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Rewiring mesenchymal stem cell lineage specification by switching the biophysical microenvironment

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The propensity of stem cells to specify and commit to a particular lineage program is guided by dynamic biophysical and biochemical signals that are temporally regulated. However, most in vitro studies rely on "snapshots" of cell state under static conditions. Here we asked whether changing the biophysical aspects of the substrate could modulate the degree of mesenchymal stem cell (MSC) lineage specification. We chose to explore two diverse differentiation outcomes: MSC osteogenesis and trans-differentiation to neuron-like cells. MSCs were cultured on soft (~0.5 kPa) or stiff (~40 kPa) hydrogels followed by transfer to gels of the opposite stiffness. MSCs on soft gels express elevated neurogenesis markers while MSCs on stiff substrates express elevated osteogenesis markers. Transfer of MSCs from soft to stiff or stiff to soft substrates led to a switch in lineage specification. However, MSCs transferred from stiff to soft substrates maintained elevated osteogenesis markers, suggesting a degree of irreversible activation. Transferring MSCs to micropatterned substrates reveal geometric cues that further modulate lineage reversal. Taken together, this study demonstrates that MSCs remain susceptible to the biophysical properties of the extracellular matrix—even after several weeks of culture—and can redirect lineage specification in response to changes in the microenvironment.

Differentiation of stem cells is not a binary event but involves several phases, where a less specialized cell becomes more specialized through several transitory states[1–4]. For instance, mesenchymal stem cells (MSCs) under specific contexts are coaxed to specify osteoprogenitor markers and then mature to pre-osteoblasts before finally committing to osteoblast and osteocyte phenotypes[5]. This gradual lineage progression may serve as an amplifying function to regulate the spatiotemporal distribution of cells that are required for a specific regeneration or homeostasis process[1]. Alternatively, subtle changes in cell state may foster transitions where a progenitor is more prone to reprogramming back to the multipotent stem cell state compared to a committed cell[6]. Emerging evidence suggests the latter scenario occurs more readily than anticipated and that cellular plasticity enables dynamic shifting of cell state through regulation of distinct epigenetic marks[7–9]. In addition to plasticity within a defined lineage program, numerous reports now indicate that MSCs may harbor the potential to trans-differentiate across germ layers[10–13]. Understanding the timescales and plasticity underlying stem cell fate determination is important for fundamental biology as well as for establishing appropriate in vitro culture conditions to direct a desired outcome.

The majority of efforts to control cell programming or reprogramming in the laboratory involve empirically derived media formulations of small molecules and proteins. More recently, the design of synthetic extracellular matrices that convey information from the microenvironment surrounding cells to regulate lineage programs has garnered attention[14,15]. Cells sense their mechanical microenvironment through the interplay of integrin mediated focal adhesions and actomyosin based cellular contractility to direct intracellular signaling programs that regulate cell functions[16–19]. This process of mechanotransduction has been shown to play a key role in modulating the lineage specification of MSCs, where the biochemical and biophysical properties of the extra-cellular matrix are integrated with soluble signals to guide signal transduction cascades that regulate gene expression and cell fate. Model extracellular matrices, where the biochemical and biophysical properties of the cell culture material can be systematically varied, have proved useful in dissecting the importance of microenvironmental signals during cell fate determination[6,10,20–27]. For example, human mesenchymal stem cells (MSCs) isolated from bone marrow or adipose tissue, when cultured on hydrogels of tunable stiffness will specify lineage programs according to native tissue-mimetic stiffness[10,25,26,28,29]. In a report by Gilbert et al., the importance of mechanotransduction in vivo was demonstrated by showing how the engraftment of skeletal muscle stem cells

