Fractal heterogeneity in minimal matrix models of scars modulates stiff-niche stem-cell responses via the nuclear exit of a mechanorepressor

P. C. Dave P. Dingal¹‡, Andrew M. Bradshaw¹, Sangkyun Cho¹, Matthew Raab¹, Amnon Buxboim¹, Joe Swift¹, and Dennis E. Discher¹*

¹Biophysical Engineering Labs in Molecular & Cell Biophysics and NanoBio-Polymers, Department of Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA.

‡Present address: Department of Bioengineering, Stanford University, Stanford, California 94305, USA.

*email: discher@seas.upenn.edu; Tel.: +1 215 898 4809
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Supplementary Discussion

Estimation of MMMS collagen concentration

From confocal stack reconstruction, we found that embedded collagen is enriched within 10 microns of the upper surface of MMMS gels of ~80 micron thickness (Supplementary Fig. S2a). For MMMS gels, surface coverage of collagen fiber bundles is ~30% based on Sirius red and/or collagen immunofluorescence staining, which matches that of mdx and DMD skeletal muscle tissue cross-sections (~20-30%; refs. 1 and 2). This leads to a corrected collagen concentration from the nominal value of 0.4 mg/mL to an effective $c \sim 12$ mg/mL. Bulk measurements of scarred mdx muscle yield 2-3 mg/ml hydroxyproline³, which is 10-20% of collagen mass, so that such scarred tissues have a mean collagen concentration of 10-30 mg/mL, which is very similar to our estimate for the MMMS system.

Adhesion and Thin-film Corrections for MMMS gel

In order to create 2D hydrogel substrates for cell culture, polyacrylamide (PA) gels were sandwiched between an allysilanated bottom coverslip and a nonreactive top coverslip (18 and 25 mm in diameter, respectively; Fisher). Embedding fibrous collagen in polyacrylamide hydrogels exhibited tackiness to the gel surface post-polymerization. That is, removal of the top coverslip required more force than without the EC fibers. This again provided evidence for interfacial localization of the fibers. To correct for tackiness of MMMS gel, the force required to remove the top coverslip was measured with a custom-made and calibrated polystyrene cantilever (spring constant $k_{sp} = 804.6$ N/m), attached at one end to a syringe pump (RS-232, Harvard Apparatus) and the other to a nylon string, which in turn was firmly attached to the top coverslip of a stage-immobilized gel. Syringe pump was set to ‘pump mode’ pulling the cantilever-string-coverslip setup at an effective velocity of 0.4 mm/s. The process of removing the top coverslip was observed with a side-view camera to determine when the pulling string should be stopped and maintained at constant force (zero velocity). Maximal deflection of the cantilever (translated to maximal force of the pulling string) was observed before coverslip was fully detached (or before cantilever relaxes back to zero deflection) and was used to calculate gel adhesion force. Adhesion force on MMMS gels was found to be $84 \pm 20\%$ higher than on pristine 0.3 kPa gels ($n = 5$).

The effective elasticity can be derived from rigid cylinder (i.e. coverslip of length $a$) pulling from a soft elastic thin film by assuming non-uniform Boussinesq-type stress distribution at the interface. The force per unit length $F/a$ (along $x$) or $P$ that propagates from the edge of the coverslip can be derived as:

$$P = D \frac{\partial^3 u_x}{\partial x^3}$$  \hspace{1cm} (1)
where $u_z$ is displacement field in the $z$-direction, $D$ is bending stiffness of the coverslip. Since coverslip bending is coupled to the deformation of the hydrogel below it, the characteristic stress decay length, $\varepsilon$, from the edge of contact is:

$$\varepsilon \sim \left(\frac{Dh_1^2}{E_1}\right)^{1/6} \quad (2)$$

where $h_1$ and $E_1$ is the height and Young’s modulus of the thin film, respectively. By taking $u_z \sim \delta_2$, where $\delta_2$ is the thin film extension length at applied pull-off force, $F$, and $x \sim \varepsilon$, we can analyze Equation (1) with (2) at the scaling level:

$$P \sim \frac{F}{a} \sim \frac{D\delta_2}{\varepsilon^3} \sim \frac{D^{1/2}E^{1/2}_1\delta_2}{h_1^{1/2}} \quad (3)$$

or simply (by removing material property constants),

$$E_1 \sim \left(\frac{F}{\delta_2}\right)^2 \quad (4)$$

for which we can estimate elasticity increase with addition of embedded collagen. Assuming adhesion work is constant (related to surface tension and interfacial area), $\delta_2$ is thus inversely correlated to $E_1$, which can be derived (ref. 4) to scale as $\delta_2 \sim E_1^{-1/3}$. From equation (4), this leads simply to

$$E_1 \sim F^6 \quad (5)$$

Thus, surface elasticity of MMMS gels, with a first approximation due to tackiness, is

$$E_{MMMS} \sim E_{0.3kPa}(1.84 \pm 20) \sim 5.8 - 22 \ kPa \quad (6)$$

AFM measurement of MMMS gel in Supplementary Fig. S4b was re-calculated based on this adhesion correction factor.

Correcting for thinness of embedded collagen fibers at the interface, a fluorescence microscopy-based approximation can also be used to approximate MMMS gel elasticity. For pure collagen gels, storage modulus $G'$ (Pa), is a function of temperature and concentration, $c$ (mg/mL). At $37 \ ^\circ\text{C}$, this was found to scale as

$$G' \approx 22 \ c^{2.1} \quad (7)$$

Since the embedded fibers are stiffer than the compliant PA around them, Equation (7) was assumed to still apply at least in the regions near fiber bundles. Plugging our collagen concentrations into Equation (7), and assuming Young’s modulus ($E$) is $2.9G'$, we predicted an effective stiffness of $E_{eff} \sim 12 \ kPa$.

**Theoretical analysis of Lateral Pulling Curves**

For an elastic substrate medium bounded on one side by a plane (e.g. PA gel on coverslip) with a point force $F$ on applied on its free surface, the distribution of displacement, $u$, along the surface ($z = 0$) has been determined in equilibrium by Landau and Lifshitz as:
\[ u_x = \frac{1+\sigma}{E} \frac{1}{r} \left\{ -\frac{(1-2\sigma)F_x}{r} + 2(1 - \sigma)F_x + \frac{2\sigma F_x}{r^2} (xF_x + yF_y) \right\} \]  

(8)

where \( E \) is Young’s modulus of the medium, \( \sigma \) is Poisson’s ratio, and \( r = \sqrt{x^2 + y^2} \). A similar formula for \( u_y \) was also found. We assumed that the lateral pulling force was applied to a small region and that the measured displacement profiles were obtained at large distances away from the initial loading point to satisfy Equation (8). In the simplest case where \( x = r \) (\( y = 0 \)), displacement far enough from the loading force (Boussinesq approximation) scales as \( u_r = a_B/r \), for some Boussinesq constant, \( a_B \). Since such scaling leads to infinite displacement at \( x = r = 0 \), we also assumed exponential scaling (\( u_r = a_0 e^{-br} \), for some characteristic decay constant \( b \); Fig. 1E, iii) to set the observed maximum displacement, \( a_0 \), at the initial point. Both scalings agree well (\( R^2 > 0.9 \)), as evidenced by the fitted scaling constants (Supplementary Fig. S4e).

**Mechanobiological Gene Circuit (MGC) Model for Matrix-directed Osteogenesis**

Lamin-A (L) and smooth muscle actin (S) message and protein circuitry is schematically presented in Supplementary Fig. S11c. Expression kinetics is described by a set of coupled rate equations for the respective transcripts (lower case) and proteins (upper case), as follows:

\[ \frac{dl}{dt} = \alpha_1 \cdot L - \beta_1 \cdot 1 \]  

(9)

\[ \frac{dL}{dt} = \gamma_1 \cdot 1 - \delta_1 \cdot \frac{L^nL}{K_L^{nL} + L^nL} \]  

(10)

\[ \frac{ds}{dt} = \alpha_2 \cdot S + \alpha_3 \cdot L - \beta_2 \cdot s \]  

(11)

\[ \frac{dS}{dt} = \gamma_2 \cdot s - \delta_2 \cdot \frac{S^nS}{K_S^{nS} + S^nS} \]  

(12)

In this parsimonious model, RNA degradation and translation were assumed linear in transcript concentration. Lamin-A protein positively regulates one of its transcription factors, RARG, and smooth muscle actin (SMA) protein positively regulates one of its transcription factors, SRF, so that each enhances its own transcription (with rate constants \( \alpha_1, \alpha_2 \)). In addition, Lamin-A protein also enhances SMA transcription via the SRF pathway (rate constant \( \alpha_3 \)). To model stress-stabilized protein assembly, we describe lamin-A and myosin-IIA protein turnover with suitable Hill models (rate constants \( \delta_1, \delta_2 \)). Specifically, Lamin-A protein turnover is suppressed by SMA-generated stress with \( K_L = S^{ns} \), while SMA protein turnover depends on matrix elasticity \( E \): \( K_S = \frac{E}{E+E_0} \). Equations (9)-(12) above were solved numerically in Mathematica (Wolfram) at steady state (all derivatives = 0). Free parameters were adjusted.
to collectively obtain best agreement with experimental data (Supplementary Fig. S8a). In particular, cooperative protein turnover was $n_S = 10.4$ and $n_L = 2$ with the half-maximum effect on SMA turnover occurring at $E_0 = 5$ kPa. All other parameters were of order of magnitude 1. As matrix stiffness and cell tension suppresses protein phosphorylation and turnover$^8,9$, steady-state levels monotonically increase with matrix $E$, consistent with the coupled mechano-regulation of lamin-A and SMA (Supplementary Fig. S8a).

**Supplementary Outlook**

Displacement profiles on stiff fiber bundles on MMMS (Fig. 1e) help answer how far away does a cell feel a scar. The profiles show that a cell 10 mm away can sense a stiff circular island of 30-mm radius, so that the effective ‘sensed area’ increases from $\sim 3000$ sq. mm to 5000 sq. mm. Thus, the 20-30% area coverage by these islands (Supplementary Fig. S6) effectively increases to $\sim 30-50\%$, notwithstanding that these stiff ‘islands’ have a fractal, rather than round, shape.

