**CELL BIOLOGY**

The nuclear piston activates mechanosensitive ion channels to generate cell migration paths in confining microenvironments

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Cell migration in confining microenvironments is limited by the ability of the stiff nucleus to deform through pores when migration paths are preexisting and elastic, but how cells generate these paths remains unclear. Here, we reveal a mechanism by which the nucleus mechanically generates migration paths for mesenchymal stem cells (MSCs) in confining microenvironments. MSCs migrate robustly in nanoporous, confining hydrogels that are viscoelastic and plastic but not in hydrogels that are more elastic. To migrate, MSCs first extend thin protrusions that widen over time because of a nuclear piston, thus opening up a migration path in a confining matrix. Theoretical modeling and experiments indicate that the nucleus pushing into the protrusion activates mechanosensitive ion channels, leading to an influx of ions that increases osmotic pressure, which outcompetes hydrostatic pressure to drive protrusion expansion. Thus, instead of limiting migration, the nucleus powers migration by generating migration paths.

**INTRODUCTION**

Cell migration in three-dimensional (3D) microenvironments plays a critical role in development, immune cell trafficking, tissue regeneration, and metastasis. During healing of bone fractures, mesenchymal stem cell (MSC) migration to the fracture sites through 3D microenvironments is critical for bone regeneration (Fig. 1A) (1, 2). MSC migration is also essential for stem cell therapies involving the delivery of MSCs in biomaterial carriers (3, 4). The 3D microenvironments in which MSCs migrate in vivo, including bone marrow and fracture hematomas, are confining and are viscoelastic, exhibiting time-dependent mechanical responses such as stress relaxation in response to deformation (5, 6). Although many studies have examined the impact of matrix viscoelasticity on MSC spreading and differentiation (5, 7–11), little is known about the physical process of how MSCs generate migration paths and migrate in confining and viscoelastic matrices.

The nucleus plays a complex role in 3D cell migration in confining matrices. The widely held view is that the stiff nucleus limits the ability of cells to squeeze through nanometer-scale pores in confining extracellular matrix (ECM) (12, 13). However, a set of studies showed that the nucleus can act as piston to generate increased hydrostatic pressure in lobopodial protrusions in fibroblasts and protease-inhibited tumor cells in reconstituted collagen gels (14, 15). The piston was found to mediate lobopodial 3D migration, though the mechanism remains unclear, as the increase in hydrostatic pressure in the protrusion would be expected to drive water out of the protrusion. Furthermore, a recent study revealed that the nucleus acts as a mechanical gauge in identifying the microchannel of least resistance in immune cells (16). Most studies of confined cell migration involve contexts in which migration paths or holes are preexisting and elastic, or in which cells use proteases to degrade a migration path (17–25). While mechanisms by which cells use proteases to generate migration paths for nuclear translocation in ECM are now well understood (20, 26, 27), the use of protease-independent mechanisms in generating migration paths are largely unclear, and the functional role of the nuclear piston in cell migration is unknown. Only recently was it found that cancer cells can use mechanical force to open up a migration path in confining ECM only when the ECM exhibited sufficient mechanical plasticity, but the role of the nucleus was not described (28). Here, we reveal a previously undescribed role of the nucleus in migration, in which MSCs use a nuclear piston to open up a migration path through confining viscoelastic microenvironments.

**RESULTS**

MSCs migrate in viscoelastic and plastic matrices by mechanically generating a migration path

Here, we examine MSC migration in 3D in engineered alginate hydrogels, used as models of a fracture hematoma and biomaterial-based stem cell delivery carriers. Alginate hydrogels are nanoporous and cannot be degraded by mammalian enzymes, serving as a model confining microenvironment and facilitating discovery of protease-independent mechanisms of migration (5, 29). While alginate itself is inert to cell adhesion, the cell adhesion peptide motif RGD (arginine-glycine-aspartate) was coupled to the alginate to promote integrin-mediated cell adhesion to the gels. Alginate hydrogel viscoelasticity and mechanical plasticity, as respectively assessed by stress relaxation tests and creep recovery tests, can be modulated independently of degradability, pore size, and initial modulus by varying alginate molecular weight (MW) and cross-linking density (5, 28, 30). Alginate hydrogels formed from low–molecular weight (low-MW) alginate showed fast stress relaxation ($\tau_{1/2}$, or the time for stress to be relaxed to half its original value in shear, was ~80 s), similar to that of a fracture hematoma, and were mechanically plastic (permanent deformation of ~30% in a 1-hour creep/1.7-hour recovery test, also in shear), while those formed from high–molecular

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MSCs migrate in fast-relaxing and plastic hydrogels but not in more elastic hydrogels

Fig. 1. MSCs migrate in confining viscoelastic matrices but not in more elastic matrices. (A) Schematics of MSC migration in a fracture hematoma and in transplanted hydrogels applied for MSC delivery for bone regeneration. (B) Schematics of alginate hydrogels composed of different molecular weights of alginate. (C) Representative stress relaxation profiles of hydrogels with fast and slow stress relaxation. The relaxation profiles of a covalently cross-linked poly(ethylene glycol) (PEG) hydrogel and a human fracture hematoma (S) are shown for comparison. (D) Representative plasticity test profiles of hydrogels with fast and slow stress relaxation. (E) Representative time-lapse image of MSC migration over time in fast-relaxing hydrogel (Fast) and in slow-relaxing hydrogel (Slow), respectively. Maximum intensity projections of R18 cell membrane dye signal are shown. (F) Representative 3D track reconstructions for cell migration in fast- and slow-relaxing hydrogels. Grid size, 10 μm. (G) Mean speeds for migrating cells in fast- and slow-relaxing hydrogels (n > 200 single cells). (H) Track length of migrating cells in each hydrogel (n > 200 single cells). ****P < 0.0001 by Student’s t test. All scale bars, 10 μm. All data are shown as means ± SEM.

