Published in final edited form as:

J Cell Physiol. 2018 April ; 233(4): 3672–3683. doi:10.1002/jcp.26236.

Lipid signaling affects primary fibroblast collective migration and anchorage in response to stiffness and microtopography

Michael A. Mkrtchjan¹, Snehal B. Gaikwad¹, Kevin J. Kappenman², Christopher Solís², Sagar Dommaraju², Long Le³, Tejal A. Desai³, and Brenda Russell^{1,2,*}

¹Department of Bioengineering, University of Illinois at Chicago, Chicago, IL

²Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL

³Department of Bioengineering and Therapeutic Sciences, University of California at San Francisco, San Francisco, CA

Abstract

Cell migration is regulated by several mechanotransduction pathways, which consist of sensing and converting mechanical microenvironmental cues to internal biochemical cellular signals, such as protein phosphorylation and lipid signaling. While there has been significant progress in understanding protein changes in the context of mechanotransduction, lipid signaling is more difficult to investigate. In this study, physical cues of stiffness (10 kPa, 100 kPa, 400 kPa, and glass), and microrod or micropost topography were manipulated in order to reprogram primary fibroblasts and assess the effects of lipid signaling on the actin cytoskeleton. In an *in vitro* wound closure assay, primary cardiac fibroblast migration velocity was significantly higher on soft polymeric substrata. Modulation of PIP2 availability through neomycin treatment nearly doubled migration velocity on 10 kPa substrata, with significant increases on all stiffnesses. The distance between focal adhesions and the lamellar membrane (using wortmannin treatment to increase PIP2 via PI3K inhibition) was significantly shortest compared to untreated fibroblasts grown on the same surface. PIP2 localized to the leading edge of migrating fibroblasts more prominently in neomycin-treated cells. The membrane-bound protein, lamellipodin, did not vary under any condition. Additionally, fifteen micron-high micropost topography, which blocks migration, concentrates PIP2 near to the post. Actin dynamics within stress fibers, measured by fluorescence recovery after photobleaching, was not significantly different with stiffness, microtopography, nor with drug treatment. PIP2-modulating drugs delivered from microrod structures also affected migration velocity. Thus, manipulation of the microenvironment and lipid signaling regulatory drugs might be beneficial in improving therapeutics geared toward wound healing.

Keywords

Mechanotransduction; PIP2; PIP3; lamellipodin; neomycin; wound healing

* corresponding author. Department of Physiology and Biophysics, University of Illinois at Chicago, MC 901, 835 South Wolcott Avenue, Chicago, IL 60612-7342, Tel: 1-312-413-0407, Fax: 1-312-996-1414, russell@uic.edu.

INTRODUCTION

Fibroblast migration is regulated by remodeling of the actin cytoskeleton and anchorage under the plasma membrane of the advancing cell. There is good basic understanding of fibroblast behavior in response to critical chemotactic cues in the microenvironment of the wound bed (Theocharidis et al., 2015; Wells et al., 2016), but the equally powerful mechanical and topographical cues that guide fibroblasts remain unappreciated. Fibroblast proliferation, phenotype, migration, and gene expression are all affected by mechanisms that depend on mechanotransduction, whereby the cell converts a mechanical cue in its microenvironment into a chemical signaling pathway. Well-studied signals include phosphorylation of proteins, such as the integrin and focal adhesion complex (Samarel, 2014; Dupont, 2015).

Lipids are more difficult to study than proteins, but nonetheless have been shown to play a key role in mechanotransduction pathways leading to remodeling of the actin cytoskeleton. The initial mechanotransduction is almost instantaneous, whereas transcriptional regulation to control cell division and other essential properties is in the time scale of hours or days. Some transduction targets of signaling pathways are the proteins present in the actin cytoskeleton that rapidly regulate cell migration and a wide variety of physiological and pathological processes, such as those involved in wound healing (Steinestel et al., 2015). The mechanisms underlying these rapid changes include lipid binding and post-translational modification by phosphorylation or acetylation of existing proteins that affect actin assembly or disassembly (Li and Russell, 2013; Li et al., 2014; Lin et al., 2013, 2015). Neomycin alters the level of an important phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2), in a cell, and PIP2 has been implicated in lamella formation (Safiejko-Mroczka and Bell, 1998, 2001), subsequently affecting cell migration (Tsujita and Itoh, 2001; Wu et al., 2014). However, other evidence suggests it is not PIP2 but phosphatidylinositol 5-phosphate (PI5P) that may affect cell migration (Haugsten et al 2003).