MSC plasticity was also exploited to mimic and clarify fibrosis mechanisms *in vivo*. MSCs recapitulated the expression changes detectable in scarred striated muscle (Fig. 1b,c). Our reductionist approach *in vitro* to scarred tissues by using MMMS helped clarify cell responses in both short and long time-scales. That a heterogeneous MMMS elicits less cell-cell heterogeneity than homogeneous substrates (Fig. 3 and Supplementary Fig. S8) highlights the importance of ECM micro-architecture in the niche and mechanochemical control of cell fate. Such a finding is difficult to explore with homogeneous and isotropic substrates alone. The findings also underscore the relative contributions of cell-intrinsic versus extrinsic variation to cell-material interactions and cell biology in general. Single-cell analyses use many types of new and sensitive detection and sequencing technologies, but they do so after plucking cells from cultures or poorly understood tissues; tissue-relevant insights can thus be obtained if matrices with bio-inspired fractal heterogeneity are used rather than homogeneous substrates. For example, a better model of cancer-associated fibrosis$^{10}$ (e.g. Fig. 1g) could be helpful for understanding such matrix remodeling and heterogeneity as a major risk factor for cancer (e.g. liver cancer) that likely affects a cancer stem cell in its niche as well as a tissue stem cell in its niche. Single-cell analyses of such important processes and cell types may be artificially limited when using homogeneous substrates that broaden phenotypic variation.

The kinetics of various mechanosensitive proteins showed that MSCs responded to our heterogeneous MMMS gels as if they were on much stiffer, homogeneous matrices – which is surprising
in that it requires MSCs to ‘remember’ stiff domains more so than soft domains of a matrix. Myosin-II polarization is implicated in the short-term mechanical memory imparted by the rigid regions of MMMS. Lamin-A changes in scarred tissues and in scar-like MMMS (Figs. 1c, 2d and Supplementary Figs. S1, S10) illustrate the plasticity of this multifunctional nucleoskeletal protein that regulates several transcription factors in normal differentiation. The 3D ex vivo decellularized heart matrices developed here also exhibit similar rheological profiles as 2D MMMS (Supplementary Figs. S6, S7), and MSCs durotax on both, reminiscent of MSC accumulation into scarred tissues. MSC migration, morphology, and protein responses on the MMMS gel all reveal a high effective $E$ despite heterogeneity, consistent with the maximum $E$ determined in suitably analyzed micromechanical measurements (Fig. 1).

A mechanobiological gene circuit (MGC) can mathematically formalize the intertwined pathways (Supplementary Discussion and Supplementary Fig. S11). Matrix stiffness induces cytoskeletal tension (evident with SMA), and this tension is applied to the nucleus, stabilizing lamin-A (Fig. 2b,d). RARG-driven, RA-repressible lamin-A expression co-regulates SRF, which co-regulates SMA. Positive mechanical feedback by SMA-rich stress fibers on lamin-A leads to fold changes of both proteins in response to matrix $E$ (Supplementary Fig. S11) – as modeled by the gene circuit. On a much slower time scale, the mechano-repressor NKX2.5 exits the nucleus and degrades to maintain a SMA-high, NKX2.5-low phenotype in long-term MSC cultures on rigid substrates – as a form of ‘mechanical memory’ (Fig. 6b). The results here are the first to show the slow dynamic changes of NKX2.5 (~days) and its (nuclear or cytoplasmic) localization with various methods (immunochemistry, mass spectrometry, and mutant overexpression).

NKX2.5 has multiple binding partners in the nucleus that would tend to retain it there. NKX2.5 binds DNA and is predicted to bind to the ACTA2 promoter. Indeed, overexpression of NKX2.5 in human MSCs decreases ACTA2 message ($p < 0.05$; Supplementary Fig. S12), consistent with decreased protein levels (Fig. 5) and with studies of promoter-reporter constructs in mouse fibroblasts. Second, Nkx2.5 binds directly or indirectly in the nucleus to several other proteins that include CDK1 (Supplementary Table S3), which is perhaps similar to a recently discovered non-transcriptional regulation by Oct4 of Cdk1, with effects on proliferation/differentiation. Third, Nkx2.5 binds in the nucleus to heterogeneous nuclear ribonuclear proteins (HNRNPA/B/R/H2) (Supplementary Table S3), which could impede pre-mRNA processing and transport as already documented for actin. Fourth, Nkx2.5 binds to the lamina-associated polypeptide-2 (LAP2, or TMPO) (Supplementary Table S3), which promotes differentiation of muscle stem cells, so that Nkx2.5 could contribute to muscle injury processes (Fig. 1c) and also crosstalk with the lamin-A gene circuit. A multiplicity of such nuclear interactions of Nkx2.5
dimers is further consistent with the cooperative nature of SMA de-repression on stiff matrix (Fig. 4b fits have Hill exponents >2): the more NKX2.5 is in the nucleus, the more it is retained to repress SMA. SMA-high cells (strong Responders) on homogeneously stiff substrates are thus more sensitive to minor changes in nuclear-localized NKX2.5, compared to weak Responders (Fig. 4b, right inset and Supplementary Fig. S9). Such sensitivity equates to the derivative, $\partial[\text{SMA}] / \partial[\text{NKX2.5}_{\text{nuclear}}]$ and helps explain why the heterogeneity of cells on homogeneously stiff matrices is greater than cells on MMMS and CC_{0.3kPa} gels (Fig. 3). For MMMS importantly, a cell on a fractal fiber island that upregulates SMA and downregulates nuclear NKX2.5, will later migrate off of this stiff island (Supplementary Fig. S5) and potentially reverse the process in a way that cannot happen on a homogeneous substrate.

The many nuclear interactions of Nkx2.5 combined with its strong NLS (Figs. 4f, 5) would tend to oppose nuclear export and cytoplasmic degradation of Nkx2.5, thus favoring its nuclear import and retention (Supplementary Fig. S12). Histones also have multiple DNA and protein interactions that strengthen nuclear retention and impede turnover: every one of the 18 histones detected in a recent proteomics study of protein turnover in fibroblasts cultured on rigid glass shows a longer half-life than the median (2.0 days for 5028 detected proteins, with histone half-lives ranging from 2.4 to 8.7 days$^{19}$). Turnover that takes many days fits our NKX2.5 kinetics for MSCs on rigid matrix (Fig. 4b). Importantly on rigid matrix (but not on soft matrix), some of the nuclear interactions of NKX2.5 could weaken and slowly tilt the balance away from nuclear retention. In particular, NKX2.5 could be displaced from chromatin binding by rigidity-driven accumulation of nuclear factors in the SRF pathway, which co-regulates ACTA2 expression$^{20}$ and is coupled to the lamin-A gene circuit$^{9}$. Interactions with nucleus-retained proteins alone may not sufficiently explain the slow NKX2.5 translocation, and regulation by other mechanotransduction pathways requires exploration. Ultimately, extensive mutagenesis beyond our in-depth analyses of the NLS domain in NKX2.5 might one day map each of NKX2.5’s many interaction partners and thereby deepen insight into mechanisms and additional pathways responsible for our observation of a slow, rigidity-driven decrease in NKX2.5’s nuclear localization. Contributions of individual amino acids to how MSCs gradually acquire long-term mechanical memory might then become clear.

MSCs are more multipotent than lineage-restricted fibroblasts$^{21}$, but SMA expression in fibroblasts is well documented in fibrosis (e.g. ref. $^{13}$). Moreover, single cell profiling of mouse fibroblasts recently implicated a role in scarring for the cell surface peptidase Cd26/Dpp4, which – when inhibited by a small molecule – diminished scarring$^{22}$, and we find CD26 in human MSCs decreases with overexpression of NKX2.5 as well as with knockdown of LMNA (Supplementary Fig. S12). While we have shown MMMS
decreases nuclear NKX2.5 and increases LMNA in MSCs, any changes in additional fibroblast proteins (e.g. CD26) due to MMMS require further study. More broadly, the fact that scarring occurs progressively only in mature stages of higher animals\textsuperscript{23} likely reflects an evolved need to generate a sufficiently stiff tissue that sustains large stresses in mature tissues. Tissue injuries initiate mechanical as well as biochemical changes that can serve as homing cues for highly plastic MSCs. For stem-cell based therapies in which differentiation-competent cells are injected into a wound, our findings provide a guide to controlling cell responses to scars that range from contractile induction to ectopic calcification\textsuperscript{24}. 
Supplementary Methods

Proteomics Analyses  Mouse tissue was prepared for analysis as described by Swift et al. Briefly, approx. 1 mm$^3$ of frozen muscle tissue was ground on dry ice and solubilized in 100 µL 1x NuPAGE LDS sample buffer (Life Technologies); samples were sonicated on ice before addition of 1% β-mercaptoethanol, followed by heat-denaturation at 80 °C for 10 min. SDS-PAGE gels (NuPAGE 4-12% Bis-Tris, Life Technologies) were loaded with matched quantities of protein (determined by Coomassie densitometry), with separations run in MOPS running buffer (Life Technologies) at 100 V for 10 minutes followed by 160 V for 25 minutes. Gel bands were excised for mass spectrometry (MS) analysis between 60 and 150 kDa. Details of protein quantification by label-free MS are described in ref. 9, with Elucidator software (Rosetta Biosystems) set to search for the hydroxylation modifications that are common in matrix proteins (proline, asparagine, aspartic acid and lysine, Δ = +15.995 Da). For specificity validation of the antibody against Nkx2.5 (molecular weight: 35 kDa), gel bands from wild-type Nkx2.5-overexpressing A549 cell lysates were excised between 30-40 kDa and analysed with MS (Supplementary Fig. S10c).

Immunoprecipitation-Mass Spectrometry (IP-MS) Analyses  Human A549 (ATCC) and primary mesenchymal stem cells (MSCs) were dissociated using Trypsin/EDTA 0.25% (Life Technologies) and immediately resuspended in serum-containing media, pelleted and washed with ice cold PBS twice. Cell pellets were then diluted in IP lysis buffer (25 mM Tris, 0.15M NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, pH 7.4) supplemented with 1% Phosphatase Inhibitor Cocktail 3 (P0044, Sigma Aldrich) and 1% Protease Inhibitor cocktail (P2714, Sigma Aldrich) at 1x10$^6$ cells/100ul of IP lysis buffer. Suspended pellets were sonicated on ice for 10 sec using a probe tip sonicator (setting at 2.0; Sonic Dismembrator Model 550, Fischer Scientific). Sonicated samples were on ice for 30 min, then centrifuged (at 4 °C, 15 min at 1500 rpm) with centrifugation to separate supernatant from discarded cell pellet.