Cross-linking density was tuned so that both exhibited similar initial elastic moduli of ~19 kPa, previously reported as the optimal modulus for osteogenesis (5, 31). Thus, the alginate hydrogels provide a confining microenvironment with independently tunable viscoelasticity/plasticity and ligand density for 3D culture.

Next, 3D migration of MSCs in the hydrogels was studied. To visualize MSC migration in the hydrogels, we stained MSCs with a membrane dye before encapsulation in the hydrogels and then performed time-lapse confocal fluorescence microscopy. Notably, MSCs were able to migrate in fast-relaxing and plastic hydrogels, while MSCs maintained rounded cell morphology and did not migrate in the more elastic hydrogels (Fig. 1E and movies S1 and S2). Cell tracking analysis indicates that cells in fast-relaxing and plastic hydrogels migrate with greater speed and persistence than those in the more elastic hydrogels (Fig. 1, F to H). Together, these data establish that matrix viscoelasticity and plasticity are critical for migration of MSCs in 3D matrices that are confining and not susceptible to degradation by proteases.

We next investigated the mechanism by which the MSCs build the migration paths to migrate in the confining gels that were fast relaxing and exhibited mechanical plasticity. MSCs initially extend long shallow protrusions into the fast-relaxing and plastic hydrogels, which then widened over time, eventually allowing migration to occur (Fig. 2, A and B). This widening process was accompanied by volume expansion in the protrusion (Fig. S2, A and B). More protrusive cells, as indicated by a lower sphericity, exhibited larger volumes than rounded cells (Fig. S2C). When body and protrusion volume were separately analyzed, the body volume was found to be similar before and after protrusion expansion, while the protrusion volume increased significantly (Fig. 2, C and D). These data indicate that the total volume of a single cell increases because of volume expansion in the protrusion and not the body. The 3D volume measurements were conducted by reconstructing 3D cell volume from a 2D stack of confocal microscope images; we previously confirmed that the volumes measured using this method match those measured using super-resolution microscopy (10). Since alginate hydrogels are not susceptible to degradation by proteases (33) and are nanoporous, it is expected that the expanding protrusion must physically open a channel in the hydrogels. To confirm this expectation and visualize the associated matrix deformation, we assessed matrix displacements using fluorescent microbeads embedding in the hydrogels. During protrusion expansion, microbeads adjacent to the protrusion moved away from the protrusion, indicating outward force generation by the protrusion (Fig. 2, E and F, and movie S3). Furthermore, when MSCs were cultured in fluorescently labeled alginate hydrogels, alginate accumulated around the length of the protrusion, confirming that protrusion expansion opened a hole in hydrogels (Fig. 2, G and H). These observations indicate that MSCs mechanically expand protrusions in viscoelastic matrices to create channels to subsequently migrate through.

A nuclear piston drives protrusion expansion

After finding that expansion of the protrusion generates force to open a migration path in the viscoelastic and plastic hydrogels, we sought to identify the mechanisms driving protrusion volume expansion. Before the expansion of the protrusion, it was consistently

observed that the nucleus moves toward and into the narrow entrance of the thin protrusions (Fig. 3A and movie S4). A previous study found that during lobopodial-based migration, the nucleus physically compartmentalizes the cytoplasm and can be pulled toward the front compartment of the cell, functioning as a piston to cause an increase in the hydrostatic pressure in that compartment (I4, I5). These observations implicate a potential mechanism driving protrusion expansion: Entry of the nucleus into the narrow protrusion increases hydrostatic pressure in the protrusion and causes expansion of the protrusion. This potential mechanism was investigated. Blebs, which occur when increased intracellular pressure drives delamination of the membrane from the actin cytoskeleton (34), were often observed at the cell membrane in protrusions following entry of the nucleus into the protrusion indicating enhanced pressure in the protrusion (Fig. 3A and movie S5). Actomyosin contractility along with the intermediate filament protein vimentin and the nucleoskeleton-intermediate filament linker protein nesprin-3 were previously found to be involved in pulling the nucleus forward and pressurizing the front of the cell (I5). Thus, the roles of vimentin and nesprin-3 in protrusion expansion and migration were first tested using small interfering RNA (siRNA) to reduce expression (fig. S3A). Nuclear location in the cell body was polarized toward the entrance of the protrusion in control cells and cells with reduced expression of nesprin-3 but not in cells with reduced expression of vimentin (Fig. 3, B and C, and fig. S3, B and C). In addition, the loss of nuclear movements toward the protrusion with reduction of vimentin was accompanied by diminished protrusion expansion and consequently inhibited the migration of the cells, establishing the role of vimentin in the nuclear piston (Fig. 3, D, E, and H to J; and fig. S3, D and E). Vimentin is connected to lamin A/C of the nucleus (35), and lamin A/C is also a key element regulating nuclear shape and rigidity during 3D migration (I3, I6). The role of lamin A/C in protrusion expansion and migration via the nuclear piston was examined using siRNA. Decreased lamin A/C expression in cells diminished protrusion expansion and migratory behaviors of cells (Fig. 3, F to J, and fig. S3A). Since nuclear movements are facilitated by both actomyosin contractility (I4) and microtubules (I5, I4), we also examined whether these actively participate in the nuclear piston behavior observed here. Inhibition of microtubule polymerization, with nocodazole, and actomyosin contractility, with myosin inhibitor ML-7, induced the loss of nuclear polarization (Fig. 4, A to C). Furthermore, each inhibition significantly reduced the migration of cells in 3D microenvironments (Fig. 4, E and F). Real-time inhibition of microtubule formation or actomyosin contractility in protruding cells resulted in an immediate relaxation of nucleus position from the entrance of the protrusion toward the center of the cell and simultaneously decreased the width of protrusion (Fig. 4, G to J). Together, these results establish that a nuclear piston, mediated by actomyosin contractility, microtubules, vimentin, and lamin A/C, drives increased pressure in protrusions and subsequent protrusion expansion for migration in confining and plastic microenvironments.