A mechanism proposed for membrane adhesion and spreading requires continuously feeding PIP2 to the fibroblast cell periphery via actin filaments (Chierico et al., 2014). Moreover, PIP2 seems to play a role in the anchorage of the cell to the underlying flat substratum (Brückner et al., 2015). Here we report that PIP2 availability affects the velocity of the leading primary cardiac fibroblast from collective migration into a wound gap model *in vitro* (Haeger et al., 2015; Mayor and Etienne-Manneville, 2016) Furthermore, the lamellar architecture varies, suggesting that one role of PIP2 accumulation is to mediate the distance from the focal adhesions to the lamellar membrane.

Additionally, bioengineering approaches are applied to manipulate the physical cues of topography and stiffness that have been shown to reprogram stem cell and fibroblasts behavior in many tissues (Doroudian et al., 2015). Here, primary rat cardiac fibroblasts are grown in culture on substrata in the physiologic stiffness range (10–400 kPa) and with cell-sized microtopography in order to assess lipid signaling effects on the actin cytoskeleton. Results show that PIP2 is regulated by these micromechanical cues to mediate changes in collective migration velocity and lamellar architecture of fibroblasts. Neomycin that affects the lipid signaling pathway was delivered by a rod-shaped polymeric microstructure and

altered cell migration *in vitro*, which may provide a useful strategy in wound healing where fibroblast migration is an important factor.

MATERIALS AND METHODS

Fabrication of substrata with varying stiffness and microtopography

Flat and microtopographical substrata of stiffness 100 kPa and 400 kPa were fabricated from polydimethylsiloxane (PDMS) (DowCorning, Midland, MI) by varying ratios of base to curing agent and spun onto cell culture glass-bottom dishes (In Vitro Scientific, Mountain View, CA), creating a surface approximately 50 μm thick, as previously described (Broughton and Russell, 2015). Softer substrata were made from polyacrylamide (PAA) per protocols modified from other groups (Tse and Engler, 2010; Poellmann and Wagoner Johnson, 2013). For PAA substrata (10 kPa and 100 kPa), 40% unpolymerized acrylamide and 2% Bis solution (Bio-Rad, Hercules, CA) were diluted in water at final concentrations of 5% acrylamide, 0.3% Bis and 30% acrylamide, 0.3% Bis, respectively. Ammonium persulfate and tetraethylmethylenediamine (Bio-Rad, Hercules, CA) were added to initiate polymerization. 10 μl of the pre-polymer solution was then added to glass bottom dishes and covered with a circular coverslip. Substrata were allowed to polymerize for 10 minutes, then coverslips were gently pried up, leaving behind a flat, circular substrate. Dishes were washed 3 times in deionized water for 10 minutes at a time to remove unpolymerized acrylamide. 100 kPa PAA and 100 kPa PDMS yielded similar migration velocities, so these data were pooled for all analyses.

Microtopographical substrata were made by unmolding the polydimethylsiloxane (400 kPa) from a parylene template to yield 15 μm high, 25 μm wide, 75 μm spaced posts as done by us previously (Motlagh et al., 2003). Microposts unmolded with softer PDMS (100 kPa) deformed into a dome shape over time and could not be used (SEM data not shown). Additionally, PAA material did not retain three-dimensional structure with parylene templates (data not shown).

Functionalization of substrata for cell culture

PDMS surfaces were functionalized with 3-aminopropyl triethoxysilane (Sigma Aldrich, St. Louis, MO) and coated with 10 $\mu\text{g}/\text{ml}$ fibronectin. To functionalize the polyacrylamide for cell adhesion, substrata were treated twice by drying sulfo-SANPAH (ThermoFisher, Waltham, MA) in HEPES (50 mM, pH 8.5) on each surface for 60 minutes at 57°C. A UV exposure box (Spectronics Corporation, Westbury, NY) with a 365 nm bulb was used to link sulfo-SANPAH to substrata. Substrata were washed 3 times then covered in HEPES containing fibronectin (10 $\mu\text{g}/\text{ml}$) at 37 °C for at least 2 hours before UV-sterilizing in water for 20 minutes.

In order to rule out issues with the quantity of fibronectin covalently attached to the substrates distribution and uniformity of fibronectin coating, coated and uncoated substrata were examined after fixation in 10% formalin and probed with 1:100 anti-fibronectin antibody (Abcam ab-26245) following standard substrate preparation. Surfaces were imaged using confocal microscopy, and no discernable differences were seen (data not shown).

