High content image analysis of focal adhesion-dependent mechanosensitive stem cell differentiation

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Running Title: “Focal adhesion proteins and mechanosensitive differentiation”

Keywords: SORBS1, ponsin, stiffness, hydrogel

Manuscript Information:

Abstract: 173 words

Body: 5216 words (including references and figure legends)

Figures: 6

Supplemental Figures: 4

Supplemental Tables: 3

Abstract

Human mesenchymal stem cells (hMSCs) receive differentiation cues from a number of stimuli, including extracellular matrix (ECM) stiffness. The pathways used to sense stiffness and other physical cues are just now being understood and include proteins within focal adhesions. To rapidly advance the pace of discovery for novel mechanosensitive proteins, we employed a combination of *in silico* and high throughput *in vitro* methods to analyze 47 different focal adhesion proteins for cryptic kinase binding sites. High content imaging of hMSCs treated with small interfering RNAs for the top 6 candidate proteins showed novel effects on both osteogenic and myogenic differentiation; Vinculin and SORBS1 were necessary for stiffness-mediated myogenic and osteogenic differentiation, respectively. Both of these proteins bound to MAPK1 (also known as ERK2), suggesting that it plays a context-specific role in mechanosensing for each lineage; validation for these sites was performed. This high throughput system, while specifically built to analyze stiffness-mediated stem cell differentiation, can be expanded to other physical cues to more broadly assess mechanotransduction and increase the pace of sensor discovery.

Introduction

Although physical properties of the niche have become widely recognized for their influence on a host of cell behaviors [1-3], significant attention has been paid to the influence of extracellular matrix (ECM) stiffness on stem cells [4-6]. While initially reported to be myosin contractility sensitive [7], their upstream mechanisms have remained unclear. Recently, however, mechanisms have been proposed involving the nucleus [8], translocation of factors to the nucleus [9], Rho GTPases [10], stretch activated channels [11], and focal adhesions, i.e. "molecular strain gauges" [12]. While numerous mechanisms may overlap, it is clear from these examples that many sensors within each category are still undetermined.

High throughput systems [13] for mechanotransduction have yet to play as significant a role as they have in other biomedical and engineering contexts, e.g. biomaterial microarrays to explore niche conditions [14,15] and microcontact printing to explore the influence of cell shape [16]; this may be due to fabrication limitations with small volume hydrogels, imaging limitations with thick hydrogels at high magnification, and biological limitations with high throughput molecular screening in stem cells. For example, hydrogels are often fabricated in larger 6- and 24-well formats [7,17,18] and have been used to investigate how a variety of niche properties influence cells [19]. Creating physiologically relevant substrates in small volumes to elicit appropriate cell behaviors is challenging but not unprecedented [20]; ensuring that the imaging plane is flat in such small wells, however, has proven difficult and has limited high resolution imaging required for many stem cell applications. Despite these challenges, it is clear that discovery of novel

mechanotransductive proteins will require screening due to the sheer number of proteins that could be involved in each mechanism type [8-12].

To create a high throughput screen of mechanotransductive proteins and their effects on stem cells, high content screening analysis of multiple cell parameters for phenotyping [21,22] is required in addition to high throughput screening systems [23]. While this combination has been used in pre-fabricated small interfering RNA (siRNA) [24] or polymer arrays [15] to examine stem cell pluripotency, their combination in a high throughput array to study mechanically sensitive stem cell differentiation has been technically challenging. Here, we have overcome the imaging challenges associated with the 96 well hydrogel array format [20] and combined it with a focal adhesion siRNA screen to determine novel mechanotransductive sensors. We report the identification of several protein hits that may regulate lineage-specific, substrate stiffness dependent differentiation.

Experimental

Cell Culture and Reagents

Human mesenchymal stem cells (Lonza) were maintained in growth medium (DMEM, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin) which was changed every four days (except in 96 well plates). Only low passage hMSCs were used for experimental studies, i.e. less than passage 9. For MAPK1 inhibition, the MAPK1 inhibitor pyrazolylpyrrole, dissolved in DMSO, was used at a final concentration of 2 nM and added to cells immediately post-plating. At 2 nM, pyrazolylpyrrole is extremely