**Nuclear piston activates ion channels to induce ion influx into protrusions**

We next sought to elucidate the molecular mechanisms connecting the nuclear piston to volume expansion of the protrusion. A simple
nuclear piston mechanism could be expected to lead to water efflux from the protrusion due to the increased hydrostatic pressure. This is the opposite of what is observed, suggesting a more complex picture. It is known that cell volume can be altered by osmotic pressure, which is driven by the difference of ion concentrations between the inside and outside of the cell membrane (37). Therefore, ion concentrations were analyzed in expanding protrusions. Sodium and calcium imaging revealed that both sodium and calcium ion concentrations increased along the length of the protrusion, with the highest concentrations found at the tip of protrusion (Fig. 5, A to D). Live-cell calcium imaging revealed that intracellular calcium ion concentration at the tip of the protrusion increased over time (Fig. 5, E and F). These implicate that influx of calcium and sodium ions at the protrusion tip increases the concentration of ions within the protrusion over time. To determine the underlying mediators of differences in ions along the protrusion, we looked at the distribution of sodium and calcium channels. Since previous studies have shown that the polarized distribution of intracellular sodium ions can be driven by polarized distribution of the sodium hydrogen exchanger-1 (NHE-1) (18), the distribution of NHE-1 ion channels along a protrusion was examined. The NHE-1 ion channels are more densely distributed at the tip of protrusion, similar to the distribution of intracellular sodium ion along the protrusion (fig. S4, A and B). Next, the distribution of transient receptor potential vanilloid–4 (TRPV4) calcium ion channels was investigated, since we previously found that mechanosensitive TRPV4 ion channels regulate MSC volume and work as a molecular sensor of matrix viscoelasticity in MSCs (10). TRPV4 calcium ion channels were evenly distributed along the protrusions, although intracellular calcium ions showed a concentration gradient (Fig. 4, C and D). However, β1 integrin membrane receptors, which can interact with and activate TRPV4 ion channels (38), exhibited a polarized distribution along the protrusion similar to that of intracellular calcium ions (fig. S4, C and D). To directly assess the roles of NHE-1 and TRPV4 ion channels in mediating an ion concentration gradient along the protrusion, we applied small-molecule inhibitors EIPA (5-(N-ethyl-Nisopropyl) amiloride) and GSK205 to inhibit the function of NHE-1 and TRPV4, respectively. When the function of NHE-1 ion channels was inhibited, the polarized distribution of NHE-1 ion channels along the protrusion disappeared and the distribution profile of sodium ions was reversed (Fig. 5, G and H, and fig. S5, A to C). Sodium ions were relatively high at the beginning and low at the end of the protrusion. Similarly, TRPV4 inhibitors also inverted the concentration profile of calcium ions, decreasing the calcium ion concentration at the leading edge of protrusion (Fig. 5, I and J). These reversed concentration profiles of calcium ions and sodium ions by each inhibition might be due to the diffusion of ions from the cell body, which have relatively higher concentrations of ions adjacent to the nucleus. Furthermore, the impact of the function of NHE-1 and TRPV4 ion channels on migration in 3D microenvironments was analyzed with siRNA knockdowns. Cell tracking analysis indicates that both cells in fast-relaxing and plastic hydrogels migrate with slower speed and less persistence when NHE-1 or TRPV4 are knocked down, relative to control cells (Fig. 5, K to N). Together, these results indicate that calcium and sodium ions flow in through NHE-1 and TRPV4 ion channels to drive protrusion expansion for migration in 3D confining environments.
We next hypothesized that the nuclear piston, or movement of the nucleus into the protrusion, serves as the key driver of ion flow into the protrusion. It is known that TRPV4 ion channels are mechanosensitive, and the movement of the nucleus into the protrusion is expected to push cytoplasmic fluid into the protrusion, which pressurizes the protrusion, as indicated by the blebs, and potentially stretches the membrane. Furthermore, it was consistently observed that movement of the nucleus into the protrusion was highly correlated with both protrusion expansion and calcium ion accumulation at the tip of the protrusion (Fig. 6A and fig. S6, A to C). To assess our hypothesis, we analyzed the distribution of intracellular calcium ions along the protrusion in the vimentin and nesprin-3 knockdown cells. In vimentin knockdown cells, which exhibited loss of nuclear piston and diminished protrusion expansion, the distribution profile of calcium ions was reversed compared to control cells, with the calcium ion concentration at the leading edge of protrusion in cells decreasing (Fig. 6, B and C). No change in calcium ion concentration were observed in nesprin-3 knockdown cells, which exhibited normal nuclear piston and protrusion expansion, relative to control cells was observed (fig. S7, A and B). Together, these results suggest that the nuclear piston movement increases intracellular pressure in the protrusion, which activates mechanosensitive ion channels to flow ions in the end of protrusion, driving the protrusion expansion.

**Theoretical modeling and experiments show that increased osmotic pressure outcompetes hydrostatic pressure to drive protrusion expansion**

To better understand the underlying physics of the nucleus-driven expansion of protrusion, and the competition between increased hydrostatic pressure in the protrusion, which would promote water efflux, and increased osmotic pressure in the protrusion, which would promote water influx, we developed a theoretical model (Fig. 6D). With the nuclear movement, the fluid in front of the nucleus is pushed into the protrusion in a piston-like manner. Subsequently, the hydrostatic pressure in the protrusion, \( P_{\text{in}} \), increases and the protrusion membrane is stretched, which, in turn, activates mechanosensitive ion channels on the protrusion membrane (Fig. 6, D and E). With the opening of the mechanosensitive channels, ions can flow in and out of the protrusion in the direction of the ion concentration gradient across the membrane (Fig. 6, D and E). The ion flux rate \( (\text{d}n/\text{d}t) \) is determined by the difference between the osmotic pressures outside \( (\Pi_{\text{out}}) \) and inside \( (\Pi_{\text{in}}) \) of the protrusion (Fig. 6, D and E). On the other hand, the protrusion expansion rate \( (\text{d}r/\text{d}t = J_1 + J_2 + J_3) \) is determined by the influx and efflux of fluid where \( J_1, J_2, \) and \( J_3 \) are respectively governed by (i) the rate of fluid transport to the protrusion by the nuclear movement, (ii) the hydrostatic pressure difference across the membrane, and (iii) the osmotic pressure difference across the membrane (Fig. 6, D and E). Note that the ion flux rate and the protrusion expansion rate are mutually dependent (see Materials and Methods).