Microrod fabrication

50% v/v or 90% v/v solutions of poly(ethylene glycol) dimethacrylate (PEGDMA) MW 750 (Sigma Aldrich, St. Louis, MO) were prepared in phosphate-buffered saline (PBS). Photoinitiator 2,2-dimethoxy-2-phenylacetophenone was dissolved in 1-n-vinyl-pyrrolidone (100 mg/mL) and added to 50% PEGDMA (20 kPa) or 90% PEGDMA (60 kPa) precursor solutions to a final 1% concentration of photoinitiator. Photolithography was used to fabricate microrods that are $100\ \mu\text{m} \times 15\ \mu\text{m} \times 15\ \mu\text{m}$ (Ayala et al., 2010).

Microrod degradation

To assess degradation, microrods were resuspended in sterile, warm saline and shaken in an incubator at 37 °C. Phase microscopic images recorded over a period of 2 months were used to determine the rate of degradation by width measurement using ImageJ software. The mean width of 20 isolated microrods was measured at each time point.

Neomycin loading of microrods

Microrods ($100\ \mu\text{m} \times 15\ \mu\text{m}^2$) have a volume of $22,500\ \mu\text{m}^3$ and the desired concentration of neomycin in the microrods needs to be equivalent to the drug delivered from solution in the media. In order to achieve the maximum drug concentration, the microrods were resuspended for 24 hours at room temperature in 500 mg/mL neomycin trisulfate salt hydrate solution (Sigma-Aldrich, cat. no. 1405-10-3, St. Louis, MO). Microrods were centrifuged, supernatant removed, and resuspended in media for immediate use. Cells were then treated with neomycin-loaded microrods and compared with the drug added directly to media.

Neomycin delivery from microrods

50% or 90% PEGDMA microrods were loaded with neomycin as described above and washed 3 times by centrifugation immediately before starting the release study. The microrods were then incubated in 300 μL of PBS at 37°C. At each time point (1, 2, 3, 6, and 12 hours), the microrods were centrifuged and 200 μL of supernatant was removed and saved for analysis. 200 μL of fresh PBS was added and allowed to incubate until the next time point. 200 μL of each sample was placed into a UV-transparent plate and quantified by absorbance at 285 nm using a SpectraMax M5 by Molecular Devices, LLC (Sunnyvale, CA). The amount of neomycin released was normalized to total number of microrods and presented as neomycin per 50k microrods.

Fibroblast cell culture

All research animals were obtained and used in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1996). Animal studies were approved by UIC institutional animal care and use committee and conducted according to the NIH Guide for the Care and Use of Laboratory Animals. Hearts were removed and cells isolated from 1- to 2-day-old Sprague-Dawley rats using collagenase type II (Worthington, Lakewood, NJ) as previously described (Boateng et al., 2003). Cells were plated in 10 cm tissue culture dishes and neonatal rat ventricular fibroblasts (NRVFs) given one hour to attach before removing surrounding media along with unattached cells. Fibroblasts were

incubated in DMEM (10% FBS, high glucose, pyruvate) (ThermoFisher, Waltham, MA) and plated at desired density on fibronectin-coated (10 µg/ml) dishes. Experiments were conducted on cells through no greater than two passages.

Removable barrier wound closure migration assay and drug treatment

The traditional scratch assay gouged the soft surface and, therefore, that method was not used for migration studies. Following substrata functionalization and fibronectin coating, cloning cylinders were placed in the dish to form a removable barrier. Cells were added to the inner and outer compartments to obtain a density in each of approximately 35,000 cells/cm² and incubated for 48 hours. Cylinders were then removed gently creating a gap, approximately 500 µm wide devoid of cells into which cells could migrate. Phase images of fibroblast migration were taken using a Zeiss Axio Observer microscope with AxioVision software (Carl Zeiss, Oberkochen, Germany) following barrier removal at one hour (t1) and again after 7 hours (t7) (Fig 1A, 1B) to study the migration on different stiffness. Analysis was done using ImageJ software. Measurements from images at t1 and at t7 yielded the size of the gap in microns. Noting that cells are migrating from both sides, the gap distance was divided by two and the cell migration velocity was calculated in microns/hr. At barrier removal, fibroblasts were subjected to treatment with drugs that reduce PIP2 availability (neomycin, 500 µM) or increase PIP2 (wortmannin, 1 µM) (Sigma-Aldrich, cat. no. 19545-26-7). For migration assays, at least three separate cultures were analyzed per experimental condition at least two random regions were analyzed per culture.