87 selective and has only been shown to inhibit MAPK1, limiting potential off-target effects
88 [25]. Non-differentiation based experiments, including western blots and durotaxis assays,
89 were performed after 24 hours while siRNA-induced protein knockdown was at a
90 maximum. Conversely, differentiation experiments took place over the course of four
91 days, since differentiation occurs as the integration of cues over time.
92
93 ***Polyacrylamide Hydrogel Fabrication in 6- and 96-Well Formats***
94 Acrylamide was polymerized on aminosilanized coverslips. A solution containing the
95 crosslinker N, N' methylene-bis-acrylamide, the monomer acrylamide, 1/100 volume
96 10% Ammonium Persulfate and 1/1000 volume of N, N, N', N'-
97 Tetramethylethylenediamine was mixed. Two different combinations of acrylamide and
98 bis-acrylamide were used to make hydrogels of 11 and 34 kilopascal (kPa; a unit of
99 stiffness). Approximately 50 μL of the mixed solution was placed between 25 mm
100 diameter aminosilanized coverslips and a chlorosilanized glass slide for 6-well plates.
101 100 μg/mL collagen I was chemically crosslinked to the substrates using the
102 photoactivatable crosslinker Sulfo-SANPAH (Pierce). Custom 96 well plates containing
103 collagen type I-conjugated polyacrylamide hydrogels crosslinked to glass bottom
104 surfaces (Matrigen) were fabricated containing equal numbers of 15 kPa wells and 42
105 kPa hydrogels to induce myogenesis and osteogenesis, respectively (Figure S1A).
106 Stiffness values were verified using an MFP3D-Bio atomic force microscope (Asylum
107 Research, Santa Barbara, CA) using previously established methods (Figure S1B) [26,27].
108 Polyacrylamide gel thickness was also verified using a BD CARV II confocal microscope
109 (Figure S1C,D).

siRNA Transfection

siRNA oligonucleotides against human vinculin, p130Cas, SORBS1 (Ponsin), SORBS3 (Vinexin), Palladin, Paxillin, and Filamin (ON-TARGETplus SMARTpool; Thermo Fisher Scientific, Waltham, MA) and a pool of four non-targeting siRNAs control oligonucleotides (ON-TARGETplus siControl; Dharmacon), diluted in DEPC water (OmniPure, EMD) and 5X siRNA buffer (Thermo Fisher Scientific, Waltham, MA), were transiently transfected into human hMSCs using Dharmafect 1 (Thermo Fisher Scientific, Waltham, MA) at an optimized concentration of 50 nM in low serum antibiotic free growth media, according to the manufacturers' protocols. Specific siRNA sequences can be found in Supplemental Table 1. Protein knockdown was characterized by western blot and immunofluorescence. After 24 hours of transfection in antibiotic-free media (2% FBS), media was replaced with standard hMSC growth media and cells replated onto appropriate substrates.

Plasmid Transfection

pEGFP-C1 sub-cloned with complete Vinculin cDNA, which had been originally excised from p1005 with EcoRI and inserted in EcoRI digested pEGFP-C1 (labeled as FL), was obtained from Dr. Susan Craig [28]. L765I mutant Vinculin plasmids were obtained via site-directed mutagenesis on FL Vinculin plasmids. All plasmids were purified using QIAGEN Plasmid Midi Kit (Qiagen). hMSCs were transfected in antibiotic-free medium with 1 mg of plasmid precomplexed with 2 μl of Lipofectamine 2000 (Life Technologies)

in 100 µl of DMEM. After 24 hours of transfection in antibiotic-free media with 2% FBS, media was replaced with standard hMSC growth media.

Immunofluorescence

hMSCs were fixed with 3.7% formaldehyde for 30 minutes at 4^{o}C and permeabilized with 1% Triton-X for 5 minutes at 37^{o}C. The cells were then stained with primary antibodies against human MyoD (sc-32758, Santa Cruz), Myf5 (sc-302, Santa Cruz, Dallas, TX), Osterix (ab22552, Abcam), CBFA1 (RUNX2) (sc-101145, Santa Cruz), pMAPK1 (ab76165, Abcam), MAPK1 (ab124362, Abcam), Vinculin (ab129002, Abcam), p130Cas (ab108320, Abcam), SORBS1 (ab4551, Abcam), SORBS3 (GTX-115362, Genetex), Filamin (ab51217, Abcam), or Paxillin (ab32084, Abcam). Corresponding secondary antibodies were conjugated to Alexa Fluor 488 (FITC) or Alexa Fluor 647 (Cy5) (Invitrogen). Nuclei were counterstained with Hoechst dye (Sigma), and the actin cytoskeleton was stained with rhodamine-conjugated phalloidin (Invitrogen). Cells not plated in 96 well plates were imaged with a Nikon Eclipse Ti-S inverted fluorescence microscope equipped with a BD Carv II camera.

