N-Cadherin adhesive interactions modulate matrix mechanosensing and fate commitment of mesenchymal stem cells

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Supplementary Methods

F-Actin Bundling, Focal Adhesion (FA), and Myosin-IIA Quantification

For F-actin/FA quantification, images were taken using a 100X 1.45 NA objective (0.12 μm/px) on a Nikon A1R Confocal Microscope. Actin anisotropy calculations on the apical/basal plane of the cell were carried out using a freely-available plugin for ImageJ, FibrilTool, previously described in[1]. Briefly, confocal slices of the apical and basal planes of the cell were taken, and the inner region of the cell (defined as excluding the outside 15% to avoid artifacts) was selected as the quantification area. Anisotropy ratios are reported on a scale from 0 (perfectly isotropic) to 1 (perfectly anisotropic). Analysis of focal adhesion characteristics were performed on paxillin images using the FAAS analysis platform[2] with input parameters of 5 threshold and a minimum adhesion size of 0.17 μm^2. Myosin-IIA content in focal adhesions was analyzed through double-immunofluorescence staining for paxillin and myosin-IIA. Images were taken using a 100X 1.45 NA objective with a 1.7x zoom (0.07 μm/px) and a pinhole of 0.15 μm (0.5AU) on a Nikon A1R Confocal Microscope. Analysis to determine Myosin-IIA accumulation in focal adhesions was performed in a manner similar to that described in[3]. First, the paxillin channel was thresholded (as described above) to create a focal adhesion mask, background levels of myosin-IIA staining were set at 1.5x the local background from around the focal adhesion mask, and these values were subtracted from the myosin-IIA channel to create a thresholded myosin-IIA signal. Data shown represent the percentage of focal adhesion area that is myosin-IIA-positive after this thresholding.

Traction Force Microscopy (TFM)

Traction force microscopy was performed as described previously[4]. Prior to polymerization, MeHA hydrogel precursor was doped with 0.2 μm diameter fluorescent microspheres at 1% v/v (#F8810; Invitrogen, Carlsbad, CA). MeHA hydrogels were UV cured to a modulus of 10 kPa (as

verified by AFM) for all TFM experiments. Small drops of a UV-curable fixative (NOA68; Norland Products, Cranbury, NJ), secured the MeHA-covered glass coverslips in a live cell imaging bath. MeHA hydrogels were subsequently washed three times with PBS and sterilized under germicidal UV light for 1 hr. MSCs were seeded on MeHA hydrogels at 3,000 cells/cm^2 and allowed to culture for 18 hour before TFM analysis was performed. Phase contrast and fluorescent images of multiple cells and embedded beads were captured at 40x magnification on a DeltaVision Deconvolution Microscope (GE Healthcare Life Sciences, Marlborough, MA). Image sequences for each cell were taken before and after cell lysis with SDS (sodium dodecyl sulfate) buffer. All imaging was performed in an environmental chamber (37^{o}C, 5% CO2). Traction force microscopy data analysis (stack alignment, particle image velocimetry, and Fourier transform traction cytometry) was performed using a freely available plugin suite for ImageJ, created by Tseng et al.[5], which was adapted from Dembo and Wang[6]. For FTTC variables, the Poisson's ratio of the MeHA hydrogel was assumed to be 0.45 and a regularization parameter of 1e^{-9} was used. Using a custom MATLAB script, traction force vector maps were analyzed to determine the average stress generation by each cell on the underlying substrate.

Adenoviral Transduction

MSCs were allowed to culture for one day prior to the addition of recombinant adenovirus. Viral titers were added to standard culture medium at 750 MOI. Control (CTL) cells had no adenovirus added, LacZ cells had a LacZ control virus introduced, and CA Rac1 cells were transduced with a constitutively active Rac1 V12 mutant construct, as in[7]. Virus was added for 24 hrs, at which point the virus was removed, cells were washed twice with PBS, followed by normal trypsinization and seeding onto 10 kPa MeHA hydrogels for further analysis. Transduction efficiency of all constructs was estimated to be close to 100%, as calculated by staining with an anti-Myc tag antibody (1:200; Abcam #1263).

Atomic Force Microscopy (AFM)

An Agilent ILM-6000 AFM was utilized to perform force spectroscopy on MeHA hydrogels to measure elastic moduli. A DNP-10 pyramidal cantilever was utilized (thermally determined stiffness of 0.2 N/m) to generate force-displacement curves of the underlying substrate. A total of 16 measurements were taken across each hydrogel. Elastic moduli at each indentation point were determined through a custom curve fitting program written in MATLAB, which determined contact point and then fit the first 500 nm of each profile with the Sneddon approximation of the Hertz model with a pyramidal tip ($\alpha=18^{\circ}$).

Western Blotting

MeHA hydrogels were cast onto 2" x 3" slides for all Western Blot analyses. Cells were lysed directly into 150uL drops of 5X SDS sample buffer containing 2% β-mercaptoethanol. Protein was separated by 10% SDS-PA gel electrophoresis and transferred to nitrocellulose membranes. Membranes were then blocked with either 5% milk or BSA in TBS with 0.1% Tween-20 (TBS-T) for at least 1 hour at room temperature. Membranes were probed with primary antibodies overnight at 4°C, as described below (**Table S1**). This was followed by 3 washes in TBS-T and incubation with HRP-linked IgG secondary antibodies for 1-2 hours at room temperature. After three more TBS-T washes, bands were detected by enhanced chemiluminescence (GE Healthcare, cat.# RPN2106), and images were acquired using the ImageQuant LAS 4000 imager system. Normalization of quantification was performed by scaling all sample values such that control scram/RGD averages were 1. GAPDH expression was used as a loading control.