For immunoprecipitation (IP) of NKX2.5, whole cell lysate was mixed overnight at room temperature with 10 µg of rabbit anti-NKX2.5 IgG (H-114 clone) or normal rabbit IgG control (sc-2027, Santa Cruz Biotechnology, Inc.) that were covalently crosslinked (with 10mM BS3 for 10 minutes) to protein A Dynabeads (10001D, Life Technologies). Dynabead-Antibody complexes were prepared and used according to manufacturer specifications. Precipitated proteins (bound to bead-antibody complex) were washed twice with Dulbecco’s Phosphate Buffered Saline (Life Technologies) with 0.01% Tween-20, and eluted by resuspending in 1x LDS buffer (Life Technologies) with 1% β-mercaptoethanol (Sigma Aldrich) and heat-denaturing at 70 °C for 10min. Protein-containing eluate was run in 12% Bis-Tris NuPAGE gel in MOPS running buffer (Life Technologies).
The IP samples were prepared for MS analyses as detailed in ref. 25 and the ‘Proteomics Analyses’ section above. Raw data from each IP-MS sample was processed separately using MaxQuant (version 1.4.1.2, Max Planck Institute of Biochemistry). MaxQuant’s built-in Label-Free Quantification (LFQ) feature was employed with full tryptic digestion and up to 2 missed cleavage sites. Peptides were searched against a FASTA database compiled from UniRef100 (June 2011) human, plus mouse and contaminants. The software’s decoy search mode was set as ‘revert’ and an MS/MS tolerance limit of 20 ppm was used, along with a false discovery rate (FDR) of 1%. Carbamidomethylation of cysteine residues (Δ = +57.021 Da) was always included in the search as ‘fixed modifications’ in order to account for the fact that samples were alkylated using iodoacetamide. N-terminal acetylation (Δ = +42.011 Da), phosphorylation (Δ = +79.9663 Da), and oxidation of methionine residues (Δ = +15.995 Da) were also included as possible modifications. Peptides that may have been derived from trypsin or keratin were considered to be contaminants and were neglected in subsequent calculations. MaxQuant output tables were then fed into a custom code in MATLAB (version 8, Mathworks, Inc) for Venn diagram sorting of identified proteins into ‘unique’ and ‘shared’ protein groups for each sample pair (within a cell type). Proteins pulled down by both control rabbit IgG and rabbit anti-NKX2.5 IgG were not included from the final list of proteins (Table S3).

**Bulk Rheology**

A cone-and-plate Bohlin rheometer (Malvern Instruments, Worcestershire, UK) was used to measure the viscoelastic spectrum (elastic modulus, G’, and viscous modulus, G”) of the hydrogels as a function of frequency. In order to confirm polymerization had come to completion, a time sweep test was also performed for 20 minutes with a controlled absolute strain of 1% at an angular frequency of 1 rad/s. PBS was then added to allow complete swelling of the gel before a frequency test was conducted from 25 rad/s down to 0.005 rad/s at 1.0% strain.

**Fractal Dimension Analysis**

Fractal dimensions were obtained from immunofluorescence images of MMMS and collagen-coated PA gels (and tissue culture plastic) to quantify collagen architecture (Supplementary Fig. S2b), as recently suggested26. We used a fractal box-count method using ImageJ software: images were transformed into binary and a built-in fractal box count plugin was used to obtain fractal dimension values. The range of fractal dimension values (within the limits of 1.0 – 2.0) was confirmed by processing a blank image and a filled image (e.g. tissue culture plastic), respectively. Collagen-coated gels exhibit fractal dimensions that are similar to a filled image, while increasingly fractal-like images approach the midpoint (~1.5).
3D reconstruction and Photobleaching in Confocal Microscopy

To visualize both PA gel and collagen-I (Fig. 1D), the gel precursor was doped with 0.02% w/v allylamine (Sigma) for subsequent fluorescein isothiocyanate (FITC, Thermo Scientific) conjugation, while collagen-I coating covalently crosslinked with sulfo-SANPAH on the gel surface was indirectly immunolabeled (secondary antibody: donkey anti-mouse Alexa-647 fluorophore, Life Technologies). To delineate registration of collagen coating on polyacrylamide gels, confocal microscopy (FV1000, Olympus) with 40x objective (UPLFLN40XO, N.A. 1.3) was used. Pinhole size was set at 125 µm to generate an optimal slice thickness of 0.76 µm. Collagen coat is ~5 µm on a fully hydrated hydrogel of ~80 µm thickness as determined by 3D confocal stack reconstruction.

Fluorescence photobleaching was also performed to determine the extent of immobilization of the collagen-I coat on the gel surface (Supplementary Fig. S4d). Gel surface fluorescence was imaged prior to bleaching. Then the confocal plane was localized on the gel surface as determined by collagen-I immunofluorescence, and laser power was increased to maximum possible amount to photobleach a circular region of interest (ROI) for 60 seconds. Gel surface with the photobleached ROI was re-acquired for another 5 minutes. Even without sulfo-SANPAH crosslinking, photobleached collagen-I remains relatively stable perhaps due to interpenetration into the inert porous gel.

Durotaxis Experiments

Gradient gels were prepared by juxtaposing 2 drops (10 uL) of different precursor solutions on a coverslip. For MSC durotaxis on CC0.3kPa/MMMS hybrid gel (Figs. S5B, S6), the formulation for 0.3 kPa PA gel was used on both sides, but with pre-mixed collagen-I on the scar-like side (Supplementary Fig. S6a). In order to delineate the regions under the microscope, fluspheres (1-µm diameter, Invitrogen) were supplemented 0.005% by weight in the soft side, while collagen fibers in the embedded collagen region were fluorescently immunolabeled. During polymerization, the gel precursor drop(s) was sandwiched with another pretreated coverslip until gel polymerizes. For knockdown experiments, Lipofectamine 2000 (Invitrogen) with 30 nM siRNA was used according to manufacturer’s instructions. The level of knockdown was assessed by Western blotting after 48 hrs. Myosin-IIB siRNA duplex sequences were obtained from Raab et al. and were synthesized by Dharmacon, Inc. Scrambled siRNA was siGENOME Non-Targeting siRNA #1 (Dharmacon). To minimize experimental variability in durotaxis experiments, both scrambled and MIIB siRNA-treated MSCs were simultaneously seeded on the hybrid gels and their migrations were visually discriminated by prior minimal labeling with hydrophobic PKH26 and PKH67 dyes, respectively, according to manufacturer’s (Sigma-Aldrich) instructions. Prior to plating into decellularized heart matrices, MSCs were transduced at passage 2 (50% confluency) with 300 PFU of lentivirus per cell for 24 hrs to stably express GFP.
Chick Embryonic Heart Matrix Decellularization and Cardiomyocyte Isolation

White Leghorn chicken eggs (Charles River Laboratories) were incubated at 37°C, rotated once per day, until the desired developmental stage was reached. Hearts were removed from chicken embryos at embryonic day 14 and the left ventricle wall was isolated and immersed in 1% SDS in deionized water for decellularization. After shaking overnight at room temperature (RT), the decellularized tissue was placed into 1% Triton-X in deionized water for 30 min and then equilibrated in PBS with 100 ug/mL penicillin and 100 uM streptomycin until used for experiments. To make a gradient of crosslinks in the matrix, the decellularized tissue was fixed on a coverslip and then placed into a chamber such that approximately one half is immersed in 100% glycerol. After allowing glycerol to penetrate into the matrix for 30 min, 3 mM of genipin (a fluorescent crosslinker, Sigma) in PBS was added on top of the glycerol to immerse the other half of the matrix, and was then incubated in the dark at 37°C for 2 hours. Glycerol was then added to overflow the genipin solution, and the resulting matrix with a crosslink gradient was washed and stored in PBS with antibiotics prior to use.

For cardiomyocyte-on-gel cultures, embryos were extracted at room temperature by windowing eggs, removing extraembryonic membranes with forceps and cutting major blood vessels to the embryonic disc tissue to free the embryo. For E2–E5 embryos, whole heart tubes (HTs) were extracted by severing the conotruncus and sino venosus. All tissues were incubated at 37°C in pre-warmed chick heart media (alpha-MEM supplemented with 10% FBS and 1% Penicillin/Streptomycin, Gibco, 12571-063) until ready for use. Cell isolation from heart tissue was performed by dicing sub-millimeter size and then digesting with trypsin/EDTA (Gibco, 25200-072). To digest, we incubated tissue in approximately 1 mL trypsin per E4 HT for 13 min rotating at 37°C, for 2 min upright to let large tissue pieces settle before carefully removing supernatant and replacing with an equal volume of fresh trypsin, and finally shaking for 15 more min. We stop digestion by adding an equal volume of chick heart media. Cells were plated at concentrations of approximately $2 \times 10^5$ cells/cm$^2$ directly on collagen-1-coated PA gels of varying stiffness.

**Time-lapse Microscopy**

Beating cardiomyocytes were immediately imaged (< 1 h) under HEPES-buffered (10 mM) phenol-red free DMEM (with 10% FBS and 1% P/S, Gibco) on a temperature-controlled (37°C) microscope stage with an inverted microscope (IX-71, Olympus) equipped with a 20x LCACH objective (NA 0.40) and a Cascade CCD camera (Photometrics). Image sequences were acquired every 0.2 s.

Imaging of MSC migration was done using an inverted Olympus IX-71 microscope with a 10x objective enclosed in a humidified chamber at 37°C and 5% CO2, 300 W xenon lamp illumination, and a high-resolution CCD camera (Photometrics CoolSnap HQ). Deltavision Softworx software was used for...
image acquisition at 10-min intervals. ImageJ was used to track the center of MSC nuclei in movie sequences, and the summed contour distance traveled divided by the time was used as our measurement of mean speed. This measurement only reflects total distance traversed but does not reflect cell persistence. We quantified the bias of the time-dependent number of steps to the left (L, soft CC<sub>0.3kPa</sub> region) and to the right (R, MMMS region) by using the equation adapted from Isenberg et al.\textsuperscript{27}:

\[ \text{Durotaxis Index} = \frac{(R - L)}{(R + L)} \]

If Durotaxis Index = 0, there is no bias to cell migration. If Durotaxis Index = 1, all of the steps are toward the stiff side. Cells that underwent mitosis and those that migrated out of the trackable region were included only prior to such events.

