Electronic Supplementary Material (ESI) for Integrative Biology

Hyaluronic Acid Matrices show Matrix Stiffness in 2D and 3D Dictates Cytoskeletal Order and Myosin-II phosphorylation within Stem Cells

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Supplementary Methods:

Cell culture

Human mesenchymal stem cells (hMSCs) were purchased from Lonza and cultured in MSC growth media consisting of low glucose DMEM (Invitrogen) supplemented with 10 % fetal bovine serum (FBS, Sigma) and 1 % penicillin/streptomycin (Invitrogen) in regular tissue culture treated flasks (Corning) at 37°C / 5.0 % CO_2. Media was exchanged every other day and cells were used up to passage number nine. For passaging of the cells, flasks were rinsed with PBS and the cells were trypsinized with 0.25 % Trypsin/EDTA (Invitrogen) for 3 min. After washing with media the appropriate number of cells were plated on hydrogels or replated in new flasks. To assure that cells are isolated, only 400 cells per cm^2 were plated on gels.

Cell viability was checked using the cell proliferation kit WST-1 (Roche Applied Biosciences) based on water-soluble tetrazolium salt. Cells were incubated with the reagent for 30 min and the amount of produced formazan dye was quantified with a spectrophotometer (SmartSpec™ Plus, Bio-Rad).

Immuno-staining, microscopy, and image analysis

Cells were fixed for 5 min in a 10 % solution of formaldehyde (Sigma) in PBS (Gibco) and subsequently permeabilized with a 0.5 % solution of TritonX 100 (Sigma) in PBS followed by rinsing in PBS. Samples were then incubated in a 1:250 solution of the appropriate primary antibody in a 3% solution of BSA (Sigma) in PBS for at least 2 hours at room temperature on a rocker. To remove any unspecificly bound antibodies the gels were incubated with a 0.5 % solution of TritonX 100 for 5 min and rinsed with PBS twice, followed by incubation with secondary antibodies (1:250 in 3% BSA in PBS) and rhodamine-phalloidin (Sigma) for at least one hour on a rocker at room temperature. Finally, the nucleus was stained using the Hoechst dye (#33342, Invitrogen) for approximately 5 min at a dilution of 1:10,000. Again to remove all non-specific bound material the samples were incubated with a 0.5 % solution of TritonX 100 for 5 min and rinsed extensively with PBS to remove residual dye for a clean background.

Images were taken on an inverted microscope (IX 71, Olympus) using a 20x air objective (LCAch, N.A. = 0.4) and a 1.6x post magnification lens, equipped with a digital Cascade 512B (Photometrix) camera, and acquired with ImagePro (Media Cybernetics). To obtain unbiased images, well separated cells were selected first in the nucleus channel and then the other fluorescence channels were recorded.

Z-stacks were recorded with a laser scanning confocal microscope (Fluoview 100, Olympus) using a 60x oil immersion objective (N.A. 1.45, Olympus). The 3D reconstruction was done with the program Voxx.[37]

Cell area *A* and spindle factor *r* (ratio of major to minor axis of a fitted ellipse) were determined using the built-in functions of ImageJ (available at http://rsb.info.nih.gov/ij). For all quantitative analysis at least 30 cells were recorded and all cell images in this paper show the cells that were representatives of the respective average values determined by a least-square method.

Western blotting and Immunoprecipitation

At least 10^5 cells were grown under each culture condition, and lysates were generated on ice with lysis buffer (150mM sodium chloride, 1% NP-40, 1% protease inhibitor cocktail, 1mM activated sodium orthovanadate, 50 mM TRIS at pH 8.0) for 30 min. For immunoprecipitation, 30mM of pervanadate solution was prepared by mixing sodium orthovanadate with H_2O_2 for 15 min at room temperature. Cells were treated with pervanadate to maintain pTyr for Westerns or IP with pTyr-specific antibodies (Santa Cruz). Cells were then washed; lysed and whole cleared lysate was mixed with anti- antibody at 4°C overnight, followed by incubation with Protein G Beads (Invitrogen) for 1hr. For Western blot, whole lysate or immunoprecipitated proteins were separated on 4-12% SDS-PAGE gels (NuPAGE 4-12% Bis-Tris, Invitrogen). They were then transferred to a polyvinylidene fluoride (PVDF) membrane with an iBlot Gel Transfer Device (Invitrogen), followed by blocking with 5% non-fat dry milk solution for 1hr. Incubation with primary antibodies was done at 4°C overnight with 1:1,000 β-actin, 1:250 pTyr and 1:1,000 NMM-IIA antibodies. For all membranes and after washing, we incubated 1:2,500 anti-rabbit and 1:1,000 anti-mouse HRP-conjugated IgG antibodies at room temperature for 1hr. The blot was developed with ChromoSensor (GenScript, Piscataway, NJ) for 5 min, followed by digital scanning to perform densitometry analysis by ImageJ (NIH).

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Supplementary Figures:

Fig. S1 ^{1}H NMR of HA-S. ^{1}H NMR spectra of HA-S in D_2O: (a) and (b) are the new resonances appearing due to the addition of the thiol group at δ = 2.72 and 2.57, respectively, while (c) represents the *N*-acetylmethyl protons of HA at δ = 1.88, used as reference peak for the disaccharide.

Fig. S2 AFM elasticity measurement and quenching of excess thiols yields stable gels. (A) Force – indentation plot of a soft HA gel. Black line represents measured data points, red broken line is the best fit using the Hertz model yielding a Young's modulus of 0.1 kPa. (B) Open squares show three different HA gel compositions measured at day 1, 2, 4, and 7 after cross-linking stiffening in an exponential manner with a characteristic time constant of approximately 1 day. The closed circles represent three HA gel compositions that where quenched after gelation retaining a stable elasticity over time for at least 4 weeks.

