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RAC1-Mediated Actin Cytoskeleton Remodeling and Monolayer Barrier Properties of Stretched Alveolar Epithelial Cells

Brian Christopher DiPaolo
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Abstract
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RAC1-MEDIATED ACTIN CYTOSKELETON REMODELING AND MONOLAYER BARRIER PROPERTIES OF STRETCHED ALVEOLAR EPITHELIAL CELLS

Brian Christopher DiPaolo

A DISSERTATION

In

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in

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Degree of Doctor of Philosophy

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Christopher S. Chen, Professor, Bioengineering
Paul A. Janmey, Professor, Physiology
David F. Meaney, Professor and Chair, Bioengineering
DEDICATION

This work is dedicated to my wonderful family and friends:

Andrew, for the strength and great power to question and confidently defend, lead, and work with a team. Dad, I'll never forget the moment you showed me the rings of Saturn through a telescope. That moment sparked a lifetime of yearning to learn and discover, a true passion for science. Thank you for teaching me so much, letting me build a crater in the backyard, for painting all my physiology models, and letting me play with rocket engines, radios, and optics. Grandmom and Grandpop DiPaolo, for your boundless interest, enthusiasm, and love for everything we did, and the importance of kindness, compassion, and humor. Grandmom and Grandpop Michener, for your zeal to explore and teach and unlimited inspiration and love. Jonathan, for the love of art, in all its glorious varieties, the value of professionalism, and robust determination. Mom, you helped me draw and color life-sized human patients on which I pretended to operate and let me save a whole turkey skeleton in the refrigerator. You inspired a lifelong exhilaration for the mysteriousness and splendor of human physiology. Thank you for being so patient and constructive. We messed the house with legos, toys, and video games a thousand times. Lisa, everyday you inspire me to love, create, and live in harmony. I am in awe of your ability to bring out the best of those around you. Susan, for your trust, immeasurable amount of instruction in so many key areas, and having shaped me into a self-sufficient investigator. Thomas, for the passion to inspire, teach, and reach out clearly and with wit. Aunts, uncles, cousins, the Lyon family, friends, Casper, Seth, Snowflake, Ted, fish and pets, for your unwavering love and companionship. These virtues I can only hope to emulate at a fraction of which you are capable. These passions, which have led me down this road, have brought me so much happiness and fulfillment. I promise to use them to help build a better tomorrow.

...and to those of our future:

Writers and creators; those able to conjure something from just bits and pieces like a magician, builder, and entertainer; those able to synthesize diverse areas of knowledge and modes of thinking, like a conductor; their prophetic ability to chart our 'real' world in advance; whisking the future air for the scent of danger or the precious light of prosperity; imagining vast, bold worlds that reveal so much about ourselves.

Teachers, patiently illuminating the world; those able to channel their extraordinary view of nature into pupils, to simplify the chaos; their deeply refined modus operandi of instilling wonder; a teachers gift of fascination that, like a seed, is selflessly and courageously planted; those able to bequeath the lifelong gift and rapture for curiosity, knowledge, and truth in a vast elegant universe that can be, on occasion, dark, mysterious, or frightening.

Scientist and engineers, pilots of Open Conspiracy, seekers of truth; those with the staunch devotion to an onerous and exacting Socratic method; those with strict adherence to the honorable faculties of patience, integrity, humility, impartiality, and critical scrutiny of self; for the intrepid exploration of the unknown, and the tapestries they have painted of our beautiful universe.

May we continue to love, inspire, enlighten, and advance; lest we fail our children.
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ABSTRACT

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Brian Christopher DiPaolo

Dissertation Supervisor: Susan S. Margulies, Ph.D.

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Rac1 inhibitor EHT-1864 strengthens monolayer barrier properties and attenuates PJAR formation during stretch but increases cell death

Rac1 agonist PDGF activates LIMK½ and cofilin and forms PJAR in UNS monolayers

PAK-1 inhibitor IPA-3 strengthens monolayer barrier properties and attenuates PJAR formation during stretch

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

INTRODUCTION

Mechanical ventilation is vital for supporting patients with pulmonary insufficiency as a result of specific life threatening conditions including acute lung injury, disease, and autonomic nervous system damage. While mechanical ventilation is critical to maintain normal blood gas levels, it has been implicated in the etiology of pulmonary barrier dysfunction (102). Each year there are an estimated 190,600 cases of acute lung injury in the United States with an associated mortality rate of 40% (69, 108). Ventilator induced lung injury (VILI) occurs in 5 - 15% of all ventilated patients (102, 132) and manifests as alveolar air leakage (6, 11), impaired pulmonary mechanics and function (8), alveolar cell dysfunction (10), deviations in alveolar fluid balance and blood-gas permeability (10), and acute respiratory failure.

VILI has been studied extensively in both animal and whole-lung models. Experimental data suggests that pulmonary injury during mechanical ventilation is a result of elevated regional lung volumes or airway pressures (47, 101, 102, 120, 121) while it has been suggested that end-inspiratory volume (maximum inflation), rather than pressure, disrupts the pulmonary barrier (45, 60). During inspiration the basement membrane of the alveoli first unfolds, then stretches, deforming the alveolar epithelial cells (AECs) (118). Studies in whole lung have shown that excessive AEC stretch due to basement membrane deformation negatively affects barrier function at the cellular level by increasing paracellular permeability, macromolecule, ion, and water transport to the airspace, resulting in alveolar edema (25, 44, 48, 49, 51, 76). Edema increases diffusing
distances, diminishing the efficiency of gas exchange, and reduces lung compliance by interfering with surfactant (46, 47).

The alveolar epithelium is composed primarily of two functionally distinct cell types which have been shown to respond to mechanical deformation. Alveolar type I cells are the primary regulators of paracellular transport (28). Type I cells are relatively thin and have a high surface area, covering >90% of the total alveolar surface (36, 134), which allows gas exchange as well as active sodium (19) and aquaporin mediated (20) water (79, 86) transport. Others have demonstrated that type I cells act as mechanosensors in the lung (9). Alveolar type II cells are situated at the corners between type I cells (32) and have been shown to be more vulnerable to deformation-induced injury when compared to type I cells (119). Type II cells cover about 7% of the total alveolar surface but outnumber ATI cells 2:1 (36). Type II cells secrete pulmonary surfactant which reduces alveolar surface tension (82). Surfactant secretion can be stimulated by mechanical deformation (59, 136) and by ATP (41, 66, 90). Specifically, type II cells, in co-culture with type I cells, released surfactant in response to stretch-induced type I paracrine ATP release (103). Additionally, others have shown that type II cells are capable of sodium (80, 133) and proton transport (85) and, in response to inflammation or wound injury, can actively spread and migrate to denuded regions and divide and differentiate into type I cells (1, 33, 52, 53, 125). Thus, the alveolus is composed of two functionally distinct cell types which respond to mechanical deformation.

Damage due to mechanical ventilation, which takes place on the cellular level, has been shown to be a result of mechanical injury (48-50, 76) and hyperoxia (68).
Excessive deformation of AEC monolayers was shown to increase paracellular permeability (25, 26), induce changes in tight junction location and structure (27), increase cell death (119), and increase plasma membrane surface area (57). It has been speculated that increased permeability due to stretch is a result of mechanical failure of molecular adhesion between adjacent cells (60). Adjacent cells are linked by actin anchored (105) tight junctions, adherens junctions (105), desmosomes (131), and gap junctions (2). The tight junction (TJ) is the most apical member of the intercellular junctional complex (56) adhering adjacent cells. This gasket like structure offers primary resistance to paracellular transport (92). The TJ (Figure 1.1) is composed of transmembrane proteins occludin and claudins 1-5 and 7 (62, 63, 65) which are detergent-insoluble, suggesting they are tightly bound to cytoskeletal elements, and junctional adhesion molecule (JAM) which is detergent-soluble (55) and not tightly bound to the cytoskeleton. The claudin family of transmembrane proteins offer primary paracellular resistance and regulate charge selectivity (7). Claudin-3, -4, and -5 are all co-expressed in primary alveolar epithelial cells in culture while claudin-1 and -2 show very little expression (129). Claudin-7 is also found specifically in type I like AECs (29, 110). Occludin, an integrin protein also found to co-localize at the TJ (63, 109), has been found to play a part in TJ barrier selectivity as well as mediate cell-cell adhesion (126). Occludin and claudins are known to anchor internally to actin (87) via zona occludins 1 (ZO-1) (54, 64, 74), ZO-2 via protein 4.1R (91), ZO-3 (74, 137), and cingulin (37).
The actin cytoskeleton is a dynamic mechanosensitive structure that bears major cellular load and assists in cell-cell adhesion (5, 22, 23, 31, 43). Previous studies have demonstrated that actin forms stress fibers in stretched cells (13, 75, 114, 117, 130). Perijunctional filamentous actin (F-actin) ring (PJAR), also referred as the perijunctional actomyosin ring (122), a specific morphological conformation of F-actin formed rapidly in biaxially stretched rat AECs (27, 43, 89, 130) and also found in endothelial cells (130). It is not yet understood if PJAR forms in response to stretch mediated pathways, and if this formation ruptures actin-TJ protein bonds and increases TJ mediated paracellular permeability found previously in AEC monolayers (25, 26). Previous studies in other cell types have implicated the actin cytoskeleton as a major mediator of monolayer permeability. Disruption of F-actin perturbs TJ barrier functionality, TJ structure (27, 88),
and distribution (21, 97, 113, 128). Stretch induced actin remodeling into PJAR and its influence on the actin-TJ protein bond is also poorly understood in type I AECs.

Studies have implicated the Rho and Rac pathways in actin remodeling and TJ-mediated monolayer permeability in other cell types. Specifically, the Rho pathway was shown to modulate actin structure, TJ protein distribution, and TJ mediated paracellular permeability in other epithelial cells types (21, 73, 97, 113, 115, 123, 124, 128). Previously, others have shown that activation of the Rho pathway influences the actin cytoskeleton (4, 13, 14, 75, 77, 114) and TJ protein and barrier properties in stretched endothelial cells and other cell types (35, 98, 104, 112, 123, 128).

Alternatively, others have implicated the Rac pathway as a modulator of actin cytoskeleton remodeling and formation of peripherally localized stress fibers in other cell types (70, 72, 96, 107, 111, 139) as well as in other stretched cell types (3, 42, 83, 84, 93). In addition, the Rac pathway has also been shown to influence monolayer permeability (21, 24, 95, 138). Others have also shown evidence that the Rho and Rac pathways interact to mediate barrier properties (15, 16). However, there is a paucity of data on the Rho and Rac pathways and their influence on actin cytoskeleton remodeling, TJ protein, and paracellular permeability in stretched AEC monolayers.

**SCOPE AND GOALS**

Previous studies have shown that pathological magnitudes of deformation result in actin cytoskeleton remodeling, changes in tight junction (TJ) properties, and in increased monolayer permeability in alveolar epithelial cell (AEC) monolayers. The overall objective of this work is to identify the biochemical signaling pathways involved
in actin cytoskeleton remodeling during mechanical deformation and establish the role of these pathways in regulation of alveolar epithelial barrier function in AEC monolayers. The overall hypothesis (Figure 1.2) driving this research is that large magnitudes of epithelial strain bring about rapid changes in the actin cytoskeleton through activation of the Rho and Rac pathways, resulting in actin-TJ bond rupture and increased paracellular permeability.

Figure 1.2: Hypothesized mechanical stretch signaling pathways involved in actin cytoskeleton remodeling and increased paracellular permeability in stretched primary rat alveolar epithelial cells (AECs). Actin cytoskeleton remodeling dynamics and PJAR formation investigations are presented in Chapter 2. Rho mediated Rho kinase (ROCK) pathway (*top*) investigations are presented in Chapter 3. Rac1 pathway (*bottom*) investigations are presented in Chapter 4. Numerous other signaling proteins may contribute to Rho and Rac1/Akt pathway regulation during stretch (12, 30, 40, 58, 61, 71, 81, 94, 99, 116, 127).

Research objectives were accomplished through a series of *in vitro* experiments using monolayers of primary rat alveolar type II cells cultured for 4 days on flexible
Silastic membranes. After 4 days, the cells had adopted comparable alveolar type I features including gene expression, phenotype, and morphology (17-20, 28, 34, 38, 39, 67, 78, 100, 106, 135) and had formed a confluent monolayer. Monolayers were stretched biaxially at physiological relevant magnitudes including at 12%, 25%, or 37% change in surface area (ΔSA), roughly corresponding to 64%, 86%, and 100% total lung capacity, respectively (119).

Chapter 2 of this dissertation presents quantitative data showing rapid actin cytoskeleton remodeling during mechanical deformation of AECs. Chapter 3 investigates the Rho pathway and its role in actin cytoskeleton remodeling. Chapter 4 investigates the Rac pathway and its effect on actin cytoskeleton remodeling, TJ structure, and monolayer permeability. Chapter 5 integrates the results, and concludes with a discussion of clinical relevance and future studies.
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CHAPTER 2: STRETCH ALTERS ALVEOLAR EPITHELIAL ACTIN CYTOSKELETON

ABSTRACT

Alveolar epithelial cells (AEC) maintain integrity of the blood-gas barrier with gasket like intercellular tight junctions (TJ) which are anchored internally to the actin cytoskeleton. We hypothesize that stretch rapidly reorganizes actin (< 10 min) into a perijunctional actin ring (PJAR) in a manner that is dependent upon magnitude, frequency, and duration of the stretch, accompanied by both spontaneous movement of actin anchored receptors at the plasma membrane and increased paracellular permeability. Primary AEC monolayers were stretched biaxially to create a change in surface area (ΔSA) of 12%, 25%, or 37% in a cyclic manner at ¼ Hz for up to 60 min, or held fixed (0 Hz) at 25% ΔSA for up to 60 min, or left unstretched. By 10 min of stretch PJARs were evident in 25% and 37% ΔSA at ¼ Hz, but not for 12% ΔSA at ¼ Hz, or at 0 Hz 25% ΔSA, or unstretched. At 60 min of stretch, monolayer permeability to the fluorescent tracer BODIPY-ouabain had increased in monolayers stretched to 25% and 37% ΔSA ¼ Hz but not in 12% ΔSA ¼ Hz. Treatment with 100 nM jasplakinolide abolished stretch-induced PJAR formation and attenuated the stretch-induced increase in monolayer permeability, however. As a rough index of actin receptor remodeling, we measured spontaneous motions of 5 µm microbeads bound to actin focal adhesion complexes on the apical membrane surfaces; within 1 min of exposure to ΔSA of 25% and 37% these motions increased substantially, increased with increasing stretch frequency, and were consistent with our mechanistic hypothesis. With 0 Hz stretch, however, the spontaneous
motion of microbeads attenuated back to unstretched levels, while PJAR remained unchanged. Stretch did not increase spontaneous microbead motion in human alveolar epithelial adenocarcinoma A549 monolayers, confirming that this actin remodeling response to stretch was a cell-type specific response. In summary, stretch of primary rat AEC monolayers forms PJARs and rapidly reorganized actin binding sites at the plasma membrane in a manner dependent upon stretch magnitude and frequency.

INTRODUCTION

Mechanical ventilation is vital for treating specific life threatening conditions but has been implicated in the etiology of pulmonary barrier dysfunction. Ventilator induced lung injury (VILI) occurs in 5 to 15% of patients requiring mechanical ventilation (50, 76) and has a mortality rate of 34 to 60% in those patients with acute respiratory distress syndrome (ARDS) (27). During mechanical ventilation, pulmonary alveolar epithelial cells (AEC) undergo biaxial stretch as the surface of the basement membrane increases (65), but the delivery of large gas volumes to localized lung regions has been implicated in the increase of blood-gas barrier permeability (24, 34). Previously, rat type I like AEC monolayers in culture were used to mimic the alveolar epithelium in vitro (4, 13, 18, 37, 48). Cavanaugh and Margulies demonstrated that high biaxial stretch (37% change in surface area, ∆SA), analogous to pathological ventilator volumes, results in an increase in paracellular permeability (11) where tight junctions (TJ) offer primary resistance to epithelial paracellular transport (45). Investigators have demonstrated an integral role of the actin cytoskeleton in cell-cell adhesion (72) and anchoring TJ protein (39), including tight junction protein zona occludens-1 (ZO-1), in other cell types (25). Others have
shown that disruption of filamentous actin (F-actin) perturbs TJ functionality as a mediator of paracellular permeability as well as TJ structure (12, 40) and distribution (5, 47, 57, 73). Moreover, cyclic stretch has been shown to alter F-actin distribution in alveolar epithelial cells (43). Taken together, these results lead to our hypothesis that during biaxial stretch the actin cytoskeleton has an integral effect on TJ mediated paracellular permeability.

When a cell is stretched, the cell transduces the mechanical signal into a cascade of biochemical signals (20, 78) resulting in actin cytoskeleton rearrangement. During uniaxial stretch, F-actin crosslinks with myosin and numerous actin-binding proteins to form thick polymerized bundles or actin stress fibers. Human pulmonary artery endothelial cells (HPAEC) cyclically elongated uniaxially rapidly form actin stress fibers aligned perpendicular to stretch direction and enhanced F-actin at the cell periphery (3, 33, 58, 62, 74). When endothelial and epithelial monolayers are stretched biaxially, actin reorganizes into stress fibers that form ‘tent-like’ structures in the direction of least strain (12, 43, 74), forming perijunctional actin rings (PJAR) or perijunctional actomyosin rings (68) composed of actin and myosin (26, 79). Lung cells experience biaxial loading routinely, but to date there is a paucity of data regarding the effect of biaxial stretch rate and magnitude on the actin cytoskeleton of AEC monolayers.

The goals of our study are to test if PJAR formation and PJAR intensity are dependent on biaxial stretch magnitude, frequency, duration, and to determine if PJAR is mechanistically related to actin dynamics and monolayer permeability in monolayers of rat type I like AECs. Our overall hypothesis is that actin redistributes rapidly (within 10 min) such that PJAR formation and fluorescent intensity are both dependent on stretch
magnitude and frequency. Our observations suggest that the actin cytoskeleton movement at the membrane increases rapidly (< 1 min), concurrent with a PJAR formation that is dependent on stretch magnitude, frequency, and time. Even with continued stretch, actin cytoskeleton rearrangement rates slow over time although PJAR remains. Finally, monolayer permeability is stretch magnitude dependent and can be modulated by actin cytoskeleton stabilization with jasplakinolide.

MATERIALS AND METHODS

Primary Rat Type I Like Alveolar Epithelial Cell Isolation

Alveolar type II cells were isolated from male Sprague-Dawley rats based on a method reported by Dobbs et al. (22) with slight modification (66). Cells were seeded at 1.0 million cells/cm² onto fibronectin coated (10 µg/cm², Invitrogen, Carlsbad, CA) flexible Silastic membranes (Specialty Manufacturing, Saginaw, MI) in custom-designed wells (65). The cells were cultured for 5 days at 37°C, 5% CO₂ in MEM (Invitrogen) with 10% FBS (Sigma-Aldrich, St. Louis, MO) replaced daily. After 5 days, the cells had adopted alveolar type I (ATI) features (4, 13, 15, 18, 48, 54) including the expression of RTI40, and had grown to a confluent monolayer. Monolayers were then serum-deprived in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech Inc., Manassas, VA) supplemented with 20 mM HEPES (Sigma) for 3 hrs (unless stated otherwise) and stretched biaxially across a range of physiological relevant magnitudes including at 12%, 25%, or 37% change in surface area (ΔSA) roughly corresponding to 64%, 86%, and 100% total lung capacity, respectively (65). Because stretch rate also significantly affects the alveolar cell monolayer viability and permeability (15, 66), both sustained tonic and
cyclic stretch modes were investigated. Sustained tonic stretch (0 Hz, held at stretch) was used to model alveoli held at partial (residual) inflation. Cyclic (sinusoidal) stretch (¼ Hz) was used to model ventilation.

*Perijunctional Actin Ring Quantification*

Primary rat AEC monolayers were stretched biaxially at 12%, 25%, or 37% change in surface area (ΔSA) cyclically at ¼ Hz for 0 (unstretched), 1, 10, or 60 min. An additional group of monolayers were stretched at 25% ΔSA and held (sustained tonic, 0 Hz) at stretch for the same durations. At the end of the stretch period, monolayers were fixed with 1.5% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate buffer saline (PBS) for 15 min, permeabilized using 0.1% Triton X-100 in PBS for 5 min, and blocked with 5% goat serum in PBS for 1 hr. Wells were double stained (in 5% goat serum in PBS for 1 hr at 23°C) for F-actin (phalloidin, Invitrogen, Carlsbad, CA) to evaluate perijunctional actin ring (PJAR) and zona occludens-1 (ZO-1; anti-ZO1 antibody, Invitrogen) to identify the location of the cell plasma membrane and evaluate peripheral ZO-1 intensity.

