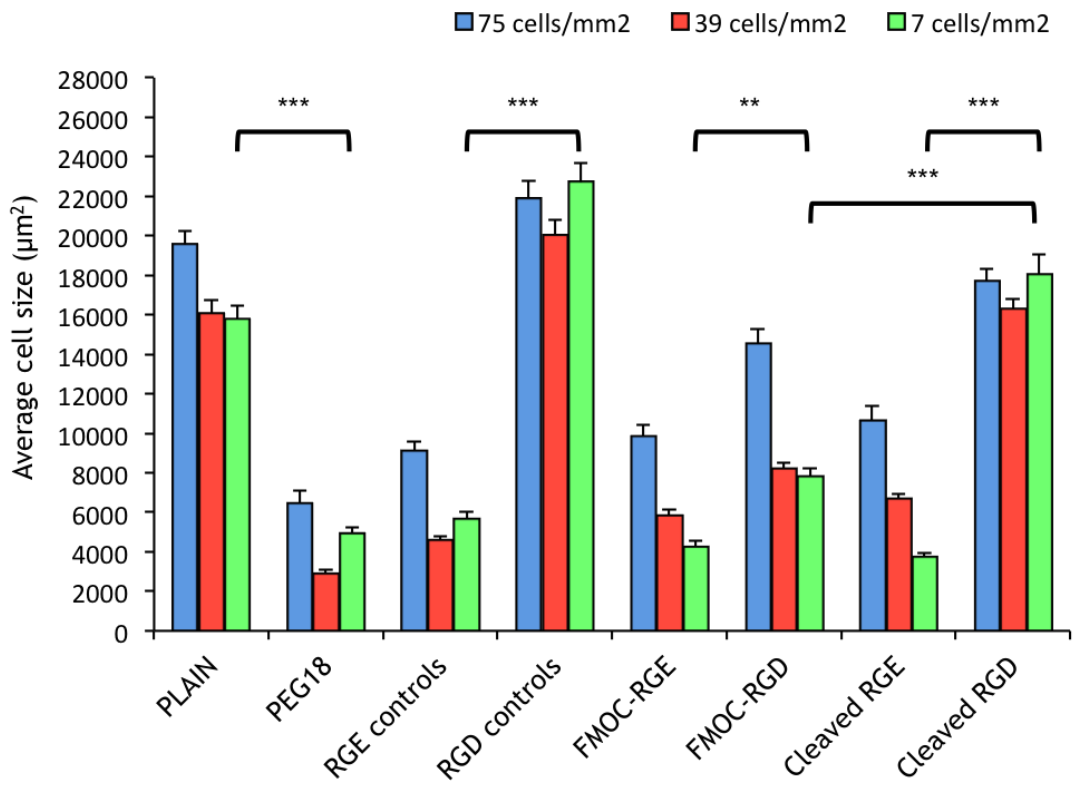
Supplementary figure 6: Changing peptide sequence to alter elastase addressability. TOF-SIMS analysis demonstrated that, with addition of elastase for 48 hours, 29% cleavage efficiency was achieved for the AARGD sequence. However, by substituting the terminal alanine for a phenylalanine as in FARGD, and increase to 50% cleavage efficiency was seen. N=4, results = mean±SD, statistics by t-test. Images are ion maps of large area scans (3 mm x 3mm). The colour scale represents relative ion counts normalised to the total ion counts. Spectra are from 1.5 mm x 1.5 mm regions of interest and normalised to the total ion count.



Supplementary figure 7: Live/dead viability assay. While adhesion and the degree of cell spreading was clearly affected on PEG_{18} and RGE controls, no dead cells were noted on the surfaces as compared with negative control where cells were killed with ethanol.



Supplementary figure 8: Stro1 MSCs seeded at a density of 7 cells/mm^2. Upper two rows refer to plain control, RGD control, FMOC-RGD and cleaved RGD where the labelled row is 24 hours and the unlabelled row is 7 days. Lower two rows are PEG_{18}, RGE controls, FMOC-RGE and cleaved RGE with labelled row at 24 hours and unlabelled row at 7 days (2 days 'low', 5 days 'high' for cleaved surfaces). Note at 24 hours, cleaved RGD and RGE are in their undigested form and therefore have the same chemistry as FMOC-RGD and FMOC-RGE. MSCs took longer to attach to PEG_{18}, RGE controls, FMOC and cleaved RGE at this density and were much smaller.



Supplementary figure 9: Quantification of MSC size using different seeding densities. MSCs were affected by substrate chemistry with cells tending to be larger on plain controls, RGD controls, FMOC-RGD and cleaved RGD surfaces compared with cells that were seeded on PEG_{18}, RGE controls, FMOC-RGE and cleaved RGE surfaces. Cell size was also affected by changes to seeding density leading to exaggerated cell responses between the surfaces. Graph represents average MSC size after 7 days of culture at initial seeding densities of 75, 39 and 7 cells/mm^2. Error bars are standard error (n=40 cells from 3 materials replicates per substrate). Stars refer to 7 cells/mm^2 seeding density only and indicate significant difference between groups as determined by one-way ANOVA and Dunn's post hoc test where *P<0.5 **P<0.1 and ***P<0.001.

