HEALTH AND MEDICINE

Nuclear softening expedites interstitial cell migration in fibrous networks and dense connective tissues

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Dense matrices impede interstitial cell migration and subsequent repair. We hypothesized that nuclear stiffness is a limiting factor in migration and posited that repair could be expedited by transiently decreasing nuclear stiffness. To test this, we interrogated the interstitial migratory capacity of adult meniscal cells through dense fibrous networks and adult tissue before and after nuclear softening via the application of a histone deacetylase inhibitor, Trichostatin A (TSA) or knockdown of the filamentous nuclear protein Lamin A/C. Our results show that transient softening of the nucleus improves migration through microporous membranes, electrospun fibrous matrices, and tissue sections and that nuclear properties and cell function recover after treatment. We also showed that biomaterial delivery of TSA promoted in vivo cellularization of scaffolds by endogenous cells. By addressing the inherent limitations to repair imposed by nuclear stiffness, this work defines a new strategy to promote the repair of damaged dense connective tissues.

INTRODUCTION

After injury or tissue damage, cells must migrate to the wound site and deposit new tissue to restore function (1). While many tissues provide a permissive environment for such interstitial [three-dimensional (3D)] cell migration (i.e., skin), adult dense connective tissues (such as the knee meniscus, articular cartilage, and tendons) do not support this migratory behavior. Rather, the extracellular matrix (ECM) density and micromechanics increase markedly with tissue maturation (2, 3) and, as a consequence, act as a barrier for cells to reach the wound interface. It follows then that healing of these tissues in adults is poor (4, 5) and that wound interfaces remain susceptible to refailure over the long term due to insufficient repair tissue formation. Similarly, fibrous scaffolds used in repair applications also impede cell infiltration when the scaffolds become too dense (6).

This raises an important conundrum in dense connective tissues and repair scaffolds; while the dense ECM and fibrous scaffold properties are critical for mechanical function, they, at the same time, can compromise cell migration, with endogenous cells locked in place and unable to participate in repair processes. This concept is supported by in vitro studies documenting that, in 3D collagen gels, the migration of mesenchymal lineage cells is substantially attenuated once the gel density and/or stiffness has reached a certain threshold (7–9). Consistent with this, our recent in vitro models exploring cell invasion into devitalized dense connective tissue (knee meniscus sections) showed reduced cellular invasion in adult tissues compared to less dense fetal tissues (3). The density of collagen in most adult dense connective tissues is 30 to 40 times higher than that used within in vitro collagen gel migration assay systems (2, 3), emphasizing the substantial barrier to migration that the dense ECM plays in these tissues.

To address this ECM impediment to successful healing, we and others have developed strategies to loosen the matrix (via local release of degradative enzymes) in an attempt to expedite repair and/or encourage migration to the wound site (10), with promising results both in vitro and in vivo (10, 11). Despite the potential of this approach, it is cognitively dissonant to disrupt ECM to repair it, and any such therapy would have to consider any adverse consequences on tissue mechanical function.

This led us to consider alternative controllable parameters that might regulate interstitial cell mobility while preserving the essential mechanical functionality of the matrix. It is well established that increasing matrix density decreases the effective pore size within dense connective tissues. The nucleus is the largest (and stiffest) organelle in eukaryotic cells (12), and it must physically deform as a cell passes through constructs that are smaller than its own smallest diameter (9). When artificial pores of decreasing diameter are introduced along an in vitro migration path (e.g., in an in vitro Boyden chamber system), cell motion can be completely arrested (13). If cells are forced to transit through these tight passages, then nuclear rupture and DNA damage can occur (14, 15). Conversely, under conditions where nuclear stiffness is low, as is the case in neutrophils (16) and some particularly invasive cancer cells (17), migration through small pores occurs quite readily.

Given the centrality of the nucleus in migration through small pores, methods to transiently regulate nuclear stiffness or deformability might therefore serve as an effective modulator of interstitial cell migration through dense tissues and scaffolds. Nuclear stiffness is defined by two primary features—the density of packing of the genetic material contained within (i.e., the heterochromatin content) and the intermediate filament network that underlies the nuclear envelope (the nuclear lamina, composed principally of the proteins Lamin B and Lamin A/C) (12, 16, 18, 19). Increasing chromatin

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condensation increases nuclear stiffness, while decreasing Lamin A/C content decreases nuclear stiffness (19, 20). Both increasing the stiffness of the microenvironment in which a cell resides (21) and the mechanical loading history of a cell promotes heterochromatin formation and Lamin A/C accumulation (22–24), resulting in stiffer nuclei. Since both matrix stiffening and mechanical loading are features of dense connective tissue maturation, these inputs may drive nuclear mechanoadaptation (25), resulting in endogenous cells with stiff nuclei that are locked in place.

On this basis, the goal of this study was to determine whether nuclear softening could enhance migration through dense connective tissues and repair scaffolds to increase colonization of the wound site and the potential for repair by endogenous cells. We took the approach of transiently decreasing nuclear stiffness in adult meniscus cells through decreasing heterochromatin content [using Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor] that promotes chromatin relaxation (26) and confirmed the importance of nuclear stiffness by reducing Lamin A/C protein content (using lentiviral-mediated knockdown). Our experimental findings and theoretical models demonstrate that nuclear softening decreases the barriers to interstitial migration through small pores, both in vitro and in vivo, resulting in the improved colonization of dense fibrous networks and transit through native tissue by adult meniscus cells. By addressing the inherent limitations to repair imposed by nuclear mechanoadaptation that accompanies cell differentiation and ECM maturation, this work defines a promising strategy to promote the repair of damaged dense connective tissues in adults.