High Content Imaging and Analysis

96 well plates were imaged on a CV1000 Cell Voyager (Yokogawa). Briefly, images were acquired through 5 z-positions with 10 µm step sizes at 25 different points in each well with three different filter sets (FITC, TXRD, and DAPI). Maximum Intensity Projections (MIPs) were constructed from the resulting stitched z-stacks to account for uneven hydrogel surfaces and analyzed using a semi-automated image analysis pipeline

in CellProfiler [29]. Nuclear outlines were obtained as primary objects with automatic Otsu Global thresholding (Figure S2A) and cell outlines were obtained using the TXRD channel as secondary objects using a Watershed Gradient algorithm (Figure S2B). The pipeline calculated morphological attributes (such as cell area, aspect ratio, and eccentricity) for each cell, as well as the mean and integrated density of the FITC channel signal in nuclei, cell outlines, and cytoplasm outlines. From these data, one could distinguish cells with nuclear expression only, cytoplasm expression only, uniform positive expression, and uniform negative expression, as shown with example cells in Figure S2C. Data analysis was performed with Microsoft Excel, GraphPad Prism, and CellAnalyst [30].

Western Blots

Cell lysates were collected by rinsing samples with cold PBS, followed by a five minute lysis in mRIPA buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1% Triton, 1% Na-DOC, 0.1% SDS) with 1 mM EGTA, 1 mM Na3VO4, 10 mM Na4P2O7, and 1 mM PMSF (protease inhibitors). Cell lysates were separated via SDS-PAGE, transferred to PVDF membranes (Bio-Rad), and washed in Buffer A (25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) + 4% SeaBlock (Thermo Fisher Scientific, Waltham, MA) overnight at 4^{o}C. Membranes were incubated with anti-Vinculin, GAPDH, Actin, ERK2, p-ERK2 (T202 and Y204), p130Cas, SORBS1, SORBS3, Filamin, or Paxillin antibodies for 1 hour, washed with Buffer A containing SeaBlock, and incubated in streptavidin horseradish-peroxidase-conjugated secondary antibodies (Bio-Rad) for 30

minutes at room temperature. Immunoblots were visualized using ECL reagent (Pierce). All western blot antibodies were obtained from Abcam (Cambridge, England).

Quantitative PCR

mRNA was isolated from hMSCs grown after 4 days with Trizol, and subsequently treated with chloroform and precipitated with isopropanol. The cell lysate was centrifuged and the pellet washed in ethanol twice, after which the pellet was allowed to dry before resuspension in DEPC water. cDNA was assembled through reverse transcriptase polymerase chain reaction (RT-PCR) for one hour at 37^{o}C, followed by a 5 minute inactivation step at 99^{o}C. 1 μL of the resulting cDNA mixture was added to 12.5 μL SYBR Green Real Time PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) containing 0.25 nM forward and reverse primers (Supplemental Table 2) and enough DEPC water to bring the total reaction volume per well to 25 μL.

Immunoprecipitation

Cell lysates were collected with a non-denaturing lysis buffer (20 mM Tris-HCl pH 8, 127 mM NaCl, 1% Nonidet P-40, 2 mM EDTA). Anti-ERK2 antibody (Abcam ab124362) was bound to protein G-conjugated Dynabeads (Life Technologies, Carlsbad, CA, USA) for 1 hour at 4^{o}C with gentle agitation. Beads were magnetically captured, the supernatant removed, and the pellet incubated overnight at 4^{o}C before Western Blot analysis.

Statistics

All experiments were performed in triplicate with the indicated number of cells analyzed per condition. Error bars are shown as standard deviation. Significance was assessed by ANOVA at a significance threshold of $p<0.05$ or lower as indicated. Values less than 0.1 were noted. For instances where data is not significantly different, N.S. is stated.