**Quantitative Immunofluorescence Microscopy**

For immunofluorescence staining, cells were fixed with 3.7% formaldehyde (Sigma) in PBS for 15 min at RT and washed with PBS twice for 5 min. Blocking was done for 1 hr with 1% BSA in PBS. All primary antibodies (see Supplementary Table S2 for dilutions) in PBS with 0.025% Tween-20 (PBST) were incubated at RT for 1 hour or overnight at 4°C. All donkey secondary antibodies (Alexa series from Life Technologies) were used to stain primary antibody-labeled fixed cells for 1 hour at RT at 1:1000 dilution in PBST. Actin filament (F-actin) staining was done in PBS with 100 ng/ml TRITC-phalloidin (Sigma) for 20 min at RT. Hoechst 33342 (Life Technologies) was used at a concentration of 1 µg/ml in PBS to stain for DNA for 5 min at RT. Quantitative immunofluorescence of immunolabeled proteins of interest and their localization was performed using an inverted microscope (IX-71, Olympus) with a 20x LCACH objective (NA 0.40) and a Cascade CCD camera (Photometrics). Image acquisition was performed under Image-Pro Plus software (Media Cybernetics, Inc.) and image analysis was done using ImageJ software. In quantitative immunofluorescence microscopy, samples were imaged under the same specifications of exposure times and gains. If two different exposure times were necessary for quantification between samples, both exposure times were performed for those samples to ensure that images were in the linear range of exposure time to fluorescence intensity. Control samples incubated with secondary antibody only (no primary antibody incubation) were also imaged as negative control under the same specifications.

**Cell Morphometrics**

Intensity analyses and cell-morphology quantification were performed in ImageJ software. From images acquired from immunofluorescent samples, F-actin and myosin IIB rear/front polarization ratios were determined by splitting the cell into two equal areas (rear and front). The ratio is the total integrated fluorescence of the rear half over the front half of the cell. Cell area and aspect ratio were quantified from myosin IIA or F-actin immunofluorescence (since they consistently stain the whole cell body). Before comparing different sample conditions, integrated intensity of a particular protein was normalized to background value (secondary antibody only control).
**Transcriptional profiling by DNA microarrays** Total RNA was extracted from cells using Trizol and purified by RNeasy (Qiagen) with on-column DNase digestion according to manufacturer’s protocol. Adherent cells were gently scraped in Trizol. Total RNA was amplified and converted to cDNA using WT-Ovation Pico kit (NuGen). Fragmented and biotin-labeled ST-cDNA was generated using WT-Ovation Exon Module (NuGen). Samples were tested with Human Gene 1.0 ST DNA microarrays (Affymetrix), used according to the manufacturer’s instructions. Expression data sets were analyzed by standard Robust Multi-array Averaging (RMA) methods.

**Histological Analysis of Osteogenic Commitment** For osteogenesis experiments, sparsely seeded MSCs (~2000 cells/cm²) were allowed to reach ~70% confluency in normal media prior to addition of osteogenic induction media (OIM, StemXVivo Osteogenic/Adipogenic Base Media and Supplement, R&D Systems). Osteogenic cultures, with fresh media every 3-4 days, were done for 1 week or 4 weeks and evaluated for Alkaline Phosphatase activity or Alizarin Red staining, respectively. All-trans retinoic acid (RA, Fisher Scientific) is an agonist drug for Retinoic Acid Receptor (RAR). Cell-on-gel cultures were treated with either control solution (0.15% ethanol, 0.15% DMSO in culture media), or with 1 µM drug solution. After 1 or 4 weeks in culture with osteogenic induction media (OIM), MSCs on gels were fixed at RT with 3.7% paraformaldehyde for 20 minutes and washed thrice in 10 mM Tris buffer (pH 7.2).

For 1-week cultures, Alkaline Phosphatase (ALP, osteogenic biomarker) activity was visualized by Fast Blue staining. While cells were being fixed, 2 mL of naphthol-AS-MX phosphate and one preweighed capsule of Fast Blue RR Salt (Sigma) were dissolved with stirring in 48 mL distilled water in the dark (Fast Blue Solution). Fixed cultures were then immersed in Fast Blue Solution for 30 min, subsequently washed in distilled water twice, and imaged immediately or mounted in aqueous mounting media for storage.

For 4 week cultures, Alizarin Red (late osteogenic biomarker) staining was performed. Cultures were fixed with 10% paraformaldehyde for 10 min, washed with PBS twice, and stained with 2% Alizarin Red S (Sigma) solution for 15 minutes, all at RT. Staining solution pH was pre-adjusted to ~4.1 – 4.3 with 0.5% ammonium hydroxide. Samples were then washed with distilled water and imaged immediately (or stored accordingly). Samples were imaged using an inverted microscope (IX-71, Olympus) with a 4x objective and a USB-compatible digital color camera (DC2000M; World Precision Instruments, Inc.) attached to the microscope c-mount tube. Color images were acquired using ScopePhoto software (ScopeTek) and were processed in ImageJ by converting to 32-bit black and white images, inverting, and measuring raw intensities. Osteogenic indices for images from both ALP and
Alizarin Red-stained samples were calculated from these raw intensities, and normalized to cell cultures under serum-only condition.

For whole-tissue Alizarin Red staining, tissues (e.g. bone and whole chick embryo samples) were fixed in ice-cold 95% ethanol for 1 hour and repeated for overnight rocking at RT. Alizarin Red was prepared at 0.5 % w/v in water and was added to fixed tissue for overnight staining. Samples were then rinsed in water and sequentially immersed in aqueous solutions with decreasing KOH and increasing glycerol concentrations: 10 mins in 1% KOH, 1 h in 20% glycerol / 0.25% KOH, 1 h in 33% glycerol / 0.25% KOH, 1 h in 50% glycerol / 0.25% KOH. Final overnight clearing and storage is in fresh 50% glycerol / 0.25% KOH solution.

**Western Analysis** Adherent MSCs on gels were trypsinized, pelleted, washed in cold PBS twice, and pelleted again prior to addition of RIPA lysis buffer (1.0 vol% NP40, 2 mM EDTA, 150 mM NaCl, 50 mM Tris-HCL, 0.1 wt% SDS, 0.5 wt% sodium deoxycholate) at 4 °C for 30 minutes. Prior to incubation, the pellet solution was sonicated to fully break down cell particulates. The pellet solution is then centrifuged at 12,000 rpm at 4 °C for 20 minutes and the collected supernatant lysate can be stored at −80 °C or immediately used. LDS (4X, Thermo Scientific) / beta-mercaptoethanol solution at 11:1 v/v is then added at 36% of lysate volume and boiled at 90 °C for 10 minutes prior to gel electrophoresis. Boiled lysates were cooled at RT, loaded onto 4-12% NuPAGE Bis-Tris gels (Life Technologies), and after gel run, transferred to blotting paper (iBlot PVDF, Life Technologies) using an iBlot gel transfer device at P3 (Life Technologies) setting for 7 min. Blots were blocked with 5% non-fat milk (American Bioanalytical) in TBST for 1 hour at RT and then desired primary antibodies in TBS were added overnight at 4 °C. HRP-conjugated secondary antibodies (ECL™, GE Healthcare) in TBST with 5% milk were added for 1 hour at RT. Two 10-min TBST washes were performed in between above steps, and a final TBS wash before precipitate development. Chromosensor (GenScript), a chromogenic substrate, was finally added to generate a precipitate for the ECL reaction that should appear visible within 5-10 min.

**Combined FISH and IF of LMNA** This method was adapted from multiple singly labeled fluorescent RNA probes developed by Dr. Arjun Raj (University of Pennsylvania) to simultaneously detect lamin-A expression in the mRNA and protein levels in situ. Reagents were kindly provided by Dr. Raj. A detailed protocol is found in ref. 28. Labeled samples were visualized in the same setup as Time-Lapse Microscopy movie sequences, but with a 100x objective with high numerical aperture to achieve a narrow depth of field and discriminate diffraction-limited mRNA spots during image processing. Roughly 40 z-stack images per cell were obtained to fully encompass cell height. Spot counting was performed in
ImageJ, by manually thresholding spot intensities and counting speckles that are in the expected diffraction limited range of 0.2 – 0.5 microns in diameter. Counts of mRNA spots reported here may be an overestimate due to the choice of imaging and image processing. Nonetheless, good mRNA-protein correlations in LMNA knockdown experiments (Supplementary Fig. S8a) validated the technique. LMNA siRNA was obtained from Harada et al.\textsuperscript{29} and transfected into MSC cultures with Lipofectamine 2000 as per manufacturer’s instructions.

**Statistical Analysis**  An independent samples t-test was used when there were only two different sample means. One-way ANOVA was used for matrix elasticity effects on cell morphometrics, protein expression, etc. Two-way Analysis of Variance (ANOVA) with Tukey post-hoc test was used for time-lapse cell migration experiments with time-dependent displacement values between conditions. Two-sample Kolmogorov-Smirnov test was used to measure whether two samples come from the same distribution. Statistical significance was determined at $\alpha = 0.05$ level. All statistical analyses were done in Microsoft Excel.
Supplementary Tables

Table S1. Polyacrylamide Gel Precursor Formulations*

| Nominal Elasticity (kPa) | Volume (µL) of 40% Acrylamide stock | Volume (µL) of 1.5% w/v Bis-Acrylamide stock | Volume (µL) of distilled water |
|--------------------------|-------------------------------------|----------------------------------------------|-------------------------------|
| 0.3                      | 75                                  | 47                                           | 874.84                        |
| 3.0                      | 112                                 | 70                                           | 812.86                        |
| 6.0                      | 100                                 | 167                                          | 733.00                        |
| 10                       | 150                                 | 93                                           | 750.68                        |
| 20                       | 200                                 | 93                                           | 696.00                        |
| 40                       | 250                                 | 200                                          | 539.47                        |

*1 µL of N,N,N',N'-Tetramethylethylenediamine (Sigma) and 10 µL of 10% w/v Ammonium Persulfate (Sigma) is added to ~1 mL gel formulations to initiate polymerization.