RESULTS

TSA treatment increases nuclear deformability and migration of meniscus cells through microporous membranes

We first determined whether TSA treatment alters chromatin organization in adult meniscal fibrochondrocytes (MFCs). Super-resolution images of the core histone protein Histone-H2B in MFC nuclei were obtained by stochastic optical reconstruction microscopy (STORM) and revealed a notable organization of Histone-H2B inside MFC nuclei (STORM; Fig. 1A), which could not be observed with conventional microscopy (conventional; Fig. 1A). It has recently been shown that super-resolution images can be segmented at multiple length scales using Voronoi tessellation (27, 28). To segment the H2B super-resolution images, we carried out Voronoi tessellation, used a threshold to remove large polygons corresponding to regions of the nucleus containing sparse localizations, and color-coded the localizations with the same color if their polygons were connected in space and shared at least one edge. This segmentation showed that H2B localizations clustered to form discrete and spatially separated nanodomains in control nuclei [([−]TSA), Nuclei treated with TSA, on the other hand, contained smaller domains. These results were quantitatively recapitulated by a decrease in the number of H2B localizations in individual domains and an overall decrease in the area of domains in MFCs treated with TSA [(+]TSA) (Fig. 1, B to D). These results are in line with a more folded chromatin confirmation in ([−]TSA) cells, which opens and decondenses after TSA treatment. These results are also consistent with recent super-resolution analysis, which showed that TSA-treated fibroblasts have small nucleosome nanodomains that are more uniformly distributed in the nuclear space compared to control fibroblasts (29, 30). This decondensation was also confirmed in TSA-treated bovine menenchymal stem cells (MSCs), where TSA treatment decreased the number and area of H2B nanodomains (Fig. S1A). This increased acetylation at H3K9 (Ac-H3K9) was apparent at the nanoscale (Fig. S1B) and via conventional fluorescence imaging of the nuclei (Fig. S1C). Conversely, there were no significant changes in H3K27me3 with TSA treatment when evaluated using STORM or conventional fluorescent microscopy (fig. S1, D and E).

In addition, TSA treatment for 3 hours [(+]TSA) also resulted in marked chromatin decondensation in MFCs seeded on aligned (AL) nanofibrous scaffolds that are commonly used for dense connective tissue repair, as evidenced by decreases in the number of visible edges in 4′,6-diamidino-2-phenylindole (DAPI)–stained nuclei compared to control cells ([−]TSA) and a reduction (~40%) in the image-based “chromatin condensation parameter” (CCP) (Fig. 1E).

To assess whether this TSA-mediated chromatin decondensation changed nuclear stiffness and deformability, we stretched MFC-seeded AL scaffolds (from 0 to 15% grip-to-grip strain) and determined the change in nuclear aspect ratio (NAR) (Fig. 1F). Nuclei that were pretreated with TSA [(+]TSA) showed increased nuclear deformation compared to control nuclei ([−]TSA) (Fig. 1G); however, TSA did not change cell/nuclear morphology (fig. S2, A to C) or cell migration on planar surfaces (Fig. 1H), and only minor changes in focal adhesions were observed (fig. S2, D and E). MFC spread area and traction force generation were also unaffected by TSA treatment when cells were plated on soft substrates (E = 10 kPa) (fig. S2, F to I). These observations suggest that TSA treatment decreases nuclear deformability by chromatin decondensation without changing overall cell migration capacity in 2D culture.

We next assessed the ability of MFCs to migrate through small pores using a commercial transwell migration assay (Fig. 1I). Cells treated with TSA [(+]TSA) (200 ng/ml) showed enhanced migration compared to controls ([−]TSA) across all pore sizes, including 3-μm pores that supported the lowest migration in controls (Fig. 1I). This improved migration with TSA treatment was dose dependent (fig. S3). Together, these data show that while TSA treatment does not change cell morphology, contractility, or planar migration on 2D substrates, chromatin relaxation increases MFC nuclear deformability, which improves cell migration through micron-sized pores.

Increased nuclear deformability enhances cell migration through dense fiber networks

Having observed increased migration through rigid micron-sized pores with nuclear softening, we next assayed whether TSA treatment would enhance migration through dense fibrillar networks. A custom microfluidic cell migration chamber was designed, consisting of a top reservoir containing basal medium (BM), a bottom reservoir containing BM supplemented with platelet-derived growth factor (PDGF) as a chemoattractant and an interposed nanofibrous poly(ε-caprolactone) (PCL) layer (labeled with CellTracker Red, ~150-μm thickness) (Fig. 2, A and B). With this design, a gradient of soluble factors is presented across the fibrous layer, as evidenced by Trypan blue diffusion over time (Fig. 2C).

MFCs were seeded atop the fibrous layer, and their migration was evaluated as a function of nuclear deformability (±TSA) and fiber alignment [AL or non-AL (NAL)]. MFCs were cultured in BM for 1 day for attachment and then were treated for 2 days either with or without TSA (Fig. 2D). Confocal imaging (Fig. 2, E and F, and movie S1, A and B) and scanning electron microscopy (fig. S4A) showed increased MFC invasion into the fibrous networks with...
TSA treatment [(+)TSA] when compared to untreated MFCs [(-)TSA]. Without TSA, MFCs remained largely on the surface of the fibers with some cytoplasmic extensions into the fibers (fig. S4B), whereas TSA treatment increased the number of nuclei entering the fiber network (fig. S4C). When quantified, infiltration was higher in the NAL group compared to the AL group (P < 0.05; Fig. 2, G and H), likely due to the increased pore size in the NAL scaffolds (6, 31), and TSA treatment improved migration to similar levels in both NAL and AL groups (P < 0.05; Fig. 2, G and H). As expected, cells in AL scaffolds showed higher aspect ratios and solidity compared to cells on NAL scaffolds, yet TSA treatment did not influence cell morphology (fig. S4D). Nuclei in NAL groups were rounder (lower NAR) than in AL groups, and TSA treatment resulted in more elongated nuclei (higher NAR) in both AL and NAL groups (fig. S4E). While promoting cell invasion, TSA treatment did not result in any change in DNA damage (as assessed by phospho-histone H2AX-positive nuclei; fig. S4F) and slightly reduced cell proliferation at this time point (fig. S4G). Thus, it appears that TSA increased nuclear deformability, resulting in enhanced cell migration into these dense fibrous networks.