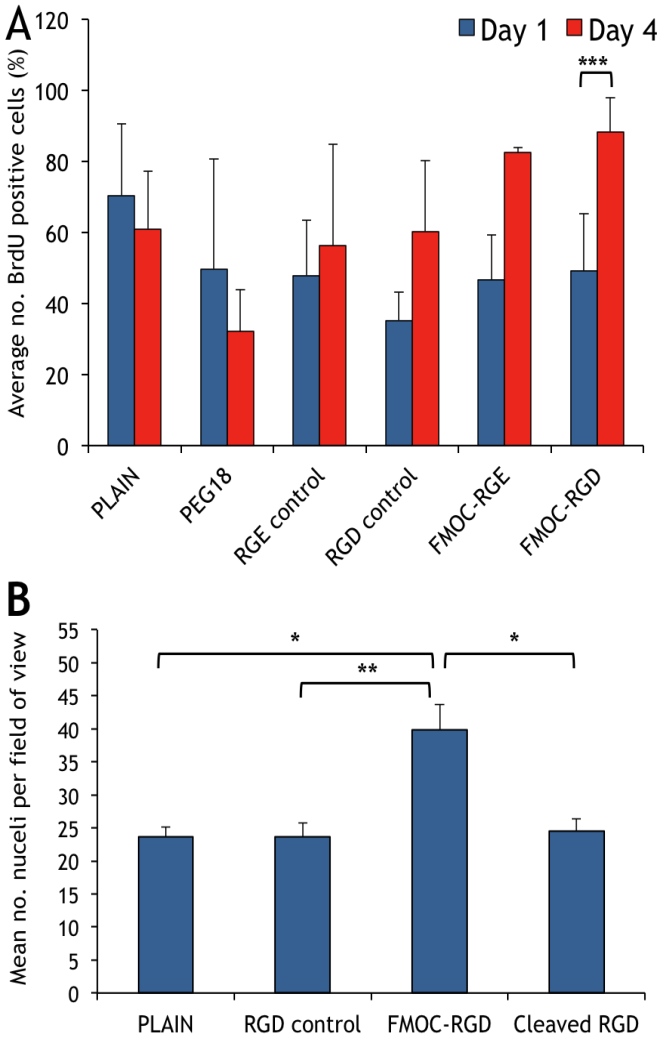
Substrate	Significance	
	Difference in rank	P value summary
PLAIN vs. PEG_{18}	145	***
PLAIN vs. RGE control	126	***
PLAIN vs. FMOC-RGD	84.9	**
PLAIN vs. FMOC-RGE	162	***
PLAIN vs. Cleaved RGE	180	***
PEG_{18} vs. RGD control	-188	***
PEG_{18} vs. Cleaved RGD	-159	***
RGD control vs. RGE control	170	***
RGD control vs. FMOC-RGD	128	***
RGD control vs. FMOC-RGE	205	***
RGD control vs. Cleaved RGE	223	***
RGE control vs. Cleaved RGD	-140	***
FMOC-RGD vs. FMOC-RGE	77.0	**
FMOC-RGD vs. Cleaved RGD	-98.8	***
FMOC-RGD vs. Cleaved RGE	95.0	***
FMOC-RGE vs. Cleaved RGD	-176	***
Cleaved RGD vs. Cleaved RGE	194	***

Supplementary figure 10: Complete list of significant differences for Stro1 MSC size at 7 cells/mm^2. Values refer to significant differences in MSC size on test substrates seeded at a density of 7 cells/mm^2. Difference in rank values were determined by one-way ANOVA (Kruskal-Wallis test) and Dunn's post hoc test. P values denote the degree of significance where stars are *P<0.5 **P<0.1 and ***P<0.01.