Distance to lamella membrane from end of actin fiber at focal adhesion

Fibroblasts grown on 10kPa, 100kPa, 400 kPa, or glass and treated with neomycin or wortmannin were fixed in 10% formalin at 7 hours after barrier removal, probed for focal adhesions with an antibody to phospho-paxillin [Y113] (Abcam, cat. ab32084, Cambridge, MA) in a 1% BSA, 0.1% Tween-20 solution. Cells were then probed with 1:400 rhodamine phalloidin and secondary antibodies (ThermoFisher, Waltham, MA), mounted in a DAPI-containing solution (Vector laboratories, Burlingame, CA) to counterstain for nuclei, and imaged on a Zeiss Axio Observer microscope with AxioVision software. The actin fiber ending closest to the center of the lamella was determined and measurements taken from it and the two to the right and left, making five measurements per cell. The distances from the paxillin at the end of the 5 actin fibers to the lamellar periphery were measured using ImageJ software and averaged for each for the conditions of stiffness and drug treatment (Fig 2B). Cells at the leading edge of the wound closure were selected in an unbiased manner for each condition with about 5 cells in at least 3 cell cultures, yielding approximately 15 cells per condition.

Localization of lamellar proteins and lipids

Cells were treated as previously described to probe for actin and nuclei. Additionally, cells were probed at a 1:200 dilution with antibodies to lamellipodin (Santa Cruz, cat. sc-68380, Dallas, TX), PIP2 (Abcam, cat. ab2335, Cambridge, MA), or PIP3 (Echelon Biosciences, cat. Z-P345, Salt Lake City, UT). Secondary antibodies, Alexa Fluor 488 and Alexa Fluor 568, (ThermoFisher, Waltham, MA) were used at a 1:400 dilution. A Zeiss LSM 710 confocal microscope or Zeiss Axio Observer were used for imaging.

Gradient of PIP2 and PIP3 near the lamella membrane with drug treatment

The distribution of PIP2 and PIP3 detected by antibodies was quantified for fibroblasts grown on glass and selected in an unbiased manner, as described above. 5 line scans were taken through the leading edge of the cells in the direction of movement into the gap created by barrier removal. The average optical density for the five line scans was determined in arbitrary units for the 10 μm from the lamellar membrane and for five 10 μm line scans within the interior region surrounding the nucleus. Ratios of lamellar/interior were calculated to compile a histogram with at least 5 cells from at least 3 separate cultures.

Additionally, a PIP2 biosensor (PLC δ (C1-PH)-GFP) was used to detect distribution (Raucher et al., 2000). Cells were transfected with 0.05 μg of plasmid per 10,000 cells using a lipofectamine 3000 transfection system (ThermoFisher, Waltham, MA) and incubated for two days prior to viewing.

Actin dynamic exchange by fluorescence recovery after photobleaching

NRVFs were infected with actin-GFP virus (Invitrogen, cat. C10582, Carlsbad, CA) by incubation 24 hours prior to live-cell imaging, per previously used protocols (Lin et al., 2013). Fluorescence recovery after photobleaching (FRAP) of actin-GFP was used to obtain the kinetic rate constant (k_{frap} , measured in s^{-1}). Fibroblasts were grown on either flat 10 kPa, 100 kPa, or 400 kPa substrata or anchored to 400 kPa microposts. Neomycin and wortmannin were applied to cells grown on glass. The region of interest (ROI) for FRAP was a uniform area along a stress fiber within the interior of the cell. The ROI was bleached to at least 40% initial intensity by a 488 nm laser. The recovery curve was fit to a nonlinear regression using OriginPro software to determine actin k_{frap} as an indication of the rate of actin assembly (Lin et al., 2013). For FRAP assays, the sample number was defined as individual cells, of which one to three cells were analyzed per culture and at least three separate cultures analyzed per experimental condition.

Statistical analysis

Data was organized using Excel software (Microsoft, Redmond, CA) and statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA), all data are expressed as means $\pm$ SEM. Statistical significance was calculated using either two-tailed Student's t-test, 1-way ANOVA or 2-way ANOVA.

RESULTS

Fibroblast gap closure and substrate stiffness

After removal of the barrier, some fibroblasts leave the denser cell sheet and migrate into the gap devoid of cells over time (Figs 1 A and B). Fibroblasts gap closure occurred at different velocities for fibroblasts grown on flat surfaces of PAA (10 kPa, 100 kPa), PDMS (100 kPa, 400 kPa) or glass. Untreated fibroblasts grown on 10 kPa PAA migrated the fastest at 5.14 $\mu\text{m/hr}$, while 100 kPa was 4.53 $\mu\text{m/hour}$, 400 kPa was 4.04 $\mu\text{m/hour}$ and on glass surfaces was 4.54 $\mu\text{m/hour}$ (Fig 1C). Untreated fibroblasts on 400 kPa were significantly slower at closing the gap compared to untreated cells on the 10 kPa surface ($P < 0.05$).