Both red (F-actin) and green (ZO-1) channels of two random microscope fields from each labeled monolayer were captured (×40 objective) on an epifluorescent scope (Nikon) using identical exposure times for all images of each type. Each field was divided into a 3 x 3 matrix of regions, and every other region (5 regions) was systematically selected for analysis. In each region, all cells with at least 50% of its area residing in the region were evaluated, typically 16 cells per field. The perijunctional F-actin fluorescent intensity of each cell was analyzed (ImageJ, ver. 1.43j) by tracing the
peripheral ZO-1 (Figure 2.4 **top inset**), superimposing this ZO-1 trace onto the same cell stained for F-actin (Figure 2.4 **bottom inset**), and enlarging the trace line thickness radially inward and outward to create a boundary zone for further analysis approximately 1.6 µm thick (average PJAR thickness from a small sample study, Figure 2.4 **bottom inset**, *white contours*). Mean F-actin fluorescent intensity in this peripheral annulus (A<sub>i</sub>) was measured. Whole cell F-actin mean fluorescent intensity (W<sub>i</sub>) was determined, including annulus and cell interior. PJAR intensity (P<sub>i</sub>) was found by taking the ratio of peripheral annulus mean intensity to whole cell mean intensity (P<sub>i</sub> = A<sub>i</sub> / W<sub>i</sub>). Similarly, perijunctional ZO-1 fluorescent intensity of each cell was analyzed by tracing the peripheral ZO-1 (Figure 2.5, *inset*) and enlarging the trace line thickness radially inward and outward to create a boundary zone for further analysis approximately 1.6 µm thick. ZO-1 intensity (Z<sub>i</sub>) was found by taking the ratio of peripheral annulus ZO-1 mean intensity to whole cell ZO-1 mean intensity (Z<sub>i</sub> = A<sub>i</sub> / W<sub>i</sub>). For each experimental group, P<sub>i</sub> and Z<sub>i</sub> were evaluated based on an average 32 cells (2 fields) per animal from at least 4 different animals. Using Dunnett’s test with 0 min stretch (unstretched, UNS) as reference, mean P<sub>i</sub> and mean Z<sub>i</sub> for each sample (monolayer) was evaluated for each stretch magnitude across time, and at each time point across stretch magnitude (12, 25, 37% ∆SA) and frequency (dynamic and tonic). Significance was defined as p < 0.05.

*Actin-mediated Binding Site Movement*

Spontaneous nanoscale motions of microbeads attached to cell surface integrin receptors were monitored to assess a mechanism of the molecular scale cytoskeletal rearrangement (2, 8, 9, 56). The receptors are linked internally to the actin cytoskeleton
Rat type I like AEC monolayers that had been maintained in culture for 4 days were then serum deprived overnight in DMEM + HEPES. Ferrimagnetic microbeads (5 µm diameter, provided by Harvard School of Public Health, Boston, MA) were coated at 150 µg peptide per 1 mg beads with either Arg-Gly-Asp (RGD, Sigma-Aldrich, St. Louis, MO) for adhesion to actin anchored (42) transmembrane integrin receptors (71, 75, 80) or acetylated low-density lipoprotein (AcLDL, Invitrogen, Carlsbad, CA), a protein complex that binds scavenger receptors but not focal adhesion complexes, as nonspecific control (52, 53, 75), then introduced onto cell monolayers. Adherent microbeads (Figure 2.2 inset top and bottom) were serially imaged through a ×20 objective (Nikon) at 1 Hz on a phase contrast epifluorescent scope in 5 min epochs, first in monolayers at rest. The monolayers were then immediately stretched biaxially to 25% or 37% change in surface area (ΔSA) and held (sustained tonic stretch) and imaged during stretch (5 min image acquisition, timeline top, Figure 2.1). Microbeads attached to unstretched (UNS) cells imaged at the same intervals for the same duration served as controls (timeline bottom, Figure 2.1). An additional group of rat type I like AEC monolayers were stretched cyclically (¼ Hz) for a total of 40 min, imaged at similar intervals for 5 min at rest, and analyzed similarly (timeline middle, Figure 2.1).
Figure 2.1: Applied stretch protocol in monolayers held in sustained tonic stretch (top), stretched cyclically (middle), or left unstretched (bottom). Monolayers were left on the scope to equilibrate for 30 min before start of stretch. Stretch starts at time = 0 min. Adherent microbeads were serially imaged in 5 min long epochs labeled MSD on the timeline. The median MSD$_{100}$ (total mean squared displacement over 100 seconds) value during stretch of each monolayer was divided by its respective median MSD$_{100}$ value just before stretch (stretch time = 0 min) to determine normalized MSD$_{100}$ (nMSD$_{100}$) of the monolayer.

A separate group of monolayers were pre-treated with 1 µM jasplakinolide (Invitrogen) or 0.1 µM latrunculin-A (Invitrogen), or DMSO as vehicle control (VC) for 10 min during bead incubation then washed to free non-adherent beads and end treatment. Five to nine monolayers per group isolated from at least 3 rats were studied under each condition, each with an average of 125 analyzed microbeads. To compare primary AEC with the human alveolar epithelial adenocarcinoma A549 cell line (ATCC, Manassas, VA), in separate studies A549 cells seeded onto silastic membranes at 0.25 million cells/cm$^2$ in MEM + 10% FBS at 37ºC, 5% CO$_2$ overnight then serum deprived with
DMEM + HEPES overnight grew to confluent monolayers and were held at sustained tonic stretch and the spontaneous nanoscale motion of microbeads was analyzed.

Each 5 min (300 frames) epoch of images was analyzed using a novel MATLAB (ver. 6.5 R13, The MathWorks, Natick, MA) program which determines the center of mass of each microbead, tracking it for the duration of the stretch (Figure 2.7 inset) while removing erroneous whole field displacement caused by microscope stage or silastic membrane movement during image capture by subtracting the median x and y displacement components of all microbeads from the x and y displacement components of each microbead respectively. Using bead coordinates we calculated the mean square displacement (MSD) of each microbead:

$$\text{MSD}(\Delta t) = [r(t + \Delta t) - r(t)]^2$$

where $r(t)$ is the microbead position at time $t$, and $\Delta t$ is the time between measurements (time lag).

When microbeads were coated with AcLDL, MSD was hypothesized to be a measure of binding site fluidity within the plasma membrane; when microbeads were coated with RGD, MSD was hypothesized to be a measure of actin remodeling within the cytoskeleton (2, 8, 9, 56). $\text{MSD}_{100}$, the total mean squared displacement over 100 seconds ($\Delta t = 100$ s, Figure 2.2), of each microbead was evaluated by averaging total MSD from every 100 s long window of time contained within the 5 min image capture epoch (200 total windows).
Figure 2.2: Microbead MSD vs. time lag in A549 cells. MSD of unstretched cells during capture plotted against time lag (Δt) at different times during 40 min of rest in A549 monolayers. MSD$_{100}$ decreased in A549 cells during 40 min rest. Top inset: Dark microbeads shown attached to A549 monolayer surface; bar = 50 µm. Bottom inset: Arg-Gly-Asp (RGD) coated microbead (white arrow) bound to phalloidin labeled F-actin cytoskeleton in rat type I like AEC monolayer; bar = 10 µm.

Median MSD$_{100}$ of all microbeads in each monolayer (average of 125 analyzed microbeads per monolayer) was then calculated and used as the measure of actin binding site movement. Median was used because MSD has a lognormal distribution (8) and to remove rare, yet potentially mean confounding, erroneous bead tracks (beads attached to monolayer impurities or adjacent beads, improperly identified beads, non-adherent beads) from the sample. The median MSD$_{100}$ value during stretch of each monolayer was divided by its respective median MSD$_{100}$ value just prior to stretch (stretch time = 0 min) to determine normalized MSD$_{100}$ (nMSD$_{100}$) of the monolayer. nMSD$_{100}$ was used to
compare across stretch regimens, durations, and cell types. Normalization was performed at each stretch duration to standardize a sample to help account for sample-to-sample variations in initial MSD\textsubscript{100} as result of variation in microscope stage temperature and monolayer handling.

To test the effect of stretch and treatment, nMSD\textsubscript{100} values were compared to time matched unstretched and untreated controls, respectively, using an ANOVA with Dunnett’s test (84) in JMP (ver. 8.0, SAS Institute Inc., Cary, NC). To test the effect of stretch time, nMSD\textsubscript{100} values were compared to their pre-stretch values using a Dunnetts 1-way ANOVA for repeated measures in JMP.

Monolayer Permeability

Paracellular permeability was assessed by monitoring the flux of the fluorescent tracer BODIPY-ouabain across the monolayer as previously described (11). Primary rat AEC monolayers were serum deprived in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Manassas, VA) supplemented with 20 mM HEPES for 3 hrs. The apical surface of the monolayer was then bathed in DMEM + HEPES containing 2 µM BODIPY-ouabain (radius ~20 Å, Invitrogen, Carlsbad, CA), a fluorescent tracer which has a high affinity for the extracellular domain of the transmembrane Na\textsuperscript{+}-K\textsuperscript{+}-ATPase (32, 60) found on the basolateral surface of type I AECs (77), for 60 min during stretch (or 60 min on unstretched monolayers). Monolayers were stretched biaxially at 12%, 25%, or 37% ∆SA cyclically at ¼ Hz for 0 (unstretched), 10, or 60 min. An additional group of monolayers were pretreated with 100 nM jasplakinolide (Invitrogen, Carlsbad, CA) for 10 min or DMSO as vehicle control then stretched at 12%, 25%, or 37% ∆SA
cyclically at ¼ Hz for 0 (unstretched) or 60 min. Following stretch, the apical surface was rinsed three times with dye-free DMEM + HEPES and imaged (Eclipse TE300 epifluorescence microscope, ×10 objective, Nikon, Melville, NY) using constant exposure, aperture, and fluorescent intensity settings. The maximum pixel intensity in the background of unstretched-untreated monolayers was measured and used as a threshold to exclude unstained regions. The area percentage of each image (3 image/well, 3 wells/animal, from at least 3 animals/group) was found using a MATLAB (version 6.5 R13, The MathWorks, Natick, MA) program (Appendix D) which calculates the area of the image above threshold intensity and divides it by the total image area. Normalized area percentage (nArea) of each image was found by dividing area percentage by the average area percentage of the unstretched-untreated group. The average nArea value was calculated for each animal and used to compare across groups. To test the effect of stretch, animal average nArea values were compared with time-matched unstretched controls using an ANOVA with Dunnett's test (84) in JMP (ver. 8.0, SAS Institute Inc., Cary, NC). To test the effect of treatment, animal average nArea values were compared with time-matched vehicle controls as well as unstretched-untreated controls using an ANOVA with Tukey-Kramer test (84) in JMP. Previously, Cavanaugh et al. showed that the BODIPY-ouabain tracer was not internalized by cells, did not undergo receptor-mediated endocytosis, did not bind due to plasma membrane rupture, and did not bind nonspecifically (11).
RESULTS

PJAR formation is rapid (< 10 min) and dependent on stretch magnitude and frequency

The phalloidin stained F-actin cytoskeleton in primary rat type I like AEC monolayers stretched biaxially reveals qualitative evidence of PJAR by 10 min persisting up to 60 min in 25% ΔSA and 37% ΔSA ¼ Hz (Figure 2.3 panel 3 and 4 respectively) stretched monolayers. In contrast, unstretched (UNS) monolayers and monolayers stretched at 12% ΔSA ¼ Hz for 60 min displayed homogenous actin morphology (Figure 2.3 panel 1 and 2 respectively). Monolayers held sustained tonic stretch at 25% ΔSA reveals evidence of PJAR at 60 min (Figure 2.3 panel 5). For comparison with microbead tracking data, qualitative F-actin in images obtained at 40 min stretch (Appendix I) are comparable to images obtained at 60 min stretch for each group.
Figure 2.3: Effect of biaxial stretch duration, magnitude, and frequency on F-actin arrangement in type I like rat AEC monolayers before and after 1, 10, and 60 minutes of stretch (time on x-axis). (1) Monolayers left unstretched (UNS). (2) 12% ∆SA ¼ Hz cyclic stretch at 60 min only. (3) 25% ∆SA ¼ Hz cyclic stretch. (4) 37% ∆SA ¼ Hz cyclic stretch. (5) 25% ∆SA sustained tonic (0 Hz) stretch. Both 25% and 37% ∆SA ¼ Hz cyclic stretch produced actin stress fibers on the cell periphery by 10 min, unlike monolayers stretched for 60 min at 12% ∆SA ¼ Hz cyclic which were similar to UNS monolayers. Monolayers held sustained tonic 25% ∆SA stretch produced actin stress fibers on the cell periphery at 60 min. Individual micrographs 56 µm width. Data at 60 min stretch is comparable at 40 min (Appendix I). Bar = 10 µm.
These qualitative observations of rapid PJAR formation correlated well with the quantitative metrics. PJAR intensity ($P_i$) was significantly higher than unstretched (UNS) by 1 min in monolayers stretched at 37% $\Delta$SA $\frac{1}{4}$ Hz and by 10 min in monolayers stretched at 25% $\Delta$SA $\frac{1}{4}$ Hz (Figure 2.4 dark-grey and light-grey respectively) but was not significantly different in monolayers stretched at 12% $\Delta$SA $\frac{1}{4}$ Hz (white) at any time. Because $P_i$ was significantly higher than UNS at 10 min in monolayers stretched at 25% $\Delta$SA $\frac{1}{4}$ Hz, but not in monolayers stretched at 25% $\Delta$SA tonic (grey-checker) we conclude that PJAR was frequency dependent. At 60 min of stretch, $P_i$ was significantly higher in monolayers stretched at 37% $\Delta$SA $\frac{1}{4}$ Hz and 25% $\Delta$SA tonic and $\frac{1}{4}$ Hz when compared to both 12% $\Delta$SA $\frac{1}{4}$ Hz and UNS, and we conclude that PJAR intensity ($P_i$) also is dependent on stretch magnitude.
Figure 2.4: PJAR intensity ($P_i$) was found by taking the ratio of peripheral annulus F-actin mean intensity to whole cell F-actin mean intensity ($P_i = A_i / W_i$) and plotted as a function of stretch magnitude, time, and frequency. $P_i$ was significantly higher than UNS by 1 min in monolayers stretched at 37% $\Delta SA \frac{1}{4} Hz$ (dark grey) and by 10 min in monolayers stretched at 25% $\Delta SA \frac{1}{4} Hz$ (light grey) but was not significantly different in monolayers stretched at 12% $\Delta SA \frac{1}{4} Hz$ (white) at any time. $P_i$ was significantly higher than UNS at 10 min in monolayers stretched at 25% $\Delta SA \frac{1}{4} Hz$ but not in monolayers stretched at 25% $\Delta SA$ tonic (grey-checker). At 60 min of stretch, $P_i$ was significantly higher in monolayers stretched at 37% $\Delta SA \frac{1}{4} Hz$ and 25% $\Delta SA$ tonic and $\frac{1}{4} Hz$ when compared to both 12% $\Delta SA \frac{1}{4} Hz$ and UNS. Unstretched (UNS, black dash line) average $P_i = 0.996 \pm 0.017$ standard error (SE). Data shown is mean ± SE with * = p < 0.05 compared to unstretched and # = p < 0.05 compared to 12% $\Delta SA \frac{1}{4} Hz$. Each data point based on an average 32 cells per animal from at least 4 different animals. The perijunctional F-actin fluorescent intensity of each cell was analyzed by tracing the peripheral ZO-1 (top inset), superimposing this ZO-1 trace onto the same cell stained for F-actin (bottom inset), and enlarging the trace line thickness radially inward and outward to create a boundary zone for further analysis approximately 1.6 µm thick (white contours). Mean F-actin fluorescent intensity in this peripheral annulus ($A_i$) was measured. Whole cell F-actin mean fluorescent intensity ($W_i$) was determined, including annulus and cell interior. PJAR intensity ($P_i$) was found by taking the ratio of peripheral annulus mean intensity to whole cell mean intensity ($P_i = A_i / W_i$). $W_i$ was found to be constant across all groups compared to unstretched (UNS) (Appendix G). Bar = 10 µm.

We hypothesized that actin cytoskeleton remodeling during formation of PJAR in stretched AEC monolayers would result in a decrease in peripherally located ZO-1.
Because peripheral ZO-1 intensity ($Z_i$) showed no dependence on stretch magnitude (Figure 2.5), ZO-1 was used to identify cell periphery for PJAR analysis (previously discussed).

![Figure 2.5: Perijunctional ZO-1 intensity ($Z_i$) was found by taking the ratio of peripheral annulus ZO-1 mean intensity to whole cell ZO-1 mean intensity ($Z_i = A_i / W_i$) and plotted as a function of stretch magnitude. $Z_i$ was constant across all stretch magnitudes. Data shown is mean ± SE. Each data point based on an average 32 cells per animal from at least 4 different animals. The perijunctional ZO-1 fluorescent intensity of each cell was analyzed by tracing the peripheral ZO-1 (inset) and enlarging the trace line thickness radially inward and outward to create a boundary zone for further analysis approximately 1.6 µm thick (white contours). Mean ZO-1 fluorescent intensity in this peripheral annulus ($A_i$) was measured. Whole cell ZO-1 mean fluorescent intensity ($W_i$) was determined, including annulus and cell interior. ZO-1 intensity ($Z_i$) was found by taking the ratio of peripheral annulus mean intensity to whole cell mean intensity ($Z_i = A_i / W_i$). $W_i$ was found to be constant across all groups compared to unstretched (UNS) (Appendix G).]

We hypothesized that actin cytoskeleton remodeling during formation of PJAR in stretched AEC monolayers would be accompanied by an increase in the movement of microbeads coated with the Arg-Gly-Asp (RGD) peptide sequence specifically binding to
apical cell surface transmembrane integrin receptors which anchor to the cytoskeleton. Qualitative PJAR formation data corroborates well with microbead tracking data, showing significant actin remodeling in 25% and 37% ∆SA tonic (0 Hz) and dynamic (¼ Hz) stretched monolayers after 1 min (Figure 2.6 and 2.7 respectively). Also, similar to quantitative PJAR intensity (P_i) data, the microbeads adhered to the RGD receptors showed an effect of stretch magnitude and frequency at 1 min of stretch, such that nMSD_{100} (mean squared displacement over the course of 100 s normalized to unstretched values) was significantly higher in both 25% and 37% ∆SA held (sustained tonic) stretched (Figure 2.6) and cyclic (¼ Hz) stretched monolayers (Figure 2.7) when compared to their unstretched values. Furthermore, at 1 min stretch nMSD_{100} was even greater in 37% ∆SA sustained tonic when compared to 25% ∆SA sustained tonic (Figure 2.6). Finally, at 1 min nMSD_{100} was greater in 25% ∆SA ¼ Hz when compared to 25% ∆SA sustained tonic stretch (Figure 2.7 vs. Figure 2.6). Thus, actin movement at the cell membrane significantly increased at 1 min of stretch when compared to the unstretched time point and, similar to PJAR intensity (P_i), was sensitive to stretch magnitude and frequency.

Although qualitative images and quantitative PJAR formation showed PJAR persistence for stretch duration, the spontaneous movement of anchored RGD-coated microbeads dropped precipitously after 1 minute, back to prestretch levels for all stretch conditions (Figure 2.6 and 2.7). The one exception was at 40 min in the 25% ∆SA ¼ Hz stretched monolayer group where nMSD_{100} was also higher than unstretched at this time point. Also, at longer stretch durations (≥ 10 to 40 min), spontaneous microbead movement showed no dependence on stretch magnitude. Finally, nMSD_{100} in
unstretched monolayers significantly decreased by 10 min, remaining constant (except at 30 min which was not different from time = 0) for the duration of time in the sustained tonic group, but not in the cyclic group.

Figure 2.6: nMSD\textsubscript{100} in primary AEC monolayers. nMSD\textsubscript{100} as a function of time for unstretched samples and 25% and 37% ∆SA held in sustained tonic (0 Hz) stretched samples. nMSD\textsubscript{100} when compared to unstretched samples was significantly higher at 1 min 25% and 37% ∆SA sustained tonic stretch, attenuating back to baseline by 10 min. nMSD\textsubscript{100} was greater in 37% ∆SA sustained tonic when compared to 25% ∆SA sustained tonic. Unstretched monolayers show significant decrease in nMSD\textsubscript{100} at 10 min, remaining constant (except at 30 min) for the duration of time. Inset: nMSD\textsubscript{100} at 1 minute of 25% ∆SA sustained tonic stretch (grey bars) or left unstretched (UNS, white bars). Stretched monolayers either treated with 0.1 µM latrunculin-A or incubated with acetylated low-density lipoprotein (AcLDL) coated microbeads showed no significant change in nMSD\textsubscript{100} when compared to their corresponding unstretched time point as well as time-matched unstretched controls. Data shown is mean ± SE from 9 monolayers per group with an average 125 beads analyzed per monolayer.
Figure 2.7: nMSD$_{100}$ in primary AEC monolayers as a function of time for unstretched samples and 25% and 37% ∆SA ¼ Hz cyclic stretched samples. When compared to unstretched samples, nMSD$_{100}$ was significantly higher at 1 min 25% and 37% ∆SA cyclic stretch, attenuating back to baseline by 10 min. nMSD$_{100}$ in 25% ∆SA ¼ Hz stretched samples became significantly higher again at 40 min. Inset: Illustrative bead motion traces over 5 min in monolayers left unstretched (UNS, left) or stretched 25% ∆SA ¼ Hz for 1 min (right). Spontaneous bead displacement in stretched monolayers was higher. Data shown is mean ± SE from 9 monolayers per group with an average 125 beads analyzed per monolayer.