Results and Discussion

High Throughput Assessment of Focal Adhesion-based Mechanosensing

Within exceedingly complex focal adhesions [31], we selected 47 candidates that bind to multiple proteins at their N- and C-terminal ends such that they could be unfolded when one end of the protein is displaced relative to the other, i.e. a "molecular strain sensor" [12]. These candidates were analyzed with ScanSite [32] to identify cryptic binding sites contained within their structure; MAPK1 binding sites were specifically selected, because of the kinase binding sites analyzed, MAPK1 was the most abundant and had the highest average inaccessibility (Figure 1A, Supplemental Table 3) [17]. Thus if force-sensitive kinase binding is a signaling mechanism at focal adhesions, MAPK1 is the most likely candidate to affect stem cell differentiation because it could impact signaling across a wide variety of cellular pathways. Of the proteins analyzed [17], five of them–Vinculin, p130Cas, Filamin, SORBS1 (Ponsin), SORBS3 (Vinexin)–had multiple binding sites to other proteins, which would allow the protein to be strained and change configuration under an appropriate amount of force, F (Figure 1B). The change in conformation could then expose a MAPK1 binding site predicted to be cryptic, but only under the appropriate amount of force; paxillin was selected as a control protein as it did not have a cryptic MAPK1 binding site (Figure S3). siRNAs were used to transiently

knock down candidate proteins, which was verified by western blot (Figure 2A) and immunofluorescence (Figure 2B-C). Although we chose to focus on MAPK1, other less prevalent cryptic kinase binding sites exist and could result in other force-induced signaling cascades.

To induce osteogenesis, hMSCs were plated into wells containing 42 kPa hydrogels within the high throughput hydrogel array and stained for osteogenic transcription factors Osterix and CBFA1; transcription factors were specifically chosen as outputs in identifying mechanosensitivity because both the expression and nuclear localization could be used as criteria for lineage commitment (Figure S2). To further reduce the false discovery rate, we only classified a protein as a mechanosensor if their knockdown impaired stiffness-induced differentiation as assessed by both transcription factors. In the osteogenesis assay, we found that p130Cas, Filamin, Paxillin, and SORBS3 (Vinexin) knockdown did not affect osteogenic differentiation signals after 4 days relative to day 0 expression and localization. Conversely, the knockdown of SORBS1 (Ponsin), which interacts with vinculin [33] and plays a role in insulin signaling [34], reduced both CBFA1 and Osterix nuclear expression by over 50%. Vinculin knockdown, which was previously shown to not affect CBFA1 expression [17], slightly reduced CBFA1 but not Osterix expression (Figure 3); no myogenic expression was found in these cells (data not shown). Thus, we concluded that SORBS1 could act as a unique stiffness-mediated sensor for osteogenic differentiation.

Untreated hMSCs were also plated on 15 kPa substrates within the high throughput hydrogel array and exhibited elevated levels of myogenic markers MyoD and Myf5 at day 4 relative to day 0. High throughput analysis indicated that siRNA knockdown of Vinculin, p130Cas, or SORBS3 resulted in a loss of stiffness-induced expression of both MyoD and Myf5 at day 4. This is in agreement with recent reports of vinculin-mediated SORBS3 mechanosensing [35]. However, Filamin, SORBS1, and Paxillin only reduced expression of one of the two myogenic markers (Figure 4). Paxillin does not contain a cryptic MAPK1 binding site, so Myf5 reduction may be due to other predicted cryptic binding domains that it contains, e.g. MAPK3; no osteogenic expression was found in these cells (data not shown). Thus, we concluded that Vinculin could act as a unique stiffness-mediated sensor for myogenic differentiation, consistent with prior reports [17].

If knockdown of the candidate focal adhesion proteins disrupts not just mechanosensitive signaling but also other normal cell behaviors, stiffness-mediated differentiation differences may not solely be related to signaling. Along with expression, CellProfiler was used to measure cell area and morphology, i.e. eccentricity, neither of which changed significantly with siRNA treatment (Figure S4A-B). Cell migration speed was also unaffected by siRNA knockdown, although SORBS3 knockdown appeared to increase migration persistence (Figure S4C). Perhaps most importantly, focal adhesion assembly in terms of size and distribution appeared unaffected in single knockdown experiments; outside of the expected loss of expression of the proteins being knocked down, no changes were observed in these focal adhesion characteristics (Figure S4D).