Table S2. Primary Antibodies*

| Protein Antigen | Host Species and Reactivity | Clone, Catalog Number, Vendor | Concentration or Dilution used and Application |
|-----------------|-----------------------------|-------------------------------|-----------------------------------------------|
| Collagen-1 (Col-1) | mouse anti-collagen type I IgG1 | COL-1, C2456, Sigma | 1:1000 (Indirect Immunofluorescence) |
| α-Smooth Muscle Actin (SMA) | mouse anti-SMA IgG2a | 1A4, A2547, Sigma | 1:1000 (Western Blot, Indirect Immunofluorescence) |
| Myosin IIB (MIIB) | rabbit anti-MIIB | Polyclonal, 3404, Cell Signaling Technology | 1:1000 (Western Blot), 1:200 (Indirect Immunofluorescence) |
| Myosin IIA (MIIA) | rabbit anti-MIIA | Polyclonal, M8064, Sigma | 1:200 (Indirect Immunofluorescence) |
| Lamin-A,C | mouse anti-lamin A,C IgG2b | 636, sc-7292, Santa Cruz Biotechnology | 1 µg/mL (Western Blot, Indirect Immunofluorescence) |
| Lamin-B | goat anti-lamin B1 | M-20 polyclonal, sc-6217, Santa Cruz Biotechnology | 1 µg/mL (Western Blot, Indirect Immunofluorescence) |
| β-Actin | mouse anti-β-Actin IgG1 | C4, sc-47778, Santa Cruz Biotechnology | 1 µg/mL (Western Blot) |
| HSP90 | mouse anti-HSP90 | AC88, ab13492, Abcam | 1 µg/mL (Western Blot) |
| HSP70 | mouse anti-HSP70 | C92F3A-5, ab47455, Abcam | 1 µg/mL (Western Blot) |
| NKX2.5 | rabbit anti-NKX2.5 | H-114, sc-14033, Santa Cruz Biotechnology | 1 µg/mL (Western Blot, Indirect Immunofluorescence) |
| Myosin phosphatase-1 (MYPT1) | rabbit anti-MYPT1 | ab24670, Abcam | 1:10000 (Western Blot), 1:2000 (Indirect Immunofluorescence) |
| Sarcomeric α-actinin | mouse anti-sarcomeric α-actinin IgG1 | EA-53, MA1-22863, Sigma | 1:1000 (Indirect Immunofluorescence) |
| β-Tubulin | goat anti-β-Tubulin | ab21057, Abcam | 1 µg/mL (Western Blot) |

*reactive to human antigen, unless otherwise specified; † used to stain rat collagen-1, also reactive to human.
Table S3A. Immunoprecipitation (IP)-Mass Spectrometry (MS) analysis of whole cell lysates from mesenchymal-like A549 cells overexpressing Nkx2.5, which is predominantly nuclear. A molecular-weight range of 30-40 kDa was cut out of SDS-PAGE gels and analyzed by MS.

| Co-Immunoprecipitated Proteins | Symbol | Gene Ontology Annotation | # Peptides (≥3) |
|--------------------------------|--------|---------------------------|-----------------|
| Homeobox protein Nkx2.5         | Nkx2.5*| Nucleus/Cytoplasm          | 9               |
| Splicing factor U2AF 35 kDa subunit | U2AF1 | Nucleus                    | 4               |
| RNA-binding motif protein, X chromosome | RBMX | Nucleus                    | 6               |
| Nuclease-sensitive element-binding protein 1 | YBX1 | Nucleus                    | 4               |
| Lamina-associated polypeptide 2, isoforms beta/gamma | TMPO | Nucleus                    | 3               |
| Heterogeneous nuclear ribonucleoprotein A/B | HNRPAB | Nucleus                    | 3               |
| ATP-dependent DNA helicase Q1   | RECQL | Nucleus                    | 3               |
| Flap endonuclease 1             | FEN1  | Nucleus                    | 3               |
| Heterogeneous nuclear ribonucleoprotein R | HNRPR | Nucleus                    | 5               |
| RuvB-like 1                     | RUVBL1| Nucleus                    | 3               |
| Heterogeneous nuclear ribonucleoprotein H2 | HNRNPC2 | Nucleus                    | 5               |
| 60S ribosomal protein L7a       | RPL7A | RNA                        | 4               |
| 40S ribosomal protein S6        | RPS6  | RNA                        | 4               |
| Eukaryotic translation initiation factor 2 subunit 2 | EIF2S2 | RNA                        | 6               |
| Aldose reductase                | AKR1B1| Cytoplasm/nucleus          | 4               |
| Protein arginine N-methyltransferase 1 | PRMT1 | Nucleus/cytoplasm          | 4               |
| Probable ATP-dependent RNA helicase DDX6 | DDX6 | RNA                        | 3               |
| Eukaryotic translation initiation factor 2 subunit 1 | EIF2S1 | RNA                        | 4               |
| Coiled-coil-helix-coiled-helix domain-containing protein 3, mitochondrial | CHCHD3 | Mitochondrion/cytoplasm/nucleus | 4               |
| Carbonyl reductase [NADPH] 1    | CBR1  | Cytoplasm/secreted/nucleus | 3               |
| Annexin A5                     | ANXA5 | Cell membrane/nucleus      | 3               |
| Cyclin-dependent kinase 1       | CDK1  | Cytoplasm/cytoskeleton/nucleus | 3               |
| Alpha-2-HS-glycoprotein         | AHSG  | Cytoplasm/secreted         | 3               |
| Glucose-6-phosphate isomerase   | GPI   | Cytoplasm/secreted         | 5               |
| Adenosylhomocysteinase          | AHCY  | Cytoplasm                  | 4               |
| Sulfide:quinone oxidoreductase, mitochondrial | SQRDL | Mitochondrion              | 3               |
| Aldo-keto reductase family 1 member C1 | AKR1C1 | Cytoplasm                  | 3               |
| Stomatlin-like protein 2, mitochondrial | STOML2 | Cell membrane/mitochondrion | 3               |
| Asparaginase--tRNA ligase, cytoplasmic | NARS | Cytoplasm                  | 3               |
| D-3-phosphoglycerate dehydrogenase | PHGDH | Cytoplasm                  | 3               |
| Dnaj homolog subfamily A member 2 | DNAJA2 | Cell membrane              | 3               |
| Dihydropyrimidinase-related protein 2 | DPHYS2 | Cytoplasm                  | 3               |
| Rab GDP dissociation inhibitor beta | GDII | Golgi/cytoskeleton/cell membrane | 3               |

*Peptide Sequences (rel. to full length; see Supp. Fig. S10c): MFPSPALTPFPFSVKDEINLEQQRSLASGDLISAREATLAPASCMMLAFLKEAYSGPEASAASGLAELRAEMGPAAPPKKCSPAFPAAPFTYPFAYGDPPDPADRPDKKELCALQKAVELDAETGDAGERPARRRRRKRPRVLFSQAOVYYELRFRKKQRLSPARQDLQASVLKLTSTTVKIFWQNRKYYKCRQORQDQDTLELLGPPPPPAARJIAVPVLVRDGKPCGLGPAAYAPAYGVGLNAYGNYADYPYPSYGAAACSPYSCAAAYPAPPAAHAPASAANSFVNFVGVGDNTVQSPGMPQGNSGVSTLHGIRAW
Table S3B. Immunoprecipitation (IP)-Mass Spectrometry (MS) analysis of whole cell lysates from MSCs on rigid substrate, which shows NKX2.5 as predominantly cytoplasmic. A molecular-weight range of 30-80 kDa was cut out of SDS-PAGE gels and analyzed by MS.

| Co-Immunoprecipitated Proteins | Symbol | Gene Ontology Annotation | # Peptides (≥3) |
|--------------------------------|--------|--------------------------|-----------------|
| Homeobox protein NKX2.5        | NKK2.5*| Nucleus/Cytoplasm         | 3               |
| Splicing factor U2AF 35 kDa subunit | U2AF1  | Nucleus                  | 4               |
| Heterogeneous nuclear ribonucleoproteins C1/C2 | HNRNPC | Nucleus                  | 7               |
| KH domain-containing, RNA-binding, signal transduction-associated protein 1 | KHDRBS1 | Nucleus                  | 4               |
| DNA-(apurinic or apyrimidinic site) lyase | APEX1  | Nucleus                  | 4               |
| Pre-mRNA-processing factor 19 | PRPF19 | Cytoplasm/cytoskeleton/nucleus | 4            |
| Signal recognition particle subunit SRP72 | SRP72  | RNA                      | 4               |
| Polyadenylate-binding protein 1 | PABPC1 | RNA                      | 3               |
| ATP-dependent RNA helicase DDX3Y | DDX3Y  | RNA                      | 10              |
| Signal recognition particle subunit SRP68 | SRP68  | RNA                      | 3               |
| Spliceosome RNA helicase DDX39B | DDX39B | RNA                      | 3               |
| Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 | PLOD1  | ER                       | 6               |
| Transketolase                  | TKT    | Cytoplasm                | 6               |
| HLA class I histocompatibility antigen, A-29 alpha chain | HLA-A  | Cell Membrane            | 4               |
| Inhibitor of nuclear factor kappa-B kinase-interacting protein | IKBP;IKIP | ER                       | 3               |
| Spermidine synthase            | SRM    | Cytoplasm                | 3               |
| TRIO and F-actin-binding protein | TRIOBP | Cytoplasm/cytoskeleton   | 3               |
| Asparagine synthetase [glutamine-hydrolyzing];Asparagine synthetase | ASNS   | Cytoplasm                | 5               |
| EF-hand domain-containing protein D2 | EFHD2  | Cell membrane            | 4               |
| Glucosidase 2 subunit beta     | PRKCSH | Cytoplasm/ER             | 3               |
| Translocon-associated protein subunit alpha | SSR1  | ER/membrane              | 3               |
| Triosephosphate isomerase       | TPI1   | Cytoplasm                | 6               |
| Threonine–tRNA ligase, cytoplasmic | TARS   | Cytoplasm                | 3               |
| Flotillin-1                    | FLOT1  | Cell membrane            | 3               |
| 4F2 cell-surface antigen heavy chain | SLC3A2 | Cell membrane            | 3               |
| Protein FAM98B                  | FAM98B | Cytoplasm                | 3               |
| ADP/ATP translocase 2          | SLC25A5| Cell membrane/mitochondrion | 7         |
| Protein disulfide-isomerase A4 | PDIA4  | ER                       | 3               |
| Prolyl 4-hydroxylase subunit alpha-2 | P4HA2 | ER                       | 3               |
| Ribose-phosphate pyrophosphokinase 1 | PRPS1  | Cytoplasm                | 4               |
| Nicotinamide N-methyltransferase | NNMT   | Cytoplasm                | 3               |
| FLJ00144 protein               | FLJ00144 | Cell membrane            | 4               |
| Putative heat shock protein HSP 90-beta 2 | HSP90AB2P | Cytoplasm                | 3               |