Stretch induced PJAR formation can be inhibited with jasplakinolide and latrunculin-A

Treatment with jasplakinolide was used to stabilize the actin cytoskeleton (6, 7). Monolayers treated with 1 µM or 100 nM jasplakinolide for 10 min and then stretched 25% ∆SA ¼ Hz showed no qualitative evidence of PJAR formation when fixed and labeled with F/G-actin (Millipore, Billerica, MA) antibody (Figure 2.8 bottom-middle and bottom-right respectively). For comparison, consider monolayers stretched at the same magnitude and duration with vehicle control (Figure 2.8 bottom left).
Figure 2.8: Effect of biaxial stretch and jasplakinolide on actin. Type I like rat AEC monolayers with antibody labeled actin left unstretched (UNS, top row) or after 10 min of 25% ΔSA ¼ Hz cyclic stretch (bottom row). Vehicle control monolayers (VC, left column) stretched at 25% ΔSA produce actin stress fibers on the cell periphery by 10 min while monolayers stretched at the same magnitude and duration treated with 1 µM (middle column) or 100 nM (right column) jasplakinolide (JAS) for 10 min to stabilize actin showed no PJAR formation. Bar = 10 µm.

Qualitative images showing inhibited PJAR formation with jasplakinolide treatment corroborated well with quantitative measures of microbead tracking data. Treatment with 1 µM jasplakinolide for 10 min also significantly attenuates the movement of integrin adhered microbeads (MSD_{100} of 2962 nm² ± 360 nm² SE), compared to untreated monolayers at the same (MSD_{100} of 5891 nm² ± 743 nm² SE).
Thus, we conclude actin stabilization with jasplakinolide pretreatment inhibits actin binding site movement and formation of PJAR in stretched monolayers.

Hypothesizing that actin reorganization requires depolymerization (inhibited by jasplakinolide) and repolymerization, we used latrunculin-A to inhibit actin repolymerization (83) in stretched monolayers. Monolayers exposed to (sustained tonic) stretch of 25% ∆SA, 0.1 µM latrunculin-A pretreatment attenuated the rapid (< 1 min) increase in the spontaneous movement of microbeads attached to integrin receptors (Figure 2.6 inset) found in untreated monolayers. Thus, pretreatment with latrunculin-A abolished stretch induced actin binding site remodeling.

**Jasplakinolide attenuates stretch-induced increase in monolayer permeability**

We hypothesized that actin cytoskeleton remodeling during formation of PJAR in stretched AEC monolayers would be followed by an increase in paracellular permeability which could be modulated with jasplakinolide. Quantitative measurement of BODIPY-ouabain tracer flux across the monolayer (nArea) corroborated well with PJAR formation data (P_i), showing an increase in monolayer permeability at 60 min of 25% and 37% ∆SA ¼ Hz stretch, but not at 60 min of 12% ∆SA ¼ Hz (Figure 2.9). Treatment with 100 nM jasplakinolide for 10 min, shown to inhibit formation of PJAR in monolayers stretched at 25% ∆SA ¼ Hz 10 min (Figure 2.8), attenuated the stretch-induced increase in nArea at this magnitude. However, treatment with jasplakinolide did not significantly change nArea in monolayers stretched at 12% and 37% ∆SA ¼ Hz for 60 min and monolayers left unstretched when compared to vehicle control (VC). Thus, similar to P_i, monolayer
permeability was sensitive to stretch magnitude and, in monolayers stretched for 60 min at 25% ΔSA ¼ Hz, could be attenuated to unstretched levels with jasplakinolide.

Figure 2.9: Normalized area stained by fluorescent tracer BODIPY-ouabain (nArea), hypothesized to be a measure of AEC monolayer permeability, was plotted as a function of stretch magnitude and treatment. nArea was significantly higher than unstretched-untreated (UNS-UNT) and unstretched-vehicle control (VC) in monolayers stretched at 25% ΔSA (light grey) and 37% ΔSA (dark grey) ¼ Hz for 60 min but was not significantly different in monolayers stretched at 12% ΔSA ¼ Hz for 60 min (white). Treatment with 100 nM jasplakinolide for 10 min (dotted grey bars) attenuated the 25% ΔSA but not the 37% ΔSA stretch-induced increase in nArea compared to vehicle control. nArea in monolayers treated with 100 nM jasplakinolide for 10 min and stretched at 12% and 25% ΔSA ¼ Hz for 60 min or left unstretched were not significantly different compared to unstretched vehicle control. Data are means ± SE; #P < 0.01 compared with unstretch; *P < 0.05 compared with unstretch; **P < 0.01 vs. Stretch; ***P < 0.01 vs. Stretch. Each data point based on 3 images/well from 3 wells/animal from at least 3 animals/group. The area percentage of each image was found by dividing the number of BODIPY-ouabain stained pixels above threshold intensity (maximum pixel intensity of background in unstretched-untreated monolayers) by the total number of image pixels. Normalized area percentage (nArea) of each image was found by dividing area percentage by the average area percentage of the unstretched-untreated group.
Actin remodeling response depends on cell type

Primary rat type I like AEC monolayer behavior was compared to monolayers of a human alveolar epithelial adenocarcinoma A549 cell line (36). Qualitatively, A549 monolayers labeled with phalloidin for F-actin exhibit PJAR in both unstretched and held (sustained tonic) stretch of 37% $\Delta$SA for up to 40 min (Figure 2.10 inset). Thus, A549 cells display no stretch-induced actin remodeling. Similarly, A549 monolayers held stretched at 25% and 37% $\Delta$SA displayed no significant response in nMSD$_{100}$ (Figure 2.10). In unstretched A549 monolayers, tracking microbeads attached to A549 integrin receptors displays a significant decrease in nMSD$_{100}$ by 20 min and continuing for the duration of stretch when compared to the 0 min time point (Figure 2.10, also Figure 2.2). Untreated, unstretched primary AEC monolayers from the sustained tonic group displayed a similar decrease in nMSD$_{100}$ by 10 min lasting for the duration. However, unlike the progressive decrease in nMSD$_{100}$ in unstretched A549 cells, nMSD$_{100}$ in untreated unstretched primary AEC monolayers did not decline further.
Figure 2.10: Normalized MSD_{100} (nMSD_{100}) as a function of stretch time in A549 monolayers stretched 25% and 37% ΔSA held in sustained tonic (0 Hz) stretch or left unstretched. nMSD_{100} in stretched monolayers did not change significantly when compared to unstretched monolayers. Unstretched monolayers showed significantly lower nMSD_{100} at 20 min to 40 min. Data shown is mean ± SE from 5 monolayers per group with an average 125 beads analyzed per monolayer. Inset: A549 cells stained with phalloidin for F-actin, unstretched (UNS) or held at 37% ΔSA sustained tonic stretch for 40 min (compare to plot black data point). A549 cells exhibit PJAR with and without stretch. Bar = 10 µm.

**Microbead tracking specificity to actin cytoskeleton movement**

To confirm that spontaneous microbead movement was specific to the reorganization of the actin cytoskeleton, additional primary AEC monolayers were incubated with microbeads coated with acetylated low-density lipoprotein (AcLDL), a protein complex that has been shown to bind scavenger receptors but not focal adhesion complexes, as nonspecific control (52, 53, 75). As hypothesized, stretch of primary AEC monolayers with AcLDL coated microbeads showed no significant change in nMSD_{100} when compared to the unstretched time point as well as time-matched unstretched
controls (Figure 2.6 inset), which similarly showed no change over time compared to time = 0 min (not shown). As further evidence of actin-specific binding, latrunculin-A and jasplakinolide attenuated MSD$_{100}$ (previous section).

**DISCUSSION**

**Methodological limitations**

The measurement of microbead movement was restricted in three ways. First, the microbeads were attached only to the apical plasma membrane, and may have not have assessed actin reorganization deeper within the cell. However, type I like AEC monolayers are relatively flat (average thickness of 3.19 µm ± 0.16 SE). Furthermore, Hu et al. tracked microbead displacements as a result of stress fiber remodeling events that were up to 30 µm away from microbead center (30). Thus, the entire actin cytoskeleton of a rat type I like AEC is well within the spatial sensitivity of the microbead tracking method. Second, a single microbead was attached to many surface expressed receptors of a cell. Thus spontaneous microbead motion represented average movement of many receptors bound internally to the actin cytoskeleton. Because an ensemble of beads was tracked over a period of time, we speculate bead motion represented the summation of all actin remodeling (e.g. depolymerization, polymerization, and spatial redistribution) events in the monolayer during the 5 minute observation windows. Third, measurement was limited to the actin that was attached (via talin, (29)) to cell surface integrin receptors, which themselves adhere to the microbeads. Therefore, the microbead motion is an indirect measure of actin movement, and that movement may
be influenced further by stress mediated integrin-actin linkage reinforcement, previously shown in fibroblasts (14).

PJAR formation is rapid (< 10 min) and dependent on stretch magnitude and frequency

Consistent with our hypothesis, PJAR formed rapidly (< 10 min) and was sustained (up to 60 min) in rat type I like AEC monolayers at high magnitude (25% and 37% ΔSA) cyclic stretch, but not at lower stretch magnitudes (12% ΔSA). Others have demonstrated comparable rapid stress fiber formation in fibroblasts (51) and endothelial cells subjected to uniaxial stretch (31, 61, 74). Formation of PJAR was previously shown in biaxially stretched primary AEC monolayers (48) and endothelial cells (74), and postulated to be a cellular response that redistributes the actin cytoskeleton to areas of least cellular strain (62, 74). Consistent with this theory, we find that rapid formation of PJAR is synchronous with the increase in spontaneous microbead movement. Others report a similar rapid integrin-mediated actin movement to stretch in human airway smooth muscle (HASM) cells, where the cell immediately takes on a fluid like behavior (9, 35, 63). Also, Trepat et al. measured cell stiffness using optical twisting cytometry and molecular-scale structural rearrangement using the spontaneous movement of beads and found a decrease in cell stiffness and an acceleration of remodeling kinetics with transient stretch (63). In addition, Krishnan et al. measured cell traction stress using cell mapping rheometry and found a decrease in cell traction force following a biaxial stretch (35). Furthermore, we find the increase in spontaneous microbead movement was dependent on stretch magnitude and frequency. Krishnan et al. and Trepat et al. also showed a stretch magnitude-dependent cell response (35, 63).
With prolonged cyclic stretch, the initial spontaneous movement of microbeads was attenuated back to unstretched levels for the duration of stretch, and steady state MSD levels were unaffected by stretch magnitude or stretch frequency. This finding suggests the mechanism of actin remodeling into PJAR took place rapidly (< 1 min) during stretch, then ceased with sustained stretch, despite persistence of PJAR. Thus, after transient fluidization at stretch onset, the cell returns to its more solid like state with sustained stretch, a finding similar to that after stretch release of HASM cells (9, 35, 63). Once formed, PJAR structure may require only a baseline actin remodeling rate for maintenance of the new organization, a rate similar to the homogeneous actin structure found in unstretched monolayers. Further investigation is needed to elucidate if rapid formation of PJAR is due to active biochemical signaling cascades, passive mechanical forces, or both.

The magnitude dependence of PJAR intensity correlates with an increase in monolayer permeability at high magnitude stretch, and no change in monolayer permeability at low magnitude stretch, also consistent with Cavanaugh et al. (10, 11). These findings strengthen the hypothesis that the actin cytoskeleton is integral to tight junction barrier maintenance in primary AEC monolayers. Others have shown PJAR and TJ are intimately linked (41) and that modification of the actin cytoskeleton results in changes in TJ mediated paracellular permeability (12, 38, 40). We speculate that the reorganization of actin might result in a physical separation of actin and TJ protein, thus diminishing the cellular ability to mediate paracellular permeability.

We hypothesized that rapid actin cytoskeletal remodeling and a stretch-induced increase in monolayer permeability would be accompanied by a decrease in
perijunctional ZO-1 intensity. Tight junction proteins occludin and claudin are known to anchor internally to actin (39) via ZO-1 (25), ZO-2 via protein 4.1R (44), ZO-3 (81), and cingulin (17). Others have demonstrated actin cytoskeletal influence on the distribution, cytoskeletal adhesion, and barrier properties of the tight junction proteins claudin, occludin, and ZO (5, 16, 47, 57, 73). There is a paucity of data on the distribution of ZO-1 in stretched AEC cells. However, perijunctional ZO-1 intensity was found to be constant across all stretch groups. Similarly, Daugherty et al. inhibited endocytosis in human fetal lung (HFL) alveolar epithelial cells and showed a change in claudin-1, -3, -4, -5, and -7 but not in ZO-1, -2, or occludin intracellular immunofluorescent intensity (19). Additionally, Tsukamoto et al. depleted ATP in Madin-Darby Canine Kidney (MDCK) cells and found decreased transepithelial resistance (TER) and increased insoluble (cytoskeletal adherent) ZO-1 and -2 with no evidence of redistribution from the perijunction (67). We conclude that stretch has no effect on ZO-1 perijunctional intensity in rat type-I like AEC monolayers.

**Stretch induced PJAR formation can be inhibited with jasplakinolide and latrunculin-A**

Previously, we reported that treatment with 1 µM jasplakinolide reduced, but did not abolish, the stretch-induced increase in monolayer permeability in primary rat AEC monolayers stretched at 37% ∆SA (10). Here we show that we abolish formation of PJAR during stretch to 25% ∆SA and attenuate the stretch-induced increase in monolayer permeability by pretreating monolayers with 100 nM jasplakinolide, an actin-stabilizing agent that inhibits depolymerization. In addition, treatment with latrunculin-A effectively inhibited the movement of actin bound receptors in biaxially stretched primary AEC
monolayers. Similarly, Trepat et al. used 0.1 µM latrunculin-A in HASM cells and showed attenuation of stretch-induced decrease in cell stiffness (9, 63). Moreover Shen et al. used latrunculin-A to depolymerize actin in Madin-Darby canine kidney cells, finding a reduction in transepithelial resistance within 5 min, an internalization of tight junction protein occludin, and an elimination of PJAR within 20 min (57). Others have demonstrated the roles of protein kinase C (23), adenylate cyclase (59), rho and rac (82), rho-kinase (1, 33, 73), myosin light chain kinase (3, 28), and cofilin (46) on actin involvement in TJ structure and function (28, 69, 70, 73) in other stretched cell types. While our results show that PJAR can be modulated, further investigation will elucidate the specific upstream pathways responsible for the formation and functional consequences of PJAR in biaxially stretched primary AEC monolayers.

Actin remodeling response depends on cell type

Unlike primary cells, A549 cell monolayers exhibited PJAR in both unstretched and stretched cell monolayers stained for F-actin. Also, the spontaneous movement of microbeads in unstretched A549 monolayers was significantly lower by 20 to 40 min when compared to time = 0 min, with nMSD_{100} at 40 min significantly less than at 20 min. This progressive decrease in microbead movement in A549 cells, shown by others previously (64), is not found in unstretched untreated rat type I like AEC monolayers. The process of stiffening has been shown to exhibit a progressive decrease in microbead movement and exhibit similarities to physical aging (9), a phenomenon found in some glassy materials (55). In aging systems, molecular networks constantly advance to microconfigurations that are progressively more stable, but do so at a speed that is slower
than any exponential process (49). We conclude that the actin arrangement and response to stretch of A549 cells is significantly different from that in primary alveolar epithelial cells.

Summary

We have demonstrated that actin rearranges rapidly in primary AEC monolayers to form perijunctional actin ring during biaxial stretch, and that formation depends on stretch magnitude and frequency. We have shown mechanistically that perijunctional actin ring (PJAR) formation was synchronous with an increase in actin binding site movement, which was attenuated to baseline levels by 10 min. We have established that monolayer permeability increases with stretch in a magnitude dependent manner and can be modulated by cytoskeletal stabilization. These data reveal that high magnitude biaxial stretch within the physiologic range increases the fluidity of the actin cytoskeleton, which reorganizes to form perijunctional actin rings. Together with our current and previous studies demonstrating that similarly large stretch magnitudes and rates adversely affect monolayer permeability (10, 11, 15), we further speculate that rapid actin cytoskeleton reorganization has a deleterious effect on paracellular permeability. Future studies will investigate the effect of actin remodeling pathway inhibitors on retaining paracellular barrier properties during stretch, to explore opportunities to prevent ventilator induced lung injury.

Data published elsewhere (21) are used with permission from The Am Physiol Soc.
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CHAPTER 3: RHO KINASE SIGNALING PATHWAYS DURING STRETCH

ABSTRACT

Alveolar epithelial cells (AEC) maintain integrity of the blood-gas barrier with gasket-like intercellular tight junctions (TJ) which are anchored internally to the actin cytoskeleton. Stretched AECs undergo magnitude and frequency-dependent actin cytoskeleton remodeling to form perijunctional actin rings (PJARs). Based on published studies in human pulmonary artery endothelial cells (HPAEC), we hypothesize Rho kinase (ROCK) activity and phosphorylation of myosin light chain (MLC) increases in stretched AECs in a manner that is dependent on stretch magnitude, and that ROCK or MLC activity inhibition will attenuate stretch-induced actin remodeling, and will increase paracellular permeability. Primary AEC monolayers were stretched biaxially to create a change in surface area (ΔSA) of 12%, 25%, or 37% in a cyclic manner at ¼ Hz for up to 60 min, or left unstretched. Western analysis was used to determine the amount of active ROCK and phosphorylated MLC. An additional group of AEC monolayers were stretched 25% ΔSA ¼ Hz for 10 min and treated with Rho pathway inhibitors ML-7, Y-27632, or blebbistatin (all 10µM for 60 min) and stained for F-actin or treated with myosin phosphatase inhibitor calyculin-A (100 nM for 30 min) and quantified for monolayer permeability to the fluorescent tracer BODIPY-ouabain. Counter to our hypothesis, ROCK activity and MLC phosphorylation decreased in AEC monolayers stretched to 25% and 37% ΔSA, and did not change in monolayers stretched to 12% ΔSA. Verifying previous findings, we found that MLC phosphorylation in HPAEC monolayers increased with 12% ΔSA, but like AECs, it decreased with 37% ΔSA, confirming cell-
type specificity. Unstretched AEC monolayers treated with Rho pathway inhibitors Y-27632 and blebbistatin formed PJARs. Myosin phosphatase inhibition with calyculin-A increased MLC phosphorylation in stretched AEC monolayers but had no effect on monolayer permeability. In summary, stretch of primary rat AEC monolayers decreased ROCK activity and MLC phosphorylation in a manner dependent on stretch magnitude.

INTRODUCTION

Previously, rat type I like AEC monolayers in culture, used to mimic the alveolar epithelium in vitro (12, 19, 27, 52, 61), were shown to form perijunctional F-actin rings (PJARs) and increase monolayer permeability in response to high magnitude biaxial stretch, analogous to pathological ventilator volumes (17, 28). However, little is known about the underlying mechanistic pathways involved in PJAR formation and the influence of PJAR formation on monolayer permeability in type I AECs.

The actin cytoskeleton is a mechanosensitive structure that bears major cellular load and assists in cell-cell adhesion (Chapter 2 and (3, 14, 15, 21, 28)). Perijunctional F-actin ring (PJAR), also referred as the perijunctional actomyosin ring (80), a specific morphological conformation of F-actin formed rapidly in biaxially stretched rat alveolar epithelial cells (AECs, Chapter 2 and (28)) and endothelial cells (84) and shown to contain the contractile protein myosin in endothelial cells (7, 86). However, it is not yet understood if PJAR contracts in response to stretch mediated pathways, and if this contraction would provide enough force to rupture actin-TJ protein bonds and increase TJ mediated paracellular permeability found previously (16, 17) and in Chapter 2. Stretch-
induced actin remodeling into PJAR and the influence of PJAR formation on monolayer permeability is also poorly understood in type I AECs.