With this model of the nuclear piston that incorporates fluid transport because of the piston, hydrostatic pressure, and osmotic pressure, we ran the model and validated the model with experiments (Fig. 7A). The model predicts that the activation of mechanosensitive ion channels, upon pressurizing the protrusion by the nuclear piston, allows an influx of ions into the protrusion that, in turn, increases osmotic pressure in the protrusion to oppose the efflux of fluid (fig. S8, B to F and H to L). This leads to a positive net amount of fluid flow into the protrusion and eventually drives cell migration.

**Fig. 4. Nuclear piston movements are mediated by microtubules and myosin contractions.** (A and B) Representative immunofluorescence images for F-actin, \( \alpha \)-tubulin, phospho-myosin light chain (pMLC), and nucleus of a protruding MSC with treatment of nocodazole (–MT), ML-7 (–MLCK), or vehicle-alone control (CNTR). (C) Polar distribution of relative location of cell nucleus in cell body of MSCs (n > 50 single cells). (D) Representative 3D track reconstructions for migration of MSC. (E) Track length of migrating cells treated with each inhibitor or vehicle-alone control (n > 200 single cells). (F) Mean speeds for migrating cells treated with each inhibitor or vehicle-alone control (n > 200 single cells). (G) Schematic of inhibition test. After finding a protruding cell with the nucleus located at the base of protrusion, inhibitors are delivered and the movement of cell nucleus is monitored. (H) Representative time-lapse images showing nuclear movements over time in cell body. (I and J) Quantification of nuclear movements (I) and of relative width of protrusion (J) after 1 hour of treatment (n > 15 single cells). All scale bars, 10 \( \mu \text{m} \). ***P < 0.001 and ****P < 0.0001 by Student’s t test and by one-way ANOVA test, respectively. All data are shown as means ± SEM.
flow into the protrusion (Fig. 8) and, subsequently, an expansion of the protrusion, which is in excellent agreement with our experimental observation (Fig. 7, B and C). Furthermore, the model predicted that in the absence of protrusion ion channels, cell protrusions cannot expand as the fluid influx \( |J_{in}| \) is almost equal to the fluid efflux \( |J_{out}| \). This prediction is consistent with experimental observations where inhibition of the ion channels or chelating of intracellular calcium ions prevents the expansion of protrusion (Fig. 7, B to D, and Fig. 8, B to F and H to L). It should be noted that while protrusion expansion, as well as cell migration speed and track length, were decreased by inhibiting ion channels or chelating intracellular ions (Fig. 7E and Fig. 8), protrusion lengthening was not impeded, indicating that other mechanisms regulate the initial extension of the thin protrusion (Fig. 7F).

Next, the model predicted the anticipated results of real-time inhibitions of mechanosensitive ion channels and actomyosin contractility (Fig. 7G and Fig. S10, A to G). After the initial expansion of the protrusion due to the forward movement of the nucleus, the model predicts that the inhibition of mechanosensitive ion channels reduces the osmotic pressure in the protrusion (Fig. S10F), so that the radius of the protrusion mildly decreases (Fig. 7H). Also, the model predicts that the nucleus continues its forward movement (Fig. 7I) as the posterior hydrostatic pressure remains higher than the hydrostatic pressure in the protrusion (Fig. S10E). Likewise, the experimental observations exhibited that TRPV4 inhibition in real time did not affect nuclear movements but did prevent the influx of ions. As a result, the radius of the protrusion decreased over time, indicating the nuclear piston to be upstream of ion channel activation (Fig. 7H to J). On the other hand, the model predicted that real-time inhibition of the contractility led to a significant drop in the posterior hydrostatic pressure (Fig. S10E), thus the nucleus moved backward (Fig. 7I) and the protrusion width decreased (Fig. 7K). These predictions were both in agreement with the experimental observations (Fig. 7, H, I, and K). Thus, the model and the experimental results elucidate how the nuclear movement, the influx/efflux of ions, and intracellular fluid work in concert to control the protrusion volume through balancing the hydrostatic and osmotic differences across the membrane.

**DISCUSSION**

Together, our data from experiments and simulations reveal the physical mechanisms of how stem cells generate paths to migrate in confining viscoelastic and plastic matrices, which have no preexisting migration path (Fig. 8). MSCs initially generate shallow protrusions...
Nuclear piston induces ion influx at the protrusion

(D) Schematics and equations for the theoretical model of the nuclear piston and protrusion expansion:

\[ J_1 = \frac{\pi(R - h)^2}{2\pi r \ell} V + \frac{2\pi R}{2\pi r \ell} \left( \frac{h^3}{12 \mu r \ell} (P_b - P_{in}) + \frac{h}{2} V \right) \]

\[ J_2 = -\alpha (\Pi_{out} - \Pi_{in}) \]

\[ J_3 = -\alpha (P_{in} - P_{out}) \]

\[ \frac{dr}{dt} = J_1 + J_2 + J_3 \]

\[ \frac{dn}{dt} = \beta (2\pi r \ell) f(\sigma) (\Pi_{out} - \Pi_{in}) \]