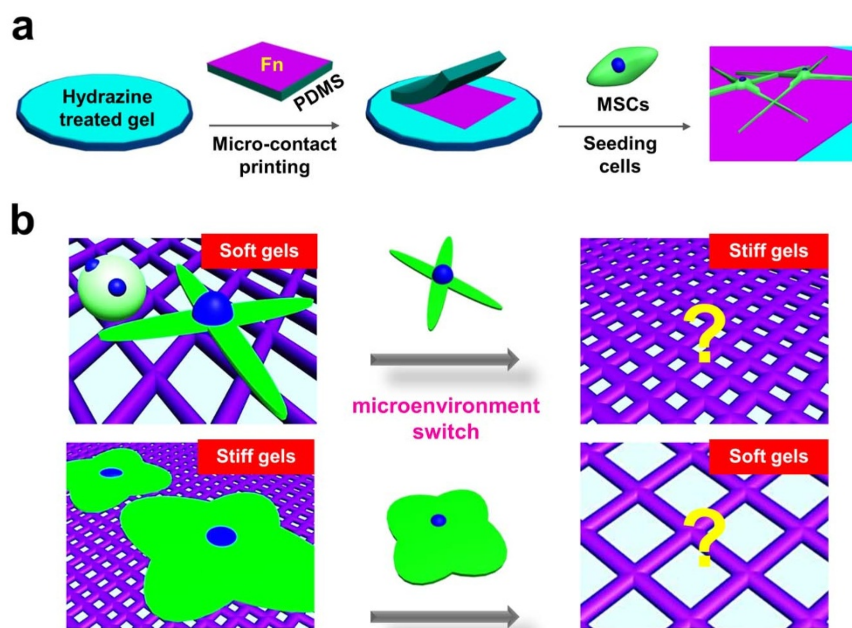

Figure 1 | Hydrogel fabrication scheme and experimental strategy. (a) Protocol for fabricating matrix protein conjugated polyacrylamide hydrogels. (b) Schematic illustration of microenvironment switch between soft (0.5 kPa) and stiff (40 kPa) substrates to monitor the dynamic changes of MSC lineage specification.

after isolation and expansion is influenced by the stiffness of the in vitro expansion substrate[30]. These reports highlight the importance of matrix mechanical properties for in vitro expansion after isolation and when designing a clinically relevant biomaterial.

While most studies aimed at elucidating the biophysical cues that regulate cell fate have involved static in vitro cultures, several recent reports have varied the properties of the substrate during culture[31–35]. Burdick and colleagues used an in situ tunable hydrogel system to study how changing matrix stiffness can modulate the degree of adipogenesis and osteogenesis in MSCs exposed to a mixed-media of soluble differentiation cues; increasing the stiffness of a hydrogel earlier will enhance osteogenesis while leaving the gel soft for longer periods promotes adipogenesis[36]. Anseth and colleagues used a dynamic softening hydrogel system to explore how the mechanical properties of the substrate are sensed by MSCs and how this information is retained over time[35]. They found that the transcriptional activators YAP and TAZ are activated in response to hydrogel stiffness that is reversible after short culture periods; however, after prolonged culture on stiff substrates YAP activation promotes irreversible lineage commitment. This is important because the majority of ex vivo MSC culture is performed on rigid (~GPa) tissue culture plastics, which may adversely affect the multipotency of MSCs[6]. Understanding MSC plasticity and the temporal regulation of lineage specification associated with the biophysical properties of biomaterials—for the canonical lineages and for putative trans-differentiation events—is an important undertaking to define the appropriate conditions to direct differentiation to specific lineages.

In this paper we use a combination of tunable stiffness hydrogels and single cell micropatterning to explore the plasticity of MSCs when cells are shifted between matrices of very different biophysical properties. Previously it has been shown that MSCs cultured on hydrogel substrates >40 kPa will commit to the osteogenesis lineage[10,25] while MSCs cultured on hydrogels <1 kPa will express markers associated with trans-differentiation to the neuronal lineage[10,26]. Here we explore the effect of rigid substrate pre-culture on the expression of neurogenic markers and the effect of soft substrate pre-culture on the expression of osteogenic markers. Monitoring the change in expression of markers associated with distinct stages of lineage commitment reveals reversible expression of early stage markers in response to both substrate stiffness and geometric constraints with less variation in markers associated with mature lineage outcomes.

Results

The influence of substrate switching on cell spreading and viability. To explore the influence of stiffness on mesenchymal stem cell (MSC) lineage marker expression, we chose to use polyacrylamide hydrogels which are an established model extracellular matrix (ECM) due to high water content and tunable stiffness by varying the ratio between Acrylamide and Bis-acrylamide. The preparation method is schematically presented in Fig. 1a. First, we prepared hydrogels (soft-0.5 kPa and stiff-40 kPa) on glass coverslips and then treated with hydrazine hydrate, modifying the surface chemistry of the gels[25,26]. Subsequently, fibronectin was oxidized and patterned onto the hydrazine treated gels by soft lithography using patterned or unpatterned polydimethylsiloxane (PDMS) stamps. The stiffness of the gels were confirmed using AFM measurements (data not shown). Since matrix stiffness can direct lineage specification of MSCs based on the similarity to the committed cells' native matrix—soft gels (~0.5 kPa) promote the expression of neurogenesis markers and stiff gels (>30 kPa) promote the expression of osteogenesis markers—we asked whether transferring MSCs from a soft to a stiff gel or vice versa would influence the expression of stiffness-directed lineage markers. After culture for 10 days the projected cell area for MSCs were ~4000 μm^2 and ~12000 μm^2 for 0.5 and 40 kPa hydrogel substrates respectively. After trypisinization and transfer between soft and stiff matrices, there were significant changes in spreading behavior. MSCs that were transferred to stiff substrates after 10 days of culture on soft substrates showed an increase in spread area from ~4000 to ~7000 μm^2 (5 days after transfer) and ~10000 μm^2 (10 days after transfer). MSCs that were transferred to soft substrates after 10 days of culture on stiff substrates showed a reduction in cell area from 12000 to ~8000 μm^2 (5 days after transfer) and ~6000 μm^2 (10 days after transfer) (Fig. 2a and b). The final spread area after 10 days from switching was comparable to MSCs cultured on the same stiffness gels without transfer. This