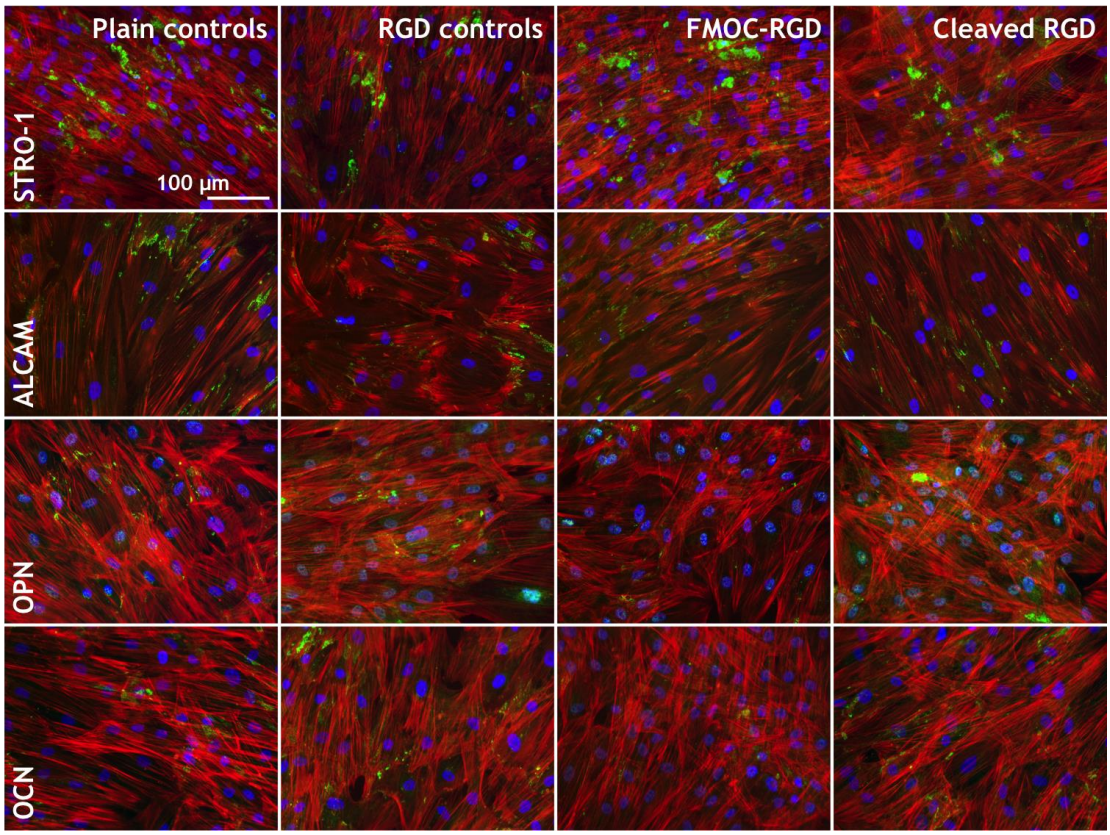
Substrate	Significance	
	Difference in rank	P value summary
(FX) PLAIN vs. RGD control	99.4	***
PLAIN vs. FMOC-RGE	71.4	*
PEG_{18} vs. RGD control	77.9	**
RGE control vs. RGD control	79.1	**
RGD control vs. FMOC-RGD	-103	***
RGD control vs. Cleaved RGE	-111	***
FMOC-RGE vs. FMOC-RGD	-75.1	**
FMOC-RGE vs. Cleaved RGE	-82.6	**
(FA) PLAIN vs. PEG_{18}	-110	***
PLAIN vs. RGE control	-100	***
PLAIN vs. FMOC-RGE	-151	***
PLAIN vs. FMOC-RGD	-86.6	***
PLAIN vs. Cleaved RGE	-115	***
PEG_{18} vs. RGD control	150	***
PEG_{18} vs. Cleaved RGD	109	***
RGE control vs. RGD control	141	***
RGE control vs. Cleaved-D	98.9	***
RGD control vs. FMOC-RGE	-192	***
RGD control vs. FMOC-RGD	-127	***
RGD control vs. Cleaved RGE	-155	***
FMOC-RGE vs. FMOC-RGD	64.7	*
FMOC-RGE vs. Cleaved RGD	150	***
FMOC-RGD vs. Cleaved RGD	85.3	**
Cleaved RGE vs. Cleaved RGD	113	***
(SMAdh) PLAIN vs. PEG_{18}	110	***
PLAIN vs. RGE control	115	***
PLAIN vs. FMOC-RGE	122	***
PLAIN vs. FMOC-RGD	104	***
PLAIN vs Cleaved RGE	187	***
PEG_{18} vs. RGD control	168	***
PEG_{18} vs. Cleaved RGE	77.0	**
PEG_{18} vs. Cleaved RGD	-134	***
RGE control vs. RGD control	-174	***
RGE control vs. Cleaved RGE	71.5	*
RGE control vs. Cleaved RGD	-139	***
RGD control vs. FMOC-RGE	181	***
RGD control vs. FMOC-RGD	163	***
RGD control vs. Cleaved RGE	245	***
FMOC-RGE vs. Cleaved RGE	64.8	*
FMOC-RGE vs. Cleaved RGD	-146	***
FMOC-RGD vs. Cleaved RGE	82.5	**
FMOC-RGD vs. Cleaved RGD	-128	***
Cleaved RGE vs. Cleaved RGD	-211	***

Supplementary figure 11: Complete list of significant differences for MSC adhesion analysis. Values refer to the differences in adhesion subtypes between different substrates