Fig. S3 HA-S is not biologically active compared to native HA. (A) Graph shows binding affinity of fluorescently labeled HA to MSCs is significantly reduced by adding HA as competing partner but not when adding HA-S, demonstrating that HA-S is not biologically active due to the chemical modification. (B) CD44 expression on hMSCs.

Fig. S4 Spreading dynamics in 2D and 3D (A) Kinetics of major (upright triangles) and minor (upside down triangles) axis and (B) spindle factor *r* for cells overlaid with a sandwich gel after 1 hour (closed symbols) and control cells receiving identical treatment except overlay (open symbols) indicated by the rose box. The dashed grey line is a fit determined from kinetics on PA gels shifted by 4 hours (sandwich preparation time) demonstrating that the sandwich procedure only delays the usual process. The dashed black line is a best fit to the sandwich data points.

Fig. S1:

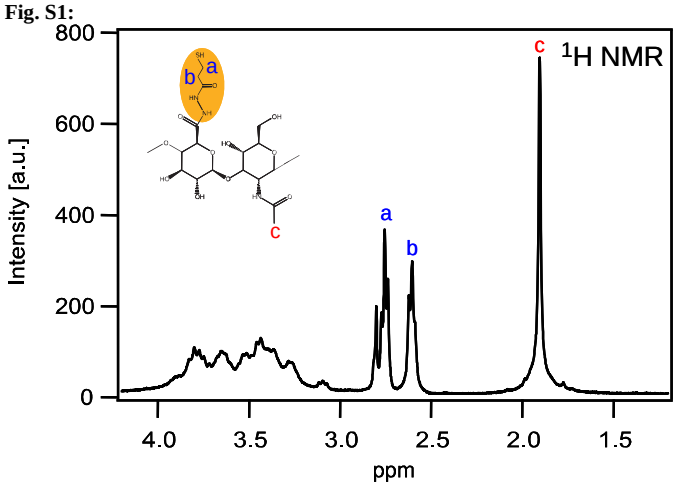


Fig. S2

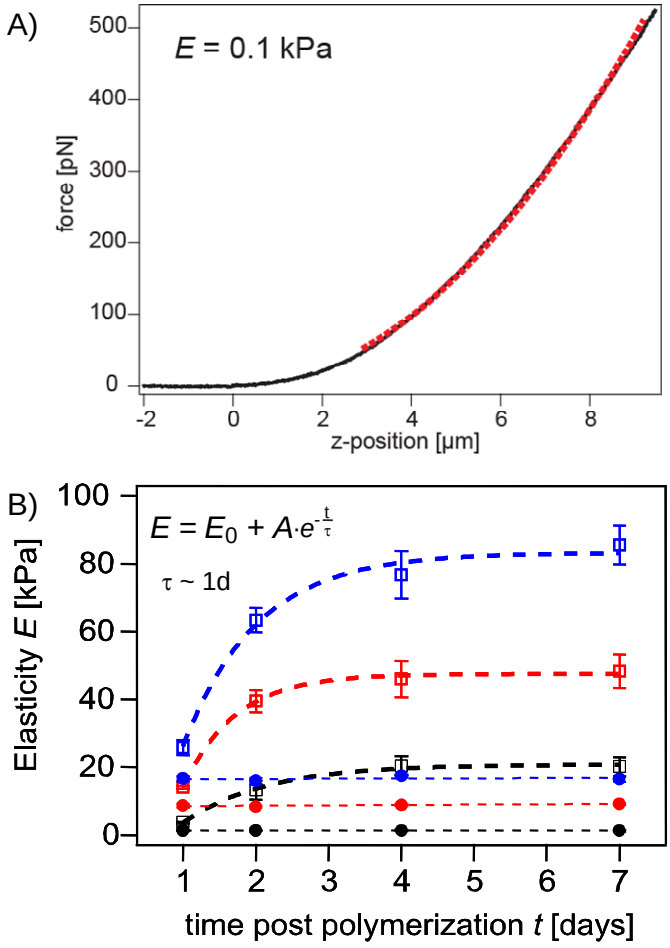
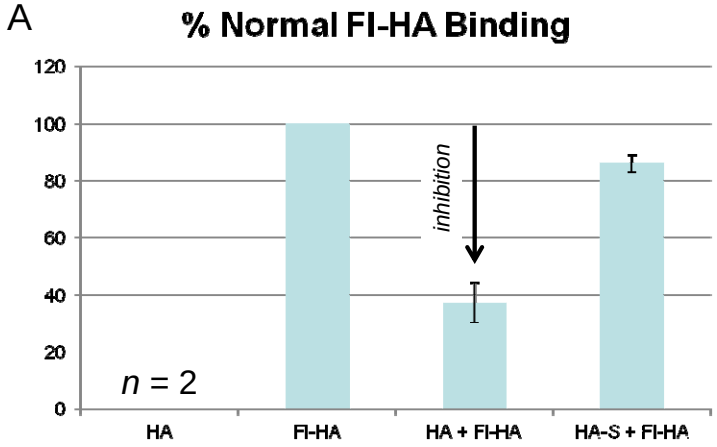


Fig. S3

A



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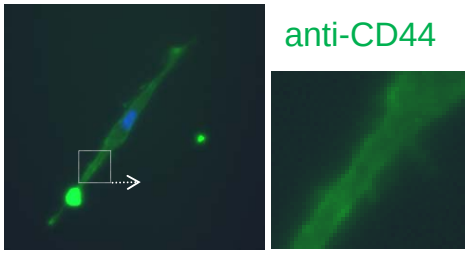

Fig. S4