Migration gap closure rate is controlled by PIP2 signaling

When treated with neomycin, significant increases in migration velocity to close the gap were observed in 10 kPa, 100 kPa, 400 kPa and glass surfaces compared to untreated cells on the same stiffness ($P = 0.05$), Fig 1C. With neomycin, the fibroblast migration velocity was significantly increased under all conditions and wortmannin treatment significantly retarded migration in 100 kPa, 400 kPa and glass surfaces compared to untreated groups. (Fig 1C).

The actin cytoskeleton and lamellar architecture are modulated by PIP2 signaling

Images of fibroblasts grown on different stiffnesses with stains for actin, paxillin, and nuclei are shown with or without drug treatments that modify PIP2 signaling (Fig 2A). The terminal focal adhesions identified by paxillin were seen at the end of actin stress fibers in fibroblasts grown on all stiffnesses and with either neomycin or wortmannin drug treatment. There were no obvious differences in amount of paxillin or actin stress fibers with stiffness alone. However, the distance between the focal adhesion and the lamellar membrane appeared to vary with stiffness and drug treatment. Quantitative measurements of these distances are shown in histograms (Fig 2B). Untreated cells were similar at all stiffnesses (10 kPa, 100 kPa, or glass). On glass, the average distance between the terminal focal adhesion and the lamellar membrane was lowest at $3.53 \mu\text{m}$, and significantly different ($P=0.03$) from untreated fibroblasts with a distance of $6.39 \mu\text{m}$. On glass, neomycin treatment also trends to a larger distance of $8.33 \mu\text{m}$, but this was not significant compared to untreated cells. On 10 kPa ($P=0.06$) and 100 kPa ($P=0.0002$), there were also significant differences with wortmannin treatment of all corresponding stiffness, each having the shortest distance compared to its untreated control.

The distribution of lamellipodin, PIP2, and PIP3 are shown by immunostaining for fibroblasts grown on glass surfaces (Fig 3). Lamellipodin was widely distributed along the cell membrane of the lamellae under all conditions, regardless of stiffness or drug treatment (Fig 3A). Quantitative evaluation of PIP3 distribution shows a difference between the lamellar region and the cell interior, but there was no difference between treatments (Fig 3B,E).

PIP2 appears to concentrate at the leading edge of the migrating fibroblast (Fig 3C) in all conditions, but lamellar PIP2 is significantly higher in the neomycin-treated cells compared to untreated. The biosensor detection method for the PIP2 domain showed a similar appearance (Fig 3D). Quantitative assessment of the gradients of at least 5 cells in 3 separate cultures shows the lamellar/interior PIP2 with neomycin treatment is 2.22 times, which is a significant increase compared to untreated control ($P=0.02$) (Fig 3F). No difference was seen with wortmannin treatment.

Microtopography: localization of cytoskeleton, PIP2, and PIP3 and actin assembly kinetics

The three-dimensional micropost topography as seen by scanning electron microscopy was maintained with 400 kPa stiffness (Figs 4A,B). Microstructure shaped into microrods are seen with phase microscopy (Figs 4C,D).

Fibroblasts grown with microrods form strong focal adhesions and have many actin stress fibers, as shown through paxillin and actin fluorescence (Fig 5A). Cells grown on the microtopographical 400 kPa surface seen with three-dimensional confocal microscopy showed a diffuse PIP3 pattern through the entire cell away from the post viewed in confocal Z-stack (Fig 5B). In contrast, PIP2 aggregates around the micropost topography (Fig 5C).

The krap kinetic rate constant of actin-GFP was measured in s^{-1} in fibroblasts on stress fibers of cells on flat or micropost topography (Figs 5 D, E). There was no significant difference for stress fibers grown on varying stiffness, or with micropost anchorage compared to an unattached cell (Fig 5E). Moreover, drug treatment with neomycin or wortmannin had no significant effect.

Degradation of microrods

Continuous shaking in saline at 37 °C did not degrade microrods over 2 months. At day 1 and at 2 months, the width of microrods was approximately 15 μm . There was no significant difference in the mean width value of the microrods, implying no degradation under these conditions (data not shown).

Neomycin delivery from PEGDMA microrods

The kinetics of neomycin release was assessed for both 50% or 90% PEGDMA microrods. As expected, 50% PEGDMA microrods released more neomycin compared to the 90% PEGDMA microrods, likely due to increased water and higher loading capacity (Fig 6A).

Neomycin released from PEGDMA microrods altered the fibroblast migration after removal of the barrier (Fig 6B). Blank microrods of different stiffness do not increase the migration velocity, which is similar to untreated at 4.54 $\mu m/hour$, (Fig 6 B). The migration velocity increases with the neomycin release from the 50% PEGDMA microrods at 10.64 $\mu m/hour$, which is higher than the direct neomycin application (7.53 $\mu m/hour$). However, the 90% PEGDMA microrods show a similar migration velocity (7.36 $\mu m/hour$) compared to the direct neomycin treatment.