Myosin II has been shown to crosslink with filamentous actin directly and numerous actin-binding proteins (34, 44), contribute to PJAR structure (86), and change its conformation (32) and activity based on mechanical stretch pathways in other cell types (8, 68, 83). It is not yet understood how significantly myosin II interacts in PJAR contraction and TJ mediated paracellular permeability during stretch. PJAR contraction has been shown to have a causal relationship with Rho mediated phosphorylation of myosin light chain 2 (MLC2) in various cell types (11, 72). Non-muscle myosin II is activated through the Rho pathway and provides contractile force via phosphorylation of MLC (Figure 3.1) at the Thr18 and Ser19 residues (36, 38, 55, 67), with force generation greater when phosphorylated at both residues compared to monophosphorylation (56). MLC can be inhibited with phosphorylation at the Ser1 and Ser2 residues (36, 39, 55, 67), additionally resulting in reduced myosin affinity for actin filaments.

MLC can be pharmacologically inhibited with blebbistatin (89), an inhibitor of myosin II ATPase. The active form of Rho, guanosine triphosphate Rho (GTP-Rho), activates Rho kinase (ROCK) (41). ROCK phosphorylates the myosin binding subunit (MBS) of myosin phosphatase (MP), inhibiting its activity, thus increasing phosphorylation of MLC of myosin II (22, 46, 66, 76) and its contractile force (56). Additionally, ROCK directly phosphorylates MLC at the Thr18 and Ser19 residues (2), with a higher affinity for the Ser19 residue (38, 76), promoting actin filament assembly (20, 26). ROCK can be inhibited by Y-27632 (33, 63, 87) and has been shown to result in decreased MLC phosphorylation (5). MP can be inhibited with calyculin-A (23, 40)
and has been shown to result in increased MLC phosphorylation (5). MLCK has also
been found to phosphorylate MLC (43) at Thr18 and Ser19 (2). MLCK is activated by
eextracellular signal-regulated kinase (ERK) (47). MLCK can be inhibited by ML-7, a
membrane permeable agent (49). Finally, ROCK mediated MLC phosphorylation has
been shown to be confined to cell central areas while MLCK mediated MLC
phosphorylation has been shown to localize at cell peripheral locations in fibroblasts (76).
We hypothesize that type I AECs will activate the Rho pathway via activation of ROCK
during stretch. We hypothesize phosphorylation of MLC2 and the formation of PJAR.
We further hypothesize inhibition of these pathways will inhibit PJAR formation and
attenuate the stretch induced increase in monolayer permeability.

Others have shown activation of the Rho pathway and have characterized its
influence on the actin cytoskeleton (1, 8-10, 45, 48, 70), tight junction (TJ) protein, and
monolayer permeability properties in stretched endothelial cells and other cell types (25,
59, 62, 68, 81, 83). Furthermore, others have implicated the Rho pathway and its effect
on actin structure, TJ protein distribution, and TJ mediated paracellular permeability in
unstretched epithelial cells (13, 37, 54, 58, 69, 71, 81-83). However, there is a paucity of
studies investigating these pathways and relationships in stretched pulmonary alveolar
cells. We plan to investigate the MLCK, ROCK, and MLC pathways and their effect on
PJAR formation in type I AECs.
The goals of our study are to test whether stretch-induced PJAR formation are dependent on the Rho kinase (ROCK) and myosin light chain (MLC) mediated pathway. Our overall hypothesis (Figure 3.1) is that rapid cytoskeletal remodeling and formation of PJAR is activated by ROCK accompanied downstream by MLC activation. We will test functional relationships by inhibiting this pathway, and expect it will inhibit PJAR formation.

MATERIALS AND METHODS

Primary Rat Type I Like Alveolar Epithelial Cell Isolation

Alveolar type II cells were isolated from male Sprague-Dawley rats based on a method reported by Dobbs et al. (30) with slight modification (79). The animal protocols used in this study were reviewed and approved by the University of Pennsylvania IACUC. The rats (190-300 g) were anesthetized (sodium pentobarbital, 55 mg/kg ip), the trachea
cannulated, the lungs mechanically ventilated, and an abdominal aortotomy was performed to exsanguinate the rat while excess blood removed via pulmonary arterial perfusion. The lungs were excised and type II cells were isolated using an elastase digestion technique followed by mechanical mincing on a tissue chopper (Sorvall, Lorton, VA) in the presence of DNase (Sigma-Aldrich, St. Louis, MO).

Cells were filtered through progressively finer Nitex mesh (Crosswire Cloth, Bellmawr, NJ) and plated on an IgG-coated (3 mg / 5 ml Tris-HCl) culture dish. After a 90 min incubation at 37°C, cells were spun down and resuspended in Minimal Essential Medium (MEM, Invitrogen, Carlsbad, CA) with Earle’s salts supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 25 µg/ml Gentamicin (Invitrogen), and 0.25 µg/ml Amphotericin B (Life Technologies). Cells were seeded at 1.0 million cells/cm² onto fibronectin coated (10 µg/cm², Invitrogen) flexible Silastic membranes (Specialty Manufacturing, Saginaw, MI) in custom-designed wells (78).

The cells were cultured for 4 days at 37°C, 5% CO₂ in MEM with 10% FBS, Gentamicin, and Amphotericin B, replaced daily. After 4 days, the cells had adopted alveolar type I (ATI) features (12, 19, 24, 27, 61, 64) including the expression of RTI40, and had grown to a confluent monolayer (Appendix A). Monolayers were then serum-deprived in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech Inc., Manassas, VA) supplemented with 20 mM HEPES (Sigma) for 3 hrs and stretched biaxially across a range of physiological relevant magnitudes including at 12%, 25%, or 37% change in surface area (ΔSA) roughly corresponding to 64%, 86%, and 100% total lung capacity, respectively (78).
**HPAEC Culture**

Human pulmonary artery endothelial cells (HPAECS, passage 8 - 10, provided by Dr. Christopher Chen, University of Pennsylvania, PA) were seeded at $0.163 \times 10^6$ cells/cm$^2$ onto collagen-I coated (5 µg/cm$^2$ overnight, BD Biosciences, San Jose, CA) flexible Silastic membranes (Specialty Manufacturing, Saginaw, MI) in custom-designed wells (78) similar to culture in (8). The cells were cultured for 48 hrs at 37°C, 5% CO$_2$ in epithelial basal medium (EBM-2, Lonza, Basel, Switzerland) supplemented with epithelial growth media (hydrocortisone, hEGF, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin, gentamicin/amphotericin-B; EGM-2, Lonza), 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% 1:1 penicillin:streptomycin.

**Measurement of MLC Phosphorylation with Western Blot**

Cells were lysed and protein content quantified similar to previous (5, 8, 63). Primary rat AEC monolayers were serum deprived in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Manassas, VA) supplemented with 20 mM HEPES for 3 hrs. Monolayers were stretched biaxially at 12%, 25%, or 37% $\Delta$SA cyclically at $\frac{1}{4}$ Hz for 0 (unstretched), 1, 10, 30, or 60 min. An additional group of monolayers were pretreated with the myosin phosphatase (MP) inhibitor calyculin-A (Cell Signaling Technology, Beverly, MA) at 100 nM for 30 min or with DMSO as vehicle control then stretched biaxially at 25% $\Delta$SA cyclically at $\frac{1}{4}$ Hz for 0 (unstretched) or 10 min. Finally, an additional group of HPAEC monolayers (with serum) were stretched at 12%, 25%, or 37% $\Delta$SA cyclically at $\frac{1}{4}$ Hz for 0 (unstretched) or 30 min.
Monolayers (3 wells/lysate) were washed with ice-cold Dulbecco's PBS twice and scraped in sodium dodecyl sulfate (SDS) sample reducing buffer (30 mM Tris-HCl, 1.25% SDS, 12.5% β-mercaptoethanol, 10% glycerol) containing 50 mM sodium fluoride, 1.0 mM phenylmethanesulfonylfluoride (PMSF), 5 mM ethylenediaminetetraacetic acid (EDTA), 2 mM sodium orthovanadate, and protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN), sonicated 5 s then boiled for 5 min. Sample concentrations were quantified using a reducing agent and detergent compatible (RC DC) protein assay (Bio-Rad) and loaded equivalently (25 µg). Protein lysates was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, NuPAGE Novex 4-12% Bis-Tris Mini Gel, Invitrogen, Carlsbad, CA), transferred to 0.2 µm pore polyvinylidene difluoride membranes (30 V for 90 min, Bio-Rad, Hercules, CA), blocked in bovine serum albumin (BSA) to minimize background, then incubated 4°C overnight with one of the following primary antibodies: anti-myosin light chain 2, anti-phospho-myosin light chain 2 (Ser19), anti-phospho-myosin light chain 2 (Thr18/Ser19) (all Cell Signaling Technology, Boston, MA), anti-phospho-myosin light chain (Ser1) (ECM Biosciences, Versailles, KY). Membranes were washed thrice in TBS/T (tris-buffered saline with 0.1% tween 20, Bio-Rad), incubated at room temperature for 90 min with horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), then rinsed six times with TBS/T.

Immunoreactive proteins were detected with enhanced chemiluminescence detection system (ECL Plus, GE Healthcare Biosciences, Piscataway, NJ). Developed film (Hyperfilm ECL, GE Healthcare) was digitized and the relative density of the protein in the bands were quantified by scanning densitometry in ImageJ (ver. 1.43j).
based on method optimization in Gassmann et al. (35). Background membrane intensity for each lane was subtracted from blot intensity. The average of three measurements was used for band density. For each band, normalized (n) protein density was found by dividing the density of the band by the unstretched-untreated (UNS-UNT) band density. Membranes were stripped (Restore, Thermo Scientific, Logan, UT) for 20 min at room temperature and reprobed with the relevant total protein antibody or with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Millipore, Billerica, MA) to ensure equivalent total protein loading across lanes (29).

Normalized density of the phosphorylated protein was divided by normalized density of the respective total protein or GAPDH signal and reported. To test the effect of stretch, animal average normalized density values (at least 3 animals/group) were compared with time-matched unstretched controls using a two-way ANOVA with post-hoc Dunnett's test (88) in JMP (ver. 8.0, SAS Institute Inc., Cary, NC). To test the effect of treatment, animal average normalized density values (at least 3 animals/group) were compared with time-matched vehicle controls as well as unstretched-untreated controls using a two-way ANOVA with Tukey-Kramer post-hoc analysis (88) in JMP.

*Rho kinase Activity Assay*

Endogenous ROCK activity was quantified using an *in vitro* kinase assay (Cell Biolabs, San Diego, CA) with recombinant myosin phosphatase target subunit 1 (rMYPT1) as substrate, similar to (6, 51). Primary rat AEC monolayers were serum deprived in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Manassas, VA)
supplemented with 20 mM HEPES for 3 hrs. Monolayers were stretched biaxially at 12%, 25%, or 37% ΔSA cyclically at ¼ Hz for 0 (unstretched), 10, or 60 min.

Monolayers (3 wells/lysate) were washed with ice-cold Dulbecco's PBS (DPBS) twice and scraped in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% SDS, 1.0%, IGEPAL CA-630, pH 7.4) with 5 mM EDTA, 2 µg/ml Pepstatin, 2 mM sodium fluoride, 2 mM sodium orthovanadate, and protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN), then sonicated 5 s. Sample concentrations were quantified using a protein assay (Bio-Rad) and loaded into the kinase assay equivalently. Lysate was kept on ice unless otherwise noted. Samples were mixed with 0.5 µg rMYPT1, 200 µM ATP, and 1 mM DTT for 30 min at 30°C. The kinase reaction was stopped with ice cold SDS-PAGE sample buffer (30 mM Tris-HCl, 1.25% SDS, 10% glycerol, 0.4% Bromophenol Blue), vortexed, then boiled for 5 min and resolved by SDS-PAGE and immunoblot (see Western blot protocol and densitometry protocol details above).

Membranes were probed for anti-phospho-MYPT1 (Thr696) to determine sample rMYPT1 phosphorylation by ROCK (ROCK activity) and for GAPDH to normalize signal to sample total protein (29). For each band, normalized (n) protein density was found by dividing the density of the band by the unstretched-untreated (UNS-UNT) band density. Normalized density of phosphorylated-MYPT1 was divided by normalized density of the GAPDH signal and reported.
MLC Immunofluorescence and F-Actin Staining

Primary rat AEC monolayers were serum deprived in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Manassas, VA) supplemented with 20 mM HEPES for 3 hrs. Monolayers were pretreated with the myosin II ATPase activity inhibitor blebbistatin, MLCK inhibitor ML-7, ROCK inhibitor Y-27632 (all 10 µM for 60 min, EMD Chemicals, Gibbstown, NJ), or with DMSO as vehicle control then stretched biaxially at 25% ∆SA cyclically at ¼ Hz for 0 (unstretched) or 10 min and stained for F-actin (phalloidin, Invitrogen, Carlsbad, CA). An additional group of monolayers were stretched at 25% ∆SA ¼ Hz for 0, 1, 10, and 60 min and immunostained for MLC using anti-myosin light chain 2 or anti-phospho-myosin light chain 2 (Thr18/Ser19) (Cell Signaling Technology, Boston, MA) as follows.

Following stretch, monolayers were fixed with 1.5% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in DPBS for 15 min, permeabilized using 0.1% Triton X-100 in DPBS for 5 min, and blocked with 5% goat serum in DPBS for 60 min. Monolayers were incubated with primary antibody in blocking buffer at 4°C overnight, washed thrice for 5 min with DPBS, then incubated with fluorescent-conjugated secondary antibody or phalloidin for 90 min at 23°C. Monolayers were washed thrice for 10 min and images were captured (Eclipse TE300 epifluorescence microscope, ×40 objective, Nikon, Melville, NY).

Monolayer Permeability

Paracellular permeability was assessed by monitoring the flux of the fluorescent tracer BODIPY-ouabain across the monolayer as previously described (17). Primary rat
AEC monolayers were serum deprived in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Manassas, VA) supplemented with 20 mM HEPES for 3 hrs. The apical surface of the monolayer was then bathed in DMEM + HEPES containing 2 µM BODIPY-ouabain (radius ~20 Å, Invitrogen, Carlsbad, CA), a fluorescent tracer which has a high affinity for the extracellular domain of the transmembrane Na\(^{+}\)-K\(^{+}\)-ATPase (42, 75) found on the basolateral surface of type I AECs (85), for 60 min during stretch (or 60 min on unstretched monolayers). Monolayers were pretreated with the myosin phosphatase (MP) inhibitor calyculin-A (Cell Signaling Technology, Beverly, MA) at 100 nM for 30 min or with DMSO as vehicle control (VC) then stretched biaxially at 25% ∆SA cyclically at ¼ Hz for 0 (unstretched), 10, or 60 min. Following stretch, the apical surface was rinsed three times with dye-free DMEM + HEPES and imaged (Eclipse TE300 epifluorescence microscope, ×10 objective, Nikon, Melville, NY) using constant exposure, aperture, and fluorescent intensity settings.

The maximum pixel intensity in the background of unstretched-untreated monolayers was measured and used as a threshold to exclude unstained regions. The area percentage of each image (3 images/well, 3 wells/animal, from at least 3 animals/group) was found using a MATLAB (version 6.5 R13, The MathWorks, Natick, MA) program (Appendix F) which calculates the area of the image above threshold intensity and divides it by the total image area. Normalized area percentage (nArea) of each image was found by dividing the super-threshold area for a condition by the average super-threshold area of the unstretched-VC group. To test the effect of stretch, animal average nArea values were compared with time-matched unstretched controls using a one-way ANOVA with post-hoc Dunnett's test (88) in JMP (ver. 8.0, SAS Institute Inc., Cary, NC).
To test the effect of treatment, animal average nArea values were compared with time-matched vehicle controls as well as unstretched-untreated controls using a two-way ANOVA with Tukey-Kramer post-hoc analysis (88) in JMP. Previously, Cavanaugh et al. showed that the BODIPY-ouabain tracer was not internalized by cells, did not undergo receptor-mediated endocytosis, did not bind due to plasma membrane rupture, and did not bind nonspecifically (17).

RESULTS

ROCK activity is reduced with stretch and is dependent on stretch magnitude

We hypothesized that actin cytoskeleton remodeling during formation of perijunctional F-actin rings (PJARs) would be preceded or accompanied by an increase in Rho kinase (ROCK) activity. Quantitative kinase activity assay data refuted our hypothesis, showing a decrease in ROCK activity at 60 min of 25% \( \Delta \text{SA} \) Hz (light grey) stretch and 10 min of 37% \( \Delta \text{SA} \) Hz (dark grey) stretch, but no change in monolayers stretched to 12% \( \Delta \text{SA} \) Hz (white), compared to unstretched (UNS) monolayers (Figure 3.2). Thus ROCK activity decreased with stretch and was sensitive to stretch magnitude and time.
Figure 3.2: Rho kinase (ROCK) activity is dependent on stretch magnitude and time. ROCK activity was down in monolayers at 60 min of 25% $\Delta \text{SA} \frac{1}{4}$ Hz stretch and 10 min of 37% $\Delta \text{SA} \frac{1}{4}$ Hz stretch but not at any time in 12% $\Delta \text{SA} \frac{1}{4}$ Hz stretch when compared to unstretched (UNS) monolayers. ROCK activity was quantified using an in vitro kinase binding assay with recombinant myosin phosphatase target subunit 1 (rMYPT1) as the substrate. Normalized (n, to UNS monolayers) phosphorylated-MYPT1 (Thr696) signal divided by n-total protein content (GAPDH) signal was used as a measure of ROCK activity. Typical pMYPT1 western blot signal shown below specific groups. Data is mean ± SE from at least 3 animals/group with ## $P < 0.01$ vs. UNS.

Table 3.1: Summary of one and two-way ANOVA results showing the effects of stretch magnitude, stretch duration, and the interaction of stretch magnitude and duration on ROCK activity and protein phosphorylation readouts. X = P < 0.05, - = no effect, and n/a = not applicable.
MLC phosphorylation is reduced with stretch and is dependent on stretch magnitude

Myosin II has been shown to directly interact with F-actin stress fibers in other cell types (86). We hypothesized that myosin light chain would co-localize with perijunctional F-actin rings (PJARs), which have been shown to form rapidly in stretched AEC monolayers (Chapter 2 and (28)). Phosphorylated-MLC (Thr18/Ser19, ppMLC, middle/yellow) and total-MLC (tMLC, bottom/cyan) co-localize with centrally located F-actin stress fibers (red/top) in unstretched (UNS) monolayers (left, Figure 3.3). With 1 min stretch to 25% ∆SA ¼ Hz, ppMLC redistributed to the perijunction and remained for up to 60 min, similar to F-actin stretch-induced PJAR formation. Total-MLC in stretched monolayers remained qualitatively similar to UNS monolayers until 60 min at which time it resembles F-actin and ppMLC, localized at the perijunction. Thus, MLC colocalizes with rapidly formed PJARs in stretched AEC monolayers.
Figure 3.3: Phosphorylated myosin light chain (MLC) co-localizes with perijunctional F-actin rings (PJARs) in stretched monolayers. Type I-like rat AEC monolayers before (unstretched, UNS, left) and after 1, 10, and 60 min of 25% $\Delta$SA ¼ Hz stretch stained for F-actin with phalloidin (top/red), dual-phosphorylated (Thr18/Ser19) MLC (ppMLC, middle/yellow) or total MLC (tMLC, bottom/cyan). In UNS monolayers, ppMLC and tMLC was located in central regions of the cell, similar to F-actin. With stretch, ppMLC localized to the perijunction within 1 min, similar to F-actin and the formation of PJARs with stretch. Even with stretch tMLC remains homogeneous except at 60 min of stretch where it predominates the perijunction. Monolayers stretch to 37% $\Delta$SA ¼ Hz for the same durations respond similarly (not shown). Bar = 10 μm.

Myosin light chain (MLC) can be activated when phosphorylated at the Thr18 and Ser19 residues (38). Phosphorylation of myosin light chain (MLC) data corroborates well with ROCK activity data, showing a decrease in phosphorylation with a stretch magnitude dependency. Monolayers stretched to 25% $\Delta$SA ¼ Hz decreased MLC dual-phosphorylation (Thr18/Ser19) at 1, 10, 30, and 60 min (light grey, Figure 3.4) and mono-phosphorylation (Ser19) at 10 min (right, Figure 3.5) compared to unstretched
(UNS) monolayers. Similarly, monolayers stretched to 37% $\Delta$SA $\frac{1}{4}$ Hz had decreased MLC dual-phosphorylation at 1, 10, 30, and 60 min (dark grey, Figure 3.4) compared to UNS monolayers. Dual-phosphorylated MLC in monolayers stretched to 25% and 37% $\Delta$SA $\frac{1}{4}$ Hz was also lower than in monolayers stretched to 12% $\Delta$SA $\frac{1}{4}$ Hz (white, Figure 3.4) at all times, which itself was not significantly different from UNS monolayers. Thus MLC phosphorylation (Thr18/Ser19 and Ser19) decreased with stretch and was sensitive to stretch magnitude.