Supplementary References

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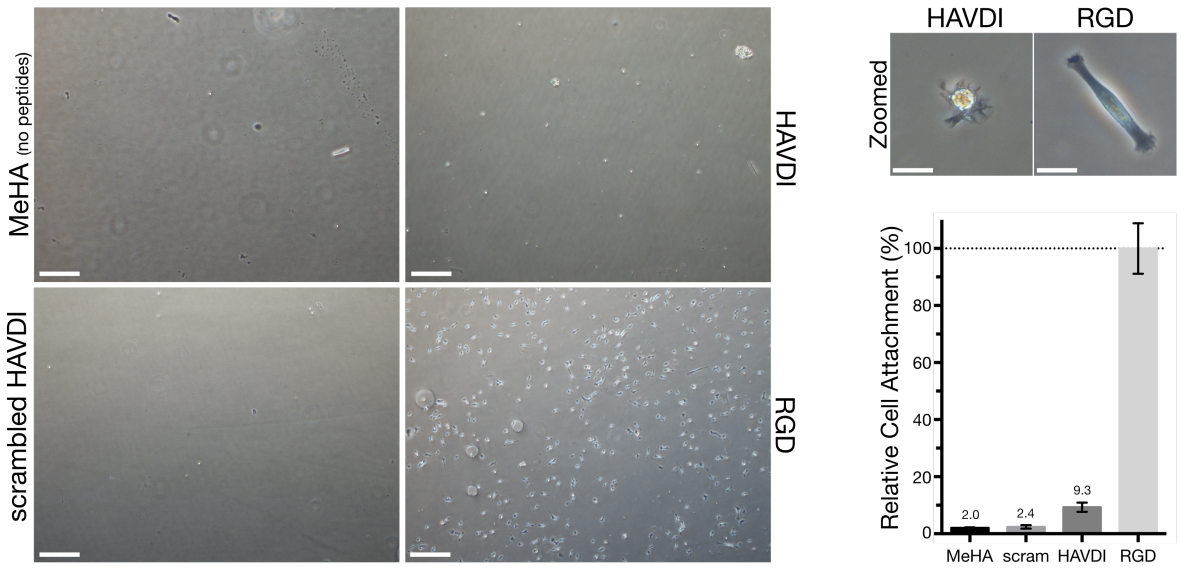
Supplementary Tables

Table S1 – Primary Antibodies for Western Blotting

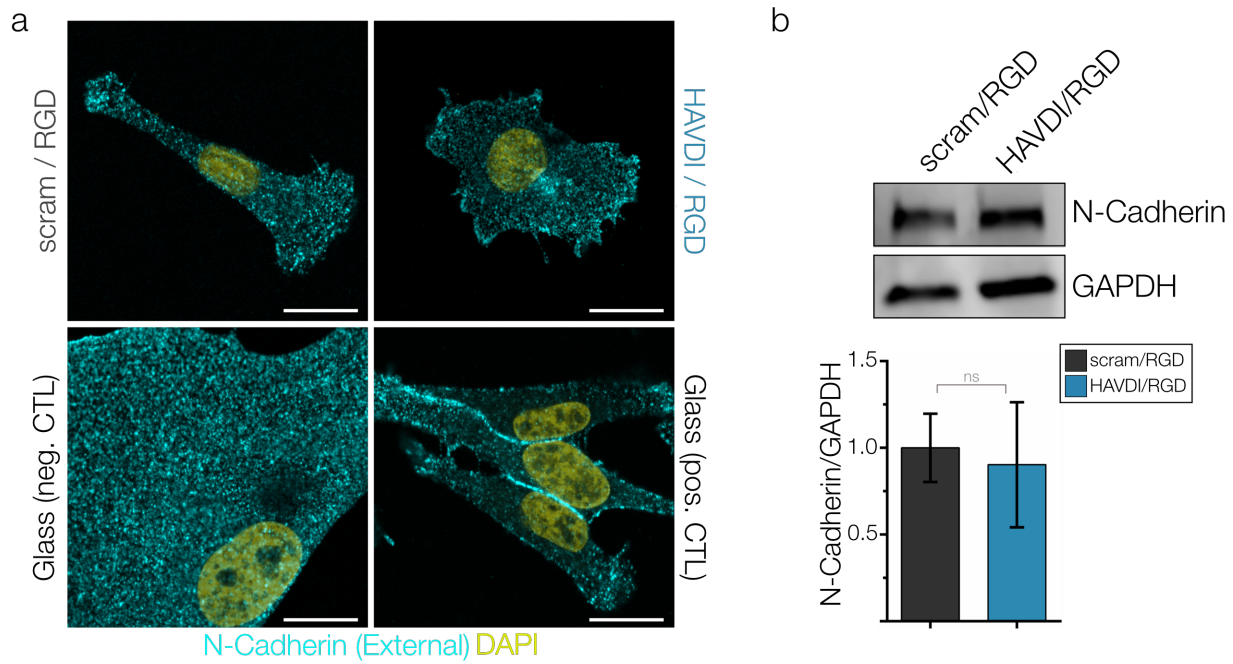
Antibody	Type	Source	Product Number	Dilution for WB
N-Cadherin	mouse monoclonal	BD Biosciences	#610921	1:500 in 5% BSA
GAPDH	rabbit monoclonal	Cell Signaling Technology	#2118	1:1500 in 5% BSA
Cofilin-pS3	rabbit monoclonal	Cell Signaling Technology	#3313	1:500 in 5% BSA
Cofilin	rabbit monoclonal	Cell Signaling Technology	#5175	1:500 in 5% milk
FAK-pY397	rabbit polyclonal	Cell Signaling Technology	#3283	1:500 in 5% BSA
FAK	rabbit polyclonal	Santa Cruz	#sc-558	1:500 in 5% milk
p130Cas-pY410	rabbit polyclonal	Cell Signaling Technology	#4011	1:500 in 5% BSA
p130Cas	rabbit polyclonal	Santa Cruz	#sc-860	1:500 in 5% milk