Validation of MAPK1-based Differentiation and Interaction with Candidate Mechanosensors

To analyze the effect of MAPK1 inhibition on substrate stiffness directed hMSC differentiation, MAPK1 was inhibited with pyrazolylpyrrole at day 0. Consistent with its use on 11 kPa [17], we found that hMSCs in the high throughput system exhibited a similar 50% reduction in nuclear-localized MyoD as well as Myf5 (Figure 5A). The effect of MAPK1 inhibition on stiffness-mediated osteogenesis is clear, but after 4 days in culture on 34 kPa substrates, pyrazolylpyrrole-treated hMSCs also exhibited reduced osteogenic transcription factor expression and localization equivalent to SORBS1 knockdown (Figure 5B). However, since pyrazolylpyrrole is a global MAPK1 inhibitor, it may inadvertently reduce lineage commitment through non-stiffness mediated mechanisms.

To verify hits directly using more targeted molecular methods, SORBS1 was immunoprecipated via MAPK1. For hMSCs cultured for 24 hours on 34 kPa PA gels, SORBS1 was detected in the pellet but not the unconcentrated whole cell lysate, suggesting that, although expressed at low levels, SORBS1 and MAPK1 interact in cells cultured on physiological-stiffness gels (Figure 6A). SORBS1 contains two predicted binding sites for MAPK1 at L500 and L1033 (Figure S2B), but among the twelve SORBS1 isoforms, only two contain the predicted L1033 binding site [36-38]. qPCR indicated that undifferentiated cells cultured on 34 kPa substrates for 24 hours did not significantly express SORBS1 isoforms containing L1033 (Figure 6B). Lacking other kinase binding domains predicted with high confidence to be inaccessible (i.e. Scansite accessibility prediction less than 0.5), the MAPK1 binding site found on SORBS1 at

L500 is the most likely candidate to act as a stretch sensitive mechanosensor. For Vinculin, which pulls MAPK1 down with immunoprecipitation on 11 kPa substrates [17], MAPK1 binding was predicted at L765 (Figure S2B). To confirm that L765 is specifically required for myogenic differentiation on 11 kPa substrates, a plasmid containing L765I-mutated Vinculin and Green Fluorescent Protein (GFP) was added back to cells that had been treated with Vinculin siRNA. While vinculin knockdown was sufficient to reduce myogenic transcription factor expression in hMSCs, addback of full-length vinculin rescued expression whereas addback of L765I-mutated Vinculin was insufficient to fully rescue expression (Figure 6C, filled vs. open arrowhead, respectively).

While these data specifically focus on screening 47 focal adhesion proteins with a "molecular strain sensor"-like structure as predicted by ScanSite, the list of proteins comprising focal adhesions is much larger and dynamic. Current estimates implicate as many as 232 different components, of which 148 are intrinsic and 84 are transient [39], as a common signature of adhesions. Recent analyses of focal adhesions have even identified more than 1300 distinct proteins within isolated adhesion complexes [40], suggesting exceedingly complex adhesion-based mechanisms for cells that must actively sense their surroundings. Focal adhesion composition and structure have also recently been shown to be relatively stable to external perturbation, including siRNA knockdown or chemical inhibition of components, suggesting that signaling transduction occurs independently of structural integrity [41]. That said, our data also focused on proteins with relatively little functional data, e.g. SORBS1, to establish proof-of-principle that we can use a high

content imaging based platform to identify candidate sensors via their influence on stem cell differentiation. After additional confirmation with rescue experiments, it is possible to validate, e.g. vinculin, or exclude, e.g. SORBS1, candidate sensors.

Beyond stem cell differentiation assays, several alternative high throughput techniques have been adapted for mechanobiology and novel mechanotransduction sensor identification [42] though they do not utilized biomimetic substrates. For example, mass spectroscopy "cysteine shotgun" assays use cysteine-binding dyes to assess differential protein labeling under stress [43] but this approach focuses on the conformational change itself and may overlook downstream signaling changes. Even when applied directly to differential unfolding in response to mechanical signals [44], one could miss transient protein unfolding during signal transduction, especially if cryptic binding domains do not contain cysteine residues. While this RNAi screening approach is more targeted, it can be adapted to fit any instance in which immunofluorescence is used to measure an output, e.g. a response to change in substrate stiffness, and can be specific for nuclear or cytoplasmic expression (Figure S2).

Conclusions

A computational approach was used to select candidate proteins that could potentially play a role in MAPK1-based mechanosensitive differentiation based on an analysis of their binding partners and presence of cryptic signaling sites, i.e. the "molecular strain gauge" structure [12]. A high throughput, high content analysis based system capable of finding hits much more quickly and efficiently was then constructed to test these

candidates, with which we identified SORBS1 and Vinculin as a potential mechanosensors in hMSCs. While this method was applied specifically to the mechanical influence of stiffness on stem cells differentiation, it can be applied to a number of applications in cell biology in which an immunofluorescently-labeled marker is differentially up- or down-regulated in response to a physical stimulus, e.g. stiffness, etc.