Fig. 6. Theoretical model and experiments suggest that nuclear piston activates ion channels, which allow an influx of ions into the protrusion. (A) Representative time-lapse images of nuclear (NC) movements and calcium ions at the tip of the protrusion in one cell over an 80-min time frame. Heatmaps for relative calcium ion concentration at the protrusion are shown merged with bright-field images. Scale bar, 10 μm. Graph on the right shows the change in protrusion width, nuclear displacement, and the concentration of intracellular calcium ions of the cell shown on the left. (B and C) Representative images and profiles of intracellular calcium ions in the protrusion of MSCs transfected with vimentin siRNA or control siRNA (n > 45 single cells). (D) Schematics and equations for the theoretical model of the nuclear piston and protrusion expansion. (E) Theoretical model predictions of hydrostatic pressure, membrane tension, the number of intracellular ion, osmotic pressure, and net flow of fluid in the protrusion over time when nucleus moves toward protrusion. Scale bar, 10 μm. Heatmaps indicate relative calcium ion concentration. *P < 0.05 and ****P < 0.0001 by one-way ANOVA test. All data are shown as means ± SEM. In profile graphs, means and SEM are indicated by solid lines and dashed lines, respectively.

into the matrices. Then, the nucleus is pushed into the protrusion like a piston, increasing intracellular pressure in the protrusion. Actomyosin contractility, vimentin, and microtubules are involved in the nuclear piston, which activates mechanosensitive ion channels to flow ions in the end of protrusion, balancing the osmotic pressure between inside and outside of protrusion. Last, the increased osmotic pressure in the protrusion reduces water efflux, promoting the expansion of the protrusion, which physically opens up a migration path in the confining viscoelastic and plastic microenvironments.

Here, we found that cells migrate in viscoelastic and plastic matrices that are nanoporous but not in matrices that were more elastic. Most natural ECMs are viscoelastic, exhibiting some degree of fluid-like behaviors and stress relaxation in response to a deformation, and viscoplastic, exhibiting irreversible deformations in response to mechanical stress or strain (5, 39, 40). In the type-1 rich collagen networks that provide mechanical support to many soft tissues, unbinding of weak bonds between fibers or between fibrils within a fiber, followed by matrix flow or sliding of fibrils and then rebinding of weak bonds, represent two sources of viscoelasticity and viscoplasticity, though many other mechanisms likely contribute (41–43). Covalent cross-links act to diminish viscoelasticity and plasticity. We have previously shown that matrix viscoelasticity and plasticity in 3D regulate processes that involve shape or volume change such as mediate cell volume expansion, matrix deposition, cell-cycle progression and mitosis, matrix remodeling, and migration of cancer cells (5, 10, 28, 30, 40, 44, 45). Particularly relevant to this study, we previously showed that matrix mechanical plasticity regulated the probability of protrusion formation and extension, and cell migration, with a sufficient level of matrix mechanical plasticity enabling protrusion formation, protrusion extension, and migration (28). Consistent with this idea, here, we show that matrix viscoelasticity and viscoplasticity regulate the migration of stem cells.

Our finding that MSCs use the nuclear piston to generate a migration path in confining matrices adds a fresh new insight into our current understanding of migration path generation. For rigid pore sizes with a diameter below ~3 μm, it is known that cancer cells and...
fibroblasts are unable to squeeze their stiff nucleus through the pore (12, 13). For immune cells, the minimum pore size for migration is lower, but the same principle applies. Thus, ECMs with smaller pore sizes are considered to be confining with cells having to generate a micrometer-size path to migrate. Smaller pore sizes are likely to be relevant to many, if not most, ECMs. However, the topic of migration path generation has been largely avoided in previous studies of confined cell migration, which often use microfabricated channels where migration paths are predefined (16–18, 21–25, 46–48). In matrices that are degradable, cells can secrete matrix metalloproteinas or other proteases to biochemically degrade the matrix and help form migration paths, and much is known about these mechanisms (12, 19, 20, 26, 27). We previously found that cancer cells can migrate through nanoporous matrices that exhibit sufficient mechanical plasticity (28). Here, we show that the nuclear piston is used to expand protrusions and generate migration paths in viscoelastic and viscoplastic matrices, independent of protease activity. From the mechanism of nuclear translocation, we found that nuclear piston movements generate a migration path by increasing pressure in the shallow protrusion. Membrane blebs, and an increase in calcium concentration in the protrusion through mechanically activated ion channels, are associated with the forward movement of the nucleus and indirectly indicate the increase of pressure in the protrusion due to the nuclear piston. Furthermore, the alginate accumulation and the dislocations of embedded microbeads around the expanding protrusion directly show that protrusion expansion generates a migration path. As biological matrices are both susceptible to degradation and are viscoelastic/viscoplastic, it is possible that the nuclear piston mechanism acts synergistically with known protease-dependent modes of migration path generation.

Mechanistically, we find some distinctions between the nuclear piston used by MSCs in confining hydrogels and the nuclear piston mechanism described previously. Previous studies demonstrated that vimentin and nesprin-3, one of the LINC complex (Linker of Nucleoskeleton and Cytoskeleton) proteins, are important components that mediate nuclear piston movement during lobopodial-based migration by cancer cells and fibroblasts in microporous collagen gels (14, 15). Unexpectedly, we found that nesprin-3 is not directly
involved in the nuclear piston movements for MSCs in nanoporous alginate hydrogels. The nesprin family is composed of four nesprins, so it is possible that another nesprin might be involved in the nuclear piston in MSCs. We do find that lamin A/C proteins are necessary for protrusion expansion and the nuclear piston mechanism. Since the lamin A/C proteins serve as a key link between the LINC complex and the nucleus, these findings are suggestive of force transmission through the nucleus during this process. In addition, while previous studies implicated actomyosin contraction at the anterior part of the cell (i.e., protrusion) as the main mechanism pulling the nucleus into the protrusion (14, 15), our results indicate that actomyosin in the posterior part of the cell may be more activated than actomyosin in the anterior part. Similar to our observation, a recent study showed that elevated contractility at the cell posterior for cancer cells in confining channels locally increases cytoplasmic pressure and promotes nuclear translocation and bleb formation (49). Collectively, these results indicate that the nuclear piston mechanism for generating the migration paths may have various different flavors, which might be dependent on cell type and whether the microenvironment is fully confining, microporous, or channel-like.