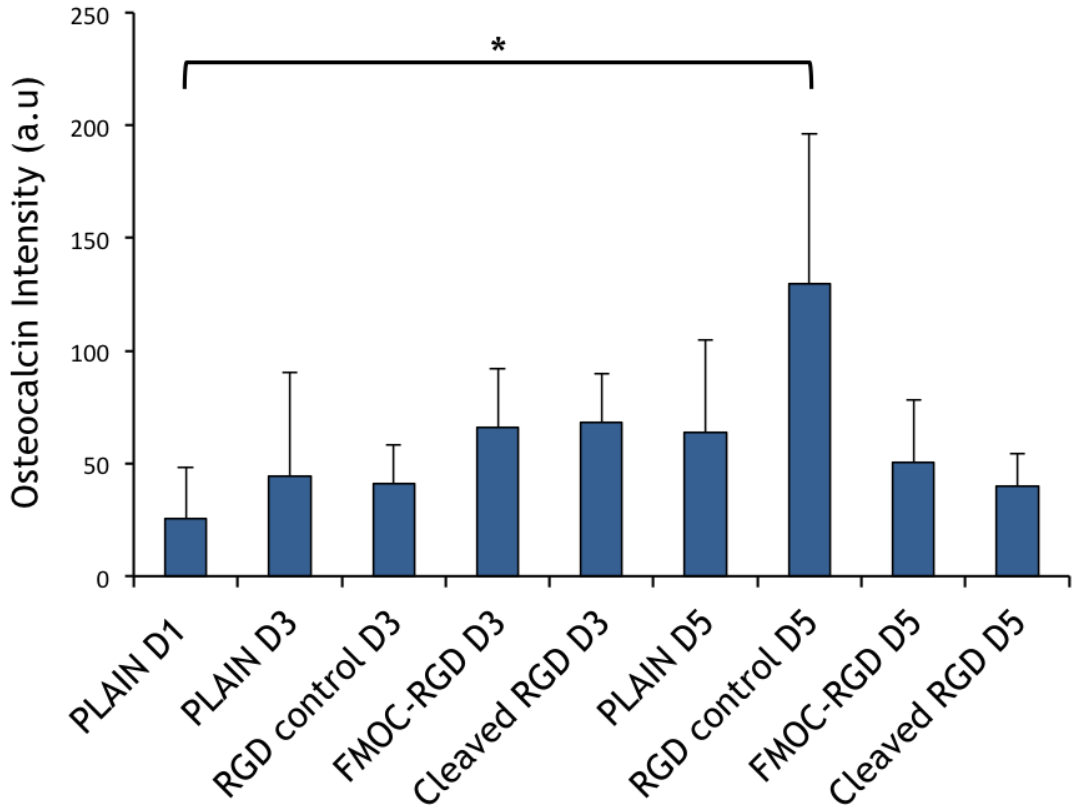
seeded at 7 cells/mm^2. Difference in rank values were determined by one-way ANOVA (Kruskal-Wallis test) and Dunn's post hoc test. P values denote the degree of significance where stars are *P<0.5 **P<0.1 and ***P<0.01. Identifiers FX, FA and SMAdh refer to individual adhesion subtype focal complex (FX), focal adhesion (FA) and supermature focal adhesion (SMAdh) respectively.



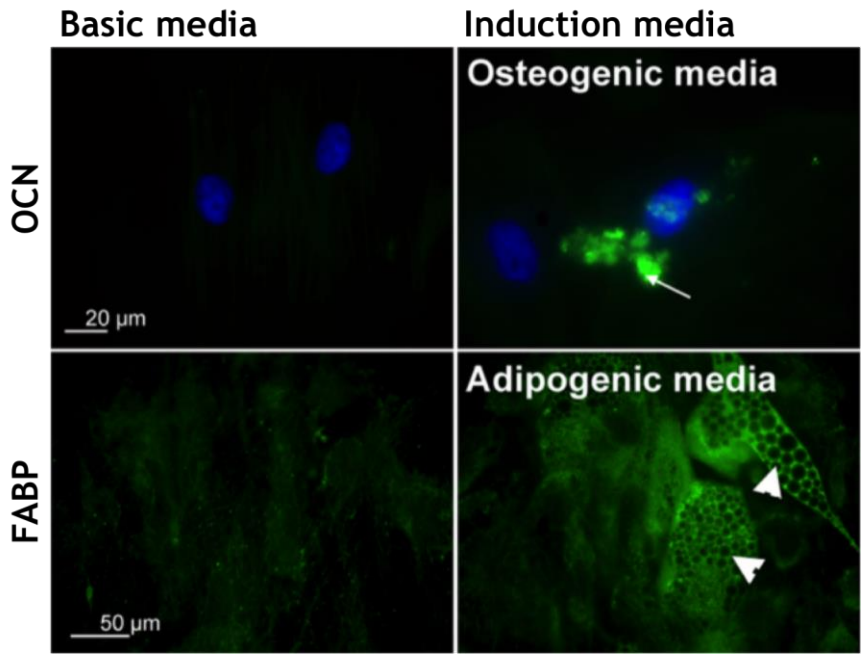
Supplementary figure 12: Cell proliferation: (A) BrdU uptake into S-phase cells showed that the only surface to significantly support enhanced proliferation between days 1 and 4 of culture, was FMOC-RGD. **(B)** After 28 days, the average number of nuclei calculated per field of view indicated that the highest rate of growth was on FMOC-RGD surfaces. Stars indicate significant difference between groups as determined by one-way ANOVA and Dunn's post hoc test where *P<0.5 **P<0.1 and ***P<0.001. Error bars represent standard deviation (n=3 from 3 material replicates per substrate for BrdU and n=20 fields of view per surface across 3 material replicates).