Supplementary Figures

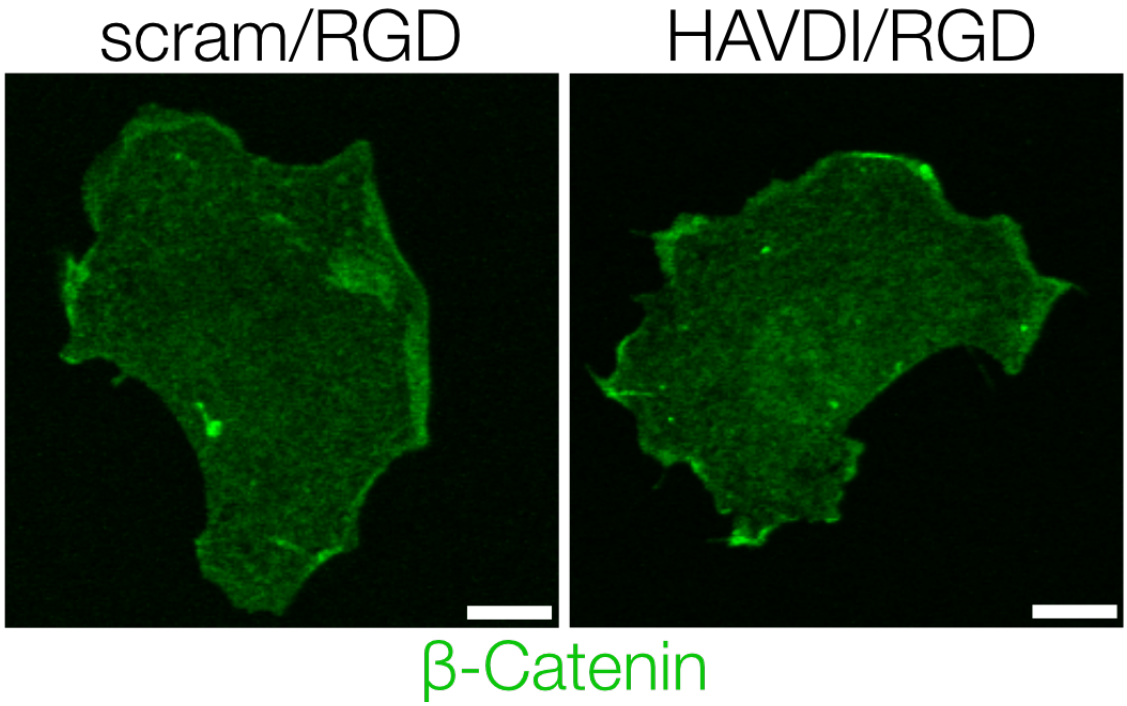
Supp. Fig. 1 – **Fluorescent tagging allows for visualization of peptide incorporation and localization in MeHA films.** Representative confocal microscope imaging of X/Z-plane images of 'HAVDI/RGD' MeHA hydrogels loaded with 1:10 peptide fractions of FITC-HAVDI and Rhodamine-RGD. Scale bar = 50μm.



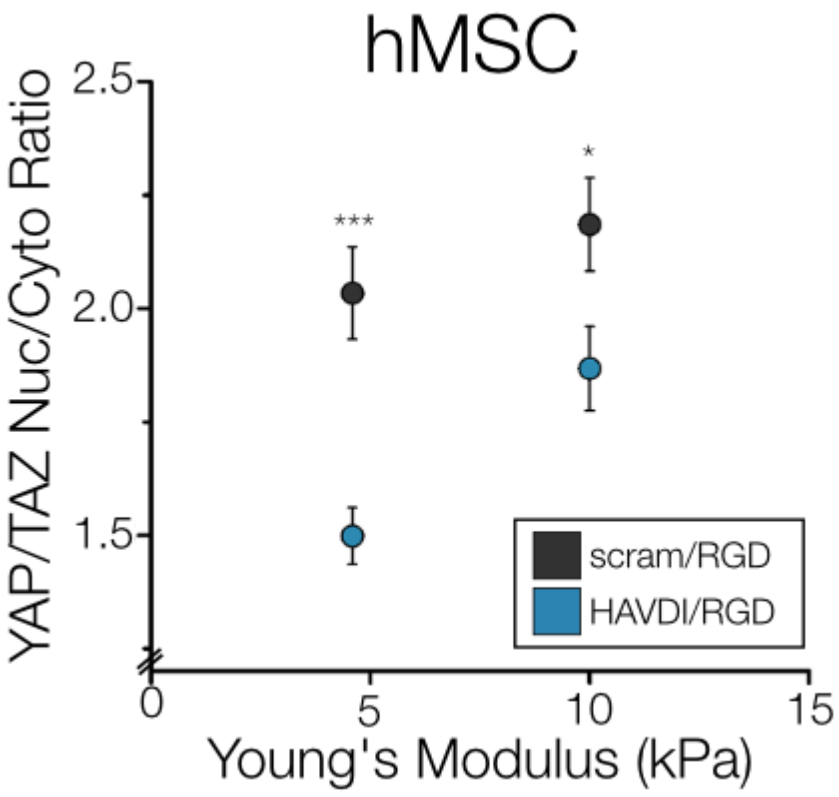
Supp. Fig. 2 – **MSC Attachment to MeHA hydrogels modified with a single peptide type.** (left) Live cell phase contrast images of MSC attachment following 18h of culture on MeHA hydrogels modified with a single peptide type, following 2x media rinses, SB= 100 μm. (top right) Representative zoomed images of spread MSCs on HAVDI and RGD substrates, scale bar = 10 μm. (bottom right) Quantifications of MSC attachment (n=3).