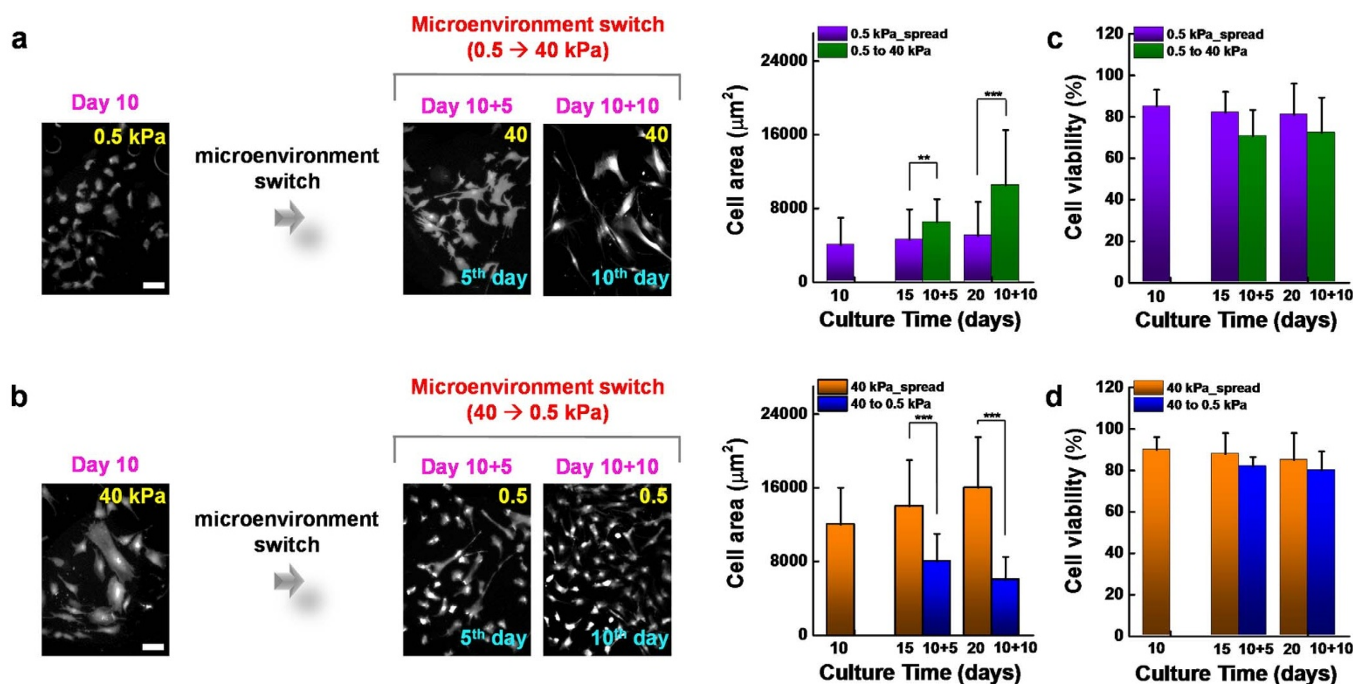

Figure 2 | Projected cell area and viability are influenced by changing the mechanical properties of the substrate. (a) – (b) Representative immunofluorescence microscopy images and quantitation of average cell area of MSCs cultured for 10 days and after microenvironment switch (0.5 ⇔ 40 kPa); scale bar: 120 μm (**P<0.005, ***P<0.0005, Fisher's exact test). (c) – (d) Cell viability of MSCs before and after substrate switch.