Figure 3.4: Myosin light chain (MLC) phosphorylation (Thr18/Ser19) is stretch magnitude dependent. Dual phosphorylation of MLC is reduced at all times in monolayers stretched to 25% and 37% $\Delta$SA $\frac{1}{4}$ Hz when compared to unstretched (UNS) and monolayers stretched to 12% $\Delta$SA $\frac{1}{4}$ Hz, which itself was not significantly different from UNS. Normalized (n, to UNS monolayers) phosphorylated-MLC (Thr18/Ser19) divided by n-total-MLC was reported. Typical ppMLC western blot signal at 10 min of each stretch magnitude shown below group. Data is mean ± SE from at least 3 animals/group with # $P < 0.05$ vs. UNS, ## $P < 0.01$ vs. UNS, *$P < 0.05$ vs. 12%, **$P < 0.01$ vs. 12%.
Myosin light chain (MLC) activity can be inhibited by phosphorylation at the Ser1 residue (39, 67), additionally resulting in reduced myosin affinity for actin filaments. Monolayers stretched for 10 min at 25% ΔSA ¼ Hz showed no change in phosphorylated (Ser1) MLC compared with unstretched (UNS) monolayers (left, Figure 3.5), corroborating with data showing MLC and F-actin colocalization during stretch. Thus stretch did not effect MLC phosphorylation at Ser1 in AEC monolayers.

Figure 3.5: Myosin light chain (MLC) phosphorylation at residues Ser1 (left) and Ser19 (right). Phosphorylation of the Ser1 residue does not change in monolayers stretched to 25% ΔSA ¼ Hz for 10 min when compared to unstretched (UNS) monolayers (left). Phosphorylation of the Ser19 residue is reduced in monolayers stretched to 25% ΔSA ¼ Hz for 10 min when compared to UNS monolayers (right). Normalized (n, to UNS monolayers) phosphorylated-MLC divided by n-total-MLC was reported. Typical pMLC western blot signal shown below group. Data is mean ± SE from at least 6 animals/group with # P < 0.05 vs. UNS.
MLC is phosphorylated with calyculin-A

Hypothesizing that stretch-induced PJAR formation and increased monolayer permeability required decreased MLC activation, we used calyculin-A to inhibit myosin phosphatase (MP) in stretched monolayers in order to recover MLC phosphorylation to UNS levels. MLC phosphorylation (Thr18/Ser19) was higher in monolayers pretreated with 100 nM calyculin-A for 30 min and stretched to 25% ΔSA ¼ Hz for 10 min (crosshatch, Figure 3.6) when compared to both vehicle control (VC) monolayers stretched to the same magnitude and time (dark grey, Figure 3.6) and monolayers left unstretched (UNS) untreated. Thus, calyculin-A increases phosphorylation of MLC in stretched AEC monolayers.

Figure 3.6: Myosin light chain (MLC) phosphorylation (Thr18/Ser19) can be activated with calyculin-A. Vehicle control (VC) monolayers stretched at 25% ΔSA ¼ Hz for 10 min show a reduction in phosphorylation (Thr18/Ser19) when compared to unstretched (UNS) VC monolayers (shown previously). Monolayers pretreated for 30 min with myosin phosphatase inhibitor calyculin-A (100 nM) then stretched at 25% ΔSA ¼ Hz for 10 min show increased MLC phosphorylation (Thr18/Ser19) when compared to both stretched VC and UNS-VC monolayers. Normalized (n, to UNS monolayers) phosphorylated-MLC divided by n-total-MLC was reported. Typical ppMLC western blot signal shown at right. Data is mean ± SE from at least 3 animals/group with # P < 0.05 vs. UNS.
Table 3.2: Summary of one-way ANOVA result showing the effect of calyculin-A treatment on MLC phosphorylation readout. X = P < 0.05, * = no effect, and n/a = not applicable.

<table>
<thead>
<tr>
<th>ANOVA</th>
<th>effect: READOUT</th>
<th>stretch mag</th>
<th>treatment CALY-A</th>
<th>interaction (mag x time)</th>
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<tr>
<td>X</td>
<td>MLC (Thr18, Ser19)</td>
<td>n/a</td>
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Increasing MLC phosphorylation above unstretched (UNS) levels in stretched monolayers using calyculin-A did not, however, attenuate the stretch-induced increase in paracellular permeability. Previously, normalized area stained by fluorescent tracer BODIPY-ouabain (nArea), hypothesized to be a measure of AEC monolayer permeability, was significantly higher in monolayers stretched to 25% ∆SA ¼ Hz for 10 and 60 min compared to unstretched untreated controls (Chapter 2). Likewise, nArea in monolayers stretched at the same magnitude for the same durations pretreated with 100 nM calyculin-A for 30 min was higher at both 10 and 60 min compared to UNS untreated monolayers and is no different when compared to time-matched vehicle controls (not shown). Thus recovering phosphorylation of MLC in stretched monolayers above UNS levels does not attenuate the stretch-induced increase in paracellular permeability.

PJAR is formed in unstretched monolayers with Y-27632 and blebbistatin

We initially hypothesized that rapid actin cytoskeleton remodeling to form PJARs would be preceded or accompanied by an increase in ROCK and MLC activity in stretched AEC monolayers. However, our data showed ROCK activity and MLC phosphorylation decreased with stretch. Quantitative ROCK activity and MLC phosphorylation data corroborate well with qualitative F-actin remodeling data, showing no effect on PJAR formation with further myosin II ATPase (blebbistatin) or ROCK
inhibition (Y-27632), or inhibition of MLCK (ML-7) in stretched monolayers (bottom row, Figure 3.7). Interestingly however, inhibition of myosin II ATPase (blebbistatin) or ROCK inhibition (Y-27632) but not MLCK inhibition (ML-7) in unstretched (UNS) monolayers produced F-actin cytoskeleton remodeling to form PJARs (top row, Figure 3.7), similar to PJAR formation found in stretched untreated monolayers (bottom left). Thus stretch-induced PJAR formation was not suppressed with inhibition of myosin II ATPase, ROCK, or MLCK. Furthermore, PJARs were formed in UNS monolayers with inhibition of myosin II ATPase or ROCK.

Figure 3.7: Effect of biaxial stretch and pathway inhibitors on F-actin. Type I like rat AEC monolayers with phalloidin labeled F-actin left unstretched (UNS, top row) or after 10 min of 25% ∆SA ¼ Hz stretch (bottom row) without treatment (UNT, left), myosin II ATPase activity inhibitor blebbistatin (column 2), MLCK inhibitor ML-7 (column 3), or ROCK inhibitor Y-27632 (right) pretreatment (all inhibitors 10 µM for 60 min). Perijunctional F-actin rings (PJARs) form in UNS monolayers treated with myosin II ATPase inhibitor and ROCK inhibitor. Stretch-induced PJAR formation (shown previously in Chapter 2) is not inhibited with any treatment. Bar = 10 µm.
MLC phosphorylation depends on cell type

Primary rat type I-like AEC monolayer behavior was compared with monolayers of human pulmonary artery endothelial cells (HPAECs). Myosin light chain (MLC) phosphorylation (Thr18/Ser19) increased in HPAEC monolayers stretched biaxially at 12% ΔSA ¼ Hz for 30 min (white), decreased in monolayers stretched at 37% ΔSA ¼ Hz for 30 min (dark grey), but did not change in monolayers stretched at 25% ΔSA ¼ Hz for 30 min (grey) when compared to unstretched (UNS) HPAEC monolayers (Figure 3.8). Phosphorylation in monolayers stretched to 12% ΔSA was greater than in both 25% and 37% ΔSA stretched monolayers. Thus MLC phosphorylation behaved differently in stretched HPAECs when compared to stretched rat type I-like AECs.

Figure 3.8: Myosin light chain (MLC) phosphorylation (Thr18/Ser19) in human pulmonary artery endothelial cells (HPAECs) is stretch magnitude dependent. Dual phosphorylation of MLC is increased in monolayers stretched biaxially at 12% ΔSA ¼ Hz for 30 min, decreased in monolayers stretched at 37% ΔSA ¼ Hz for 30 min, but did not change in monolayers stretched at 25% ΔSA ¼ Hz for 30 min when compared to unstretched (UNS) monolayers. Phosphorylation in monolayers stretched to 12% ΔSA was greater than in both 25% and 37% ΔSA stretched monolayers. Normalized (n, to UNS monolayers) phosphorylated-MLC (Thr18/Ser19) divided by n-total-MLC was reported. Typical ppMLC western blot signal shown below group. Data is mean ± SE from at least 3 animals/group with ## P < 0.01 vs. UNS and **P < 0.01 vs. 12%.
DISCUSSION

Results summary

In summary we find ROCK activity and MLC phosphorylation decrease with high magnitude stretch of primary AEC monolayers. This inactivation is stretch-magnitude dependent, similar to MLC phosphorylation in stretched HPAEC monolayers. We have shown that rescuing MLC phosphorylation during stretch has no effect on monolayer permeability. Finally, unstretched monolayers with ROCK and MLC activity inhibited show PJAR formation.

Methodological limitations

Western blot, used to quantify the amount of phosphorylated and total protein concentrations, is limited in a few ways. Quantification is limited by antibody availability, known phosphorylation sites on the target protein, and previous data on functionality of phosphorylation sites. Furthermore, we were only able to quantify total protein lysate from whole monolayers. This represents the ensemble of chemical signaling of a few million cells and thus lacks local, cell to cell signaling. Additionally, cells can employ spatial compartmentalization as well as subcellular binding to direct biochemical signaling. Subcellular western blot data would further our understanding of the phosphorylation state of MLC at the perijunction as well as elucidate the mechanistic link between ROCK and MLC in stretched AEC monolayers.
ROCK activity is reduced with stretch and is dependent on stretch magnitude

Previous studies have investigated the ROCK/MLC pathway during stretch in other cell types. Human pulmonary artery endothelial cells (HPAEC) cells exposed to 18% elongation at 30 cycles per minute show rapid (<10 min) increase in MLC phosphorylation, activation of p38 and ERK ½ mitogen-activated protein (MAP) kinases, and remodeling of actin to form PJAR (8). Cyclic 18% elongation at 25 cycles per minute shows Rho activation and peripheral localization as well as actin stress fiber formation perpendicular to distention vector by 20 min in a separate preparation (70). Perpendicular stress fiber formation has also been illustrated in bovine aortic endothelial cells stretched at 10% elongation at 1 Hz (45).

When ROCK is inhibited in these stretched cells via Y-27632, actin stress fibers were found to orient parallel to the stretch vector (45). Injection of RhoA has been shown to induce the assembly of stress fibers and focal contacts (4, 65). Albinsson was able to show that cofilin-2 is phosphorylated in vascular wall cells that are stretched and that this could be inhibited by inhibiting ROCK with Y-27632 (1). Cyclic elongation of endothelial cells was found to phosphorylate MLC by 10 min via ROCK and MLCK (8, 10, 48). Phosphorylation of MLC was found to rapidly alter actin arrangement by constriction of PJAR, increase detergent soluble (independent of actin) TJ protein content (59, 81, 83), increase TJ permeability (62), and redistribute ZO-1 and occludin (68). Equibiaxial cyclic stretch in vascular endothelial cells shows increased occludin and ZO-1 protein expression (25). Finally, we have shown previously that 37% ΔSA cyclic biaxial stretch produces a decrease in peripheral occludin band intensity (18). Numerous
studies establish the link between stretch and MLCK, ROCK, and MLC activation, PJAR formation, and monolayer permeability in endothelial cells.

The ROCK/MLC pathway has also been shown to impart major influence on TJ protein coupling and monolayer permeability in Madin-Darby canine kidney (MDCK) cells, Caco-2 cells, and T84 cell lines. Bruewer et al. used dominant-negative (DN) and constitutively active (CA) forms of GTPases RhoA, Rac1, and Cdc42 in MDCK cells to show their effect on TJ protein location and actin structure. Briefly, CA RhoA redistributed occludin, ZO-1, claudin-1, claudin-2, and JAM-1 and intensified perijunctional actin ring (PJAR) while claudin-1 and claudin-2 redistributed with CA Rac1 and DN Rac1. Furthermore, DN RhoA and CA Rac1 abolished the PJAR. Finally, all CA GTPases increased detergent solubility of claudin-1 and claudin-2 (13) indicating a loosening of the bonds between these TJ proteins and actin. Hecht et al. investigated MDCK cells expressing myosin light chain kinase (MLCK) lacking inhibitory domain (thus continuously active). Cells expressing the construct displayed a transepithelial resistance (TER) that was less than 10% that of the wild type in addition to a threefold increase in MLCK induced myosin light chain (MLC) phosphorylation (37). Shen et al. used latrunculin-A (LatA) to depolymerize actin in MDCK cells, finding a reduction in TER within 5 min, an internalization of occludin, and an abolishment of PJAR within 20 min (69). Turner et al. used Caco-2 cells to investigate MLCK and MLC influence on TER. Inactivation of Na⁺-glucose co-transporter (SGLT1) showed a 24% increase in TER and was reversible by MLCK inhibition. SGLT1 activation showed 2.08 fold increase in MLC phosphorylation and was reversible by MLCK inhibition via ML-9 (82).
Activation of protein kinase C (PKC) increased TER within 15 min with concurrent increase in phosphorylation of MLCK (up 64% at 15 min and 51% at 2 hrs) and decrease in phosphorylation of MLC (down 12% at 15 min and 41% at 2 hrs) (81), providing supporting evidence that PJAR relaxation via MLC influences TJ permeability. Nusrat et al. used a polarized human intestinal cell line T84 exposed to the DC3B toxin to induce ADP-ribosylation of RhoA. The toxin induced three synchronic changes including the disassembly of filamentous actin (F-actin) at the cell apex and PJAR, an increase in TJ permeability to 10 kDa dextran, and ZO-1 internalization from the membrane to the cytoplasm (58). Walsh et al. also used the T84 line to test the effect of ROCK inhibition via Y-27632 and found a decrease in PJAR intensity, a decrease in TER, and a co-localization of a subpool of ROCK and ZO-1 (83). Finally, Simonovic et al. used calyculin-A to inhibit myosin phosphatase (MP) in infected intestinal epithelial cells and found the inhibitor prevented infection-induced occludin relocalization and resistance reduction (71). In summary, these studies show parallel changes and associations between PJAR morphology, TJ protein distribution and permeability, modulated by Rho, MLCK, ROCK, and MLC in cell lines. Previously, we reported stretch magnitude and frequency dependent PJAR formation (28) and a stretch-induced increase in paracellular permeability (17) in alveolar epithelial cell (AEC) monolayers. We hypothesized that ROCK was activated with stretch and that the stretch-induced increase in monolayer permeability could be attenuated with pharmacological inhibition of ROCK. Counter to our hypothesis, we found that ROCK activity is decreased with stretch of AEC monolayers in a stretch-magnitude dependent manner.
**MLC phosphorylation is reduced with stretch and is dependent on stretch magnitude**

Myosin light chain (MLC) phosphorylation data corroborated well with ROCK activity data, showing a stretch magnitude depended decrease in MLC phosphorylation (Thr18/Ser19) with high magnitude stretch (25% and 37% ∆SA) but no change at low magnitude stretch (12% ∆SA). MLC mono-phosphorylation at the Ser19 residue, a site shown to have higher affinity for ROCK mediated phosphorylation (38, 76), likewise decreased phosphorylation in stretched monolayers. Similarly Mizutani et al. demonstrated a decrease in MLC phosphorylation (Thr18/Ser19) in NIH-3T3 cells stretched for 1 - 2 hrs uniaxially at 10% (57). Thus, MLC phosphorylation in AEC monolayers stretched to high magnitudes decreased and we speculate this results in reduced MLC activity and contractile force. Additionally, MLC was found to colocalize with F-actin in stretched monolayers. Others have shown MLC colocalization with F-actin in other cell types (7, 86) and we speculate that MLC anchoring to the actin cytoskeleton is necessary for force generation. Together with previous data showing a rapid increase in actin rearrangement during stretch (28) and MLC colocalization with stretch-induced PJARs, we speculate that monolayer tension may diminish in stretched AEC monolayers, previously demonstrated in other stretched cell types (15, 50, 77).

**MLC is phosphorylated with calyculin-A**

To test the effect of increased MLC activity in stretched AEC monolayers, we used calyculin-A, an inhibitor of myosin phosphatase. As hypothesized, calyculin-A increased MLC phosphorylation in stretched monolayers. Similarly, Bhadriraju et al. used calyculin-A in A10 rat aortic smooth muscle cells to increase MLC phosphorylation
and contractility (5). Inhibition of the stretch-induced decrease in MLC phosphorylation with calyculin-A, however, did not attenuate the stretch-induced increase in monolayer permeability. Thus, restoring cellular tension to above unstretched levels with calyculin-A is not beneficial to monolayer barrier maintenance in stretched AEC monolayers.

**PJAR is formed in unstretched monolayers with Y-27632 and blebbistatin**

Stretch induced PJAR formation was unaffected by MLCK inhibition with ML-7, ROCK inhibition with Y-27632, and MLC activity inhibition by blebbistatin. Interestingly, PJARs were formed in unstretched monolayers treated with the ROCK inhibitor and the MLC activity inhibitor, but not with the MLCK inhibitor. Previously, Bhadriraju et al. demonstrated a reduced ROCK activity and MLC phosphorylation, which could not be rescued by constitutively active RhoA, in primary bovine pulmonary artery endothelial cells with diminished actin-mediated extracellular matrix (ECM) adhesion (6). Additionally, cells treated with blebbistatin to decrease cellular tension showed decreased focal adhesions as well as reduced ROCK and MLC activity by 20 min (6). Others have shown that injection of RhoA induces assembly of stress fibers and focal contacts (4, 65). Furthermore, endothelial cells treated with sphingosine 1-phosphate (Sph-1-P) exhibit a thick cortical actin ring and enhanced barrier and adhesion properties (74).

Taken together with our data showing decreased ROCK and MLC activity with stretch, these previous studies lead us to speculate that AEC monolayer adhesion to the ECM is reduced with high magnitude biaxial stretch. Previously we reported the formation of PJARs with high magnitude stretch (28). Coupled with our data showing
PJAR formation in unstretched monolayers with inhibition of ROCK and MLC activity, we speculate that formation of PJAR is the result of a cellular response to high magnitude stretch that reduces cellular tension by inactivating the Rho pathway. Future studies will investigate if AEC monolayer adhesion to the ECM is reduced with stretch and if reduction is due to mechanical tearing, active biochemical signaling, or both. Furthermore, if AEC monolayer adhesion to the ECM is reduced with stretch, it is not known if strengthening adhesion would serve to increase paracellular barrier integrity, similar to the Sph-1-P endothelial model (31), or if decreased ECM adhesion is a survival response that aids in reducing monolayer tension during high magnitude stretch of AEC monolayers.

**MLC phosphorylation depends on cell type**

Unlike primary AECs, HPAEC monolayers stretched for 30 min at 12% ∆SA Hz showed increased MLC phosphorylation, similar to results published previously by Birukov et al. in HPAECs stretched for 30 min of 18% cyclic elongation (8). Furthermore, we demonstrate that MLC phosphorylation in HPAECs is stretch magnitude dependent, similar to our findings in AEC monolayers, with decreased MLC phosphorylation at high magnitude (37% ∆SA) stretch. We speculate that high biaxial stretch magnitudes (25% and 37% ∆SA) used in AEC monolayer models, while physiologically relevant (78), are significantly higher than strain magnitudes typically experienced by endothelial cells utilized in numerous studies which show ROCK and MLC activation with stretch. Thus, AEC monolayers respond differently by reducing activation of ROCK and MLC. We conclude that MLC phosphorylation in stretched
HPAEC monolayers is significantly different from that in primary alveolar epithelial cells at low magnitude stretch, though similar in response at high magnitudes.