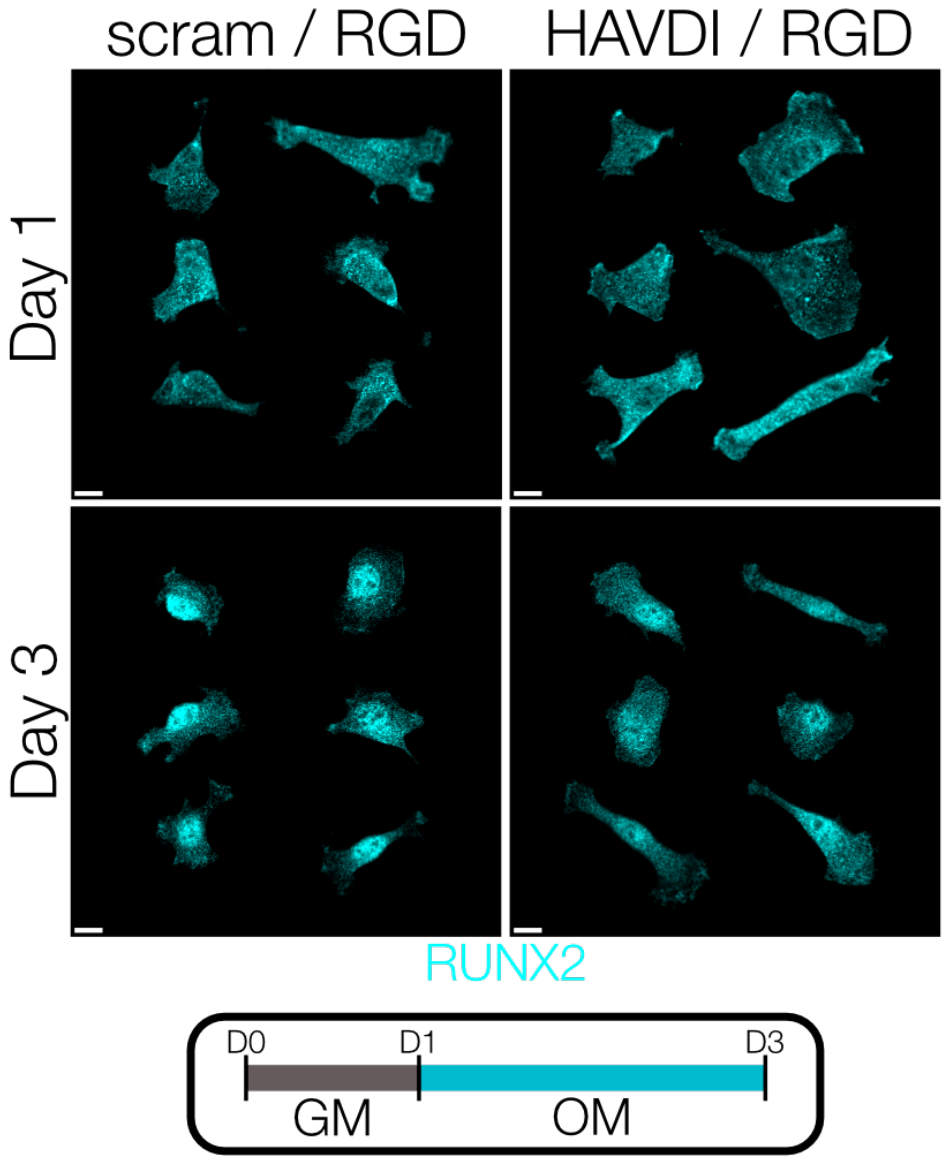
Supp. Fig. 3 – **External N-Cadherin Immunostaining and Quantification. (a)** Representative confocal images of extracellular-only N-Cadherin immunofluorescence from the basilar plane of MSCs cultured on 10 kPa MeHA substrates or on glass coverslips. (b) Western blots of N-Cadherin levels in MSCs cultured on 10 kPa MeHA substrates (n=3 blots, p=0.2984, mean ± SEM). Scale bars = 10μm.



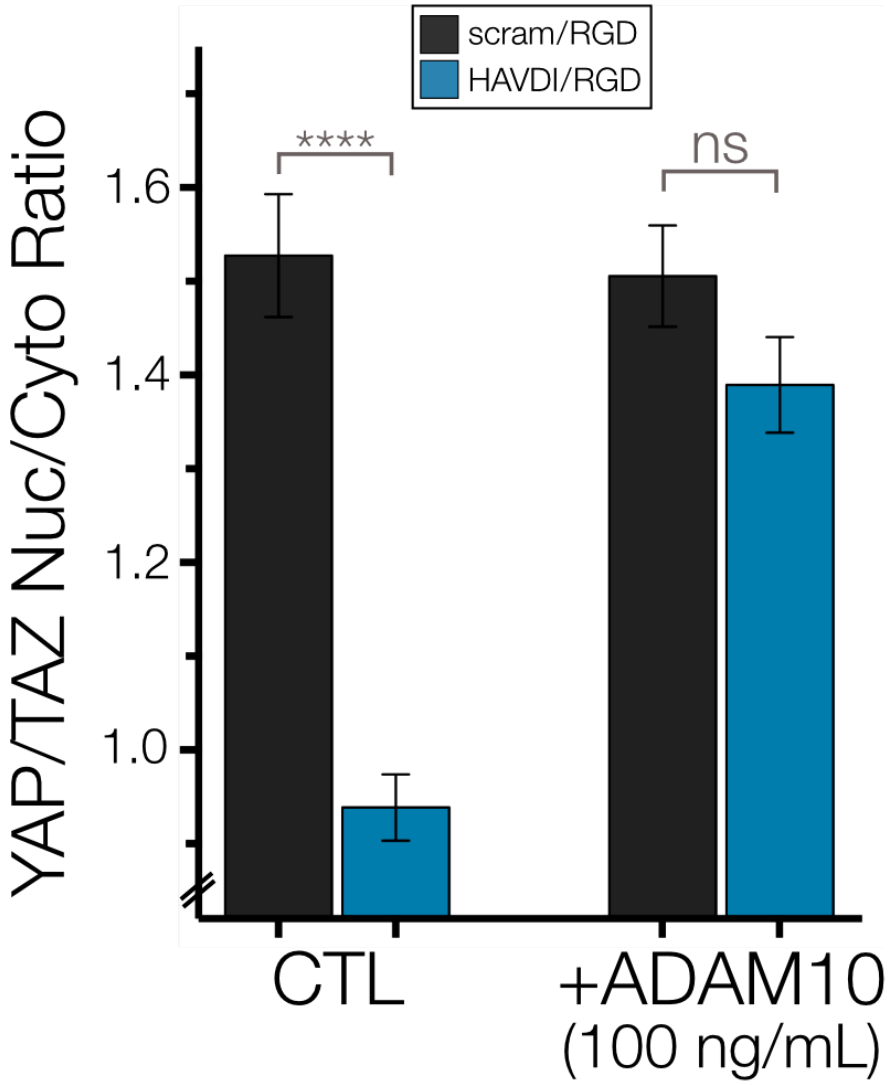
Supp. Fig. 4 – β-Catenin immunostaining in MSCs on scram/RGD and HAVDI/RGD hydrogels. Representative confocal microscopy images of basal plane β-Catenin immunostaining in MSCs cultured on 10 kPa hydrogels. Scale bar = 10μm.