Acknowledgements

The authors would like to acknowledge Dr. Jamie Kasuboski for assistance with high content imaging and Dr. Brenton Hoffman for helpful comments on the manuscript. This work was supported by grants from the National Institutes of Health (DP2006460 to A.J.E.) and National Science Foundation (1463689 to A.J.E) as well as a National Science Foundation predoctoral fellowship (to L.G.V.)

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Figures:

Figure 1: ScanSite Results for 47 Different Focal Adhesion Proteins. (A) Each data point represents a predicted binding partner. The y-axis displays the number of times this binding partner was identified during the analysis of the 47 focal adhesion proteins, while the x-axis shows the average accessibility of the binding site. Predicted surface inaccessible binding sites have accessibility values below 1 (gray region). (B) Schematic of force-induced conformational changes by a "molecular strain sensor" where proteins bound to the sensor stretch the it by transmitting a force across the protein. The resulting conformational change exposes the once cryptic binding site at an optimal force, F* (middle schematic). Above or below that value results in excessive deformation of the binding site to prevent binding or not enough stretch causing the site to remain cryptic, respectively.

Figure 2: Confirmation of siRNA-induced Knockdown. (A) Western blots of lysates collected 2 days post siRNA treatment. (B) Immunofluorescence images of proteins being knocked down. (C) Quantification of mean immunofluorescence intensity from knockdown cells. For Vinculin, p130Cas, SORBS3, SORBS1, Filamin, and Paxillin in (C), $n > 10$ cells in triplicate.

Figure 3: Osteogenic Differentiation and Focal Adhesion Protein Knockdown. Normalized mean intensity levels of (A) CBFA1 and (B) Osterix immunofluorescence staining after four days of culture of osteogenically favorable 42 kPa substrates. Representative images show cell outlines along with (C) CBFA1 and (D) Osterix

expression. Filled arrowheads indicate nuclei that maintained transcription factor expression whereas open arrowheads indicate nuclei that lost expression. (E) Heat map indicating fold-change in expression of the indicated osteogenic markers from day 0 wild type cells. For WT, Vinculin, p130Cas, Filamin, SORBS3, Paxillin, SORBS1, and d0 WT in (A) and (B), n=298, 35, 28, 44, 29, 28, 20, and 40, respectively.

Figure 4: Myogenic Differentiation and Focal Adhesion Protein Knockdown. Normalized mean intensity levels of (A) Myf5 and (B) MyoD immunofluorescence staining after four days of culture of myogenically favorable 15 kPa substrates. Representative images show cell outlines along with (C) Myf5 and (D) MyoD expression. Filled arrowheads indicate nuclei that maintained transcription factor expression whereas open arrowheads indicate nuclei that lost expression. (E) Heat map indicating fold-change in expression of the indicated myogenic markers from day 0 wild type cells. For WT, Vinculin, p130Cas, Filamin, SORBS3, Paxillin, SORBS1, and d0 WT in (A) and (B), n=39, 31, 43, 24, 30, 35, 29, and 9, respectively.

Figure 5: MAPK1 inhibition and Myogenic and Osteogenic Differentiation. MAPK1 inhibitor pyrazolylpyrrole (MAPKi) or siRNA for the indicated proteins was applied to cells at the beginning of the 4-day time course on both (A) 11 kPA and (B) 34 kPa substrates and stained for (A) MyoD (white) or Myf5 (gray) and (B) CBFA1 (white) or Osterix (gray) as indicated on day 4. Mean nuclear fluorescence is plotted normalized to untreated cells. **$p<0.01$ and ***$p<0.001$ relative to untreated cells stained for the same transcription factor.

Figure 6: Molecular validation of Mechanosensitive Protein Interactions. (A) SORBS1 blots of lysates without (top) or with immunoprecipitation (middle and bottom) via a MAPK1 antibody. Supernatant and pellet fractions of the immunoprecipitation are shown (middle and bottom, respectively). Prior to lysis, cells were cultured on 34 kPa substrates. (B) qPCR of SORBS1 using primers that target a conserved portion of the gene (labeled All SORBS1) versus a region only found in the two full length isoforms (labeled L1033). Data is normalized to the GAPDH and then the All SORBS1 condition. Input RNA was collected from hMSCs on 34 kPa substrates for 24 hours. (C) Add back of Full-Length (FL) or mutated Vinculin plasmid (L765I) to Vinculin siRNA-treated cells showing GFP and MyoD expression after 4 days on 11 kPa substrates. Filled and open arrowheads indicate where nuclear localized MyoD expression is or should be.