In addition, the identification of mechanosensitive ion channels in cell migration adds the known list of key molecular players driving the nuclear piston and involved in cell migration generally. It was known that the nuclear piston mechanism involved actomyosin contractility, vimentin, and nesprin-3 to pull the nucleus forward and pressurize the front of the cell (14, 15). We show that mechanosensitive ions subsequently play a critical role in driving the osmotic pressure changes that overcome the increase in hydrostatic pressure in the protrusion to drive protrusion expansion. While we focused on TRPV4 and NHE-1, a recent study found that the mechanosensitive ion channel TRPM7 plays a central role in how cancer cells sense hydraulic pressure and cross-sectional area in microfabricated microchannels (24). This suggests that other mechanosensitive ion channels could also be involved in regulating the nuclear piston. Together, these findings highlight the important, and previously underappreciated, role of mechanosensitive ion channels in cell migration.

Last, our finding that matrix viscoelasticity and plasticity facilitate the migration of stem cells has broad implications for the design of biomaterials in clinical applications. Various applications in regenerative medicine involve the delivery of stem cells, or recruitment of local host cells (50). Furthermore, the emerging field of biomaterial-based immunoengineering often involves the use of biomaterials to recruit, infiltrate, and program immune cells (51). Thus, our findings point toward hydrogel viscoelasticity and visco-plasticity as a critical design parameter that can be modulated to optimize performance in these applications.

**MATERIALS AND METHODS**

**Alginate preparation**

Sodium alginate (average MW, 280 kDa, high-MW; Protanal LF 20/40, FMC Biopolymer) was used directly for the high-MW alginate gels. This alginate was also modified by irradiation with an 8-Mrad cobalt source to produce low-MW alginates (average MW, 35 kDa) (52). RGD oligopeptides coupled to the alginates using standard carbodiimide chemistry (52). The alginates were reconstituted at 1% (w/v) in 0.1 M MES buffer. Sulfo-N-hydroxysuccinimide (Thermo Fisher Scientific), N-(3 dimethylaminopropyl)-N′-ethylcarbodiimide (EDC; Sigma-Aldrich), and RGD oligopeptide (GGGGRGDSP, peptide 2.0) were sequentially added in the alginate solution, and then the reaction continued for 20 hours until adding hydroxylamine hydrochloride (Sigma-Aldrich) to quench. The alginate was dialyzed in deionized water for 2 to 3 days, purified with activated charcoal, sterile-filtered, lyophilized, and stored in a −20°C freezer until it was used. For cell encapsulation, alginate was reconstituted at 3% (w/v) in serum-free Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies). The final concentration of RGD peptide in the 2% (w/v) alginate hydrogels was 1500 μM. For preparation of fluorescent alginates, fluorescein amine isomer (Acros Organics) was coupled to RGD-coupled alginate using carbodiimide chemistry at a concentration of 37.74 μM as described above.

**Mechanical characterization**

Unconfined compression tests were made with an Instron 5848 mechanical tester (Instron) (30). Alginate hydrogels and poly(ethylene glycol) (PEG) hydrogels for compression characterization were prepared with disk-shaped hydrogels (6 mm in diameter and 2 mm thick). The disks of hydrogels were incubated overnight in DMEM (InVitrogen). The samples were compressed with a rate of 1 mm/min until 15% strain and held constant. Stress and strain of the hydrogels was recorded over time. The initial modulus of the hydrogel was calculated with the slope of stress-strain curve between 5 and 10% strain. The time of stress relaxation of each alginate (τ1/2) was measured as the time for which the initial stress at 15% strain decreased to half of its original value. The stress relaxation profile of a human fracture hematoma in Fig. 1C was obtained from previously published work (5). Since volume of the gels or fluid flow out of
the gel does not occur in shear, shear relaxation tests were conducted with an AR-G2 rheometer to show differences in stress relaxation only due to ionic cross-linker unbinding and polymer flow and not the fluid flow out of the samples (30). For sample preparation, the mixtures of alginate hydrogels were directly deposited between two plates of the rheometer as soon as mixing. After fully gelling the alginate mixtures indicated by reaching an equilibrium of both loss and storage modulus, a constant shear strain of 15% strain was then applied for 10,000 s and measured stress relaxation property of hydrogels.

Mechanical plasticity of the hydrogels was characterized using creep recovery tests in a rheometer (40). Hydrogel samples for plasticity tests were prepared by directly depositing alginate solutions between two plates of the rheometer immediately after mixing, before gelation. To prevent the dehydration of hydrogel, mineral oil (Sigma-Aldrich) was applied to the surface of the gel disk at the edge of the plates. Gelation was tracked by periodically recording the storage and loss modulus. After the storage modulus reached an equilibrium value, this time sweep was followed by a creep recovery test. First, a constant shear stress (150 Pa) was applied for 3600 s and then unloaded (0 Pa) for 6400 s (1.7 hours) to recover the samples from the absence of stress. The strain derived from loading and unloaded (0 Pa) for 6400 s (1.7 hours) to recover the samples from the absence of stress. The strain derived from loading and unloaded stress was recorded as a function of time. The plasticity of each hydrogel was measured with the ratio of strain after recovery test to the maximum strain at the end of loading.