To verify that nuclear softening is the primary mechanism for enhanced migration into fibrous networks, we also knocked down Lamin A/C in MFCs before seeding. In previous studies, cells lacking Lamin A/C showed increased nuclear deformability and increased mobility in collagen gels and through small pores in Boyden chambers (13, 32). Consistent with these studies (12, 19, 33), reduction of Lamin A/C protein levels in MFCs and MSCs (fig. S5, A to C) increased nuclear deformability in response to applied stretch (fig. S5D). When MFCs with Lamin A/C knockdown were seeded onto fibrous networks, a greater fraction entered into the scaffold and reached greater infiltration depths (fig. S5, E to G). To further illustrate that nuclear stiffening reduces migration, we cultured MSCs in
transforming growth factor–β3 (TGF-β3)–containing media for 1 week before seeding onto the fibers. As we reported previously (23), these conditions induce differentiation in MSCs, resulting in stiffer nuclei with increased chromatin condensation and decreased nuclear deformability. Compared to undifferentiated MSCs, these differentiated MSCs were found largely on the scaffold surface (fig. S6, A to D) and had a lower infiltration rate and depth. While many factors change during cell differentiation, these findings also support that a less deformable nucleus is an impediment to interstitial cell migration. Together, these studies support that a stiff nucleus is a limiting factor in the invasion of the small pores of dense fibrous networks.

To investigate the combined role of porosity and nuclear softening on migration, we next fabricated fibrous networks through the combined electrospinning of both PCL and poly(ethylene oxide) (PEO), where PEO acts as a sacrificial fiber fraction to enhance porosity (6, 31). Consistent with our previous findings, cell infiltration percentage and depth progressively increased as a function of increasing PEO content (Fig. 2, I and J). When nuclei were softened with TSA treatment, we observed greater infiltration into low-porosity scaffolds (PEO content, <25%), but no difference in high porosity scaffolds (Fig. 2, I and J). This suggests that increasing nuclear deformability is only beneficial in the context of dense networks, where the nucleus impedes migration.

**Computational model for cell migration through dense fiber networks**

To better define the relationship between pore size and nuclear stiffness on cellular migration, we developed a computational model to predict the critical force ($F_c$) required for the nucleus to enter a small channel (Fig. 3). This model was motivated by studies of cellular transmigration through endothelium in the context of cancer invasion, where the surrounding matrix properties (stiffness), endothelium properties (stiffness and pore size), and the cell properties (in particular, the nuclear stiffness) appear to regulate transmigration (34). Here, we considered cell migration into a narrow and long channel to mimic migration into a porous fiber network, where network properties are defined by fiber density (Fig. 3A). When the cell enters the channel, the resistance force encountered by the nucleus...
increases monotonically as the cell advances, reaching a maximal resistance force (defined as the critical force, $F_c$). Following this, the nucleus snaps through the opening, leading to a drop in the resistance force, which vanishes after the nucleus fully enters the channel (Fig. 3B and movie S2). Thus, the cells must generate a sufficient force to overcome this critical force to migrate into a channel. As the channel size ($r_g$) becomes smaller and the ECM modulus ($E_{ECM}$) becomes greater, the critical force required for the nucleus to enter the channel increases (Fig. 3C and fig. S7). As this required force increases, it eventually exceeds the force generation capacity of the cell, resulting in a situation where the cell cannot enter the pore.

To better understand the influence of PEO content (affecting both the channel size and ECM modulus) and dose of TSA (affecting nuclear modulus) on cell migration, we used the normalized critical force data obtained from the model. Our previous work (6) defined the influence of PEO content on matrix mechanical properties and pore size; the effect of TSA on nuclear stiffness has also been measured quantitatively by other groups (26). Using these data, we predicted the critical force at different PEO contents for both TSA-treated and control cells (Fig. 3D). Results from this model showed that critical force decreased monotonically as PEO content increased, given that a higher PEO content results in larger pores (31). This indicates that infiltrated cell numbers should increase as the PEO content increases, consistent with our experimental results. Likewise, since TSA results in a softer nucleus (26), the critical force drops significantly compared to control conditions. This is particularly important at low PEO contents (denser networks), where the critical force for TSA-treated nuclei drops markedly. In networks with larger pores, the difference in critical force between TSA-treated groups vanishes. We included the model to gain, in general, insight into how a change in nuclear deformability (with TSA) might broadly affect cell migration in 3D and chose a simple configuration to gain some initial insight. While this model is simple (i.e., it does not represent the geometry of our fiber networks or native tissue), its predictions were consistent with our experimental findings, where the percentage of infiltrated cells was higher with TSA treatment at 0% PEO but the difference between groups disappeared at 50% PEO (Fig. 2I). The model also predicted that the NAR (after fully embedded in the channel) should increase as the nucleus becomes softer or the ECM becomes stiffer (both resulting in a larger normalized ECM modulus, $E_{ECM}/E_n$).