suggests that MSC spreading characteristics can recover in response to new mechanical microenvironments. We performed a cell viability assessment before and after the substrate switch (Fig. 2c and d). Cells cultured on stiff substrates displayed higher viability than those cultured in soft substrates. In addition, cell viability decreased when cells were transferred to substrate of opposite stiffness (e.g. soft to stiff and stiff to soft) compared to cells cultured with no environment change. Interestingly, cell viability after transfer depended on the mechanics of the final substrate where viability decreased by 15% (soft to stiff) and 8% (stiff to soft). Total cell viability after transfer (stiff to soft, total 20 days culture) showed similar level of viability of cells cultured on soft substrates only for 20 days. MSCs proliferated on both hydrogel substrates but tended to grow faster on the 40 kPa hydrogels. After transfer to the alternate environment the cells continued to proliferate (Supplementary Figure S3).

The plasticity of lineage specific marker expression. To assess the expression of lineage specific markers in response to the mechanical properties of our polyacrylamide gels, we chose to immunostain MSCs for early and late stage markers associated with neurogenesis (β3tubulin and MAP2) and osteogenesis (runx2 and osteopontin) (Fig. 3 and Supplementary Fig. S4). Cells cultured on soft substrates show elevated expression of neurogenic markers (~6-fold higher for β3 tubulin and ~3-fold higher for MAP2 than cells cultured on stiff substrates) while cells cultured in stiff substrates tend to express elevated osteogenic markers (~7-fold higher for runx2 and ~2-fold higher for osteopontin than cells cultured on soft substrates), and regardless of stiffness cells stably express the neurogenic and osteogenic markers at nearly constant levels from 10 to 20 days. Since recent studies have demonstrated plasticity in marker expression in response to substrate mechanics[35], we transferred MSCs after culture for 10 days from soft to stiff and stiff to soft, and performed immunofluorescence characterization after 5 days and 10 days culture on the new substrates. The choice of 10 days for the transfer point was guided by our previous studies which demonstrated maximum neurogenic expression in MSCs cultured on soft polyacrylamide hydrogels[26]. Furthermore, osteogenic marker expression plateaued at 10 days and there was no significant difference in matrix mineralization under these conditions as determined by Alizarin Red staining (Supplementary Fig. S5). Transferred MSCs (stiff to soft) showed decreased levels of osteogenic marker expression and increased expression level of neurogenic markers depending on culture time relative to cells maintained in culture on stiff substrates. However, when cells were transferred to soft gels after 10 days on stiff gels, the expression of nuclear runx2 remained elevated compared to MSCs that were cultured on soft gels for 10 days. In contrast, transferred MSCs (soft to stiff) tended to decrease the expression of β3tubulin and increase the levels of runx2 to levels that are comparable to cells that were cultured on the stiff gels alone. This suggests that stiff gels promote a degree of irreversible runx2 activation that is insensitive to changes in substrate stiffness. We observed the same trends in lineage specification modulation in response to stiffness changes for the late markers osteopontin and MAP2; however the changes were less pronounced. To further verify the observed fluctuations in lineage specification, we performed gene expression analysis using quantitative real-time polymerase chain reaction (PCR) (Supplementary Fig. S6). We observed the same trend as with the immunofluorescence results: we see a higher degree of neurogenic and lower degree of osteogenic transcript expression for cells (stiff to soft), and elevated levels of osteogenic and lowered levels of neurogenic transcripts for cells (soft to stiff).

The effect of cell shape on modulating the plasticity of lineage specific markers. Recently we demonstrated how cell geometry can be controlled across hydrogel substrates using microcontact printing to refine the degree of osteogenesis[25] and neurogenesis[26] in adherent MSCs. Since transferring MSCs from stiff to soft substrates did not lead to a complete lineage reversal, we asked whether transfer to patterned substrates, with shapes that are expected to enhance the stiffness-directed lineage, could further direct the lineage switch. MSCs were cultured on unpatterned soft or stiff substrates for 10 days, and then transferred to different stiffness substrates containing patterns of fibronectin (circle, oval, star, or unpatterned; 5000 μm^2) (Supplementary Fig. S7). As shown

Figure 3 | Matrix stiffness modulates the degree of MSC lineage specification. (a) Expression of osteogenic (runx2 and osteopontin) and neurogenic (β-tubulin and MAP2) markers before and after switching the substrate (0.5 ⇔ 40 kPa) (*$P<0.05$, **$P<0.005$, ***$P<0.0005$, Fisher's exact test). (b) Representative immunofluorescence microscope image of MSCs cultured on the unpatterned fibronectin coated substrates after immunostaining for nuclei, osteopontin, MAP2 and filamentous actin; staining for MSC nuclei (blue), actin (cyan-green), osteopontin (orange), MAP2 (red). Scale bar: 35 μm.