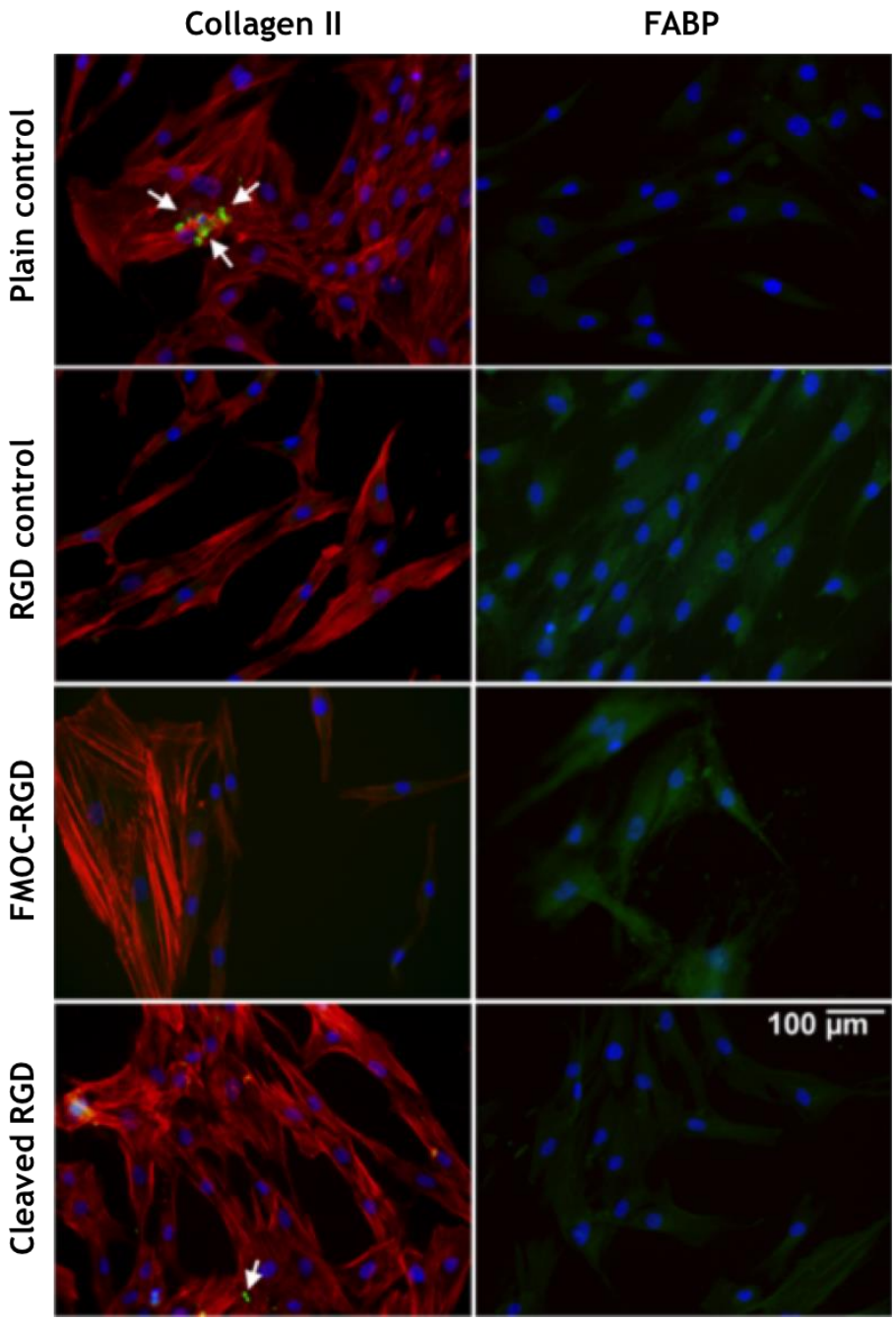
Supplementary figure 13: Immunofluorescence images of Stro1 MSCs at day 7. MSCs cultured on plain controls, RGD controls, FMOC-RGD and cleaved RGD surfaces expressed different levels of STRO-1, ALCAM, OPN and OCN. While STRO-1 and ALCAM were still expressed on all surfaces, OPN and OCN were only seen on RGD controls and cleaved RGD surfaces at this early time point. Colours are red =actin, green = STRO-1/ALCAM/OPN/OCN, blue = nuclei.