**Summary**

We have demonstrated that ROCK activity and MLC phosphorylation decrease with high magnitude stretch of primary AEC monolayers. This inactivation is stretch-magnitude dependent, similar to MLC phosphorylation in stretched HPAEC monolayers. We have shown that rescuing MLC phosphorylation during stretch has no effect on monolayer permeability. Finally, unstretched monolayers with ROCK and MLC activity inhibited show PJAR formation. Together, with our previously published studies demonstrating that large stretch magnitudes rapidly reorganize the actin cytoskeleton to form PJAR, we speculate that PJAR formation is the result of a cellular response to reduce cellular tension. Future studies will investigate pathways involved in actin-coupled extracellular matrix adhesion, to test the effect of increasing monolayer adhesion on retaining paracellular barrier properties during stretch in order to investigate opportunities to prevent ventilator-induced lung injury.
REFERENCES


CHAPTER 4: RAC MEDIATED MECHANISTIC SIGNALING PATHWAYS AND PHARMACOLOGICAL FACTORS DURING STRETCH

ABSTRACT

Alveolar epithelial cells (AECs) maintain integrity of the blood-gas barrier with gasket like intercellular tight junctions (TJ) which are anchored internally to the actin cytoskeleton. AEC monolayers stretched biaxially undergo rapid magnitude and frequency-dependent actin cytoskeletal remodeling to form perjunctional actin rings (PJARs). We hypothesize that Rac1 and downstream effectors LIMK½, cofilin, Akt, and SSH1L are activated in stretched AECs. Furthermore we hypothesize that Rac1 pathway inhibition will attenuate stretch-induced actin remodeling, loss of occludin, and preserve paracellular permeability during stretch. Primary AEC monolayers were stretched biaxially to create a change in surface area (ΔSA) of 37% in a cyclic manner at ¼ Hz for 10 or 60 min or left unstretched. Additional monolayers were pretreated with Rac1 pathway inhibitors wortmannin (10 and 100 nM 60 min), EHT-1864 (10 µM 60 min), or IPA-3 (10 µM 60 min) or agonists PIP3 (5 µM 60 min), PDGF (10 ng/ml 15 min), or calyculin-A (10 nM 30 min), and then stretched. Unstretched treated and untreated AECs served as controls. Western analysis was used to determine the amount of active Rac1, occludin, and phosphorylated Akt, LIMK½, Rac1, cofilin, and SSH1L. F-actin arrangement was visualized using phalloidin. Monolayer permeability was assessed by monitoring the flux of the fluorescent tracer BODIPY-ouabain across the monolayer. Stretch-induced cell death was quantified using ethidium-homodimer-1 to determine if cell death is affected by changes in Rac1 pathway or monolayer permeability. Without
any treatments, biaxial stretch rapidly increased Rac1, Akt, LIMK½, and coflin activity but did not affect SSH1L. Furthermore, stretch in untreated monolayers also resulted in reduced occludin content, rapid actin remodeling and PJAR formation, and increased monolayer permeability and cell death. Compared to stretched vehicle control monolayers, Rac1 pathway inhibitors wortmannin, EHT-1864, and IPA-3 reduced Rac1, Akt, LIMK½, and coflin activity, attenuated actin remodeling and preserved monolayer permeability in stretched monolayers, without affecting cell death. Exogenous PIP3 increased Akt phosphorylation in unstretched monolayers and reduced cell death, but increased monolayer permeability (only at 10 min) in stretched monolayers. Rac1 pathway agonists PDGF and calyculin-A induced actin cytoskeleton remodeling to form PJARs in unstretched monolayers. In summary, stretch of primary rat AEC monolayers increased Rac1 pathway activity, cytoskeleton remodeling, and monolayer permeability, all of which could be attenuated with Rac1 pathway inhibitors.

INTRODUCTION

Previously, rat type I like AEC monolayers in culture, used to mimic the alveolar epithelium in vitro (17, 30, 41, 97, 123), were shown to form perijunctional F-actin rings (PJARs) and increase monolayer permeability in response to high magnitude biaxial stretch, analogous to pathological ventilator volumes (26, 50). However, little is known about the underlying mechanistic pathways involved in PJAR formation and the influence of PJAR formation on monolayer permeability in type I AECs.

Numerous studies have investigated the Rho and Rac1 pathways and their roles in actin cytoskeleton remodeling and perijunctional F-actin ring (PJAR) formation. Some
have shown activation of the Rho pathway with stretch and have characterized its influence on the actin cytoskeleton (4, 13-15, 87, 91, 140), tight junction (TJ) protein content and distribution, and monolayer permeability properties in stretched endothelial cells and other cell types (39, 118, 128, 138, 155, 165). Others have implicated the Rho pathway and its effect on actin structure, TJ protein distribution, and TJ mediated paracellular permeability in unstretched epithelial cells (18, 74, 100, 117, 139, 142, 155, 156, 165). However, our previous studies have shown that the Rho pathway, including Rho kinase (ROCK) and myosin light chain (MLC), becomes inactivated in rat type I like AEC monolayers stretched biaxially to high magnitudes (Chapter 3).

Alternatively, other investigators have implicated the Rac pathway as a modulator of actin cytoskeleton remodeling and formation of peripherally localized stress fibers (69, 72, 116, 134, 137, 173). Previous studies have investigated the Rac signaling cascade in non-AEC cell types during stretch (98) or with pharmacological inhibition (23, 169). Because there is a paucity of data investigating the Rac pathway in pulmonary AECs, in this chapter we investigate the Rac, Akt, LIMK, and cofilin pathways and their effect on PJAR formation, TJ mediated paracellular permeability, and cell death in type I AECs. The following three paragraphs cover the model of the Rac1 signaling pathway, starting upstream and proceeding to downstream targets. The Rac1 signaling pathway and inhibitors are summarized below in our hypothesis (Figure 4.1). Rac1 can be modulated through a number of upstream proteins including membrane bound integrin receptors which link to extracellular matrix fibronectin via focal adhesion kinase (FAK) and phosphatidylinositol-3-kinase (PI3K) (32, 44, 45, 137). Rac1 has been implicated in the formation of actin rich lamellipodia and membrane ruffles at the cell periphery in cells
with exogenously applied Rac1 as well as in spreading cells that are responding to local injury (69, 116, 134). Active guanosine-5'-triphosphate (GTP) bound (29, 115, 148) Rac1 can be inhibited in vivo by EHT-1864 (141). EHT-1864 is an exogenous inhibitor which promotes the loss of Rac1 bound nucleotide and has a high affinity for Rac1 (48, 122). Rac1 can also be inhibited when phosphorylated at the Akt specific Ser71 site, resulting in inhibited GTP-binding activity without a change in GTPase activity (94). Rac1 can be activated with exogenous platelet-derived growth factor (PDGF), resulting in the formation of new stress fibers by 10 min (132, 134, 141), or through the PI3K-Akt signaling pathway (23, 73). Phosphatidylinositol 3,4,5 triphosphate (PIP3), the catalytic product of PI3K (78), activates Rac (92) by entering in a complex with epithelial growth factor receptor pathway substrate 8 (Eps8), Abl interacting protein 1 (Abi1), and son of sevenless 1 (Sos-1) (78). Rac1 can also feedback to activate PI3K (34, 110). PIP3 can be delivered exogenously (64, 159, 168) with histone-mediated membrane permeability (124, 168). PI3K can be inhibited with wortmannin (42, 150, 172), which irreversibly binds and competes for the ATP binding pocket with the highest specificity when compared to other PI3K inhibitors, including LY294002 (159, 164). Thus PI3K, which is coupled to extracellular stimuli, has been shown to be intimately tied to the Rac1 signaling pathway.

Rac influences numerous downstream proteins including p21-activated kinase (PAK-1), LIM kinase (LIMK), and coflin. PAK-1 is activated when bound to Rac (90, 102, 171). PAK-1 can be inhibited with IPA-3, a covalently binding inhibitor that results in allosteric, non-ATP competitive inhibition (16, 43, 162). LIMK-1/2 (LIMK½) can be phosphorylated at the Thr508/Thr505 site by active PAK-1 (53, 143, 173) with phosphorylation of LIMK required for activity (40, 53, 101, 119, 145). LIMK can also
be regulated by heat shock protein 90 (Hsp90) (10, 96) or bone morphogenetic protein (BMP) (58) while LIMK2, specifically, can also be phosphorylated by Rho kinase (ROCK) (119, 145) or transforming growth factor-β1 (160). Furthermore, active LIMK has been shown to phosphorylate actin depolymerizing factor (ADF)/cofilin at the Ser3 site, resulting in cofilin inhibition (2, 8, 53, 106, 173). Actin turnover and polymerization is mediated by two major proteins; Cofilin and actin related protein 2/3 (Arp2/3). Arp2/3 complex has been shown to be an actin nucleation factor (108, 109). Cofilin has been shown to mediate actin turnover and reorganization into F-actin by increasing disassociation rate (31). Cofilin itself is activated (107) by the phosphatase slingshot-1 (SSH1) via Ser3 dephosphorylation of cofilin-1 (93, 111, 114, 120). Phosphorylation of SSH-1L at the Ser978 site facilitates 14-3-3 binding, rendering the phosphatase inactive (54, 89). Thus it is important to identify the phosphorylation state of LIMK and cofilin in stretched AEC monolayers to determine if they are involved in Rac1 mediated actin remodeling.

Protein kinase B (PKB/Akt), shown to control survival and inhibit apoptosis (19, 20, 25, 59, 60, 176) when activated by phosphorylation at the Thr308 (5) and Ser473 sites (82, 136), has been shown to be affected by PIP3 and Rac1 and require phosphorylation of ezrin at Tyr353 (66). Akt interacts directly with actin and this interaction could be modulated by Rac1 agonist PDGF (28). Akt is phosphorylated by 3'-phosphoinositide-dependent kinase-1 (PDK1) and recruited to the membrane by PI3K in agonist or mutant activated PI3K cells (158, 159). Additionally, PIP3 was shown to activate Akt (20, 60, 64, 151) and could be inhibited with wortmannin (20, 60, 84) or endogenous PTEN phosphatase (24). Expression of Rac, as well as Rac1 agonist PDGF, enhances Akt
phosphorylation at Ser473 (28, 76). Finally, phosphorylated (Ser473 and Thr308) Akt colocalized with Rac at the leading edge of fibroblasts (76) and was found to be essential for Rac regulated cell motility (76, 92), but not Rho (67). Thus it is important to identify the phosphorylation state of Akt in stretched AEC monolayers in order to elucidate the effect of Akt on the Rac1 signaling pathway and stretch-mediated apoptosis.

Previous studies have investigated the downstream components of the Rac1/Akt pathways during stretch in other cell types. Rac1 has been implicated in the formation of actin rich lamellipodia and membrane ruffles at the cell periphery (69, 116, 134) and shown to modulate actin cytoskeleton remodeling and formation of peripherally localized stress fibers (69, 72, 116, 134, 137, 173). Stretched endothelial cells showed an approximately 5-fold increase in Rac1-GTP when compared to unstretched cells (98). Similarly, stretch of human airway smooth muscle cells (HASMCs) showed a rapid and transient increase in GTP-bound Rac1 (105). Akt has been shown to control survival and inhibit apoptosis (19, 20, 25, 59, 60, 176). Liu et al. used endothelial cells with moderate physiological levels of cyclic (6-10% 1 Hz) stretch and showed an inhibition of apoptosis at 30 min concurrent with activation (phosphorylation) of Akt, which could be inhibited with 100 nM wortmannin (99). Previously, wortmannin was shown to inhibit PI3K (42, 150, 172) by irreversibly binding and competing for the ATP binding pocket with the highest specificity when compared to other PI3K inhibitors, including LY294002 (159, 164). Similar to Liu et al., Akt was activated (phosphorylated) in portal vein smooth muscle cells stretched for 5 min or 6 hrs (3). Likewise, Dimmeler et al. shows an activation of Akt in endothelial cells under laminar shear stress (49). Other studies, however, have shown a down-regulation of Akt in response to mechanical stress (127).
There is a paucity of studies investigating these pathways and relationships in stretched alveolar epithelial cells.

Numerous studies have investigated the Rac1/Akt pathways influence on monolayer permeability in other cell types. Rac1 depletion reduced the TNF-induced increase in permeability of human umbilical vein endothelial cells (HUVECs) (23, 169). Bruewer et al. showed that constitutively active (CA) Rac1 abolished PJAR, redistributed, and increased claudin-1 and claudin-2 detergent solubility indicating a loosening of the bonds between these TJ proteins and actin, in Madin-Darby canine kidney (MDCK) (18). Nagumo et al. showed that constitutively active cofilin rapidly restructures the F-actin cytoskeleton, independent of myosin light chain kinase (MLCK) activation, resulting in a decrease in transepithelial resistance (TER) in Caco-2 cells (113). Cofilin has been shown to mediate actin turnover and reorganization into F-actin by increasing disassociation rate (31). Blocking cofilin dephosphorylation (activation) with EGTA, a Ca$^{2+}$ chelator, also reduced the capsaicin-induced attenuation of TER in Caco-2 cells (113). Finally, PI3K has been shown to be upstream of Rac1 (32, 44, 45, 137) and Akt (66) (Figure 4.1) in other cell types. PI3K inhibition with siRNA targets for p110α, -β, -γ, -δ isoforms increased barrier function in HUVECs (23). Little, however, is known about these pathways and their influence on the monolayer permeability of stretched alveolar epithelial cells.

The goals of our study are to test whether stretch-induced PJAR formation and increased monolayer permeability are dependent on the Rac/PI3K mediated pathway. Our overall hypothesis (Figure 4.1) is that rapid cytoskeletal remodeling and formation of PJAR is activated by Rac1 accompanied downstream by Akt, LIMK½, and cofilin
activation, and that inhibition of any element of this Rac1 pathway will inhibit PJAR formation, and attenuate the stretch-induced increase in monolayer permeability.

Figure 4.1: Hypothesized mechanical stretch signaling pathway involved in actin cytoskeleton remodeling and increase paracellular permeability in stretched primary rat alveolar epithelial cells (AECs). Numerous other signaling proteins may contribute to Rac1/Akt pathway regulation during stretch (10, 34, 46, 58, 61, 71, 96, 110, 119, 145, 160).

MATERIALS AND METHODS

Primary Rat Type I Like Alveolar Epithelial Cell Isolation

Alveolar type II cells were isolated from male Sprague-Dawley rats based on a method reported by Dobbs et al. (52) with slight modification (153). The animal protocols used in this study were reviewed and approved by the University of Pennsylvania IACUC. The rats (190-300 g) were anesthetized (sodium pentobarbital, 55 mg/kg ip), the trachea cannulated, the lungs mechanically ventilated, and an abdominal aortotomy was performed to exsanguinate the rat while excess blood removed via pulmonary arterial perfusion. The lungs were excised and type II cells were isolated using an elastase digestion technique followed by mechanical mincing on a tissue
chopper (Sorvall, Lorton, VA) in the presence of DNase (Sigma-Aldrich, St. Louis, MO). Cells were filtered through progressively finer Nitex mesh (Crosswire Cloth, Bellmawr, NJ) and plated on an IgG-coated (3 mg / 5 ml Tris-HCl) culture dish. After a 90 min incubation at 37ºC, cells were spun down and resuspended in Minimal Essential Medium (MEM, Invitrogen, Carlsbad, CA) with Earle’s salts supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 25 µg/ml Gentamicin (Invitrogen), and 0.25 µg/ml Amphotericin B (Life Technologies). Cells were seeded at 1.0 million cells/cm² onto fibronectin coated (10 µg/cm², Invitrogen) flexible Silastic membranes (Specialty Manufacturing, Saginaw, MI) in custom-designed wells (152). The cells were cultured for 4 days at 37ºC, 5% CO₂ in MEM with 10% FBS, Gentamicin, and Amphotericin B, replaced daily. After 4 days, the cells had adopted alveolar type I (ATI) features (17, 30, 37, 41, 123, 131) including the expression of RTI40, and had grown to a confluent monolayer (Appendix A). Monolayers were then serum-deprived in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech Inc., Manassas, VA) supplemented with 20 mM HEPES (Sigma) for 3 hrs and stretched biaxially across a range of physiological relevant magnitudes including at 12%, 25%, or 37% change in surface area (ΔSA) roughly corresponding to 64%, 86%, and 100% total lung capacity, respectively (152).

**Experimental Design**

Primary rat AEC monolayers were serum deprived in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Manassas, VA) supplemented with 20 mM HEPES for 3 hrs. Each condition was studied in cells from 3 monolayers per rat, from at least 3 rats. Monolayers were pretreated with one of the following inhibitors or agonists as

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summarized in Table 4.1: To inhibit PI3K we used wortmannin (WORTMAN, Sigma-Aldrich, St. Louis, MO) at 10 or 100 nM for 60 min. Wortmannin was previously shown to inhibit PI3K in other cell types (42, 150, 172) by irreversibly binding and competing for the ATP binding pocket with the highest specificity when compared to other PI3K inhibitors, including LY294002 (159, 164). We have demonstrate that activity of Akt, LIMK½, and coflin, all downstream of PIK3, is reduced with wortmannin (Figure 4.10). To activate Akt/Rac1 we used the agonist phosphatidylinositol 3,4,5-triphosphate di-C₈ (PIP3, Echelon, Salt Lake City, UT) dissolved in 1.67 µM histone H1 shuttle (Echelon, as described previously in (168)) at 5 µM for 60 min (dark grey bar and crosshatch bar Figure 4.8). PIP3 was previously shown to be the catalytic product of PI3K (78) and activate PKB (20, 60, 64, 151). To inhibit Rac1 we used EHT-1864 (Tocris Bioscience, Ellisville, MO) at 10 µM for 60 min (dark grey bar, Figure 4.14), previously shown to promotes the loss of Rac1 bound nucleotide and have a high affinity for Rac1 (48, 122). To activate Rac1 we used agonist platelet-derived growth factor (PDGF-AA, Cell Signaling Technology, Beverly, MA) dissolved in 20 mM citrate (132, 134, 141) at 10 ng/ml for 15 min. To inhibit LIMK we used the PAK-1 inhibitor IPA-3 (Tocris Bioscience) at 10 µM for 60 min (light grey bars, Figure 4.17), previously shown to inhibit PAK-1 by covalently biding to PAK-1 and resulting in allosteric inhibition (16, 43, 162). Protein phosphatase type 1 and 2A (PP1/PP2A) antagonist calyculin-A (CALY-A, Cell Signaling) at 10 nM for 30 min, previously shown to greatly dephosphorylate coflin (activate) (75, 121, 175), was used to activate coflin (white bar, Figure 4.20). ATP was depleted with 2 mM 2-deoxy-D-glucose + 10 µM antimycin A (Sigma-Aldrich) for 60
min. Dimethyl sulfoxide (DMSO) was used as vehicle control (VC). Finally, monolayers were also left untreated (UNT).

Following pretreatment or vehicle control exposure, monolayers were stretched to 12, 25, or 37% change in surface area (ΔSA) cyclically at ¼ Hz for 0 (unstretched), 1, 10, 30, or 60 min as shown in Table 4.1.

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Table 4.1: Experimental design matrix. Primary rat AEC monolayers were serum deprived then pretreated with one of the inhibitors or agonists listed above, vehicle control (VC) dimethyl sulfoxide (DMSO), or left untreated (UNT) for the duration shown. Monolayers were then stretched biaxially to 12, 25, or 37% change in surface area (ΔSA) cyclically at ¼ Hz for 0 (unstretched), 1, 10, 30, or 60 min. Finally, monolayers were lysed and probed for specific phosphorylated and total proteins (western blot), fixed and stained (immunocytochemistry), quantified for monolayer permeability (permeability), or quantified for cell death (cell death) as described subsequently. In the readout columns, X denotes that results are reported at all stretch times noted, while a 0 or 10 m denote that data was collected only at 0 or 10 min of stretch.
Following treatment and stretch, monolayers were immediately processed through one of the four readouts shown in Table 4.1: Western blot, immunocytochemistry, monolayer permeability quantification, or cell death quantification. These methods are described in detail in the following sections.

**Measurement of Protein Phosphorylation with Western blot**

Cells were lysed and protein content quantified similar to previous (11, 13, 129). Primary rat AEC monolayers were pretreated and stretched as previously described in the Experimental Design section Table 4.1 Western blot column. Monolayers (3 wells/lysate from at least 3 animals/group) were washed with ice-cold Dulbecco's PBS twice and scraped in sodium dodecyl sulfate (SDS) sample reducing buffer (30 mM Tris-HCl, 1.25% SDS, 12.5% β-mercaptoethanol, 10% glycerol) containing 50 mM sodium fluoride, 1.0 mM phenylmethanesulfonylfluoride (PMSF), 5 mM ethylenediaminetetraacetic acid (EDTA), 2 mM sodium orthovanadate, and protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN), sonicated 5 s then boiled for 5 min. Sample concentrations were quantified using a reducing agent and detergent compatible (RC DC) protein assay (Bio-Rad) and loaded equivalently (25 µg). Protein lysates were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, NuPAGE Novex 4-12% Bis-Tris Mini Gel, Invitrogen, Carlsbad, CA), transferred to 0.2 µm pore polyvinylidene difluoride membranes (30 V for 90 min, Bio-Rad, Hercules, CA), blocked in nonfat dry milk (Bio-Rad), then incubated 4°C overnight with one of the following primary antibodies: anti-phospho-Akt (Ser473), anti-phospho-Akt (Thr308), anti-Akt, anti-phospho-Rac1 (Ser71), anti-Rac1, anti-phospho-LIMK½ (Thr508/Thr505), anti-LIMK½, anti-phospho-cofilin
(Ser3), anti-cofilin (all Cell Signaling Technology, Boston, MA), anti-phospho-SSH1L (Ser978), anti-SSH1L (ECM Bioscience, Versailles, KY), or anti-occludin (Invitrogen).