**Fig. 3. Computational model for cell migration through dense fiber networks.** (A) Schematic showing a nucleus (blue) above a narrow channel representing the small pores in a dense fiber network (orange). The geometric parameters are the radius of the nucleus ($r_n$) and the half width of the channel ($r_g$). The stiffness parameters are the modulus of the nucleus ($E_n$) and the fiber network ($E_{ECM}$). The nucleus is treated as a spheroid for simplicity. (B) Simulation of a nucleus moving into and through the channel in the dense fiber network. The normalized resistant force ($F/E_{n}r_{n}^2$) encountered by the nucleus is plotted as a function of the normalized displacement of the nucleus ($u_{n}/r_{n}$). The maximum normalized resistance force is defined as the critical force. (C) The critical force as a function of the normalized ECM modulus (with respect to $E_n$) and normalized channel size (with respect to $r_n$). The critical force is larger as the ECM becomes stiffer or the channel becomes smaller. (D) The critical force decreases as the PEO content increases. TSA treatment also decreases the critical force, particularly for dense networks (low PEO content). (E) Normalized NAR after entry into the channel increases as the ECM becomes stiffer or the nucleus becomes softer (both lead to a larger normalized ECM modulus, $E_{ECM}/E_n$).

**Persistence of TSA-mediated chromatin relaxation and its impact on collagen production of meniscus cells**

The above data demonstrate that TSA treatment decreases chromatin condensation for a sufficient period of time to permit migration. However, prolonged exposure to this agent may have deleterious
effects on cell phenotype and function. To assess this, we queried how long changes in MFC nuclear condensation persist after TSA withdrawal. MFCs were treated with TSA for 1 day as above, followed by five additional days of culture in fresh BM (Fig. 4A). Consistent with our previous findings, TSA decreased chromatin condensation and CCP after 1 day of treatment (Fig. 4, B and C). Upon removal of TSA, CCP values progressively increased, reaching baseline levels by day 5 (Fig. 4, B and C). A similar finding was noted in H2B localizations and domain area via STORM imaging, where these values returned to baseline levels within 5 days of TSA withdrawal (fig. S8, A to C). Similarly, nuclei in MFCs treated with TSA showed increased deformation compared to control MFC nuclei that were not treated with TSA (Fig. 4D) and increased Ac-H3K9 levels (Fig. 4, E and F), but these values gradually returned to the baseline levels within 5 days with TSA removal (Fig. 4, D to F). Over this same time course, proliferation was decreased in TSA-treated cells but returned to baseline levels within 5 days of TSA withdrawal on both tissue culture plastic (TCP) and on AL nanofibrous scaffolds (fig. S8, D and E). No change in levels of apoptosis (caspase activity) was observed over this time course (fig. S8F). Further, to investigate phenotypic behavior of cells after TSA treatment in the context of tissue repair, we next assayed whether cells exposed to TSA showed alterations in fibrochondrogenic gene expression and collagen production in MFCs. Although the sample size was small in this study, we did not detect a significant change in gene expression for any of the major collagen isoforms or proteoglycans normally produced by meniscus cells (fig. S9A). To further assess this, MFCs were treated with TSA for 1 day, followed by culture in fresh BM or TGF-β3 containing chemically defined media (to accelerate collagen production) for an additional 3 days. Collagen produced by these cells and released to the media was not altered by TSA treatment (fig. S9B). Together, these data support that TSA treatment decreases chromatin condensation by increasing acetylation of histones in MFCs but this change is transient and baseline levels are restored gradually after TSA is removed, without alterations in collagen production.

**Nuclear softening enhances cell infiltration in engineered constructs and native tissue**

Given that transient TSA treatment softened MFC nuclei, resulting in enhanced interstitial cell migration, and did not perturb collagen production in the short term, we next investigated longer-term maturation of a tissue engineered construct with TSA treatment. For this, MFCs were seeded onto AL-PCL/PEO 25% scaffolds and cultured in TGF-β3 containing chemically defined media for 4 weeks with/without TSA treatments (once a week for 1 day) as illustrated in Fig. 5A. In controls [−TSA], collagen deposition occurred mostly at the construct border (Fig. 5B), but both deposition and cell distribution were improved with TSA treatment [(+TSA)] (Fig. 5, B and C). Quantification showed that ~50% of cells were located within 50 µm of the scaffold edge in controls [−TSA], while TSA treatment [(+TSA)] increased the number of cells deeper within the scaffold (250- to 400-µm range; Fig. 5D).

Toward meniscus repair, it is important to evaluate MFC migration through the dense fibrous ECM of meniscus tissue in the context of TSA treatment. For this, adult meniscus explants (Φ 5 mm) were cultured for ~2 weeks, donor cells in these vital explants were stained with CellTracker, and the explants were placed onto devitalized tissue substrates and cultured for an additional 48 hours, with/without TSA treatment [−(+TSA)] (Fig. 6A). During this 48-hour period, the cells derived from the donor explants adhered to the tissue substrates (Fig. 6B). In control groups [−(−TSA)], cells were
found predominantly on the substrate surface, whereas TSA-treated MFCs were found below the substrate surface (Fig. 6, B and C). Quantification showed that both the percent infiltration and the infiltration depth were significantly greater with TSA treatment (Fig. 6D).