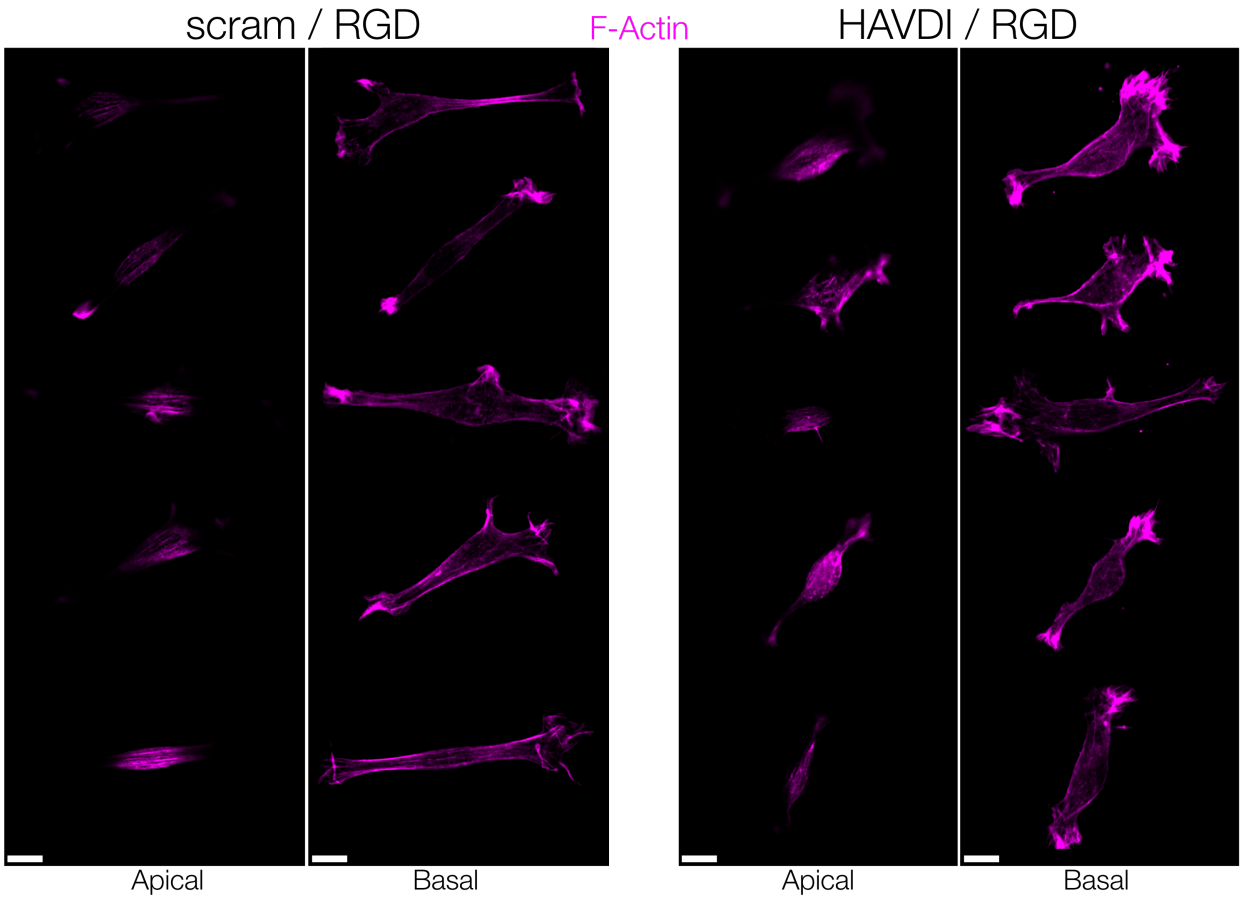
Supp. Fig. 5 – YAP/TAZ ratios in hMSCs cultured at intermediate substrate stiffnesses. YAP/TAZ nuclear to cytoplasmic ratios of human MSCs cultured on 5 and 10 kPa substrates (n>22 cells/group, *=p<0.05, ***=p<0.001 by 1-Way ANOVA with Bonferroni post-hoc, mean ± SEM).