in the preceding section, after 10 days of culture the spread cells on the soft substrates show elevated expression of neurogenic markers (β3tubulin and MAP2) while cells that spread on stiff substrates express elevated osteogenic markers (runx2 and osteopontin). For patterned cells where the mechanical properties change from soft to stiff, the extent of staining for neurogenic or osteogenic markers by circular cells was similar to spread cells (Fig. 4a and b). Interestingly, transferred cells (soft to stiff) in oval and star shapes showed higher expression of osteogenic markers compared to cells in other shapes; patterned star shaped cells displayed over 2-fold enhancement in runx2 expression compared to spread or circular cells. In fact, after transfer of MSCs from soft gels to star shapes on stiff gels, runx2

Figure 4 | Cell shape directs lineage specification of MSCs after microenvironment change. (a) Quantitation of osteogenic (runx2 and osteopontin) and neurogenic (β-tubulin and MAP2) markers for a population of cells cultured with and without a microenvironment change from soft to stiff. (b) Representative immunofluorescence images. (c) Expression of osteogenic (runx2 and osteopontin) and neurogenic (β-tubulin and MAP2) markers for a population of cells cultured with and without a microenvironment change from stiff to soft. (d) Representative immunofluorescence images; staining for MSC nuclei (blue), actin (cyan-green), runx2 and osteopontin (orange), β-tubulin and MAP2 (red). Scale bar: 35 μm. (*$P<0.05$, ***$P<0.005$, Fisher's exact test).

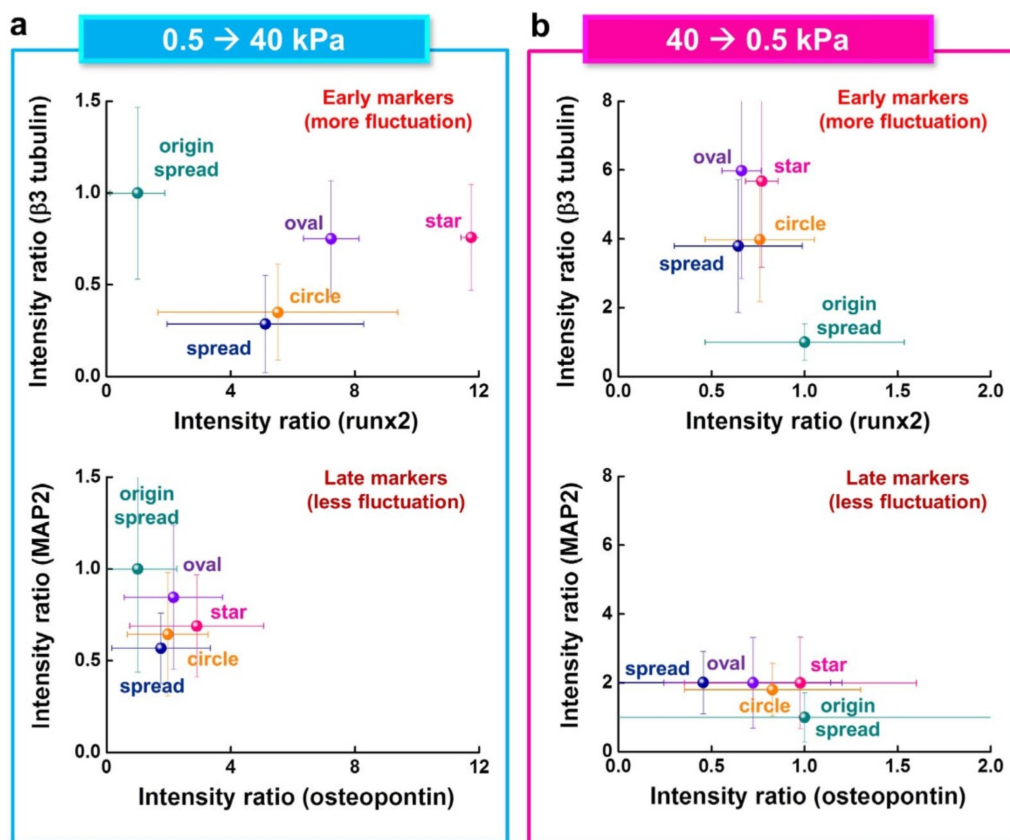

Figure 5 | Geometric cues differentially reprogram early and late markers of neurogenesis and osteogenesis for (a) soft to stiff and (b) stiff to soft.