Next, we developed an assay to evaluate endogenous cell migration within native tissue. For this, tissue explants (Φ, 6 mm) were excised from adult menisci, and the cells on the periphery of the explants were devitalized using a two-cycle freeze-thaw process (freezing in −20°C for 30 min, followed by thawing at room temperature for 30 min, repeated twice on day −2; fig. S10A). This resulted in a ring of dead cells at the periphery of the tissue and a vital core. Processed explants were then treated with TSA for 1 day (day −1) and cultured in fresh media for an additional 3 days (fig. S10A). At the end of culture, living cells along the explant border were quantified. In controls that had not been treated by freeze-thaw (Ctrl), live cells occupied the periphery (fig. S10, B and D). With the two-cycle freeze-thaw process, there was a significant decrease in the number of live cells in this region (fig. S10, B and D), while cells in the center of the explant remained vital (day −2; fig. S10, B and D). With TSA treatment [(+)TSA], the number of vital cells that had migrated from the vital core to the periphery was significantly increased (day 3; fig. S10, C and D).

Last, to demonstrate the clinical potential of these findings, we developed an integrated biomaterial implant system to improve tissue repair in vivo (10, 35) via TSA delivery (Fig. 6E). Here, TSA was released from the PEO fiber fraction of a composite nanofibrous scaffold when this fiber fraction dissolves when placed in an aqueous environment. To first demonstrate bioactivity of the scaffold, we directly included small segments of these TSA-releasing composite scaffolds in the top chamber of the microfluidic migration device to treat seeded MFCs (Fig. 6F). Consistent with findings from soluble delivery, the percentage of infiltrated cells increased with the addition of the TSA-releasing composite scaffold (Fig. 6G): scaffolds releasing ~200 ng of TSA resulted in similar cell migration as direct addition of TSA (200 ng/ml) to the chamber (Fig. 6G). These results show our ability to deliver TSA to the wound site in a controlled fashion. To determine whether these TSA-releasing scaffolds could improve interstitial migration of endogenous meniscus cells in an in vivo setting, we subcutaneously placed meniscal repair constructs in nude rats with empty (PCL/PEO) or TSA-releasing scaffolds (PCL/PEO/TSA) interposed between the cut surfaces and histologically evaluated cellularity of the tissue and implant at 1 week (Fig. 6H). Results showed that interfacial cellularity was markedly higher for repair constructs with the scaffolds releasing ~100 ng of TSA (PCL/PEO/TSA) compared to control scaffolds (PCL/PEO; fig. 6I), with cells occupying the full thickness of the TSA-releasing scaffold (Fig. 6I). Together, these data indicate that biomaterial-mediated nuclear softening of endogenous meniscus cells increases their capacity for interstitial migration through the tissue and into the scaffold in an in vivo setting.

**DISCUSSION**

In this work, we posited that healing of dense connective tissues such as the knee meniscus could be expedited by transiently decreasing nuclear stiffness. To test this hypothesis, we interrogated the interstitial migratory capacity of adult meniscal cells through dense fibrous network and adult tissue microenvironments, with or without nuclear softening. We also developed a simple computational model to better understand how changes in matrix and nuclear parameters interact to affect cell migration through dense networks. Our experimental results show that a transient increase in nuclear deformability with TSA treatment enhances meniscus cell migration through otherwise impenetrable networks (fibrous scaffolds and native tissue). While it is difficult to unambiguously prove that nuclear stiffness is the only operative factor in this improved migration and infiltration, our data support this idea. In addition, these cells were able to resume their normal phenotype and reestablish functional matrix production over the long term, even with repeated episodes of nuclear softening. While these studies support the feasibility of transient exposure to TSA to improve migration, genome-wide analyses are required to elucidate the full effects of TSA treatment on the epigenome and transcriptome in MFCs and how this recovers to baseline after TSA withdrawal. Once this is accomplished, the use of this Food and Drug Administration–approved small molecule (TSA) coupled with localized delivery (10) may present a promising strategy to promote the repair and/or regeneration of damaged dense connective tissues in adults. Large animal trials evaluating this technology in the context of meniscus repair are now underway. Ultimately, improving cell migration in dense connective tissues such as the meniscus has the potential to provide treatments...
for a widespread unmet clinical need, transforming how these injuries are treated.

**MATERIALS AND METHODS**

**Preparation of nanofibrous scaffolds**

PCL nanofibrous scaffolds were fabricated via electrospinning as in (6). Briefly, a PCL solution (80 kDa; Shenzhen Bright China Industrial Co. Ltd., China; 14.3% (w/v) in 1:1 tetrahydrofuran and N,N-dimethylformamide) was extruded through a stainless steel needle (2.5 ml/hour, 18-gauge, charged to +13 kV). To form NAL scaffolds, fibers were collected on a mandrel rotating with a surface velocity of <0.5 m/s. For AL scaffolds, fibers were collected at a high surface velocity (~10 m/s) (36). In some studies, to enhance cell infiltration, PCL/PEO (PEO, 200 kDa; Polysciences Inc., Warrington, PA) composite AL fibrous scaffolds were produced by coelectrospinning two fiber fractions onto the same mandrel, as in (6). For this, solutions of PCL (14.3%, w/v) and PEO (10%, w/v, in 90% ethanol) were electrospun simultaneously onto a centrally located mandrel (~10 m/s, 2.5 ml/hour). Resulting composite scaffolds were produced with PEO content of 0, 25, and 50% by scaffold dry mass. To visualize fibers, CellTracker Red (0.0005%, w/v) was mixed into the PCL solutions before electrospinning. Scaffolds were hydrated and sterilized in ethanol (100, 70, 50, and 30%; 30 min per step) and incubated in a fibronectin (20 μg/ml) solution overnight to enhance initial cell attachment. TSA-releasing scaffolds contained a semipermanent (very slow degrading) fiber population (PCL) and a transient (water soluble) fiber population (PEO). The PEO fibers released TSA as
they dissolve. To form this fiber fraction, TSA was added to the PEO solution (1% wt/vol) 2 days before spinning. PCL (10 ml) and PEO/TSA (10 ml) solutions were loaded into individual syringes and electrosprun simultaneously by coelectrospinning onto a common centrally located mandrel, as above. Estimates of TSA content (mass per scaffold) were based on electrospinning parameters and the mass of each fiber fraction (Fig. 6E).