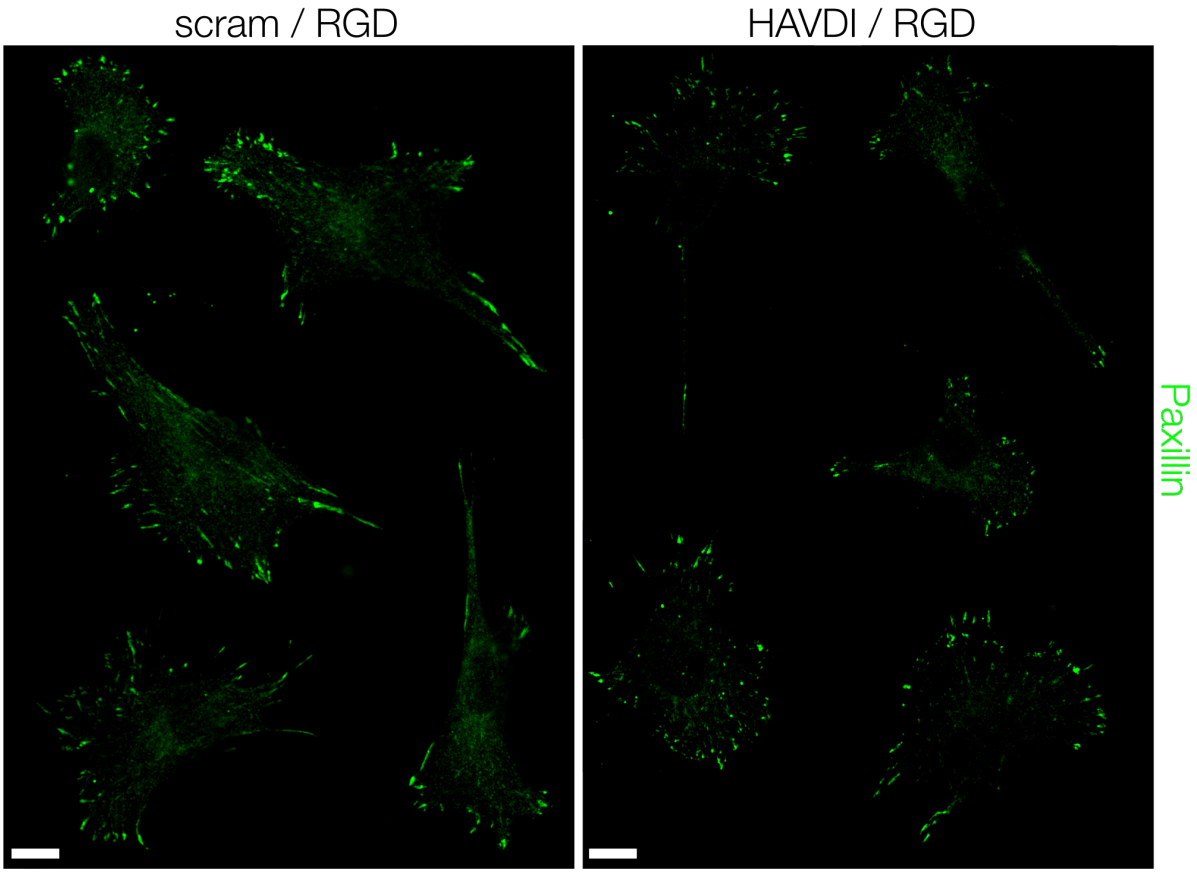
Supp. Fig. 6 – **Early osteogenic differentiation of MSCs.** Representative mosaic confocal microscopy images of RUNX2 immunostaining in MSCs cultured on 10 kPa hydrogels following 1 day of culture in growth media (GM) and two days of culture in osteoinductive media (OM). Scale bar = 10 μm.



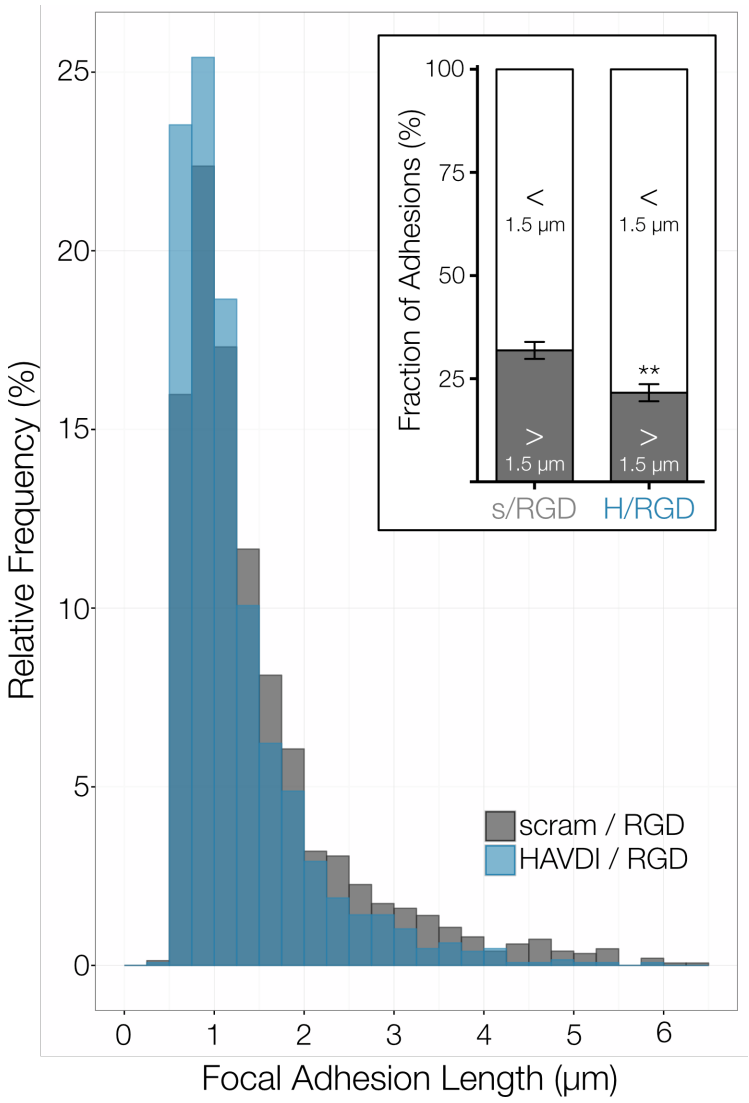
Supp. Fig. 7 – Treatment with ADAM10 abrogates HAVDI-driven alterations in ECM mechanosensing. YAP/TAZ ratios in MSCs cultured on 10 kPa scram/RGD and HAVDI/RGD hydrogels following the addition of 100 ng/mL recombinant ADAM10 for 4hr (n>106 cells/group, **** = $p<0.0001$ by 1-Way ANOVA with Kruskal-Wallis post-hoc, mean +/- SEM shown).