Membranes were washed thrice in TBS/T (tris-buffered saline with 0.1% tween 20, Bio-Rad), incubated at room temperature for 90 min with horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), then rinsed six times with TBS/T. Immunoreactive proteins were detected with enhanced chemiluminescence detection system (ECL Plus, GE Healthcare Biosciences, Piscataway, NJ). Developed film (Hyperfilm ECL, GE Healthcare) was digitized and the relative density of the protein in the bands were quantified by scanning densitometry in ImageJ (ver. 1.43j) based on method optimization in Gassmann et al. (65). Background membrane intensity for each lane was subtracted from blot intensity. Membranes were stripped (Restore, Thermo Scientific, Logan, UT) for 20 min at room temperature and reprobed with the relevant total protein antibody or with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Millipore, Billerica, MA) to ensure equivalent total protein loading across lanes (51).

The average of three measurements was used for band density. For each band, normalized (n) protein density was found by dividing the density of the band by the unstretched-untreated (UNS-UNT) band density. Normalized density of the phosphorylated protein was divided by normalized density of the respective total protein or GAPDH signal and reported.

To test the effect of stretch, animal average normalized density values (3 wells/lysate from at least 3 animals/group) were compared with time-matched unstretched-untreated controls using a one-way ANOVA with a post-hoc Dunnett's test
(174) in JMP (ver. 8.0, SAS Institute Inc., Cary, NC). To test the effect of treatment, animal average normalized density values (3 wells/lysate from at least 3 animals/group) were compared with time-matched vehicle controls as well as unstretched-vehicle controls using a two-way ANOVA with Tukey-Kramer post-hoc analysis (174) in JMP (ver. 8.0, SAS Institute Inc., Cary, NC).

*Rac1 Activation Assay*

Rac1 activity was quantified using an *in vitro* pull-down activity assay (Cell Biolabs Inc., San Diego, CA) with glutathione S-transferase linked p21-activated protein kinase p21-binding domain (GST-PAK1 PBD) agarose beads, as previously described (23, 68). Primary rat AEC monolayers were pretreated and stretched as previously described in the Experimental Design section Table 4.1 Western blot Rac1-GTP column. Monolayers (3 wells/lysate from at least 3 animals/group) were washed with ice-cold Dulbecco's PBS twice, and scraped in sodium dodecyl sulfate (SDS) sample reducing buffer (30 mM Tris-HCl, 1.25% SDS, 12.5% β-mercaptoethanol, 10% glycerol) containing 50 mM sodium fluoride, 1.0 mM PMSF, 5 mM EDTA, 2 mM sodium orthovanadate, and protease inhibitor cocktail (Complete Mini, Roche, Indianapolis, IN), then sonicated for 5 s. Sample concentrations were quantified using a reducing agent and detergent compatible (RC DC) protein assay (Bio-Rad) and loaded into the assay equivalently. Lysate was kept on ice unless otherwise noted. Samples (125 µg) were mixed with GST-PAK1 PBD beads (5 µg) in a pull-down buffer (125 mM HEPES, 750 mM NaCl, 5% nonyl phenoxypolyethoxylethanol (NP-40), 50 mM MgCl₂, 5 mM EDTA, 10% Glycerol, 1.0 mM PMSF, 10 µg/ml leupeptin, and Complete Mini protease inhibitor
cocktail, pH 7.5), thoroughly vortexed, and incubated at 4°C for 60 min. Samples were then centrifuged (14,000 × g, 4°C for 10 s) and washed with pull-down buffer thrice, resuspended in 2X SDS sample reducing buffer, boiled 95°C for 5 min, then centrifuged (14,000 × g, 4°C for 10 s). Pull-down samples were resolved by SDS-PAGE and immunoblot (see Western blot and densitometry protocol details above). Membranes were probed for monoclonal anti-Rac1 to determine the amount of active Rac1 and for GAPDH to ensure pull-down assay purity. For each sample, whole cell lysate was also directly loaded at equivalent volumes on a separate SDS-PAGE and immunoblot (see Western blot protocol and densitometry protocol details above) and probed for anti-Rac1 to normalize activity assay signal to sample total protein and for GAPDH (Millipore, Billerica, MA) to ensure equivalent total protein loading across lanes (51). For each band, normalized (n) protein density was found by dividing the density of the band by the unstretched-untreated (UNS-UNT) band density. Normalized density of pull-down Rac1 (active Rac1) was divided by normalized density of whole-cell total Rac1 and reported.

Monolayer Permeability

Paracellular permeability was assessed by monitoring the flux of the fluorescent tracer BODIPY-ouabain across the monolayer as previously described (26). Primary rat AEC monolayers were serum deprived in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Manassas, VA) supplemented with 20 mM HEPES for 3 hrs. The apical surface of the monolayer was then bathed in DMEM + HEPES containing 2 µM BODIPY-ouabain (radius ~20 Å, Invitrogen, Carlsbad, CA), a fluorescent tracer which has a high affinity for the extracellular domain of the transmembrane Na⁺-K⁺-ATPase
(83, 147) found on the basolateral surface of type I AECs (167), for a duration of 60 min for all samples (during stretch or on unstretched monolayers).

Primary rat AEC monolayers were pretreated and stretched as previously described in the Experimental Design section Table 4.1 permeability column. Following stretch, the apical surface was rinsed three times with dye-free DMEM + HEPES and imaged (Eclipse TE300 epifluorescence microscope, ×10 objective, Nikon, Melville, NY) using constant exposure, aperture, and fluorescent intensity settings. The maximum pixel intensity in the background of all unstretched-untreated monolayers was measured and used as a threshold to exclude unstained regions. The area percentage of each image (3 image/well, 3 wells/animal, from at least 3 animals/group) was found using a MATLAB (version 6.5 R13, The MathWorks, Natick, MA) program (Appendix F) which calculates the area of the image above threshold intensity and divides it by the total image area. Normalized area percentage (nArea) of each image was found by dividing area percentage by the average area percentage of the unstretched-untreated group (or unstretched-vehicle control in treatment studies). To test the effect of stretch magnitude and duration, animal average nArea values were compared with time-matched unstretched-untreated controls using a two-way ANOVA with a post-hoc Dunnett's test (174) in JMP (ver. 8.0, SAS Institute Inc., Cary, NC). To test the effect of treatment (inhibitors or exogenous agonists), animal average nArea values were compared with time-matched vehicle controls as well as unstretched-vehicle controls using a three-way ANOVA with Tukey-Kramer post-hoc analysis (174) in JMP. Previously, Cavanaugh et al. showed that the BODIPY-ouabain tracer was not internalized by cells, did not undergo
receptor-mediated endocytosis, did not bind due to plasma membrane rupture, and did not bind nonspecifically (26).

**Immunofluorescence and F-Actin Staining to Evaluate Perijunctional Actin Ring**

Primary rat AEC monolayers were pretreated and stretched as previously described in the Experimental Design section Table 4.1 immunocytochemistry column. Following stretch, monolayers were fixed with 1.5% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in DPBS for 15 min, permeabilized using 0.1% Triton X-100 in DPBS for 5 min, and blocked with 5% goat serum in DPBS for 60 min. Monolayers were incubated with primary antibody in blocking buffer at 4°C overnight, washed thrice for 5 min with DPBS, then incubated with fluorescent-conjugated secondary antibody and also stained for F-actin (phalloidin, Invitrogen, Carlsbad, CA) for 90 min at 23°C. Monolayers were washed thrice for 10 min and mounted on slides with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) containing antifade reagent to stain for nuclei. Images were captured (Eclipse TE300 epifluorescence microscope, ×40 objective, Nikon, Melville, NY).

Monolayers were stained for F-actin with phalloidin and immunostained for zona occludens-1 (ZO-1) using anti-ZO1 antibody (Invitrogen) to identify the location of the cell periphery. Monolayers were then quantified for perijunctional actin intensity (see below) similar to previous (50). Additional monolayers were stained for F-actin and immunostained for phosphorylated coflin using anti-phospho-cofilin (Ser3) or phosphorylated LIMK½ using anti-phospho-LIMK½ (Thr508/Thr505, Cell Signaling Technology, Boston, MA).
To evaluate the PJAR, both red (F-actin) and green (ZO-1) channels of two random microscope fields from each labeled monolayer were captured (×40 objective) on an epifluorescence scope (Nikon) using identical exposure times for all images of each type. Each field was divided into a 3 x 3 matrix of regions, and every other region (5 regions) was systematically selected for analysis. In each region analyzed, all cells with at least 50% of its area residing in the region were evaluated, typically 16 cells per field. The perijunctional F-actin fluorescent intensity of each cell was obtained (ImageJ, ver. 1.43j) by tracing the peripheral ZO-1 (Figure 4.11 top inset), superimposing this ZO-1 trace onto the same cell stained for F-actin (Figure 4.11 bottom inset), and enlarging the trace line thickness radially inward and outward to create a boundary zone for further analysis approximately 1.6 µm thick (average PJAR thickness from a small sample study, Figure 4.11 bottom inset, white contours). Mean F-actin fluorescent intensity in this peripheral annulus \( A_i \) was measured. Whole cell F-actin mean fluorescent intensity \( W_i \) was determined, including annulus and cell interior. PJAR intensity \( P_i \) was found by taking the ratio of peripheral annulus mean intensity to whole cell mean intensity \( P_i = \frac{A_i}{W_i} \). For each experimental group \( P_i \) was evaluated based on an average 32 cells (2 fields) per animal from at least 3 different animals. To test the effect of treatment, animal averaged \( P_i \) values were compared with time-matched vehicle controls as well as unstretched-untreated controls using a two-way ANOVA with Tukey-Kramer post-hoc analysis (174) in JMP.
Cell Death Quantification

Primary rat AEC monolayers were pretreated and stretched as previously described in the Experimental Design section Table 4.1 cell death column. Monolayers were then stained for dead cells using 2 µM ethidium homodimer-1 (Invitrogen) similar to previous (152). Following the stretch or no-stretch period, monolayers (Figure 4.2) were washed free of dye and imaged (Eclipse TE300 epifluorescence microscope, ×10 objective, Nikon, Melville, NY) for dead stain (red) and phase (grey). A subset of images from each group was used to determine the total number of cells in an image field. Total number of cells per phase field was counted in ImageJ (ver. 1.43j) and was found to be constant across all groups (872 cells/field ± 16 cells/field SE). The number of dead cells per field was counted using a MATLAB (ver. 6.5 R13, The MathWorks, Natick, MA) program (Appendix F) which identifies and counts ethidium homodimer-1 stained nuclei (dead cells) in the dead channel of each image. Percent dead (% dead) was found by dividing the number of dead cells by the average total number of cells in a field.

To test the effect of stretch, animal averaged % dead values (3 images/monolayer, 3 monolayers/animal, from at least 3 animals/group) were compared with time-matched unstretched-untreated controls using a two-way ANOVA with post-hoc Dunnett’s test (174) in JMP (ver. 8.0, SAS Institute Inc., Cary, NC). To test the effect of treatment, animal averaged % dead values were compared with time-matched vehicle controls as well as unstretched-untreated controls using a three-way ANOVA with Tukey-Kramer post-hoc analysis (174) in JMP.
RESULTS

Rac1, Akt, LIMK½, and cofilin activity increased with stretch

We hypothesized that actin cytoskeleton remodeling during formation of perijunctional F-actin rings (PJARs) and decreased Rho kinase (ROCK) activity, shown previously in chapters 2 and 3, would be accompanied by an increase in Rac1 activity, an increase in phosphorylation of Akt and LIMK½, and a decrease in phosphorylation of cofilin. Quantitative GTP-Rac1 (active) pull-down assay data confirmed our hypothesis, showing an increase in GTP bound Rac1 at 10 min of 37% ΔSA ¼ Hz (dark grey bar) stretch when compared to unstretched-untreated (UNS-UNT) monolayers (Figure 4.3). Thus, we find that Rac1 becomes activated (GTP bound) with stretch.
Figure 4.3: Rac1 activity, occludin content, and phosphorylation of Akt, Rac1, LIMK½, and coflin in stretched AEC monolayers. Rac1 activity (Rac1-GTP, dark grey bar), Akt phosphorylation (Ser473 and Thr308, crosshatch bars), Rac1 phosphorylation (Ser71, striped bar), and LIMK½ phosphorylation (Thr508/5, light grey) all increased in monolayers at 10 min of 37% ΔSA ¼ Hz stretch when compared unstretched-untreated (UNS-UNT, dashed line) monolayers. Cofilin phosphorylation (Ser3, white bar) and occludin content (65 and 50 kDa, dotted bars) decreased with stretch. SSH1L phosphorylation (Ser978, checker bar) did not change with stretch. Normalized (n, to UNS monolayers) pull-down GTP-Rac1 signal divided by n-total-Rac1 signal was used as a measure of Rac1 activity. n-phosphorylated signal divided by n-total signal was used as a measure of Akt, Rac1, LIMK½, coflin, and SSH1L phosphorylation. n-occludin signal divided by n-GAPDH signal was reported for both 65 kDa and 50 kDa molecular weight occludin. Typical western blot signals shown below groups. Occludin 50 kDa located below more intense 65 kDa band. Data is mean ± SE from at least 3 animals/group with # P<0.05 and ## P<0.01 vs. UNS-UNT.
Table 4.2: Summary of one and two-way ANOVA results showing the effects of stretch magnitude, stretch duration, and the interaction of stretch magnitude and duration on monolayer permeability, cell death, and protein phosphorylation readouts. X = P < 0.05, - = no effect, and n/a = not applicable.

Akt (PKB), previously shown to activate when phosphorylated at the Ser473 and Thr308 residues (82, 136) or with increased Rac activity (28, 76), supported Rac1 data at 37% ∆SA ¼ Hz, showing an increase in phosphorylation in monolayers stretched for 10 min to 37% ∆SA ¼ Hz (crosshatch bars, Figure 4.3) but revealed a stretch magnitude effect because Akt phosphorylation in monolayers stretched to 25% ∆SA (crosshatch bars, Figure 4.4) were not significantly different from UNS-UNT monolayers.
We hypothesized that the Ser71 residue of Rac1, previously shown to inhibit Rac1 ability to bind GTP when phosphorylated by Akt (94), would become phosphorylated with stretch. Rac1 phosphorylation at Ser71 data supported Akt data, showing an increased phosphorylation in monolayers stretched for 10 min at 37% ∆SA $\frac{1}{4}$ Hz (striped bar) when compared to UNS-UNT monolayers (Figure 4.3). Thus Rac1 becomes phosphorylated at Ser71 with stretch, though it does not result in a net decrease of Rac1 total-activity during stretch (dark grey bar, Figure 4.3).

We hypothesized that LIMK$\frac{1}{2}$, previously shown to activate when phosphorylated at the Thr508/5 residue by p21-activated kinase (PAK-1) (40, 53, 101, 112, 113),
downstream of Rac1, would become phosphorylated with stretch. Indeed, LIMK½ phosphorylation increases in monolayers stretched for 10 min of 37% ΔSA ¼ Hz (light grey bar) when compared to UNS-UNT monolayers (Figure 4.3). Furthermore, phosphorylated LIMK½ (green) is localized to the perinuclear regions of the cell in monolayers stretched for 10 min at 37% ΔSA ¼ Hz (bottom left) when compared to non-localized phosphorylated LIMK½ in unstretched monolayers (top left, Figure 4.18). Thus, we find that LIMK½ is activated (phosphorylated) and sequestered away from the perijunction in stretched monolayers.

We hypothesized that cofilin, shown previously to depolymerize F-actin (2, 8, 31, 106) and become inactivated when phosphorylated at the Ser3 residue by LIMK (8, 53, 173), would become dephosphorylated with stretch-induced actin remodeling and PJAR formation. Cofilin is dephosphorylated in monolayers stretched for 1 and 10 min at 25% (light grey bars) and 37% (dark grey bars) ΔSA ¼ Hz, 30 min at 37% ΔSA, and 60 min at 12% ΔSA (white bar) when compared to UNS-UNT monolayers (Figure 4.5). Furthermore, deactivated (phosphorylated) cofilin (green) is localized to the perinuclear regions of the cell in monolayers stretched for 10 min at 37% ΔSA ¼ Hz (bottom left) when compared to non-localized phosphorylated cofilin in unstretched monolayers (top left, Figure 4.19). Thus we find that cofilin is activated (dephosphorylated) with stretch in a magnitude and duration dependent manner and that inactivated (phosphorylated) cofilin is sequestered away from the perijunction stretched monolayers.
We hypothesized slingshot-1L (SSH1L) phosphatase, shown previously to bind 14-3-3 and become inactive when phosphorylated at Ser978 (54) and also shown to dephosphorylate (activate) cofilin at Ser3 (54, 111, 114), would become dephosphorylated with stretch. We found no change in SSH1L phosphorylation in monolayers stretched for 10 min at 37% ΔSA ¼ Hz (checker bar) when compared with UNS-UNT monolayers (Figure 4.3).
Occludin, a tight junction (TJ) protein integral to TJ integrity and barrier function (62, 103, 170), linked internally to the actin cytoskeleton via ZO-1 (56, 63, 81), was previously shown to decrease in AEC monolayers stretched for 60 min at 37% ∆SA ¼ Hz (27). These results were confirmed in untreated monolayers stretched at shorter duration (10 min) 37% ∆SA ¼ Hz (dotted bars) when compared to UNS-UNT monolayers (Figure 4.3). Similarly, and also comparable to previous results (27), occludin separates as two distinct bands with SDS-PAGE; a light band at ~50 kDa and a dark band at ~65 kDa. Thus, we find occludin content is reduced rapidly in stretched monolayers.

Cell death and monolayer permeability increase with increasing stretch

Cell death after 60 min of stretch was previously shown to be stretch magnitude dependent in AEC monolayers (152). Now we have confirmed previous results, showing that cell death increases with stretch magnitude significantly after either 10 or 60 min of stretch (Figure 4.6). At 37% ∆SA ¼ Hz (dark grey) cell death shows a time dependency, with increased cell death at 60 min stretch when compared to 10 min. Finally, we find stretch magnitude dependency at 10 min of stretch, similar to 60 min, with higher cell death in monolayers stretched to 37% ∆SA when compared to 25% ∆SA. Thus, we find rapid stretch magnitude and time dependent cell death in stretched AEC monolayers.
Figure 4.6: Percent dead cells is dependent on stretch magnitude and time. Percent dead (% dead) was significantly higher in monolayers stretched for 10 or 60 min of 25% and 37% ΔSA ¼ Hz when compared to unstretched (UNS) monolayers. Monolayers stretched to 37% ΔSA have higher % death compared to monolayers stretched to 25% ΔSA at both 10 and 60 min. Furthermore, % death is higher at 60 min of 37% ΔSA stretch when compared to the 10 min stretch duration. The number of ethidium homodimer-1 staining (dead) cells in a microscope field was divided by the average total number of cells in a field to find % death. Data is mean ± SE from at least 3 animals/group with ## P<0.01 vs. UNS, ** P<0.01 and * P<0.05 vs. 25%, and && P<0.01 vs. 10 min.

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Table 4.3: Summary of three-way ANOVA results showing the effects of stretch magnitude, stretch duration, treatment, and the interaction of these effects on the cell death readout. X = P < 0.05, - = no effect, and n/a = not applicable.

We hypothesized that actin cytoskeleton remodeling during formation of PJAR in stretched AEC monolayers (Chapter 2) would be followed by a rapid increase in paracellular permeability. Previously, AEC monolayers stretched for 60 min show
increased permeability to fluorescent tracer BODIPY-ouabain, hypothesized to be a measure of AEC monolayer paracellular permeability (26). We were able to confirm these results, showing increased BODIPY-ouabain permeability (nArea) in monolayers stretched for 60 min at 25% \( \Delta \text{SA} \) (light grey bars) and 37% \( \Delta \text{SA} \) (dark grey bars) \( \frac{1}{4} \text{ Hz} \) but not at 12% \( \Delta \text{SA} \) (white bars) (Figure 4.7). Additionally, we find that rapid stretch (10 min) produces the same stretch magnitude response to nArea. Thus, we find that rapid stretch produces a stretch magnitude dependent increase in AEC monolayer permeability.