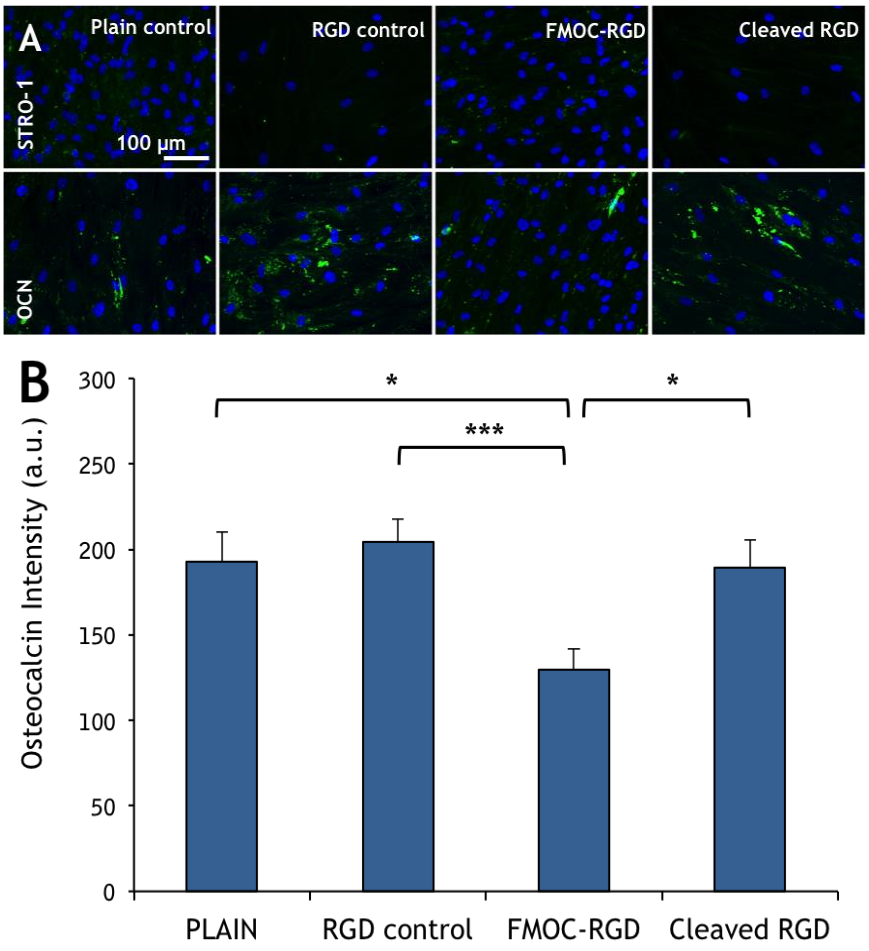
Supplementary figure 14: Osteocalcin (OCN) image analysis at 3 and 5 days cultures. During the first 5 days of culture, only background levels of osteocalcin were recorded for all surfaces except the RGD control at day 5. Star indicates significant difference between plain controls at day 1 and RGD control determined by one-way ANOVA and Dunn's post hoc test where *P<0.5. Error bars are standard deviation (n=3 from 3 material replicates per surface). Note that a.u is arbitrary units.