Cell isolation and culture

MFCs were isolated from the outer zone of adult bovine (20 to 30 months; Animal Technologies Inc.) or porcine menisci (6 to 9 months; Yucatan, Sinclair BioResources). For this, meniscal tissue segments were minced into ~1-mm³ cubes and placed onto TCP and incubated at 37°C in a BM consisting of Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum and 1% penicillin/streptomycin/fungizone (PSF). Cells gradually emerged from the small tissue segments over 2 weeks, after which the remaining tissue was removed and the cells were passed one time before use. MSCs were isolated from juvenile bovine bone marrow as in (37) and expanded in BM. To induce MSC fibrochondrogenesis, passage 1 MSCs were seeded on AL PCL scaffolds and cultured in a chemically defined serum free medium consisting of high glucose DMEM with 1× PSF, 0.1 μM dexamethasone, ascorbate 2-phosphate (50 μg/ml), l-proline (40 μg/ml), sodium pyruvate (100 μg/ml), insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenium acid (6.25 ng/ml), bovine serum albumin (BSA; 1.25 mg/ml), and linoleic acid (5.35 μg/ml) (Life Technologies, NY, USA). This base medium (Ctrl) was further supplemented with TGF-β3 (10 ng/ml) to induce differentiation (Ctrl/Diff, R&D Systems, Minneapolis, MN). Cell-seeded constructs were cultured in this medium for up to 7 days.

STORM imaging

MFCs or MSCs were plated into eight-well Lab-Tek 1 cover glass chambers (Nunc), followed by preculture in BM for 2 days. At this time point, cells were treated with TSA for 3 hours, followed by fixation in methanol-ethanol (1:1) at −20°C for 6 min. After a 1-hour incubation in blocking buffer containing 10 weight % BSA (Sigma-Aldrich) in phosphate-buffered saline (PBS), samples were incubated overnight with anti-H2B (1:50; abcam1790, Abcam), anti-H3K4me4 (1:100; MA3-11199, Thermo Fisher Scientific), or anti-H3K27me3 (1:100; PA5-31818, Thermo Fisher Scientific) at 4°C. Next, samples were washed and incubated for 40 min with secondary antibodies custom labeled with activator-reporter dye pairs (Alexa Fluor 405–Alexa Fluor 647, Invitrogen) for STORM imaging (29, 38). All imaging experiments were carried out with a commercial STORM microscope system from Nikon Instruments (N-STORM). For imaging, the 647-nm laser was used to excite the reporter dye (Alexa Fluor 647, Invitrogen) to switch it to the dark state. Next, a 405-nm laser was used to reactivate the Alexa Fluor 647 in an activator dye (Alexa Fluor 405)–facilitated manner. An imaging cycle was used in which one frame belonging to the activating light pulse (405 nm) was alternated with three frames belonging to the imaging light pulse (647 nm). Imaging was carried out in a previously described imaging buffer [Cysteamine (#30070-50G, Sigma-Aldrich), GLOX solution: 1 glucose oxidase (0.5 mg/ml), 1 catalase (40 mg/ml) (all from Sigma-Aldrich), and 10% glucose in PBS] (39). STORM images were analyzed and rendered using custom-written software (Insight3, gift of B. Huang, University of California, San Francisco, USA) as previously described (39). For quantitative analysis, a previously described method was adapted that segments super-resolution images based on Voronoi tessellation of the fluorophore localizations (27, 28). Voronoi tessellation of a STORM image assigns a Voronoi polygon to each localization, such that the polygon area is inversely proportional to the local localization density (40). The spatial distribution of localizations is represented by a set of Voronoi polygons such that smaller polygon areas correspond to regions of higher density. Domains were segmented by grouping adjacent Voronoi polygons with areas less than a selected threshold, and imposing a minimum of three localizations per domain criteria generates the final segmented dataset.

Assessment of the CCP and nuclear deformability

MFCs (P1) were seeded onto AL PCL (0% PEO) scaffolds in BM for 2 days. To induce chromatin decondensation, TSA, a HDAC inhibitor (26) was added to the media for 3 hours. Chromatin condensation state and nuclear deformability were evaluated 3 hours after TSA treatment. For chromatin condensation analysis, constructs were fixed in 4% paraformaldehyde for 30 min at 37°C, followed by PBS washing and permeabilization (with 0.05% Triton X-100 in PBS supplemented with 320 mM sucrose and 6 mM magnesium chloride). Nuclei were visualized by DAPI (ProLong Gold Antifade Reagent with DAPI, P36935, Molecular Probes, Grand Island, NY) and imaged at their mid-section using an confocal microscope (Leica TCS SP8, Leica Microsystems Inc., IL). Edge density in individual nuclei was measured using a Sobel edge detection algorithm in MATLAB to calculate the CCP as described in (24).