DISCUSSION

Fibroblast lamella formation is known to be regulated by substrate stiffness with remodeling of the actin cytoskeleton through many signaling pathways including PIP2 (Safiejko-Mroczka and Bell, 1998, 2001). For the first time, a mechanism linking migration from the leading edge of a cell in a wound closure assay *in vitro* suggests dependence on the lamella architecture and actin cytoskeleton remodeling is related to the underlying lipid signaling pathway. Drug treatments to alter phospholipid availability show that cell migration depends on the level of production and distribution of PIP2. The distance between focal adhesions and the lamellar membrane was significantly shortest with wortmannin treatment (a PI3K inhibitor) compared to untreated fibroblasts grown on the same surface. The micromechanics imposed on the cell by increased substrate stiffness or by microtopography leads to strongly anchored fibroblasts, with PIP2 increased near the micropost. The use of the PIP2 scavenger, the antibiotic neomycin, may affect its wound healing properties, since it doubles cell migration velocity on 10 kPa, a stiffness likely to occur in a wound.

Micromechanics, cell migration and the actin cytoskeleton

Mechanical feedback between mechano-sensing and cytoskeleton of cells is important for collective migration in cell colonies (Lange and Fabry, 2013). Durotaxis is the term given to the net migration of cells on a flat surface with a stiffness gradient. Since cells decrease in velocity as the surface stiffens, the net result over time is an accumulation of cells at the stiff end (Harland et al., 2011). It is interesting to note that cells develop tension internally by myosin motor force generation against the actin cytoskeleton. This net local tension explains the effect of crowds and sheets via collective migration versus individual cells in the migration of sheets (Ng et al., 2012; Haeger et al., 2015; Mayor and Etienne-Manneville, 2016).

This study focuses on the mechanisms underlying the mechanical cues to cell migration in addition to cell anchorage. Actin binding proteins control actin polymerization and formation of parallel bundles in stress fibers by formin or in branched networks of the spreading lamella by Arp2/3 (Hotulainen and Lappalainen, 2006; Koster and Mayor, 2016; Grikscheit and Grosse, 2016). Cells have a cortical layer with proteins binding the actin filaments to the membranes through PIP2 (Brückner et al., 2015). They also have dorsal stress cables for actin treadmilling to propel the cell forward. Immunofluorescent images of actin stress fibers show that distance of the actin cable anchored by a focal adhesion to the lamella membrane depended on PIP2 availability. Decreasing PIP2 availability could lead to decreased WASP assembly activity, which increases Arp2/3 activation (Zhang et al., 2012).

The role of PIP2 signaling

The role of signaling pathways and cell migration in response to mechanical changes are most often reported for the TGF- β pathway after receptor binding, mediated by kinases and phosphatases (Samarel, 2014). Less is known about the lipid signaling pathways such as PIP2. Here, cell migration was retarded by wortmannin treatment, a PI3K inhibitor, effectively increasing the presence of PIP2 by blocking the addition of a phosphate group to the 3' position of the phosphoinositide. Conversely, the PIP2 scavenger, neomycin, decreased the availability of PIP2, leading to near doubling of the velocity of cellular migration on 10 kPa substrata. Interpretation of the role of PIP2 is complex because it is distributed on membranes throughout the cell and also binds to hydrophobic pockets of numerous actin binding proteins. For example, ezrin, FAK, talin, vinculin, and paxillin associate with each other and the cell membrane. Some of these partnering proteins control actin polymerization at sites far from the membrane, such as the muscle actin capping protein, CapZ (Li and Russell, 2013, Lin et al., 2015). Thus, lowering PIP2 may have a secondary effect on the actin stress fibers within the cell anchored for focal adhesions to a micropost or those involved in actin polymerization and treadmilling.

Cell migration depends on pushing the cell forward, so the effects of PIP2 on migration are likely to be at the actin binding complexes mediated by PIP2 located in the inner membrane bilayer by its hydrophobic fatty acid tails. PIP2 can be transported via actin stress cables (Chierico et al., 2014). Thus, neomycin reduction of PIP2 could prevent the linkage from the cortical actin via a protein like ezrin to the PIP2 in the membrane. Lack of ezrin bound in the membrane bilayer decreased the surface tension of the cell and allowed fluidity, which might

enhance migration (Brückner 2015). Furthermore, the Arp2/3 complex binds to PIP2 so that neomycin scavenging would be predicted to prevent the normal actin mesh from forming in the lamella (Koster and Mayor, 2016). Interestingly, the stress fiber binding partner is formin, and a lack of PIP2 would prevent the anchorage of the stress bundle to the membrane, disabling a filopodia from pulling a membrane sheath around it (Koster and Mayor, 2016). Moreover, phosphatidylinositol metabolism has been shown to induce membrane ruffling, and PIP2, which serves as a membrane dock for cytosolic phospholipase A, would have increased presence in wortmannin-treated cells (Moes et al., 2010).