expression is significantly higher than unpatterned MSCs cultured on stiff gels alone for 10 days. In addition, MSCs that were transferred from soft gels to oval and star shapes on stiff gels—geometries which have previously been shown to enhance neurogenic marker expressions[26]—displayed a significantly smaller reduction in β3tubulin expression (~1.4-fold) compared to spread or circular cells (> 3-fold declines). When MSCs were transferred from stiff to soft substrates, there was no appreciable difference in osteogenic markers across unpatterned and patterned cells. However, MSCs that were transferred from stiff gels to soft gels patterned with oval and star shapes displayed higher changes in neurogenic marker expression (~6-fold for β3tubulin and ~2-fold for MAP2) compared to cells that were unpatterned or in circle shapes.

Next we analyzed trends in expression for early and late stage markers for differentiation (Supplementary Fig. S9 and S10). Fig. 5 summarizes the results when MSCs are transferred from soft to stiff or stiff to soft substrates (cultured for 10 days (initial stiffness) + 10 days (final stiffness)) for patterned and unpatterned cells. It is clear that the expression of early markers for neurogenesis (β3tubulin) and osteogenesis (runx2) display significantly more fluctuations compared to late markers (MAP2 for neurogenesis and osteopontin for osteogenesis). For instance, cells transferred from soft to stiff substrates and captured in star shapes showed ~12-fold increase in runx2 compared to a ~3-fold increase in osteopontin. Similarly, cells transferred from stiff to soft substrates and captured in oval shapes show ~6-fold increase in β3tubulin compared to only 2-fold increase in MAP2.

Discussion

The commitment of adult stem cells to a particular lineage is a complex process involving subtle changes in gene expression patterns as the multipotent cell progresses through intermediate progenitor states. Committed progenitors have also been shown to reprogram to more primitive multipotent states under defined conditions. Lineage specification and reversal in vivo is likely context dependent and guided by combinations of biochemical and biophysical cues in the extracellular microenvironment. Understanding how the properties of in vitro cell culture substrates and prospective cellular delivery materials directs fate-specific differentiation in the laboratory is essential for stem-cell based therapies.

In this paper we explored the plasticity of lineage specification of mesenchymal stem cells (MSCs) cultured on hydrogels of variable stiffness. Understanding MSC plasticity in vitro is important because these cells are one of the most promising adult stem cell types for regenerative therapies. MSCs have been shown to reverse the specification of lineage specific markers in response to changes in soluble media components[10]. Since the physical properties of the MSC microenvironment has been shown to exert an influence on lineage specification, we asked whether changes in the biophysical properties of the substrate over time would redirect the expression of lineage specific markers. We chose to examine two very different MSC fate decisions: the widely studied differentiation of MSCs to osteoblasts, and the more controversial transdifferentiation of MSCs to cells of neuronal lineage. We chose these particular stiffness-directed outcomes because they show the largest difference in native tissue mechanical properties (0.5 kPa for neural tissue and 40 kPa for pre-calcified bone). We employed both early and late markers for neurogenesis (β3-tubulin and MAP2) and osteogenesis (runx2 and osteopontin) to ascertain the degree of which physical cues of the substrate guide lineage specification and reversal after transfer to a new microenvironment. β3-tubulin is a marker for immature neurons that is expressed prior to the neuron-specific protein, microtubule-associated protein 2 (MAP2)[37]. Runx2 is an important transcription factor expressed in early osteoblast progenitors that precedes the expression of bone-associated markers like osteopontin (mid/late osteoblast)[5]. After culture on soft gels for 10 days, MSCs

show reduced area, extension of neuronal-like processes and elevated expression of β3-tubulin and MAP2. In contrast, MSCs cultured on stiff gels show high spreading and elevated expression of runx2 and osteopontin. After culture on soft gels for 10 days, MSCs were transferred to stiff gels and cultured for 5 and 10 days. At both time points there was a decrease in neurogenic markers and an increase in osteogenic markers, where at the 10 day time point expression levels of osteogenic markers were comparable to MSCs that were cultured exclusively on stiff gels for 20 days. In contrast, MSCs that were first cultured on stiff gels and then transferred to soft gels only showed a modest decrease in runx2 after 10 days on the new substrate suggesting that active nuclear runx2 remains operable after the microenvironment switch. This finding is consistent with a recent report by Anseth and colleagues that demonstrated one week culture on rigid surfaces promotes nuclear runx2 expression that remains active after the surrounding matrix is softened[35]. There was an increase in the expression of neurogenic markers when MSCs cultured on stiff gels were transferred to soft gels, albeit not as high as MSCs that were cultured exclusively on soft gels for 20 days.