Encapsulation cells in hydrogels

D1 MSCs (CRL-12424, American Type Culture Collection) were cultured to 70% confluency and encapsulated in alginate hydrogels. Cells were trypsinized with 0.05% trypsin/EDTA, washed with Dulbecco’s phosphate-buffered saline (DPBS), centrifuged, and resuspended in growth medium containing 10% fetal bovine serum (GE Healthcare). The number of cells was counted using a Vi-CELL Coulter counter (Beckman Coulter). Cells were homogeneously mixed with the alginate solution before adding calcium cross-linkers (CaSO4). The final cell concentration was 10 million cells/ml in the cell-laden mixture. The mixture was then deposited between two glass plates spaced 1 mm apart. The hydrogels were allowed to gel for 45 min and then disks of hydrogel were punched out using a biopsy punch. The hydrogel disks containing cells were then cultured in gels for 1 to 2 days in induction medium containing L-ascorbic acid (50 μg/ml; MilliporeSigma), 10 mM β-glycerophosphate (MilliporeSigma), and 0.1 μM dexamethasone (MilliporeSigma). After 1 day, bright-field microscopy was used to capture cell morphologies. For visualizing displacement of hydrogels, fluorescent alginate beads were used, instead of normal alginate or fluorescent microbeads (Thermo Fisher Scientific, catalog no. F8811) were added into the cell-alginate mixture.

Live-cell imaging

For live-cell studies using D1 MSCs in alginate, samples were prepared with a procedure similar to above. For migration studies, the membranes of MSCs were labeled with octadecyl rhodamine B chloride (1:1000 dilution; R18, Thermo Fisher Scientific) before encapsulating cells in alginate. The stained cell-laden alginate hydrogels were punched out, transferred into the wells of a chambered coverglass (Labtek), and covered with low-melting temperature agarose (catalog no. 50302, Lonza, Swiss) to prevent the hydrogel from floating. Induction medium was added in well with vehicle alone or inhibitor. After hydrogels were fully swelled for 6 hours in an incubator, MSCs were imaged in an incubated chamber (37°C and 5% CO2) at 15- or 30-min intervals with a confocal microscope (Leica CM1950 20/0.40–numerical aperture (NA) objective) overnight. For imaging nuclei, a similar procedure was followed; cells in hydrogels were incubated for 1 hour with a nucleus dye (Hoechst 33342, Invitrogen) and washed three times with DMEM, and the hydrogels were covered with agarose for live-cell imaging.

For live inhibition studies, the chambers were covered with plastic wrap to prevent dehydration of medium. Individual cells with nuclei positioned at the entrance of protrusion were marked using the mark-and-find feature of the Leica software. Small-molecule inhibitors were then delivered to each well, and multiple cells were imaged at every time point. Specific drug concentrations were described at the section of inhibition tests.

Intracellular ion imaging

For imaging intracellular ions in MSCs, both Fura Red, AM (33 μM; Thermo Fisher Scientific) and Fluo-3 AM (20 μM; Thermo Fisher Scientific) were applied for imaging intracellular calcium (53, 54) and cytosolic Na+ indicator. After hydrogels were incubated for 1 hour and washed three times with DPBS and incubated in the induction medium before live-cell imaging. Intracellular ions were live-imaged at 15- or 30-min intervals with a confocal microscope (Leica CM1950 20/0.8-NA objective) overnight. Sodium ion concentration in cells was measured with the intensity of sodium indicator, and calcium ion concentration was measured using the ratio of the Fluo-3 intensity to the Fura-red intensity (53-55).

Immunohistochemistry

For immunohistochemical staining, the cell-laden hydrogel samples were taken out of media, washed in phosphate-buffered saline (PBS) containing calcium (cPBS, GE), and fixed with 4% paraformaldehyde (Alfa Aesar) in DMEM at room temperature for 45 to 60 min. Then, the samples were washed three times in PBS, incubated in 30% sucrose (Thermo Fisher Scientific) in PBS overnight, and then placed in a mixture of sucrose and optical cutting temperature compound (OCT), containing 50% OCT (Tissue-Tek, Sakura) and the other 50% of a 30% sucrose solution, for 4 hours. The samples were then embedded in OCT and frozen. The gels were then sectioned with a thickness of 30 to 60 μm using a cryostat (Leica CM1950), and the staining was processed using standard immunohistochemistry protocols.

The following antibodies and reagents were applied for immunohistochemistry: anti–NHE-1 (1:200; ab67314, Abcam), anti–TRPV4 (1:200; ab39260, Abcam), anti–β1 integrin (1:300; ab24693, Abcam), anti–vimentin (1:200; ab92547, Abcam), anti–nesprin-3 (1:200; ab74261, Abcam), anti–α-tubulin (1:200; DM1A, Cell Signaling Technology), and Phospho-Myosin Light Chain (1:200; ab3381, MilliporeSigma). DAPI (4’,6-diamidino-2-phenylindole) (1:1000; Invitrogen) and Alexa Fluor 488 Phalloidin (1:80; Invitrogen) were used to stain the nucleus and the actin cytoskeleton, respectively. The following secondary antibodies were used: Goat anti-Rabbit IgG (immunoglobulin G) Alexa Fluor 647 (Invitrogen), Goat anti-Mouse IgG Alexa Fluor 647 (Invitrogen), Goat anti-Rabbit IgG Alexa Fluor 555 (Invitrogen), and Goat anti-Mouse IgG Alexa Fluor 555 (Invitrogen). ProLong Gold antifade reagent (Life Technologies) was applied to prevent photobleaching.
Inhibition/siRNA knockdown tests
For inhibition the function of TRPV4 and NHE ion channels, GSK205 [10 μM; Calbiochem (10, 56)] and EIPA [20 μM; R&D Systems (18)] were applied, respectively. Baap-AM [10 μM; Thermo Fisher Scientific (24, 57)] was used to chelate intracellular calcium ions in MSCs. For inhibition microtubule polymerization and myosin activity, nocodazole [10 μM; Tocris Bioscience (15, 16)] and ML-7 [25 μM; Tocris Bioscience (5, 10)] were applied to the induction media, respectively. Inhibitor concentrations were based on those used in previous studies.