*Peptide Sequences (rel. to full length; see Supp. Fig. S10d): MFPSPALTPTPSVKDILNLDAQQRSLAAAGELSARLEATLPLSCESSMLAACKPEAYAGPEAAAPGPELRELGRAPSAPKASAFPAAPAFYPRAAYSDDPDPAKPRAEKKLECAKAVELEKTEADNPRARRRRRPRRRRLFSQAQVYELERSPKQQQRYLSPAPRERQDLASVLKSTQSIRFQONRYKCKQRGRQDTQELVGLPPPPPPARRIAVPLVVRGLPKCLGDASAPYAPAYGVLNPYGYNAYPAYPGYGGAGACPSYQYSTAAAYPAGPSAPQPKTARANNNFVNFQGDNNAVQSPGPFQSNSGVSTLHIGRAW
Supplementary Figures
Figure S1

Mechanobiological Gene Transcripts in Human and Mouse (mdx) Muscular Dystrophy

a) i) LMNA vs COL1

ii) ACTA2 vs COL1

iii) CALD1 vs COL1

iv) RARG vs COL1

v) SRF vs COL1

vi) NKX2-5 vs COL1

Matrix Proteins

Nuclear Lamina Proteins

b) C57 vs mdx

C57 vs mdx

C57 vs mdx

C57 vs mdx
Figure S1 | Transcriptional and proteomic profiling, mechanics and histology of muscular dystrophy tissue. a, Tissue samples from patients with muscular dystrophy and mouse muscular dystrophy (mdx) model showed higher COL1 and correspondingly higher levels of i) LMNA, ii) ACTA2, and iii) CALD1. (Bottom) Transcription factors relevant in mechanotransduction pathways either iv) scale with (RARG), v) does not scale (SRF) with vi) or scale against (NKX2-5) increases in COL1 in dystrophic tissues. Inset: ACTA2 and NKX2-5 are anti-correlated. mdx = mouse muscular dystrophy model; FSHD = facioscapulohumeral muscular dystrophy; EDMD = Emery-Dreifuss muscular dystrophy; LGMD = limb-girdle muscular dystrophy; BMD = Becker’s muscular dystrophy; DMD = Duchenne muscular dystrophy. b, Quantification of matrix proteins in mouse muscle tissue by label-free mass spectrometry (MS, ref. 9). The signal strength of each ECM protein was normalized by lamin-B1 to account for variations in cell density between samples; mdx mouse tissue is shown relative to a control of C57 mouse tissue (y-axis shows fold-change). A Kruskal-Wallis location equivalence test showed the mdx mouse to have significantly more lumican, collagen-1 and collagen-3 than the control (n = 4). c, MS measurements also suggested that mdx mice had correspondingly higher i) lamin-A,C; ii) lamin-A to -B stoichiometry (n = 4; not significant).
Figure S2

--- Embedded fibers localize near the interface as a thin film ---

a

--- Embedded fiber bundles are heterogeneous and polarized ---

b

--- Mesh size dictates fiber size ---

c

--- Bulk rheology of PA gels ---

d

i) $G'$ (Pa)

$G'$ and $G''$ (Pa)

ii) $G'$, $G''$ (Pa)

Gel size dictates the timescale for cell traction generation.
Figure S2 | Characteristic fiber bundle size, registry and rheology of MMMS gels.  a, (Left) Top view of MMMS gel with 1-µm fluorescent beads (red) to delineate gel surface. (Right) Collagen-1 fiber bundles (green) localize as a heterogeneous thin film within ~10 µm near the gel surface (arrow).  b, Left, Fractal dimensions of fiber bundles, and collagen-coated surfaces compared to homogeneous tissue culture plastic (TCP). Mean ± s.e.m. ***p < 0.001. Right, Aspect ratio values of EC fiber bundles of varying cell-relevant projected areas indicate polarized bundle shapes. Inset: Immunofluorescence image of EC fiber bundles.  c, Embedded fiber bundles in polyacrylamide (PA) gels decreased in number as mesh size decreased in stiffer PA gels. Inset: Sirius red staining. Scale bars, 50 µm.  d, i) Time-sweep measurement showing development of bulk elastic moduli of various PA gels; embedding collagen-1 at 0.4 mg/mL (MMMS, green) does not increase its nominal elastic modulus (0.3kPa, blue).  ii) Bulk elastic (G') and viscous (G'”) moduli of MMMS and pure PA gels are measured via cone-plate rheometry at frequencies (0.0008 – 8 Hz) and strain (1%) that mimic traction forces exerted by a cell²¹ (grey area).
Figure S3

MSC does not spread on embedded collagen-only 0.3kPa gel

**a** i)  

![Cell Area (μm²)](image)

MSC does not spread on embedded collagen-only 0.3kPa gel

**b** i)  

![2d & 7d images](image)

Embedded Col1 in MMMS gel is stable
Figure S3 | Embedding Collagen in soft gel is stable but is not sufficient to induce cell spreading. a, i) MSC cell area (mean ± s.e.m.) on soft (CC 0.3kPa) and stiff (CC 40kPa) gels were significantly higher than on non-coated MMMS gel (embedded collagen-only 0.3kPa). ii), Myosin IIA (MIIA, green) and Collagen-1 (Col1, red) immunofluorescence of MSCs on gels. *p < 0.05, ***p < 0.001, compared to cells on CC0.3kPa gel. b, i) Immunofluorescence images of α-smooth muscle actin (SMA, red) in MSCs on MMMS cultured for 2 (left) and 7 (right) days showed that Col1 fiber bundles (green) were stable in the gel. ii) Cell number (blue) increased from 2 to 7 days, while SMA/cell and Col1 intensity remained the same. ***p<0.001, compared to 2 days on MMMS gel. Scale bars, 100 µm.
Surface rheology of functionalized PA gels

Collagen-1 coating depends on Sulfo-SANPAH crosslink density

Displacement profiles near MMMS fiber bundles and of a 10kPa PA gel are similar
Figure S4 | Surface rheology and functionalization of PA gel.  

a, Atomic force microscopy (AFM) measurements showed similar elastic moduli of bare PA (green) and CC-PA (red) gels.  
b, Atomic force microscopy measurements of functionalized PA gels; (top figure panel) Elasticity map (40 µm x 40 µm) of gel surfaces. (Bottom) Distribution of AFM-derived elasticity values of CC$_{0.3kPa}$ gels (blue), MMMS (mean $E = \sim 0.8$ kPa, green), and CC$_{40kPa}$ (red) gels. Adhesion (grey) and thin-film corrections (black) predict an order-of-magnitude higher stiffness.  
c, (Left) Covalent attachment of collagen-1 (Col1) on a PA gel ($E = 3$ kPa) depended on the concentration of Sulfo-SANPAH, a heterobifunctional crosslinker. Bound Col1 intensity was measured via immunofluorescence from confocal stacks through the gel. (Right) Absence of Col1 (+Antibody only) exhibited some Sulfo-SANPAH binding and reaction with fluorescent anti-Col1 antibodies diffusing in the gel.  
d, Photobleaching of immunolabeled Col1 coated via covalent crosslinking (red) or pure adsorption (blue) showed no recovery.  
e, Fit constants of surface pulling profiles assuming exponential decay (red, $a_0 e^{-b r}; R^2 > 0.90$) or classic Boussinesq decay$^7$ (black, $a_0/r; R^2 > 0.90$). Pulling profiles of fibers in MMMS gels behaved similar to those of 10kPa gels. *p < 0.001 (red), 0.05 (black); compared to CC$_{0.3kPa}$ gel.
Figure S5

Cell and nucleus shapes polarize on MMMS

a) Nucleus Shapes

![Graph showing cell aspect ratio vs. matrix elasticity](image)

- Untreated
- Blebbistatin

MMMS ($E_{eff} = 10-14$ kPa)

inhibited contractility

Matrix Elasticity, E (kPa)

b) i) Fiber bundle axis

![Images of cell polarization over time](image)

big cell

small cell

ii) Bundle axis aligned

not aligned

iii) small Cell, small Nucleus

33% 0°

-30° -30° -60° -90° 0° 30° 60° 90°

time-step (10*min)

55% 0°

-30° -30° -60° -90° 0° 30° 60° 90°

time-step (10*min)

Cells with high spread area polarize along local fiber bundle axis

---

Untreated Cell

Aspect Ratio

Matrix Elasticity, $E$ (kPa)

Blebbistatin

0 20 40

2 3 4

MMMS

TCP

inhibited contractility

Cells with high spread area polarize along local fiber bundle axis

---
Figure S5 | Matrix elasticity-dependent cell polarization. a, Quantification of cell aspect ratio was derived from nonmuscle myosin II A immunofluorescence staining of MSCs cultured for 2 days on gels. Myosin-II activity was inhibited with Blebbistatin (50 µM, red) for 30 minutes prior to fixation (n = 2, mean ± s.e.m). Representative nucleus shapes (top) are derived from Hoechst 33342-stained nuclear DNA. b, i) MSCs that spread highly (top panel) on polarized fiber bundles also polarize more along the bundle’s long axis and persist for several hours longer than MSCs with smaller spread areas (bottom panel). Colored arrowheads indicate cells tracked over time. Scale bar, 100 µm. ii) Alignment between a migrating MSC and a fiber bundle based on the angle difference, θ, of their respective major axes. iii) Real-time tracking of θ of small (mean cell/nuclear spread area <5000/400 µm²) and big cells (>5000/400 µm²) (8 cells each from a representative experiment).
**Figure S6**

Early response of Myosin IIB directs migration on heterogeneous matrix

**a**

Non-adhesive

Disperse Cells

**b**

% Surface Area Coverage

Distance (mm)

**c i)**

CC \(_{0.3\text{kPa}}\) region

Transition region

MMMS region

**c ii)**

Net Displacement (\(\mu m\))

(Transition region)

**d i)**

Durotaxis Index

Time (min)

**d ii)**

Unbiased Migration

Myosin IIB

HSP70

**d iii)**

Effective Change in Elasticity, \(\Delta E\) (kPa)

VSMC (Isenberg et al.)