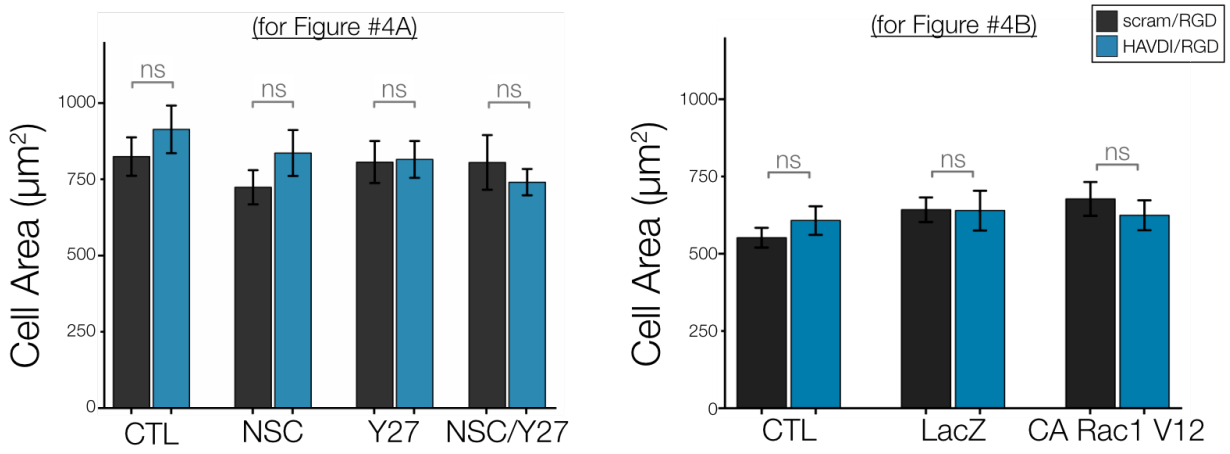
Supp. Fig. 8 –- F-Actin anisotropy of MSCs on MeHA hydrogels. Representative mosaic confocal microscopy images of apical and basilar F-Actin immunostaining in MSCs cultured on 10 kPa hydrogels following 1 day of culture in growth media. Scale bar = 10 μm.



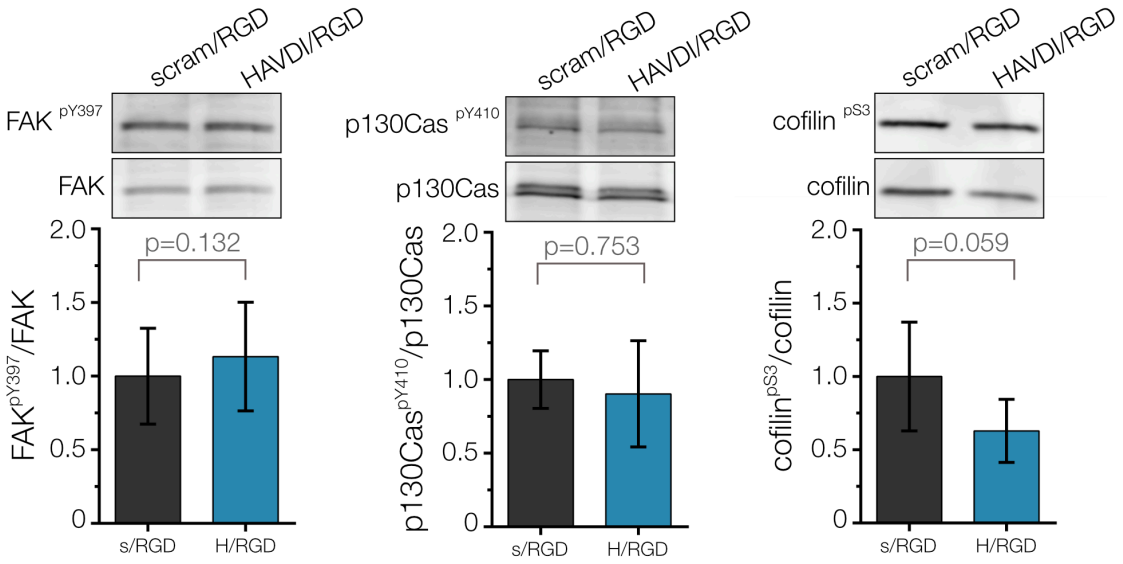
Supp. Fig. 9 – Paxillin immunostaining to assay focal adhesion characteristics. Representative mosaic confocal microscopy images of paxillin immunostaining in MSCs cultured on 10 kPa hydrogels. Scale bar = 10 μm.



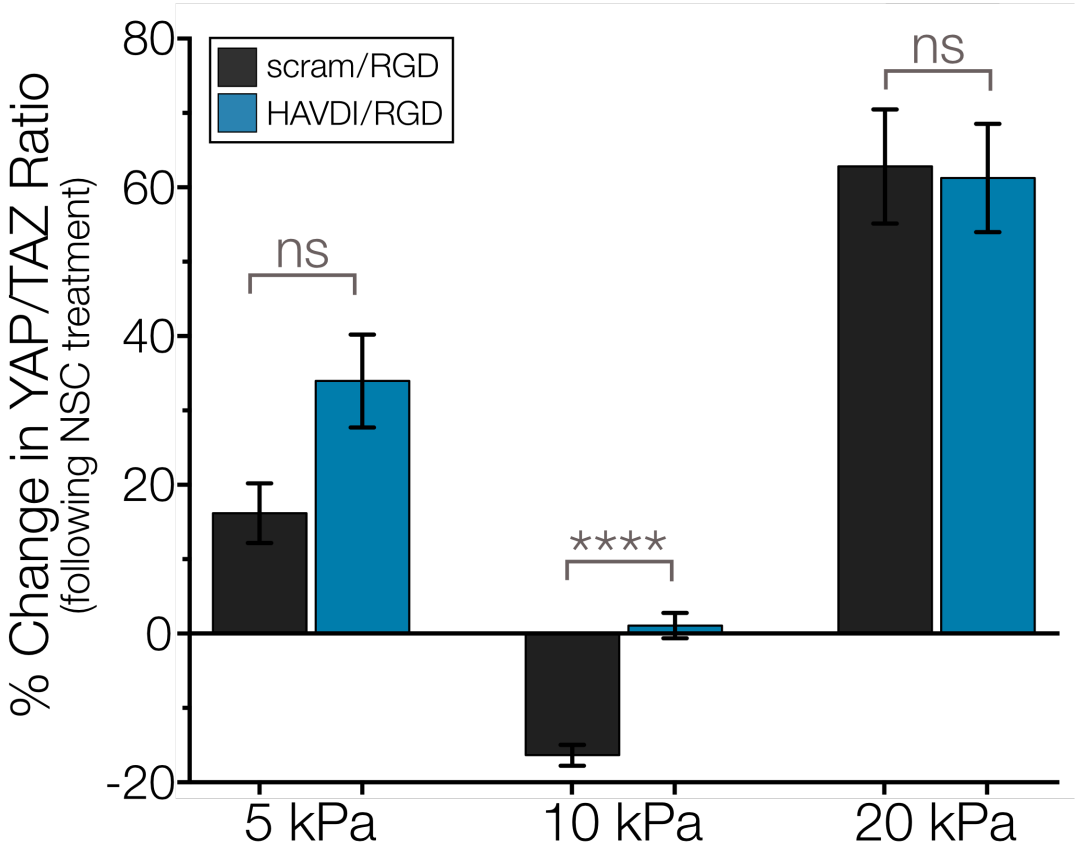
Supp. Fig. 10 – Depletion of the large focal adhesion population with culture on HAVDI/RGD hydrogels. Histograms of focal adhesion length (determined via Paxillin staining) for MSCs culture on scram/RGD and HAVDI/RGD substrates for 18h, n>1275 adhesion/group for n=18 cells/group. (inset) Percentage of focal adhesion population over 1.5 μm in length for each cell (n=18 cells/group, ** indicates p<0.01 by Student's t-test).