To assess nuclear deformability, the NAR (NAR = a/b) was evaluated before (0%) and after 9 and 15% grip-to-grip static deformation of constructs. Nuclear shape was captured on an inverted fluorescent microscope (Nikon T30, Nikon Instruments, Melville, NY) equipped with a charge-coupled device camera at each deformation level. NAR was calculated using a custom MATLAB code. Changes in NAR were tracked for individual MSC nuclei at each strain step as in (41).

Assays of in vitro cell migration

To assess MFC migration on 2D substrates, a “scratch” assay was performed with or without TSA treatment. For this, passage 1 MFCs were plated into a six-well tissue culture dish (2 × 10⁵ cells per well) and cultured to confluence (for 2 to 3 days). Confluent monolayers were then scratched with a 2.5-μl pipette tip, and cell debris was removed via PBS washing. Images were taken using an inverted microscope at regular intervals and wound closure computed using ImageJ.

In addition, as an initial assessment of MFC migration, 96-well transwell migration assay kits (Chemicon QCM 96-well Migration Assay; membrane pore size, 3, 5, or 8 μm) were used to assess cell migration. Briefly, human recombinant PDGF-AB (100 ng/ml in 150 μl of BM; Prospect Bio) was added to the bottom chamber, and passage 1 MFCs (50,000 cells per well) were seeded into the top chamber. Cells were allowed to migrate for 18 hours at 37°C with/without TSA treatment. In some studies, different dosages of TSA (0 to 800 nM) were applied (at a pore size of 5 μm).

Fabrication and application of a poly(dimethylsiloxane) migration assay chamber

To assess initial cell migration through dense nanofiber networks, a custom–poly(dimethylsiloxane) (PDMS) “migration assay chamber” was implemented (Fig. 2A). Top and bottom pieces containing holes (top, 6, 7, 6 mm in diameter; bottom, 6, 5, 6 mm in diameter) and a channel (bottom, 2 mm in width and 20 mm in length) were
designed via SOLIDWORKS software for 3D printed templates (Acura SL 5530, Protolabs), and these were cast from the templates with PDMS (Sylgard 184, Dow Corning). To assemble the multilayered chamber, bottom PDMS pieces, the periphery of PCL electrospun fiber networks, and top PDMS pieces were coated with uncured PDMS base and curing agent mixture (10:1 ratio) and placed on cover glasses sequentially. For firm adhesion of each layer, chambers were incubated at 40°C overnight. The final device consisted of a top reservoir containing BM and a bottom reservoir containing BM + PDGF (100 ng/ml) as a chemoattractant (Fig. 2A). To simulate chemoattractant diffusion from bottom to top reservoirs, trypan blue 0.4% solution (MP Biomedicals) was introduced to one of the side holes to fill the bottom reservoir, and the central top reservoir was filled with PBS. Cell migration chambers were kept in incubator (37°C, 5% CO₂), and images were obtained at regular intervals (Fig. 2D).

Fluorescently labeled (CellTracker Red) AL or NAL nanofibrous PCL scaffolds (thickness, ~150 μm) were interposed between the reservoirs, and MFCs (2000 cells, passage 1) were seeded onto the top of each scaffold, followed by 1 day before culture in BM. Cells in chambers were cultured in BM with/without TSA for an additional 2 days. At the end of 3 days, cells were fixed and visualized by actin/DAPI staining. Confocal z-stacks were obtained at x40 magnification, and maximum z-stack projections were used to assess cellular morphology (cell/nuclear aspect ratio, area, circularity, and solidity). The percentage of infiltrated cells was quantified from confocal z stacks, with cells located beneath fibrous categorized as "infiltrated" (Fig. S3C) and the infiltration depth measured on cross-sectional images using ImageJ. For scanning electron microscopy imaging, additional samples were fixed and dehydrated in ethanol (30, 50, 70, and 100%, 60 min per step) and then hexamethyldisilane for terminal dehydration under vacuum.

Model formulation
Details on the model have been described previously (34). Briefly, to understand the influence of both intracellular and extracellular cues on cell migration through the fibrous ECM, we considered a model in which a cell with a spherical nucleus of radius \( r_n \) is invading ECM through a deformable gap (with radius \( r_g \)) smaller than the diameter of the nucleus (Fig. 3A). For simplicity, the nucleus is modeled by a neo-Hookean hyperelastic material in which a cell with a spherical nucleus of radius \( r_n \) is invading ECM through a deformable gap (with radius \( r_g \)) smaller than the diameter of the nucleus (Fig. 3A). For simplicity, the nucleus is modeled by a neo-Hookean hyperelastic material. To understand the influence of both intracellular and extracellular cues on cell migration through the fibrous ECM, we considered a model in which a cell with a spherical nucleus of radius \( r_n \) is invading ECM through a deformable gap (with radius \( r_g \)) smaller than the diameter of the nucleus (Fig. 3A).

| Parameter | Description | Typical Value | Source |
|-----------|-------------|---------------|--------|
| \( E_n \) | Nuclear Young’s modulus | ~10 kPa | Caille et al. (42) |
| \( v_n \) | Poisson’s ratio for nucleus | 0.3 | Typical value for compressible neo-Hookean material |
| \( E_{ECM} \) | ECM modulus | ~1–20 kPa | Typical tissue modulus (3) |
| \( v_{ECM} \) | Poisson’s ratio for ECM | 0.3 | Typical value for compressible neo-Hookean material |
| \( r_n \) | Nuclear radius | ~1–5 μm | Typical nucleus radius |
| \( r_g \) | Tissue pore radius | ~0.5–5 μm | Wolf et al. (9) |