-1

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**Figure S6 | MSC Durotaxis on gradient of embedded-collagen fibers.** MSCs’ possible contributions to repair of injured tissue such as an infarcted heart are controversial\(^{32}\), but evidence for cell trafficking from soft marrow to an injured site such as a stiff scar is compelling\(^{11}\). Such migration will typically involve a large gradient in stiffness (such as an infarct border zone of \(\sim 9\) kPa/mm\(^{33,34}\)), and with several other homogeneous gel systems, various cell types including MSCs have been seen to ‘durotax’ from soft to stiff matrix\(^8,27,33,35\). Here, a gradient of increasing embedded-collagen fiber bundles was made to resemble a transition from homogeneous soft tissue to a heterogeneous scar. a, A hybrid CC\(_{0.3\text{kPa}}/\text{MMMS}\) gel, with both nonfibrillar and scar-like regions, as an in vitro system to study MSC durotaxis.

b, Quantification of Sirius Red staining along the transition region of CC\(_{0.3\text{kPa}}/\text{MMMS}\) hybrid gel shows a 2 – 4 mm gradient of fiber bundles that saturates to a 20-30\% surface coverage, which is similar to scar fractions seen in diseased tissue\(^1,2\). c, i) Live cell imaging allowed us to track cells on either side of the Transition zone. Normalized migration trajectories and final positions (squares) of scrambled or myosin IIB siRNA-treated MSCs on CC\(_{0.3\text{kPa}}/\text{MMMS}\) hybrid gel after 20 hours of imaging. Migration far from the gradient was random in both CC\(_{0.3\text{kPa}}\) and MMMS regions. Durotaxis occurs if more cells move to the MMMS side (right) more than the CC\(_{0.3\text{kPa}}\) side (left). ii) Net positive displacement in the Transition region indicates MSC migration toward MMMS region over time. Statistical difference between scrambled (green) and myosin-IIB knockdown (red) was determined by two-way analysis of variance with Tukey’s HSD test at \(\alpha = 0.05\) significance level.

d, i) Cell centroid displacements every 15 min towards either the CC region or MMMS region were used to calculate a Durotaxis Index\(^8,27\). Durotaxis index over time of MSCs treated with either scrambled (red) or myosin-IIB siRNA (siMIIB, green) on the Transition region. No significant change in mean speed was seen in both scrambled (0.38 \(\mu\)m/min) and siMIIB-treated MSCs (0.40 \(\mu\)m/min). ii) Average durotaxis indices of scrambled and siMIIB-treated MSCs on various regions of the hybrid gel (*\(p < 0.05\), when compared to siMIIB). Inset: Western blot of siRNA-treated cells indicating \(\sim 75\%\) reduction of myosin-IIB. iii) Durotaxis index versus effective elasticity change \(\Delta E\) on different hydrogel gradient systems. Monotonic increase of Durotaxis Index with increasing gradient strength has been found previously for vascular smooth muscle cells (VSMC, ref. \(^{27}\)). Importantly, matrix heterogeneity did not obscure durotaxis probably because the turnover of cytoskeletal structures (e.g. MIIB) takes hours\(^8\). Cells thus average over fractal heterogeneities and ‘remember’ only the stiffer fiber bundles – which is not addressable with isotropic substrates that are of unknown or questionable relevance to scars. Haptotaxis did not contribute because MSCs did not spread on MMMS without the additional collagen coating (Supplementary Fig. S3a). Moreover, the minimum area fraction of ‘scar’ for imparting a mechanical memory is estimated to be 10-15\% since MSCs durotax into an increasingly heterogeneous scar-like matrix, with migration results similar to those on homogeneous gels. Mean ± s.e.m from \(n = 2\) independent experiments.
Figure S7

--- Embryonic heart matrix is composed mainly of collagen-1 ---

**a**

Fresh Day-13 Chick Heart → Decellularized Insoluble Mass

1% SDS 12 hrs → Trypsinize to Peptides → Chicken Genome → LC-MS/MS Sequencing

Total Peptide Ion Currents from each protein:

| Protein                                      | %   |
|----------------------------------------------|-----|
| Collagen alpha-1(I) chain (COL1A1)           | 58  |
| Collagen alpha-2(I) chain (COL1A2)           | 22  |
| Collagen alpha-1(III) chain (COL3A1)         | 7.8 |
| Collagen alpha-1(II) chain (COL2A1)          | 2.6 |
| Collagen alpha-2(V) chain (COL5A2)           | 0.71|
| Fibrillin-2 (FBN2)                           | 4.4 |
| Fibrillin-1 (FBN1)                           | 2.0 |
| Fibrillin-1/2 (FBN1/2)                       | 1.1 |
| Fibrillin-2/3 (FBN2/3)                       | 0.33|
| Periostin (POSTN)                            | 0.37|
| Heparan sulfate proteoglycan core (HSPG)     | 0.31|
| Vimentin (VIM)                               | 0.031|

Extracellular Matrix Proteins

Cellular Protein

--- MSCs durotax in 3D heart-derived matrix ---

**b**

i) Decellularized Heart strip on coverslip → Genipin, fluorescent crosslinker

ii) iii) Apparent Elasticity (kPa) → Genipin intensity (a.u.) → MSCs/Area (norm.) → Genipin intensity (a.u.)

Genipin, fluorescent crosslinker

MSCs durotax in 3D heart-derived matrix
Figure S7 | MSCs durotax in 3D heart-derived matrix. a, Decellularized day-13 embryonic chick heart that was trypsin-digested and sequenced in Mass Spectrometry revealed the compositional breakdown of extracellular matrix proteins, which was dominated by Collagen-1 based on total peptide ion current. Scale bar, 2 mm. b, To confirm and extend findings in Supplementary Fig. S6, a 3D model for scarred matrix was made. i) (Left) Decellularized strip of a left ventricular tissue from day-13 chick embryo is supported on a coverslip and half of the collagen-I-dominated matrix was stiffened by a fluorescent protein-crosslinker, Genipin.. (Right) MSCs expressing GFP (green) migrate within the matrix. DNA, blue. Scale bar, 50 µm. ii) Gradient in Genipin fluorescence intensity for the indicated region matches the measured gradient in apparent matrix elasticity measured by AFM. The apparent E of this 3D matrix determined by AFM approximates the results from AFM for CC_{0.3kPa} and MMMS gels (0.3–1 kPa; Supplementary Fig. S4b). iii) MSCs in the ex vivo matrix were plated homogeneously. After 1–2 days, cell numbers were depleted in the gradient region as a wave of MSCs accumulated in the crosslinked, stiff region. The findings are consistent with the net effect of durotaxis-based accumulation, as illustrated with the MMMS.
Flow cytometry analysis of responding cell population
Figure S8 | Heterogeneous MMMS elicits less cell-cell heterogeneity than stiff homogeneous gels. a, i) Measurements of lamin-A here likewise showed an increase on MMMS (see also Fig. 2D), consistent with our recent report that lamin-A is mechanosensitive to matrix stiffness⁷, likely through cytoskeletal stress exerted on the nucleus⁹, 36. Quantitative immunofluorescence analysis of SMA and lamin-A expression in MSCs cultured for 7 days on gels (n = 4; mean ± s.e.m.) revealed tight coupling of cytoskeletal and nucleoskeletal tension as predicted by the Mechanobiological Gene Circuit (MGC) model (dashed line; see Supplementary Discussion), with representative immunofluorescence images of lamin-A (scale bars, 10 µm). Inset: Lamin-A is known to regulate SRF, while SRF regulates ACTA2; however, a 65% knockdown of LMNA led to only 35% reduction of ACTA2. ii), Combined fluorescence in situ hybridization (FISH) and immunofluorescence (inset image) of MSCs showed lamin-A mRNA (LMNA, red) spot counts correlate with protein expression (green) in the single-cell level. (Inset plot) LMNA normalized to GAPDH mRNA spots (blue) indicate extent of knockdown. Scale bar, 10 µm. iii), Quantitative immunofluorescence analysis of SMA and lamin-A levels of MSCs on gels with or without Retinoic Acid (RA, 1 µM) treatment for 7 days on gels (n = 2; mean ± s.e.m.). Retinoic acid (RA) treatment also greatly reduced lamin-A levels⁹ on stiff substrates but SMA decreased much less, consistent with the NKX2.5 mechanorepressor pathway being independent of the RA/Lamin-A/SRF signaling axis. b, Mechanosensitive proteins MIIB and SMA show good correlation (data from Fig. 3E; n = 2). As in Fig. 3C, note the most heterogeneous cell response on homogeneous CC₄⁰kPa gels. c-d, Passage-6 MSCs were cultured on gels for 7 days, trypsinized, fixed, permeabilized and immunostained for SMA, Myosin IIA (MIIA) and its phosphorylation at Serine1943 (phospho-S1943: a marker of MIIA disassembly and loss of mechanosensitivity⁸). High-throughput intracellular flow cytometry of responding populations (out of 9500 cells per condition) confirms heterogeneities in the cells responding to gel stiffness. c, SMA heterogeneity is highest on homogeneous CC₄⁰kPa gels (KS test: p << 0.05 for all 3 comparisons). d, On the other hand, phospho-S1943 heterogeneity is highest on CC₀.₃kPa gels (KS test: p << 0.05 for all 3 comparisons), with no obvious differences in total MIIA levels (inset). Upper right inset shows immunofluorescence images of cells analyzed by flow cytometry; scale bar, 50 µm. Note that differences in SMA levels here are not as pronounced as with immunofluorescence image analyses in Fig. 3C due to high-passage of cells and/or the needed trypsinization (which disassembles cytoskeleton) with permeabilization (which might extract disassembled SMA [42 kDa], but not MIIA [220 kDa]) of cells.
MSCs at day 2 respond to MMMS and stiff matrix by spreading, increasing SMA, and losing nuclear NKX2.5.

(a) Figure S9

(b) Graph showing the relationship between nuclear NKX2.5 and smooth muscle actin (SMA).Responders are distinguished by a smaller increase in nuclear NKX2.5.