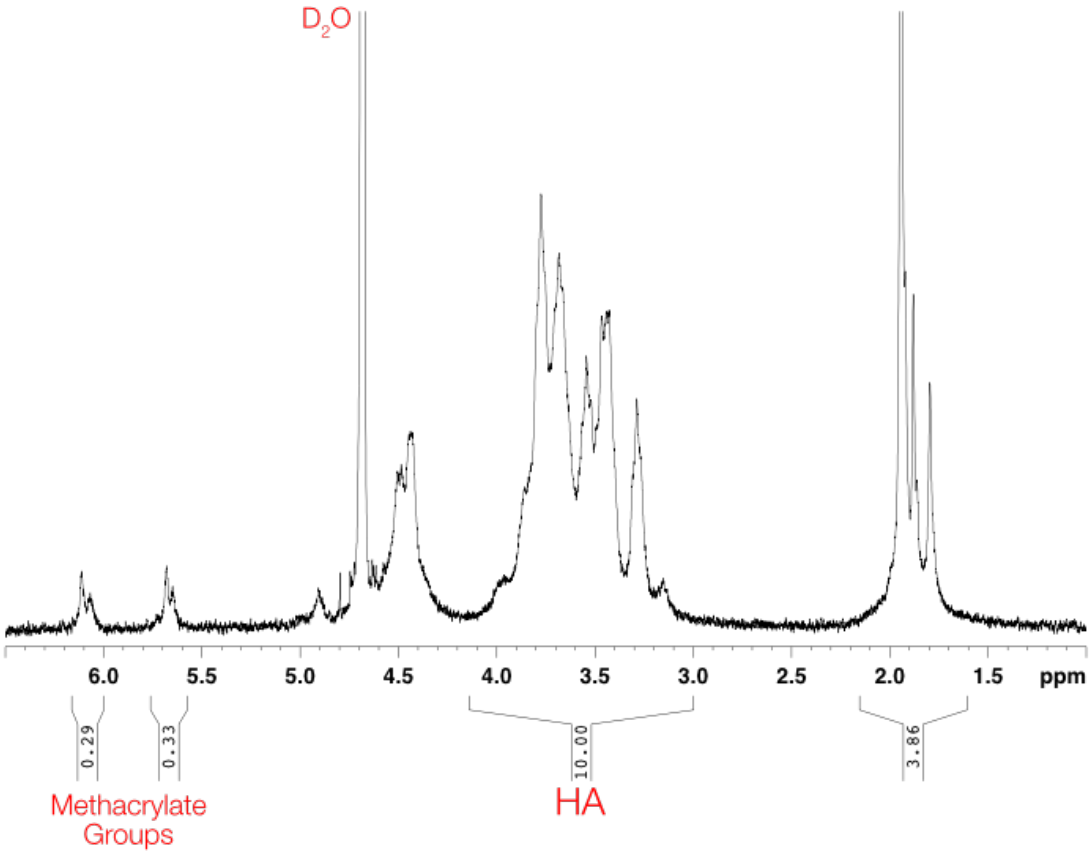
Supp. Fig. 11 – **Cell spread area following perturbations to Rho and Rac signaling.** MSC spread areas corresponding to Figure 4A-B. Comparisons were made between MSCs on scram/RGD hydrogels and HAVDI/RGD hydrogels (n>51 cells/group, Stats by 1-Way ANOVA with Bonferroni post-hoc testing).



Supp. Fig 12 – Western blotting for upstream mediators of substrate stiffness sensing. Western blotting for pFAK/p130Cas/pCofilin in MSCs cultured on 10 kPa MeHA hydrogels for 18 hours. Densitometry plotted as a ratio of phospho-to-total levels and both groups were normalized such that average values of scram/RGD controls were equal to 1 (n=3, Stats by paired t-test, mean ± SEM).



Supp. Fig 13 – **Rac1 activation contributes to HAVDI-driven YAP/TAZ signaling on 10 kPa MeHA-RGD substrates, but not on 5 or 20 kPa MeHA substrates.** Percent change in YAP/TAZ nuclear-to-cytoplasmic ratios in MSCs cultured on scram/RGD and HAVDI/RGD hydrogels following treatment with 50 μM NSC-23766 for 1 hr to block Rac1 activity (n>53 cells/group, ****=p<0.0001 by 1-Way ANOVA with Kruskal-Wallis post-hoc, mean ± SEM).



Supp. Fig 14 – ^{1}H NMR Spectra for methacrylated hyaluronic acid (MeHA). Spectra indicates ~ 31% modification of the HA backbone with methacrylate groups.