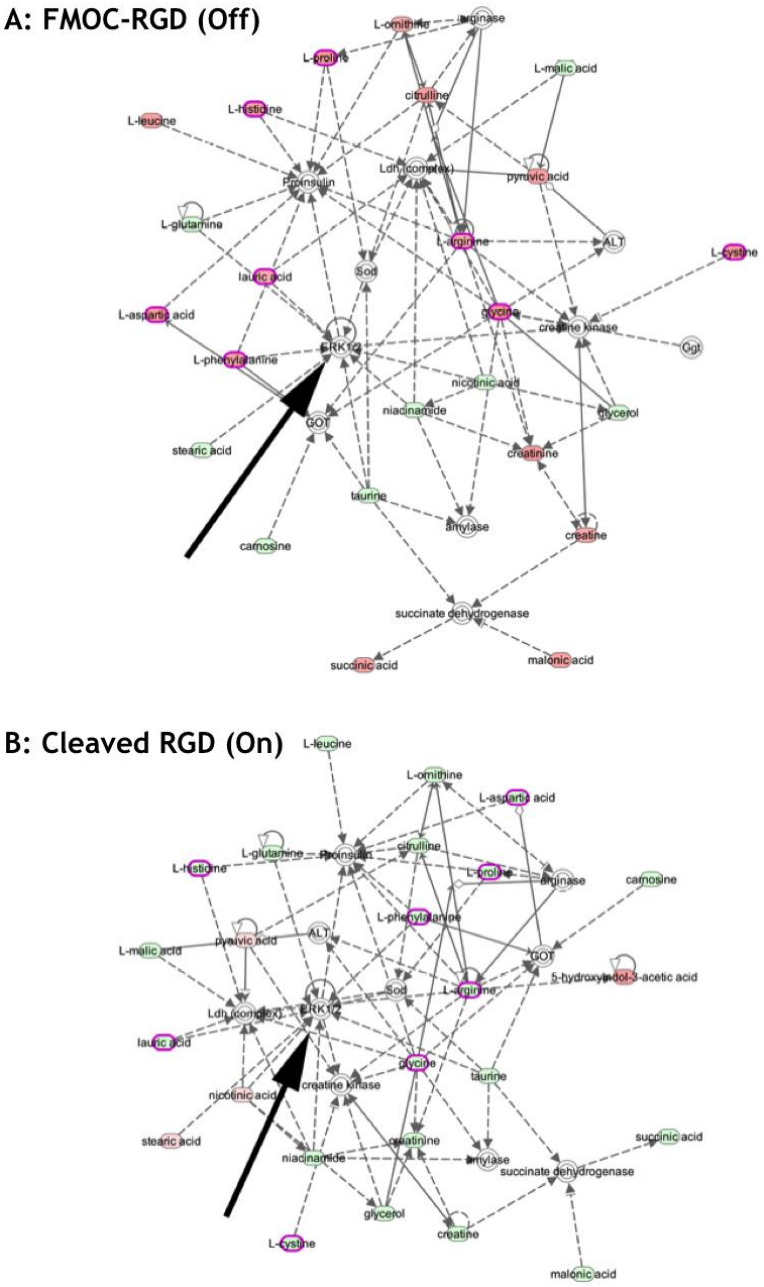
Supplementary figure 15: Induction of differentiation after 21 days of culture on FMOC-RGD. Immunostaining for osteocalcin (OCN) and fatty acid binding protein (FABP) involved in osteogeneis and adipogenesis respectively. Images show that the cells did not express OCN when cultured in basal media, and only background level of FABP were detected. However, with induction media, intense OCN nodules (arrow) were observed in osteogenic media, and FABP staining surrounding lipid droplets (arrowheads) was clearly seen in adipogenic media. Blue = nucleus, green = OCN or FABP.



Supplementary figure 16: Expression of adipogenic and chondrogenic phenotypes after 14 days of culture. Immunostaining for collagen II and fatty acid binding protein (FABP) involved in chondrogenesis and adipogenesis respectively. Collagen II expression was only noted on plain controls (arrows) and only background levels of FABP were noted on the other surfaces. Red = actin, blue = nucleus, green = Collagen II or FABP.

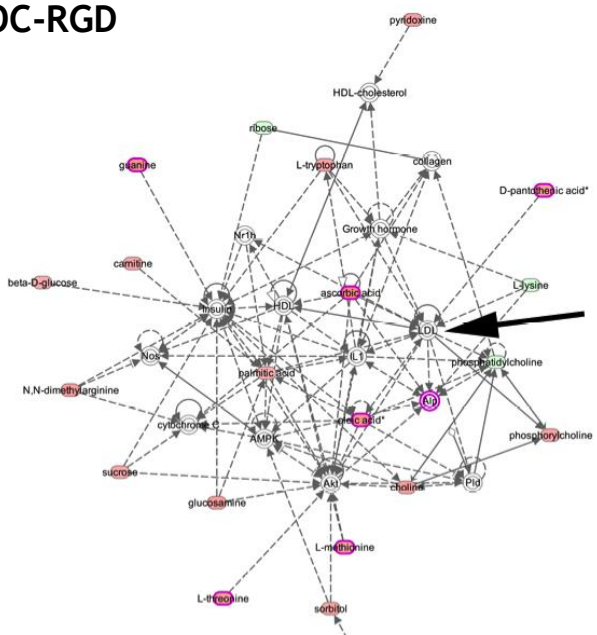


Supplementary figure 17: Analysis of MSC growth and differentiation at day 28. (A) Immunofluorescence images of Stro1 MSCs at day 28. STRO-1 was only observed on the plain control and FMOC-RGD (low) at this late time point. In comparison, OCN levels were somewhat increased on plain controls and mostly increased on RGD controls and cleaved RGD (high) surfaces. Green = STRO-1 or OCN and blue = nuclei. **(B)** OCN expression was quantified using image analysis and confirmed the trend seen in image A. Statistics by one-way ANOVA and Dunn's post hoc test where *P<0.05, **P<0.01 and ***P<0.001, n=3 material replicates; note that AU = arbitrary units and scale is in thousandths.