MSCs cultured on deformable substrates adopt morphologies that are characteristic of cells from lineages with comparable mechanical properties. For instance, MSCs cultured on soft gels will adopt neuronal-like shapes with dendritic processes while MSCs cultured on rigid substrates will adopt cuboidal shapes characteristic of osteoblasts. Controlling the shape of single cells in culture using micropatterning has been shown to influence the mechanosensitivity of MSCs to lineage specification[6,23,25–27,38,39]. Common to these studies is the apparent importance of geometric features that modulate the degree of actomyosin contractility. For instance, increased aspect ratio and subcellular concave regions at the cell perimeter increase cytoskeletal tension and promote osteogenesis[23,25]. In addition, we have found that anisotropic geometries promote the expression of neurogenic markers[26]. From our initial studies the transfer of MSCs from a stiff substrate after 10 days culture to a soft substrate did not lead to a decrease in runx2 comparable to MSCs cultured on the soft substrate alone. Therefore, we investigated whether controlling cell shape across the substrate in features that have been shown to promote neurogenesis and osteogenesis in adherent MSCs would influence the lineage outcome after a microenvironment switch. Cells that were initially cultured on soft or stiff gels and transferred to gels of the opposite stiffness showed a trend in lineage marker expression that was dependent on cell shape. Transfer from soft gels to high aspect ratio ovals and shapes approximating a 5-pointed star on stiff gels led to an enhancement in osteogenic marker expression, presumably because these shapes have been shown to promote osteogenesis through increased actomyosin contractility compared to isotropic shapes without perimeter curvature[24,25,39]. Surprisingly, transfer to stiff gels in these geometries led to partial maintenance of neurogenic marker expression, even after 10 days culture, when compared to unpatterned or circular shapes that promoted a significant decrease. MSCs that are initially patterned in oval and star shapes on stiff gels show low levels of β3-tubulin. Taken together, this suggests that transfer of cells that are expressing elevated levels of neurogenic markers to islands displaying anisotropic features may help maintain the neuronal phenotype, even when presented with an antagonistic stiffness. MSCs that are transferred from stiff gels to oval and star shapes on soft gels show an increase in β3-tubulin expression. This finding is consistent with our previous work that demonstrated the importance of anisotropic geometries in guiding the extension of neuron-like processes[26].

This study reveals that lineage specification to diverse outcomes is reversible by switching the biophysical parameters of stiffness and cell geometry. In particular, the early markers for osteogenesis (runx2) and neurogenesis (β3-tubulin), respond more readily to changes in the biophysical characteristics of the substrate, compared to the late markers osteopontin and MAP2. Even after 20 days in culture, there is little variation in the magnitude of expression for both early and late markers. This suggests the biophysical aspects of the cellular microenvironment only promote early differentiation events. Since differentiation in vivo involves the dynamic temporal regulation of discreet cellular states in response to a host of biophysical and biochemical signals, we speculate that the presentation of physical cues alone serve to prime stem cells to a reversible progenitor state that is poised to receive further signals to guide the progression to full commitment. Selection of appropriate materials that harness lineage specific biophysical conditions may serve as a good starting point for cell-based therapies, where endogenous in vivo signals integrate to direct full differentiation.

Methods

Materials. Laboratory chemicals and reagents were purchased from Sigma Aldrich unless otherwise noted. Tissue culture plastic ware was purchased from Thermo Fisher Scientific. Cell culture media and reagents were purchased from Gibco. Human MSCs and differentiation media were purchased from Lonza. Rabbit anti-Runx2 was purchased from abcam (ab23981) Technologies, rabbit anti-Osteopontin was purchased from abcam (ab8448), mouse anti-β3 tubulin was purchased from Sigma (T8660), and chicken anti-MAP2 was purchased from abcam (ab5392). Tetramethylrhodamine-conjugated anti-rabbit IgG antibody, Alexa488-phalloidin, Alexa647-conjugated anti-mouse IgG, Alexa647-conjugated anti-chicken IgG, and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. Glass coverslips (18-mm circular) for surface preparation were purchased from Fisher Scientific.