(c) Additional analysis from Fig. 4B, indicating a small increase in nuclear NKX2.5 for strong responders and more weak responders.
Figure S9 | NKX2.5 and SMA heterogeneity on MMMS and stiff gels. a, Randomly selected MSCs cultured for 2 days on soft CC\textsubscript{0.3kPa}, MMMS and stiff CC\textsubscript{40kPa} gels. Relative to cells on soft gel, most cells on MMMS and stiff gels have high SMA levels and predominantly cytoplasmic NKX2.5. b, Single-cell image analyses of nuclear/total NKX2.5 and SMA (unfilled circles, 30 - 40 cells per condition). Mean (± s.e.m.) expressions of non-responder (unfilled squares) and responder (filled squares) subpopulations are shown per condition. Non-responder cells are determined as in Fig. 3C (see also Methods). **p < 0.001 compared to CC\textsubscript{0.3kPa} SMA; %%%p < 0.001 compared to MMMS SMA; ##p < 0.001 compared to CC\textsubscript{0.3kPa} nuclear/total NKX2.5. c, Left, due to highly cooperative nature of NKX2.5-mediated mechano-repression (see curve fit in Fig. 4B), a small increase in nuclear NKX2.5 greatly decreases the number of strong responders (Right) without much affect on numbers of weak or non-responders in terms of SMA levels. This explains why SMA-expression variations in MSCs on homogeneous CC\textsubscript{40kPa} gels is noisier than in cells on MMMS gels (see also Figs. 3C–F).
Figure S10

—— NKX family —— Localization of Nkx2.5 in mechanosensitive cardiomyocytes ——

(a) Normalized Abundance

| Protein | Value |
|---------|-------|
| Nوخ1.2  | 0.33  |
| Nوخ2.1  | 0.10  |
| N وخ2.2  | 0.12  |
| N وخ2.3  | 0.17  |
| N وخ2.4  | 0.74  |
| N وخ2.5  | 1.00  |
| N وخ2.6  | 0.27  |
| N وخ3.1  | 0.15  |
| N وخ3.2  | 0.55  |
| N وخ6.1  | 0.27  |
| N وخ6.2  | 0.32  |
| N وخ6.3  | 0.20  |
| N وخ6.4  | 0.84  |

(b) i) Embryonic day 5 cardiomyocytes

ii) Nkx2.5 Total Intensity

(c) Validation of NKX2.5 antibody by mass spectrometry

(d) Mass Spectrometry-detected Nkx2.5 peptides (30 - 40 kDa)

(e) Nkx2.5 in A549 cells confirm importance of NLS to nuclear localization

(f) NLS mutants:

Wild-type: RRRRRR
NLS1: AAAAAB
NLS2: AAAAAN
NLS3: AAAAAA
Figure S10 | NKX2.5 abundance and localization. a, Microarray profiling revealed relative abundances within the NKX family transcriptome in human MSCs, with NKX2-5 to be the most abundant. b, i) Matrix stiffness modulates Nkx2.5 expression in embryonic cardiomyocytes. Immunofluorescence staining of Nkx2.5 (left) and sarcomeric α-actinin (right, red) in embryonic day-5 (E5) chick cardiomyocytes plated on CC_{0.3kPa} PA gel for 3 days. Hoechst 33342 for DNA, blue. Scale bar, 50 μm. ii) Quantification of total Nkx2.5 intensity (top) and nuclear-to-total Nkx2.5 intensity (bottom) in E5 chick cardiomyocytes cultured on soft (0.3 kPa) and stiff (40 kPa) gels for 3 days (n = 2, mean ± s.e.m). *p < 0.05; compared to CC_{0.3kPa} gels. c, Left, Western analysis of wild-type Nkx2.5 overexpressed in A549 cells, with the expected molecular weight (MW) of 35 kDa, as well as higher-MW bands that may be post-translationally modified. Degradation was also found evident in Nkx2.5-overexpressed cells (bands < 35 kDa). Right, We analyzed gel bands (in the 30–40 kDa range) by mass spectrometry (MS) and observed 7 peptides unique to Nkx2.5 that covers 28.6% of the full-length protein sequence and a median intensity that ranks 50th out of 496 detected proteins in that band region. d, MS also detected 3 NKX2.5 peptides in immunoprecipitated (IP) MSC lysates (analyzed in the 30–80 kDa range). e, Nkx2.5 was found to co-localize with MYPT1 in lamellipodia (arrowheads) of Nkx2.5/MYPT1-overexpressing A549 cells. f, Overexpressed wild-type Nkx2.5 is strongly localized in the nuclei of A549 cells that, compared to MSCs, express little to no endogenous NKX2.5; alanine mutations of the nuclear localization signal (NLS) cause loss of nuclear enrichment. Scale bar, 10 μm.
Retinoic Acid inhibits calcification on scar-like and stiff matrices

Mechanorepressor NKX2.5 delays SMA expression by decoupling from SRF Pathway

**Mechanobiological Gene Circuit (MGC)**

- **Insoluble Factors:**
  - Scar Matrix
  - Micro-stiffness

- **Soluble Factors:**
  - e.g. Retinoic Acid (RA)

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**Legend:**
- gene
- protein
- protein interactions

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**Figure S11**

- **a** Alkaline Phosphatase Staining (1 wk)
- **b** Alizarin Red Staining (4 wks)

Staining Controls
**Figure S11 | Stiffness of MMMS increases lamin-A and osteo-commitment of MSCs.** Our analyses of striated muscle diseases prone to scarring revealed an increase in lamin-A (Fig. 1C, S1), which is a nucleoskeletal protein implicated in various differentiation and maturation processes\(^9\), \(^{38}\), \(^{39}\). **a**, Lamin-A regulates nuclear entry of a retinoic acid (RA) receptor (RARG), which is highly expressed in bone\(^9\), and osteogenesis is also positively regulated by the SRF pathway\(^{40}\), which appears stiffness-activated. Previous studies further showed that MSCs injected into infarcted hearts can sometimes form calcified structures\(^{24}\) that would be detrimental to the beating heart. Given the long-term maintenance of a scar-like phenotype for MSCs (Figs. 2C, 6A), we studied osteogenesis on the MMMS gels. MSCs on gels cultured in serum-only (UT) or in osteogenic induction media (OIM) with or without RA (1 µM) for 1 week and stained for Alkaline Phosphatase (ALP), an early marker of osteogenic commitment. Compared to MSCs cultured on soft gels for a week with osteogenic induction media (OIM), MSCs on MMMS and CC\(_{40kPa}\) gels exhibited greater Alkaline Phosphatase (ALP) activity. **b**, Left, MSCs on gels cultured for 4 weeks and stained for Alizarin Red, a late marker for bone; calcification was evident on the stiffer matrices. Osteogenic indices (top right of each image, see Supplementary Information) are relative to UT MMMS gels \(n = 2\). Scale bars, 100 µm. MSCs on CC\(_{0.3kPa}\) gel were negative for both osteogenic markers, and the lack of significant Alizarin Red staining on MMMS gels confirms observations that its \(E_{\text{eff}}\) is well below what is needed for osteogenic commitment either *in vitro*\(^{41}\) or *in vivo*\(^{24},^{34}\). In all cases, RA treatment inhibited both ALP activity and Alizarin Red staining, consistent with reduction of stress-stabilized lamin-A. The results suggest that the rigid substrates here tend to be osteogenic, and that therapeutically relevant doses of RA could be useful – when needed – for inhibiting osteogenesis. Right, Alizarin Red staining of adult mouse tibia (mature bone) and chick embryo at day-4 (prior to bone development) are positive and negative controls, respectively. **c**, MGC model (see Supplementary Discussion for derivation) predicts that local increases in matrix stiffness, can stress-stabilize and couple cytoskeletal and nucleoskeletal tension. Retinoic acid (RA) disrupts stress-stabilized lamin-A assembly (but only partially disrupts downstream SMA) through retinoic acid receptor - gamma (RARG) (ref. 9), as NKX2.5 independently functions as a mechanosensitive repressor of SMA.
Figure S12

**a**

Replicate 1
Replicate 2
Replicate 3

\[ P_{\text{upreg}} = 0.002 \]

\[ P_{\text{downreg}} = 3.6 \times 10^{-12} \]

**b**

\[ P_{\text{upreg}} = 5.8 \times 10^{-11} \]

\[ P_{\text{downreg}} = 3.4 \times 10^{-21} \]

**c**

Rounded Nucleus
(Soft Substrate, and \( t = 0 \) on Stiff Substrate)

Stiff Substrate
- multiple nuclear interactions and slow degradation can delay loss

**d**

Fibrogenic genes
Epithelial genes
(Rinkevich et al 2015)

\[ \text{COMP} \]
Figure S12 | NKX2.5 overexpression and mechanism of its delayed nuclear exit. a, Gene expression levels (log₂-intensity values) for NKX2-5 and ACTA2, were obtained from microarray (Affymetrix Human Gene 1.0 ST) analysis at the probe level (n = 3 technical replicates at 1/3-, 1-, and 3-fold of the standard load for arrays). Other genes that increase (CD74) or decrease (COMP) with overexpression of NKX2.5 were consistent with b, findings in mouse fibroblasts²⁰ (with or without addition of other transcription factors: MYOCD, MEF2C, and TBX5). MSCs were transduced with rtTA/tetO-NKX2.5 (OE) or rtTA only (WT) and treated with doxycycline (see also Fig. 5A) for 1 week. The probability of upregulation of a transcript (e.g. NKX2-5, CD74) was simply calculated with the formula: \( P_{\text{upreg}} = \frac{0.5 \times \text{counts above diagonal}}{0.5 \times \text{counts below diagonal}} \). A similar formula was used for downregulation (e.g. ACTA2, COMP). Similar results are obtained when raw intensities are normalized by a housekeeping GAPDH probe. c, Left, Predicted transcription factor binding of NKX2.5 and Serum Response Factor (SRF) using MatInspector (www.genomatix.de; ref. ⁴²) on a region near transcription start site of ACTA2. Previously known consensus sites for NKX2.5 (ref. ¹³) and SRF (refs. ⁴³) are also shown. Two NKX2.5 binding sites have been shown to synergistically repress ACTA2¹³, possibly in a dimerized form¹⁸. This repressive loop is reminiscent of the well-studied LacI repressor: when a series of repressive loops are upstream of an induced promoter in interphase cells, transcription doubles by half-hour after induction as the chromatin visibly de-condenses⁴⁴. In other words, de-condensation does not occur in seconds. For the NKX2.5 system here, a repressive-dimer loop may need to be lost before SRF can drive ACTA2 transcription, and this de-condensation could take 1–2 orders of magnitude longer. Right, More importantly, multiple nuclear interactions (Table S3A) contribute to retention of NKX2.5 in the nucleus. At sufficiently long times (several days) of sustained cytoskeletal tension, the nuclear pool of NKX2.5 is depleted and eventually degraded as ACTA2 is activated. d, Left, Effect of NKX2.5 overexpression on fibrogenic and epithelial genes²² in late passage MSCs, particularly on cell-surface peptidase DPP4 (CD26) downregulation (n = 3); Right, LMNA knockdown (KD) also leads to reduced DPP4 expression (n = 3). Mean ± s.e.m.
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