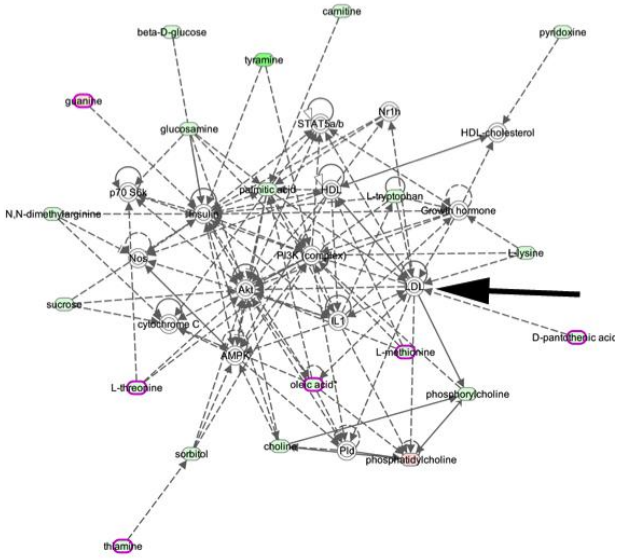


Supplementary figure 18: Network analysis of metabolites affecting growth-related biochemistry. Ringed metabolites are highlighted by Ingenuity® Pathway Analysis as being implicated in cell growth and are generally up-regulated on the FMOC-RGD (low) surface and down-regulated on the cleaved RGD (high) surface (red=up, green=down). Extracellular signal related kinase (ERK1/2, arrow) has been implicated in retention of MSC multipotency[2].

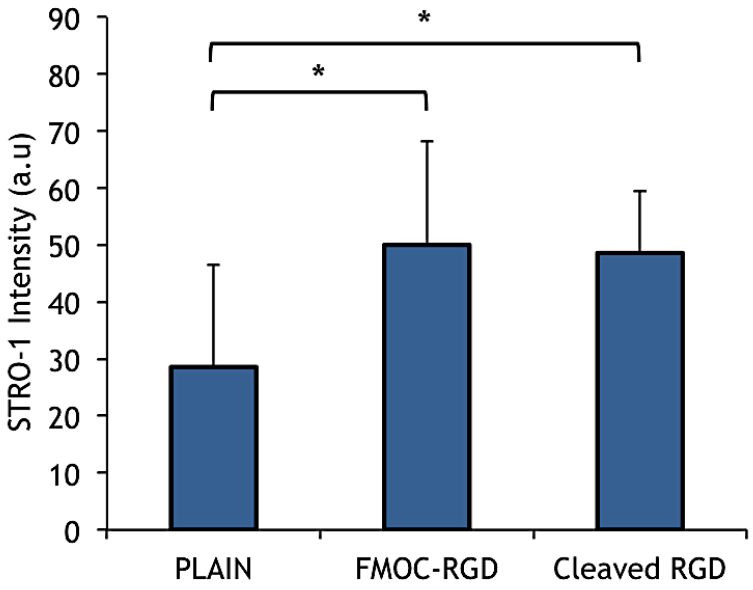
A: FMOC-RGD (Off)



B: Cleaved RGD (On)



Supplementary figure 19: Network analysis of metabolites affecting growth-related biochemistry. Ringed metabolites are highlighted by Ingenuity® Pathway Analysis as being implicated in cell growth are generally up-regulated on the FMOC-RGD (low) surface and down-regulated on the cleaved RGD (high) surface (red=up, green=down). Low density lipoprotein (LDL, arrow) has been implicated in retention of MSC multipotency previously[2].



Supplementary figure 20: STRO-1 image analysis for long-term growth before switch. MSCs were cultured for 4 weeks (2 week 'low' and 2 week 'high' for cleaved RGD). STRO1 was quantified using image analysis and indicated that MSCs cultured on FMOC-RGD surfaces retain STRO-1 compared to plain controls. However, the reduction in STRO-1 on cleaved RGD surfaces was not statistically different. Statistics by one-way ANOVA and Dunn's post hoc test where *P<0.05, n=3 material replicates; note that AU = arbitrary units.

SUPPLEMENTARY REFERENCES

1. Lhoest, J. B.; Wagner, M. S.; Tidwell, C. D.; Castner, D. G. Characterization Of Adsorbed Protein Films By Time Of Flight Secondary Ion Mass Spectrometry. *J. Biomed. Mater. Res.* 2001, 57, 432-440.
2. Tsimbouri, P. M.; Mcmurray, R. J.; Burgess, K. V.; Alakpa, E. V.; Reynolds, P. M.; Murawski, K.; Kingham, E.; Oreffo, R. O.; Gadegaard, N.; Dalby, M. J. Using Nanotopography And Metabolomics To Identify Biochemical Effectors Of Multipotency. *ACS Nano* 2012, 6, 10239-49.