Supplemental Figures:

Figure S1: Characterization of 96 Well Plates. (A) Image of 96 well glass-bottom plate used for cell culture and high content imaging. (B) Plot of atomic force microscope-determined stiffness of 5% and 10% acrylamide hydrogels, i.e. myogenic- and osteogenic-inducing hydrogels, respectively. (C) Schematic illustrating the diffusion based technique used to determine hydrogel thickness. Red circles indicate Texas Red-conjugated beads. (D) Plot of hydrogel thickness for 5% and 10% acrylamide hydrogels.

Figure S2: CellProfiler Pipeline. (A) Nuclei are identified first from the DAPI channel. (B) Using the nuclei as seed regions, cell outlines are identified for each nucleus. (C) Representative images and their average nuclear and cytoplasmic fluorescence are shown to indicate marker expression and distribution information obtained through CellProlifer. Cells with high nuclear expression of the transcription factor are considered to have expressed and localized the factor correctly.

Figure S3: ScanSite Analysis of Candidate Mechanosensors. (A) Plots of surface accessibility reveal regions corresponding to predicted MAPK1 domains. Green highlighted regions denote surface inaccessible binding sites predicted to bind MAPK1 domains. (B) List of sites and surface accessibility values for given predicted MAPK1 binding domains. Note that a surface accessible site has a value above 1 and a completely inaccessible site has a value of 0 in this analysis.

Figure S4: Secondary Metrics from High Content Image Analysis. (A) Average cell area and (B) cell eccentricity, calculated as the ratio between the distance between the two foci of a fitted ellipse and the major axis length of the cell, are plotted as a function of siRNA treatment. For WT, Vinc, p130, Fil, SORBS3, Pax, and SORBS1 in (A) and (B), n=39, 31, 43, 24, 30, 35, 29, and 35, respectively. (C) Cell migration rose plots on tissue culture plastic for each knockdown condition. (D) Staining for Vinculin and Paxillin in the indicated siRNA conditions reveals no substantial differences in focal adhesion morphology as a result of siRNA induced knockdown.

Supplemental Tables

Supplemental Table 1: siRNA sequences used for transient knockdown.

Gene	Accession Number	Sequences
Vinculin	P18206	CAGCAUUUAUUAAGGUUGA, GCCAAGCAGUGCACAGAUA, GAGCGAAUCCCAACCAUAA, UGAGAUAAUUCGUGUGUUA
p130Cas	P56945	GGUCGACAGUGGUGUGUAU, GGCCACAGGACAUCUAUGA, GCAAUGCUGCCCACACAUC, CCAGAUGGGCAGUACGAGA
Paxillin	P49023	GAGCUAACAUCCAUAUUUA, GUGCAACUGUCUUUAAUAU, CCAGUAACUUUCACAUGUA, GAGUUUAUCUGGAGUGUAG
Filamin	P21333	GCAGGAGGCUGGCGAGUAU, GCACCCAGACCGUCAAUUA, GCACAUGUUCCGUGUCCUA, GAAUGGCGUUUACCUGAUU
SORBS1	Q9BX66	CAAGAGCAUUUACGAAUAU, GAGAUGAGCUACAUUGAUG, UAUACCAGCUGAUUACUUG, GAAGAGCACUCAGGACUUA
SORBS3	O60504	GAGAGGCUGUGGCCCAGUA, CAUCUUCCCUGCUAAUUAU, CCAAGGAGCUGACUCUGCA, CCUAACACCUCUCAGAUAC

Supplemental Table 2: Forward and reverse primers for qPCR.

Primer set 1: targeting all isoforms of SORBS1	
Forward primer: GAAGGTAGTCAAGAGGTCGGC	T_m: 60.14 °C
Reverse primer: GGGGGTTCCCAGTCATTTCTT	T_m: 59.92 °C
Primer set 2: targeting SORBS1 isoforms containing L1033	
Forward primer: CACCTCGCCTTGTCACCAA	T_m: 60.23 °C
Reverse primer: GTGGGACGATCTGACCAACT	T_m: 59.39 °C