Lamellar protrusion initiates and defines the direction of cell movement in a PI3K dependent manner (Welf et al., 2012). Increased migration of breast cancer cells was due the reduction of a pro-migratory molecule like Pfn1 through enhanced lamellar targeting of Ena/VASP proteins. Stable lamellar protrusion is a characteristic feature of gliding cell movement and involves lamellipodin (Bae et al., 2009). However, we did not find any difference in lamellipodin distribution under any condition.

Contact inhibition, anchorage and stress fiber formation

Contact inhibition and cell anchorage are generally viewed as the key mechanisms for the prevention of cell migration. This is usually thought to be by a chemical process where the ligand and receptor interact to form focal adhesions. Surprisingly, a physical object without chemical cues is also effective. At the micron scale, anchored micropost projections blunted migration and blocked cell proliferation of fibroblasts in culture while similarly shaped unanchored objects did not (Boateng et al., 2003). The authors concluded cell properties are driven by the reactive forces first recognized in Newton's Third Law. Our lab has previously demonstrated an increased abundance of focal adhesion formation when cells bind to the microposts, leading to better anchorage of stem cells, fibroblasts, and cardiac myocytes in culture (Motlagh et al., 2003; Doroudian et al., 2013; Broughton and Russell, 2015). The mechanical and topographical cues act through mechanotransduction signaling pathways to form focal adhesions (Samarel, 2014).

PIP2 also localizes around the vertical projection on microtopographical surfaces. Strongly attached focal adhesions support actin assembly and stress fiber formation. A change in actin dynamics and thin filament assembly with mechanical loading was detected in cardiac myocytes by FRAP methods (Li and Russell, 2013), where increased PIP2 led to higher kfrap values. Since a similar loading mechanism might have been present in fibroblasts with substrate stiffness or topography. However, FRAP actin dynamics for stress fibers were the same on all substrata regardless of stiffness, topography or drug treatment suggesting that the rates are the same. The explanation for failure to detect any difference might be that ROI was in the stable central part of the stress fiber and not analyzed at the growing tip near the periphery where actin polymerization is occurring. Measurements at the ends of the stress fibers or in the lamella were attempted but were unsuccessful due to rapid structural changes at the cell periphery.

Relevance for wound healing

Wound healing is a major clinical issue. It is worth noting commercially available neomycin balms are used to prevent infection of cuts. In addition to its function as an antibiotic, here an added beneficial side effect is described, namely in its interaction with PIP2 to promote migration. Neomycin has a short half-life (2 hrs) and has undesirable systemic side effects like nephrotoxicity and neurotoxicity from percutaneous absorption (Gannu et al., 2014). Neomycin delivered locally from microporous microrods might be advantageous in clinical use. MGF eluted over several days from PEGDMA microrods (Doroudian et al., 2014) improved cardiac function after ischemia injury (Peña et al., 2015). PEGDMA microrods were loaded with high concentrations of neomycin and maintain release over 12 hours. Hence, development of these neomycin microrod devices for a single therapeutic application to a wound might enhance the migratory behavior of cells over a period of time, resulting in improved wound closure and better scar formation.

Acknowledgments

Olivia Thomson, Research Resources Center-Electron Microscopy Services Lab, University of Illinois Chicago Scanning Electron Microscopy with a Hitachi S-3000N VPSEM. We thank Jieli Li MD, PhD for the initial suggestion of neomycin in migration studies.

Contract grant sponsor: National Institutes of Health; Contract grant number HL 62426

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Figure 1. Fibroblast migration in a wound closure model is regulated by substrate stiffness and PIP2 availability

Fibroblasts were grown on flat surfaces with stiffness varying from 10 kPa to over 1 GPa (glass) and subjected to treatment with drugs that reduce PIP2 availability (neomycin, 500 μM) or increase PIP2 via PI3K inhibition (wortmannin, 1 μM). Phase images of cells grown on glass at (A) one hour after barrier removal (t_1) and (B) at 7 hours after barrier removal (t_7) to show distance migrated over time (bar = 250 μm). (C) Migration velocity was significantly highest on the softest surface (10 kPa) of untreated fibroblast compared to 400 kPa (+ in black bars, $P < 0.05$). Migration velocities on all stiffnesses showed significant increase in migration velocity with neomycin treatment with doubling on 10 kPa. Velocities

were significantly retarded with wortmannin treatment on all surfaces. Means $\pm$ SE; * $P < 0.05$, ** $P < 0.01$, # $P < 0.001$, ## $P < 0.0001$ compared to untreated of equal stiffness, n = at least 7 cultures for each condition.