Assessment of cell migration and collagen production in tissue engineered constructs
For long-term evaluation of matrix production after TSA treatment, MFCs were seeded on PCL/PEO 25% AL nanofibrous scaffolds (P1, 10⁵ cells, 1 cm by 1 cm by 0.1 cm) and were cultured in TGF-β3 containing chondrogenic media for 4 weeks. TSA was applied once each week for 24 hours. After 4 weeks, constructs were fixed with 4% paraformaldehyde and embedded in CryoPrep frozen section embedding medium (optimal cutting temperature (OCT) compound, Thermo Fisher Scientific, Pittsburgh, PA). Using a cryostat microtome (Microm HM-500 M Cryostat, Ramsey, MN), constructs were sectioned to 8 μm in thickness through their depth and stained with Picrosirius Red and DAPI to visualize collagen and nuclei, respectively. Stained sections were visualized and imaged by brightfield and fluorescent microscopy (Nikon Eclipse TS 100, Melville, NY). To quantify cell infiltration in the scaffolds, the number of migrated cells as a function of scaffold depth was determined for each experimental group (n = 3 scaffolds per group) using ImageJ.

Assessment of interstitial cell migration through native tissue
To isolate fresh MFCs, cylindrical tissue explants (6 mm in diameter and 3 mm in height) were excised using biopsy punches from the middle zone of the meniscus, and these explants incubated in BM for ~2 weeks to allow cells to occupy the periphery. To fabricate devitalized tissue substrates, additional cylindrical tissue explants (8 mm in diameter) were embedded in OCT sectioning medium (Sakura Finetek, Torrance, CA) and axially cut (to ~50 μm in thickness) using a cryostat microtome. These devitalized sections were placed onto positively charged glass slides and stored at ~20°C until use. After ~2 weeks of in vitro culture, the living explants were incubated in 5-chloromethylfluorescein diacetate (5 μg/ml) (CellTracker Green, Thermo Fisher Scientific, Waltham, MA) in serum-free media (DMEM with 1% PSF) for 1 hour to fluorescently label cells in the explants. The explants were placed atop tissue substrates to allow for cell egress onto and invasion into the sections, and slides with explants were incubated at 37°C with/without TSA treatment in BM for 2 days, at which point maximum z-stack projections were acquired using a confocal microscope (Leica TCS SP8, Leica....
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In addition, to observe endogenous meniscus cell migration in the native ECM, a tissue-based migration assay was developed. Cylindrical meniscus tissue explants (6 mm in diameter and ~6 mm in height) were excised from the middle zone of adult menisci. To kill the cells on the border of the tissue, explants were frozen at ~20°C for 30 min and then thawed at room temperature for 30 min; this process was repeated twice (two-cycle) (day −2; fig. S10A). After devitalizing the periphery, explants were cultured in BM for 1 day, and TSA was added for 1 day (day −1; fig. S10A). After TSA treatment, explants were washed with PBS (day 0; fig. S10A), followed by culture in fresh BM for an additional 3 days. At day 3, LIVE/DEAD staining was performed, and explants cross sections were imaged (day 3; fig. S10A). Images were acquired from eight regions distributed evenly around the boundary (Leica TCS SP8, Leica Microsystems Inc., IL). The number of live cells located within 1 mm of the boundary was determined using ImageJ.

To evaluate the impact of biomaterial-mediated TSA delivery on endogenous meniscus cell migration in an in vivo setting, a nude rat xenotransplant model was used, as in (10). All animal procedures were approved by the Animal Care and Use Committee of the Corporal Michael Crescenz VA Medical Center. Before subcutaneous implantation, horizontal defects were created in adult bovine meniscal explants (8 mm in diameter and 4 mm in height, n = 3 donors; Fig. 6H). Electrospun PCL/PEO scaffolds with/without TSA were prepared (6 mm in diameter with a 2-mm-diameter central fenestration (Fig. 6H)). Electrospun PCL/PEO scaffolds with/without TSA were prepared (6 mm in diameter with a 2-mm-diameter central fenestration (Fig. 6H)).

**Statistical analyses**

Statistical analysis was performed using Student t tests or analysis of variance (ANOVA) with Tukey’s honestly significantly different post hoc tests (SYSTAT v.10.2, Point Richmond, CA). For datasets that were not normally distributed, nonparametric Mann-Whitney or Kruskal-Wallis tests were performed, followed by post hoc testing with Dunn’s correction using GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA, USA). Results are expressed as the means ± SEM or SD, as indicated in the figure legends. Differences were considered statistically significant at P < 0.05.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/25/eaax5083/DC1

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Acknowledgments: We acknowledge S. Gullbrand, D. H. Kim, and E. Henning for technical support. Funding: This work was supported by the NIH (R01 AR056624), the Department of Veterans Affairs (I01 RX000174), the NSF Science and Technology Center for Engineering Mechanobiology (CMMI-1548571), and the Penn Center for Musculoskeletal Disorders (P30 AR069619). Author contributions: S.-J.H., K.H.S., X.C., A.P.P., B.N.S., F.Q., V.B.S., M.L., J.A.B., and R.L.M. designed the studies. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments. S.-J.H., K.H.S., X.C., A.P.P., and B.N.S. performed the experiments.

The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 5 April 2019
Accepted 28 April 2020
Published 19 June 2020
10.1126/sciadv.aax5083

Citation: S.-J. Heo, K. H. Song, S. Thakur, L. M. Miller, X. Cao, A. P. Peredo, B. N. Seiber, F. Qu, T. P. Driscoll, V. B. Shenoy, M. Lakadamyali, J. A. Burdick, R. L. Mauck, Nuclear softening expedites interstitial cell migration in fibrous networks and dense connective tissues. *Sci. Adv.* 6, eaax5083 (2020).