Surface preparation. Polyacrylamide gels were fabricated on a glass cover slip (15 mm) as reported previously[25,26]. We used the protocol of making hydrogels with varying stiffness by applying a mixture of Acrylamide and Bis-acrylamide according to the desired stiffness, and for the polymerization, 0.1% Ammonium Persulfate (APS) and 0.1% of Tetramethylethylenediamine (TEMED). 20 μl of the mixtures were pipetted onto the hydrophobic treated glass slides, and the amino-silanized coverslips were added with the treated side down. After appropriate polymerization time for each stiffness condition, the gel-coated cover slips were gently detached. Hydrazine hydrate 55% (Fisher Scientific) was utilized for 1 h to convert amide groups in polyacrylamide to reactive hydrazide groups. Sodium periodate was incubated with the glycoproteins to yield free aldehydes. The gels were washed for 1 h in 5% glacial acetic acid (Fluka/Sigma) and for 1 h in distilled water. To create patterned surfaces, PDMS (Polysciences, Inc.) stamps were fabricated by polymerization upon a patterned master of photoresist (SU-8, MicroChem) created using UV photolithography through a laser printed mask. 25 μg/mL of fibronectin in PBS was applied for 30 min to the top of patterned or unpatterned PDMS, and then dried under air, and applied to the surface.

Cell source and culture. Human mesenchymal stem cells (MSCs) from bone marrow were thawed from cryopreservation (10% DMSO) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) low glucose (1 g/mL) supplemented with 10% fetal bovine serum (MSC approved FBS; Invitrogen), and 1% penicillin/streptomycin (p/s). Media was changed every 3 or 4 days and cells were passaged at nearly 80% confluency using 0.25% Trypsin:EDTA (Gibco). Passage 4–7 MSCs were seeded on patterned and non-patterned surfaces at a cell density of ~5000 cells/cm^2. For transfer between hydrogels of different stiffness, MSCs cultured for 10 days on 0.5 or 40 kPa substrates were suspended by using 0.25% trypsin and reseeded onto opposite stiffness substrate (0.5 to 40 and 40 to 0.5). After transfer, the cells were cultured for 10 days and media was changed every 3 or 4 days.

Immunocytochemistry. Cells on surfaces were fixed with 4% formaldehyde (Alfa Aesar) for 20 min, permeablized in 0.1% Triton X-100 in PBS for 30 min and blocked with 1% bovine serum albumin (BSA) for 15 min. Primary antibody labeling was performed in 1% BSA in PBS for 2 h at room temperature (20°C) with rabbit anti-Runx2 and anti-Osteopontin, mouse anti-β3 tubulin, and chicken anti-MAP2 (1 : 200 dilution). Secondary antibody labeling was performed using the same procedure with Tetramethylrhodamine-conjugated anti-rabbit IgG antibody along with Alexa Fluor 488-phalloidin (1 : 200 dilution), Alexa647-conjugated anti-mouse or chicken IgG antibody, and 4′,6-diamidino-2-phenylindole (DAPI, 1 : 5000 dilution) for 20 min in a humid chamber (37°C). Immunofluorescence microscopy was conducted using a Zeiss Axiovert 200 M inverted research-grade microscope (Carl Zeiss, Inc.), and immunofluorescent images were analyzed using ImageJ to measure the fluorescence intensity of single cells. The number of cells measured is over 20 cells per each condition and we confirmed the results three times. We set the threshold exposure time with the condition having lowest intensity among the samples so that we could compare the relative intensities of each condition. The relative intensity of the fluorescence was determined by comparing each intensity value to the average intensity of one condition. For Figure 3, average osteogenic marker intensity (runx2 and osteopontin) of spread cells after microenvironment change from 40 to 0.5 kPa (10 + 10 and 10 + 5 days, respectively) and average neurogenic marker intensity of β3 tubulin and MAP2 of spread cells after microenvironment change from 40 to

0.5 kPa (10 + 10 days) and from 0.5 to 40 kPa (10 + 10 days), respectively were selected. For Figure 4, average osteogenic marker intensity of spread cells cultured for 10 days on 0.5 kPa and average neurogenic marker intensity of spread cells cultured for 10 days on 40 kPa were selected. Each intensity value was obtained by subtracting cytoplasmic intensity from nuclei intensity. The absolute value was used for all markers because β3 tubulin expression showed cytoplasmic staining. Cell viability was assessed by using a live/dead (viability/cytotoxicity) staining kit (life technologies) and cell viability was calculated by multiplying % viability before and after mechanical microenvironmental changes.

Statistical analysis. Statistical analysis was performed using one-way ANOVA and student's t-test and values of $p < 0.05$ were considered statistically significant.

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Author contributions

J.L. designed and conducted experiments, and analyzed data. A.A.A. conducted experiments. K.A.K. designed experiments and analyzed data. All authors contributed to writing the manuscript.

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