For knocking down the expression of vimentin and nesprin-3, MScs were transfected with 50 nM vimentin siRNA SMARTpool (L-061596-01-0010, Dharmacon), 50 nM Syns3 siRNA SMARTpool (L-052180-01-0010, Dharmacon), 50 nM NHE-1 siRNA SMARTpool (L-048336-01-0020, Dharmacon), 50 nM TRPV4 siRNA SMARTpool (L-040742-00-0020, Dharmacon), and 50 nM Lamin A/C siRNA SMARTpool (L-040758-00-0020, Dharmacon), respectively. Control cells were transfected with ON-TARGETplus Non-Targeting Control Pool (D-001810-10-20, Dharmacon). All cells were transfected for 3 days using DharmaFECT Transfection Reagent (T-2001-01, Dharmacon), and knockdown efficiency was analyzed by Western blotting.

Western blot
siRNA-transfected cells were extracted and centrifuged. Cell pellets were lysed in Pierce radioimmunoprecipitation assay buffer (89900, Thermo Fisher Scientific) with Protease Inhibitor Cocktail Tablets (11836170001, Roche) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (04906845001, Roche). The protein concentration in each sample was measured using the Pierce BCA Protein Assay Kit (23227, Thermo Fisher Scientific). Lamin B1 Sample Buffer (1610747, Bio-Rad) was used to dilute samples to 3.5 μg/μl. Thirty-five micrograms of total protein was added in each lane of 4 to 15%, 15-well gradient gels. The gels were run for 35 min and the proteins were transferred to nitrocellulose (Bio-Rad) at 100 V for 45 min. The membrane was blocked in 5% milk for 1 hour, incubated overnight in primary antibodies against Vimentin (ab92547, Abcam; 1:1000), Nespri3 (ab74261, Abcam; 1:500), Lamin A/C (2032S, Cell Signaling Technology; 1:1000), NHE-1 (SC-136239, Santa Cruz Biotecology; 1:1000), TRPV4(ab39260, Abcam; 1:1000), P38 (SC-535, Santa Cruz Biotecology; 1:1000), and glyceraldehyde-3-phosphate dehydrogenase (ab181602, Abcam; 1:1000). Blots were incubated with secondary antibodies against the primary for 1 hour and imaged using a LI-COR Odyssey imaging system (LI-COR Biotechnology).

Three-dimensional image analysis
To measure cell volume and sphericity, cells in hydrogels were fixed with 4% paraformaldehyde and stained with R18 and DAPI. Three-dimensional image stacks were taken using a confocal microscope (Leica SP8, A 20×/0.8-NA dry objective). The 3D stacks for live-cell imaging were taken with a 1-μm z-axis interval by considering the pixel distance of the x-y plane.

To compare protrusion volume to width, the identical image stacks were used to measure the protrusion volume and width. The 3D image stacks were converted into a 2D image by accumulating all images. The protrusion width was then measured at the middle of the protrusion with the accumulated 2D images, and the protrusion length was measured with a line following the protrusion from leading edge of protrusion to the edge of body part.

For live-cell images to take 3D stacks, labeled cells in hydrogels were imaged using a confocal microscope (Leica SP8, A 20×/0.8-NA dry objective). The 3D stacks for live-cell imaging were taken with a 1-μm z-axis interval by considering the pixel distance of the x-y plane.

To measure track length and speed of migration in 3D hydrogels, 3D stack images were recorded with a 5- to 10-μm z-axis interval for 20 hours and the cell centroids in the 3D stack images were tracked using Imaris software. Track length and speed of migration were automatically calculated by Imaris software. Migration trajectories were reconstructed with a custom MATLAB code.

Two-dimensional image analysis
For measuring the distribution of intracellular ion and proteins, ImageJ was used with the 2D image accumulating all slices of 3D stack images. The distribution profiles were obtained by measuring signal intensity profile of each target. Along the protrusion, the intensity profiles were measured three times with a line following the protrusion from the leading edge of protrusion to the edge of body part.

Nuclear location in the cell body was measured using ImgeJ with DAPI and phalloloid signals. As described above, body area in the entire single cell was found with the ellipsoid and the center of the ellipsoid was found as the center of the body. The centroid of nucleus was found with DAPI signal. A vector was then obtained from the center of cell body to the center of nucleus. Another vector was obtained from the center of cell body to the middle of protrusion entrance. Polarization of nuclear location in cell body was calculated as the angle between these two vectors.

For mapping the displacement of matrix, drift of fluorescent microbead image was corrected using ImageJ and microbeads embedded in hydrogels in images were tracked with a particle image velocimetry (PIV) plugin in ImageJ (PIVlab; MATLAB) using three cross-correlation windows (128 × 128, 64 × 64, and 32 × 32 pixel-size interrogation window). The mesh sizes of the windows were manually chosen accordingly for the local bead concentration. The results from this PIV analysis provided a vector field of matrix displacements and a heatmap. The control was quantified by measuring the dislocation of beads located 30 μm away from each cell.

The accumulation of matrix was measured as the ratio intensity of fluorescent alginate within 2 μm of the border of protrusion to the intensity of the background hydrogel 30 μm away from each cell. The control was quantified as the ratio intensity of fluorescent alginate within 10 μm of the cell border to the background intensity of the hydrogel.

Statistical analysis
GraphPad Prism was used for all statistical analyses. Specific method of statistics, P values, and sample numbers of each comparison are reported in figure legends. All quantifications for statistical analysis...
were from at least three independent experiments with two biological replicates per experiment.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/2/eabd4058/DC1

**REFERENCES AND NOTES**


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The nuclear piston activates mechanosensitive ion channels to generate cell migration paths in confining microenvironments

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