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Figure 2. Focal adhesion and actin stress fibers in leading lamella of migratory fibroblasts with varying stiffness and altered PIP2 level

Fibroblasts were grown on PAA substrate of 10 kPa or 100kPa, or glass (> 1 GPa) stiffness and viewed with a fluorescent microscope. (A) Untreated fibroblasts (left panels), neomycin treatment (central panels), and wortmannin treatment (right panels) have similar cytoskeletal structure with actin stress fibers (red) ending in focal adhesions (green). Actin, red; nucleus, blue; focal adhesion, paxillin, green (bar = 10 μ m). (B) Diagram to show sampling method for the distance between the focal adhesion and the lamella membrane by 5 white lines per cell. (C) Histogram with focal adhesion distance from leading edge membrane to show

variation with stiffness and drug treatment. Means $\pm$ SE; ** $P < 0.01$ compared to untreated cells on same stiffness with over 15 cells per condition.

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Figure 3. Localization of actin, lamellipodin, PIP2, and PIP3 with varying stiffness and altered PIP2 level

Fibroblasts grown on glass and viewed with a fluorescent microscope shown untreated (left panels), with neomycin treatment (central panels), and with wortmannin treatment (right panels). Actin, red; nucleus, blue; lamellipodin, PIP2, and PIP3 green with their respective antibodies (bar = 10 μ m). (A) Lamellipodin is distributed on the leading edge membrane under all conditions. (B) PIP3 antibody shows diffuse pattern with elevation at lamella under all conditions. (C) PIP2 antibody shows localization near the leading edge of the fibroblast. Significantly, neomycin-treated cells show even greater localization at lamellar edge. (D) A PIP2 biosensor (green) transfected living fibroblasts grown on glass and shows a distribution

similar to the PIP2 antibody detection. (E, F) Histogram to show the ratio of intensity of PIP3 or PIP2, respectively from ten microns near the leading edge to ten microns in the cell interior. PIP3 shows no difference between treatments. Ratio of lamellar to interior PIP2 is significantly higher in neomycin-treated cells compared to untreated. Means $\pm$ SE; * $P < 0.05$, $n = 23$ cells for PIP3, $n = 32$ cells for PIP2.

Figure 4. Micropost topography and microrods

Micropost topography fabricated by unmolding PDMS (400 kPa) from the template. Micropost surface is viewed by scanning electron microscopy from 45° angle (A) with low magnification showing tetragonal array (75 μm apart, 15 μm high and 15 μm diameter) (bar = 75 μm) and (B) at higher magnification (bar = 15 μm). Phase image of PEGDMA microrods showing shape (C) at low magnification (bar = 75 μm) and (D) at high magnification (bar = 100 μm).

Figure 5. Microtopography and distribution of actin, PIP2, and PIP3

(A) Fibroblasts grown with microrod (between dashed lines) show strong focal adhesion formation (white arrow). (B, C) Fibroblasts on microtopography (PDMS, 400 kPa), show cells attached to micropost (top indicated by white line). Actin, red; nucleus, blue; PIP3, or PIP2 (B, C respectively), green (bar = 15 μm). PIP3 pattern is diffuse to cell periphery but PIP2 shows increased localization around microposts. (D) For actin dynamics, a stress fiber region of interest (red box) was bleached to approximately 40% initial intensity by a 488 nm laser at high power and observed during recovery in cells grown on flat 10 kPa, 400 kPa, or glass surfaces, microtopography, or on glass with neomycin or wortmannin treatment (bar =

15 μm). (E) Time course of recovery of fluorescence intensity determined kfrap kinetic constant was not significantly difference on different stiffnesses, microtopography, or with drug treatment. FRAP experiments in different conditions were conducted on cells from at least 3 separate cultures. Means $\pm$ SE.

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Figure 6. Release of neomycin from PEGDMA microrods

(A) Cumulative release of neomycin from 50,000 microrods in a 12-hour time period. Diamond from 50% PEGDMA, circle from 90% PEGDMA microrods. (B) Migration velocity of primary rat cardiac fibroblast grown on glass was significantly higher with neomycin delivered as a drug to the media or released from 50% PEGDMA microrods. Migration was not significantly different for blank microrods or from 90% PEGDMA microrods. Means $\pm$ SE; ** $P < 0.01$, **** $P < 0.0001$, $n =$ at least 5.