![Figure 4.7: Normalized area (nArea) stained by fluorescent tracer BODIPY-ouabain, hypothesized to be a measure of AEC monolayer paracellular permeability, was plotted as a function of stretch magnitude and time. nArea was significantly higher than unstretched (UNS, dashed line) in monolayers stretched for 10 and 60 min at 25% \( \Delta \text{SA} \) (light grey) and 37% \( \Delta \text{SA} \) (dark grey) \( \frac{1}{4} \text{ Hz} \) but was not significantly different at any time in monolayers stretched at 12% \( \Delta \text{SA} \) \( \frac{1}{4} \text{ Hz} \) (white). nArea was higher in monolayers stretched to 25% and 37% \( \Delta \text{SA} \) when compared to monolayers stretched to 12% \( \Delta \text{SA} \) at both stretch durations. Data are means ± SE; ##P < 0.01 compared with unstretched; **P < 0.01 compared with 12% \( \Delta \text{SA} \). Each data point based on 3 images/well from 3 wells/animal from at least 3 animals/group. The area percentage of each image was found by dividing the number of BODIPY-ouabain stained pixels above threshold intensity (maximum pixel intensity of background in all unstretched-untreated monolayers) by the total number of image pixels. Normalized area percentage (nArea) of each image was found by dividing area percentage by the average area percentage of the unstretched-untreated group.](image-url)
Exogenous PIP3 decreases cell death but does not affect actin remodeling or monolayer permeability

Phosphatidylinositol 3,4,5 triphosphate (PIP3), the catalytic product of phosphatidylinositol-3-kinase (PI3K) (78), can be delivered exogenously (64, 159, 168). We hypothesized that PIP3 would increase Rac1 and coflin activity and result in increased monolayer permeability in unstretched monolayers. PIP3 (5 µM 60 min), dissolved in 1.67 µM histone H1 shuttle, increased Rac1 activity in unstretched (UNS) monolayers (dark grey bar) when compared to vehicle control (VC) monolayers (Figure 4.8). However, PIP3 did not affect PJAR formation (Figure 4.21) nor did it affect coflin downstream in stretched and unstretched monolayers. UNS monolayers and monolayers stretched to 37% ΔSA ¼ Hz showed no change in phosphorylated coflin (white bars) when compared to their respective VC groups (Figure 4.8).
Figure 4.8: Effect of exogenous PIP3 (5 µM 60 min) on protein phosphorylation in stretched AEC monolayers. Rac1 activity (Rac1-GTP, dark grey bar), Akt phosphorylation (Thr308 only, crosshatch bar), and Rac1 phosphorylation (Ser71, striped bar) was increased in unstretched monolayers when incubated with exogenous PIP3 compared to unstretched-vehicle control (UNS-VC, dashed line). Cofilin phosphorylation (Ser3, white bars) was unchanged with PIP3 treatment in both UNS and stretched monolayers. Occludin in unstretched monolayers (65 and 50 kDa, dotted bars) was unchanged when incubated with exogenous PIP3 (light crosshatch bars, 5 µM 60 min) compared to VC. While stretch decreased occludin content in untreated monolayers, occludin in PIP3 treated stretched monolayers was not different compared to UNS-VC. Normalized (n, to UNS monolayers) pull-down GTP-Rac1 signal divided by n-total-Rac1 signal was used as a measure of Rac1 activity. n-phosphorylated signal divided by n-total signal was used as a measure of Akt, Rac1, and cofilin phosphorylation. n-occludin signal divided by n-GAPDH signal was reported for both 65 kDa and 50 kDa molecular weight occludin. Typical western blot signals shown below groups. Data is mean ± SE from at least 3 animals/group with # P<0.05 vs. UNS-VC and ## P<0.01 vs. UNS-VC.
Table 4.4: Summary of one, two, and three-way ANOVA results showing no significant effect from vehicle control (treatment vehicle column) on monolayer permeability, cell death, and protein phosphorylation readouts when compared to the untreated (UNT) group. X = P < 0.05, ✔ = no effect, and n/a = not applicable.

Table 4.5: Summary of one, two, and three-way ANOVA results showing the effects of stretch magnitude and stretch duration for PIP3 treatment, and the interaction of these effects on monolayer permeability and protein phosphorylation readouts. X = P < 0.05, ✔ = no effect, and n/a = not applicable.

Monolayer permeability was largely unaffected by exogenous PIP3. nArea did not change in UNS monolayers incubated with PIP3 (light crosshatch bar) when compared to VC (white bar, Figure 4.13). PIP3 did, however, increase nArea in monolayers stretched for 10 min at 37% ∆SA ¼ Hz when compared to stretched VC monolayers, but not at 60 min stretch which was similar to VC. While stretched-VC monolayers showed decreased occludin content (spotted bars, Figure 4.8) compared to UNS-VC, monolayers treated with PIP3 showed no attenuation of occludin content (65 kDa and 50 kDa) in monolayers stretched for 10 min at 37% ∆SA ¼ Hz when compared to UNS-VC monolayers. Thus, exogenous PIP3 activates the Rac1 pathway but does not
significantly affect cofilin phosphorylation or monolayer permeability in stretched or unstretched monolayers.

We hypothesized that PIP3 would increase phosphorylation of Akt and reduce cell death (Figure 4.1). Like Rac1, Akt phosphorylation (*crosshatch bars*) increases (Thr308 only) in unstretched (UNS) monolayers treated with PIP3 when compared to vehicle control (VC) monolayers (Figure 4.8). Phosphorylation of the Ser473 residue of Akt did not change with PIP3 treatment however. Likewise, phosphorylation of the Ser71 residue on Rac1 (*striped bars*) did increase with PIP3 treatment when compared to VC monolayers (Figure 4.8), though phosphorylation of Rac1 did not attenuate net Rac1 activity (*dark grey bar*, Figure 4.8) in UNS PIP3 treated monolayers. Increased Akt phosphorylation, previously shown to inhibit apoptosis in other cell types (19, 25, 176), corroborates well with cell death data, showing decreased cell death in monolayers treated with PIP3 (*light crosshatch bars*) and stretched for 10 and 60 min at 37% \( \Delta S_A \) ¼ Hz (Figure 4.9). Thus, we find that exogenous PIP3 activates Akt in unstretched monolayers and inhibits cell death in stretched AEC monolayers. Finally, PIP3 activates the Rac1 pathway but does not significantly affect cofilin phosphorylation, PJAR formation, or monolayer permeability in stretched or unstretched AEC monolayers.
Figure 4.9: Percent dead cells is dependent on stretch time and treatment. Percent dead (% dead) in unstretched monolayers (UNS, inset) was increased in monolayers incubated with Rac1 inhibitor EHT-1864 (dark dotted bars, 10 µM 60 min) but was not changed in untreated monolayers (UNT) or monolayers incubated with exogenous PIP3 (light crosshatch bars, 5 µM 60 min), PI3K inhibitor wortmannin (striped bars, 10 and 100 nM 60 min) or exogenous PDGF (light grey bars, 10 ng/ml 15 min) when compared to vehicle control (VC). Percent death increased at 10 (left) and 60 (right) min of 37% ΔSA ¼ Hz stretch (white bars) when compared to UNS monolayers, and could be attenuated with PIP3 treatment or exacerbated with EHT-1864 treatment when compared to VC monolayers at both stretch durations. The number of ethidium homodimer-1 staining (dead) cells in a microscope field was divided by the average total number of cells in a field to find % death. Data is mean ± SE from at least 3 animals/group with ## P<0.01 and # P<0.05 vs. UNS-VC, ** P<0.01 and * P<0.05 vs. 37% 60 min VC, and && P<0.01 vs. 37% 10 min VC.

PI3K inhibitor wortmannin strengthens monolayer barrier properties and inhibits actin remodeling during stretch.

To contrast our exogenous PIP3 studies, we inhibited PI3K during stretch using wortmannin. We hypothesized that inhibition of PI3K using wortmannin would result in decreased Akt, LIMK½, and cofilin activity, decreased actin remodeling. We
hypothesized this would reduce the amount of actin-TJ protein bond rupture and result in increased paracellular barrier strength in stretched AEC monolayers. Downstream of Rac1, LIMK½ phosphorylation (*light grey bars*, Figure 4.10-A and B) was reduced by wortmannin (10 nM and 100 nM 60 min) in unstretched (UNS) monolayers when compared to vehicle control (VC) monolayers. LIMK½ phosphorylation was also reduced in monolayers stretched for 10 min at 37% ΔSA ¼ Hz and treated with 10 nM wortmannin when compared to stretch-VC (A) and no different in stretched monolayers treated with 100 nM wortmannin when compared to UNS-VC (B). Similarly, wortmannin inactivated (phosphorylated) cofilin (*white bars*, Figure 4.10-A and B) in UNS monolayers as well as monolayers stretched for 10 min at 37% ΔSA ¼ Hz, which showed increased phosphorylation in monolayers treated with 100 nM wortmannin when compared to stretched-VC (B) and no difference in stretched monolayers treated with 10 nM when compared to UNS-VC (A).
Figure 4.10: Effect of PI3K inhibitor wortmannin on protein phosphorylation in stretched AEC monolayers incubated with 10 nM (A) and 100 nM (B) for 60 min. Akt (crosshatch bars), Rac1 (striped), and LIMK½ (light grey) phosphorylation in unstretched monolayers decreased when incubated with 10 nM (Akt Ser473 only, A) and 100 nM (B) compared to vehicle control (VC). While stretch increased Akt, Rac1, and LIMK½ phosphorylation in untreated monolayers, Akt, Rac1, and LIMK½ phosphorylation was attenuated in stretched monolayers with wortmannin when compared to stretched VC monolayers. Cofilin phosphorylation (Ser3, white) in unstretched monolayers was increased with wortmannin. Cofilin phosphorylation in stretched monolayers was increased with wortmannin (100 nM only) while stretched monolayers with 10 nM wortmannin were no different when compared to UNS-VC monolayers. Occludin (A only, dotted bars) in stretched monolayers was increased in monolayers with wortmannin (50 kDa only) compared to stretched-VC monolayers and no different compared to UNS-VC in monolayers with wortmannin (65 kDa only). Normalized (n, to UNS monolayers) phosphorylated signal divided by n-total signal was used as a measure of Akt, Rac1, LIMK½, and cofilin phosphorylation. n-occludin signal divided by n-GAPDH signal was reported for both 65 kDa and 50 kDa molecular weight occludin. Typical western blot signals shown below with wortmannin concentration noted in nM. Data is mean ± SE from at least 3 animals/group.
Table 4.6: Summary of one, two, and three-way ANOVA results showing the effects of stretch magnitude and stretch duration for wortmannin (10 nM \textit{top}, 100 nM \textit{bottom}) treatment, and the interaction of these effects on monolayer permeability and protein phosphorylation readouts. X = P < 0.05, - = no effect, and n/a = not applicable.

Western blot data supports qualitative F-actin intensity (P$_i$) data showing significant PJAR intensity in monolayers stretched for 10 min at 25\% (\textit{light grey} ∆SA $\frac{1}{2}$ Hz but not in monolayers stretched at the same magnitude and treated with 100 nM Wortmannin 60 min (\textit{striped bar}, Figure 4.11).
Figure 4.11: PJAR intensity ($P_i$) was found by taking the ratio of peripheral annulus F-actin mean intensity to whole cell F-actin mean intensity ($P_i = A_i / W_i$) and plotted as a function of stretch magnitude and treatment. $P_i$ was significantly higher than unstretched (UNS) in monolayers stretched at for 10 min at 25% ($\Delta SA = 25\%$) and 37% ($\Delta SA = 37\%$) $\Delta SA$. $P_i$ was attenuated by treatment with the PAK-1 inhibitor IPA-3 ($\text{light crosshatch bars}$, 10 µM 60 min) in monolayers stretched at 25% and 37% $\Delta SA$ and by treatment with PI3K inhibitor wortmannin ($\text{dark striped}$, 100 nM 60 min) in monolayers stretched at 25% $\Delta SA$. Data shown is mean ± SE with ## $p<0.01$ compared to unstretched and ** $p<0.01$ and * $p<0.05$ compared to stretch-VC. Each data point based on an average 32 cells per animal from at least 4 different animals. The perijunctional F-actin fluorescent intensity of each cell was analyzed by tracing the peripheral ZO-1 ($\text{top inset}$), superimposing this ZO-1 trace onto the same cell stained for F-actin ($\text{bottom inset}$), and enlarging the trace line thickness radially inward and outward to create a boundary zone for further analysis approximately 1.6 µm thick ($\text{white contours}$). Mean F-actin fluorescent intensity in this peripheral annulus ($A_i$) was measured. Whole cell F-actin mean fluorescent intensity ($W_i$) was determined, including annulus and cell interior. PJAR intensity ($P_i$) was found by taking the ratio of peripheral annulus mean intensity to whole cell mean intensity ($P_i = A_i / W_i$). $W_i$ was found to be constant across all groups compared to unstretched (UNS) (data not shown). Bar = 10 µm.
Figure 4.12: Effect of biaxial stretch and pathway inhibitors on F-actin. Type I like rat AEC monolayers with phalloidin labeled F-actin (red) left unstretched (UNS, top) or after 10 min of 37\% (middle row) or 25\% (bottom row) ΔSA ¼ Hz stretch with vehicle control (VC, left column), Rac1 inhibition with EHT-1864 or PI3K inhibition with wortmannin (middle column), or PAK-1 inhibition with IPA-3 (right). UNS monolayers exhibited an intercellular network of F-actin. Perijunctional F-actin rings (PJARs) formed in untreated stretched monolayers. Inhibition of Rac1 (EHT-1864, 10 µM 60 min) in stretched monolayers significantly reduced PJAR formation, with discrete areas of peripherally located stress fiber and overall homogeneous F-actin staining. Inhibition of PI3K (wortmannin, 100 nM 60 min) in stretched monolayers exhibited no actin cytoskeletal remodeling when compared to UNS monolayers. Finally, inhibition of PAK-1 (IPA-3, 10 µM 60 min) in stretched monolayers significantly reduced PJAR formation while cells retained perinuclear stress fibers also found in UNS monolayers. Monolayers also labeled for nuclei (dsDNA, blue) with DAPI. Bar = 10 µm.
Qualitative F-actin data agrees with quantitative F-actin images, showing F-actin remodeling and PJAR formation in VC monolayers stretched to 25% $\Delta S_A = \frac{1}{4}$ Hz for 10 min (bottom left) while stretched monolayers treated with 100 nM wortmannin 60 min (bottom middle) showed inhibition of F-actin remodeling and a lack of PJAR formation (Figure 4.12).

Wortmannin reduced LIMK and cofilin activity and inhibited F-actin remodeling and PJAR formation. We hypothesized this would reduce the amount of actin-TJ protein bond rupture and result in increased paracellular barrier strength in stretched AEC monolayers. Wortmannin (10 nM 60 min) increased the amount of occludin (dotted bars, Figure 4.10-A) in monolayers stretched for 10 min at 37% $\Delta S_A = \frac{1}{4}$ Hz. Occludin (65 kDa) was not different in wortmannin treated stretched monolayers when compared to UNS-VC. Occludin (50 kDa) was increased in wortmannin treated stretched monolayers when compared to stretched-VC. Similarly, wortmannin decreased area stained (nArea) by fluorescent tracer BODIPY-ouabain, hypothesized to be a measure of AEC monolayer permeability. Monolayers stretched for 10 and 60 min of 25% $\Delta S_A = \frac{1}{4}$ Hz or 10 min of 37% $\Delta S_A = \frac{1}{4}$ Hz showed reduced nArea, no longer different compared to UNS-VC, in monolayers treated with 10 nM or 100 nM wortmannin (striped bars) when compared to time-matched stretched-VC (Figure 4.13). Monolayers stretched for 60 min of 37% $\Delta S_A = \frac{1}{4}$ Hz showed reduced nArea when treated with 100 nM wortmannin. Thus, we find that 10 nM wortmannin attenuated the stretch-induced decrease in occludin content as well as modulated the stretch-induced increase in monolayer permeability.
Figure 4.13: Normalized area stained by fluorescent tracer BODIPY-ouabain (nArea), hypothesized to be a measure of AEC monolayer paracellular permeability, was plotted as a function of stretch magnitude, time, and treatment. nArea was higher in monolayers stretched for 10 and 60 min at 25% (light grey bars, middle) and 37% (dark grey, right) ∆SA ¼ Hz when compared to unstretched (UNS) monolayers. Unstretched monolayers (left) were unaffected by incubation with exogenous PIP3 (light crosshatch bars, 5 µM 60 min), PI3K inhibitor wortmannin (striped bars, 10 and 100 nM 60 min), Rac1 inhibitor EHT-1864 (dark dotted bars, 10 µM 60 min), and PAK-1 inhibitor IPA-3 (not shown) when compared to vehicle control (VC). nArea was attenuated by wortmannin in monolayers stretched for 10 and 60 min to 25% and 37% ∆SA ¼ Hz when compared to stretched-VC monolayers (except in monolayers with 10 nM wortmannin stretched 60 min to 37% ∆SA ¼ Hz). nArea was attenuated by IPA-3 in monolayers stretched for 60 min to 25% ∆SA ¼ Hz (dark crosshatch bar) and by EHT-1864 in monolayers stretched for 10 and 60 min to 37% ∆SA ¼ Hz when compared to stretched VC monolayers. Furthermore, nArea in these three stretched-treated groups was not significantly different when compared to UNS-VC (except in monolayers with wortmannin stretched for 60 min at 37% ∆SA ¼ Hz). nArea was increased by PIP3 in monolayers stretched for 10 min to 37% ∆SA ¼ Hz when compared to stretched-VC monolayers. Each data point is mean ± SE based on 3 images/well from 3 wells/animal from at least 3 animals/group. The area percentage of each image was found by dividing the number of BODIPY-ouabain stained pixels above threshold intensity (maximum pixel intensity of background in all unstretched-untreated monolayers) by the total number of image pixels. Normalized area percentage (nArea) of each image was found by dividing area percentage by the average area percentage of the unstretched-untreated group. ## P<0.01 and # P<0.05 vs. UNS-VC, ** P<0.01 and * P<0.05 vs. stretch-VC.

We hypothesized that inhibition of PI3K with wortmannin during stretch would result in decreased Akt phosphorylation and increase cell death. We found Akt
phosphorylation (*crosshatch bars*) is decreased in unstretched (UNS) and stretched monolayers treated with 10 nM or 100 nM wortmannin (Figure 4.10-A and B). Akt phosphorylation in stretched monolayers treated with 10 nM wortmannin showed no difference when compared to UNS-VC monolayers. Thus we find that wortmannin decreases Akt phosphorylation in both UNS and stretched monolayers. Likewise, we found reduced phosphorylation of the Akt mediated Ser71 site on Rac1. Wortmannin (10 nM and 100 nM) reduced Rac1 phosphorylation (*striped bars*) in both UNS monolayers and monolayers stretched for 10 min at 37% ∆SA ¼ Hz (Figure 4.10-A and B). While wortmannin (10 nM and 100 nM) did decrease Akt phosphorylation, it did not increase cell death (% dead) as hypothesized in either UNS monolayers or those stretched for 10 and 60 min to 37% ∆SA ¼ Hz (*striped bars*, Figure 4.9).

**Rac1 inhibitor EHT-1864 strengthens monolayer barrier properties and attenuates PJAR formation during stretch but increases cell death**

Previously, EHT-1864 was shown to inhibit Rac1 activity in other cell types (48, 122, 141). We hypothesized that Rac1 inhibition with EHT-1864 would attenuate actin remodeling and PJAR formation, decrease activity of Akt and LIMK½, and result in increased barrier properties in stretched AEC monolayers. Unstretched (UNS) monolayers treated with EHT-1864 showed decreased Rac1 activity (*dark grey bars*) when compared to vehicle control (VC) monolayers (Figure 4.14). While stretch increased Rac1 activity in VC monolayers, treatment with EHT-1864 significantly decreased Rac1 activity when compared to stretched-VC monolayers. LIMK½ phosphorylation data supported Rac1 activation data. Unstretched monolayers treated
EHT-1864 showed decreased LIMK½ phosphorylation (light grey bars) when compared to VC (Figure 4.14). Similarly, while VC monolayers stretched for 10 min at 37% ΔSA ¼ Hz showed increased LIMK½ phosphorylation, stretched monolayers treated with EHT-1864 were not significantly different when compared to UNS-VC monolayers. Finally, EHT-1864 did not affect SSH1L phosphorylation (checker bars) in both UNS and stretched monolayers (Figure 4.14). Thus, as hypothesized EHT-1864 reduces Rac1 and LIMK½ activity in both stretched and UNS monolayers.
Figure 4.14: Effect of Rac1 inhibitor EHT-1864 (10 µM 60 min) on protein phosphorylation in stretched AEC monolayers. Rac1 activity (Rac1-GTP, dark grey bars), Rac1 phosphorylation (Ser71, striped bar), and LIMK½ phosphorylation (Thr508/5, light grey bar) in unstretched monolayers was decreased when incubated with EHT-1864 compared to unstretched vehicle control (UNS-VC). Rac1 activity in stretched monolayers, while increased in UNT monolayers, was attenuated with inhibitor EHT-1864 when compared to VC monolayers. Akt phosphorylation (Ser473 and Thr308, crosshatch bars) in stretched monolayers was no different from UNS-VC in monolayers with EHT-1864. Rac1 phosphorylation in stretched monolayers was no different from UNS-VC in monolayers with EHT-1864. LIMK½ phosphorylation in stretched monolayers was no different from UNS-VC in monolayers with EHT-1864. Radioclinin (Ser978, checker bars) was unchanged in stretched and UNS monolayers. Occludin (65 and 50 kDa, dotted bars) in unstretched monolayers was unchanged with EHT-1864 compared to VC. Occludin in stretched monolayers was no different compared to UNS-VC in monolayers with EHT-1864 (65 kDa only). Normalized (n, to UNS monolayers) pull-down GTP-Rac1 signal divided by n-total-Rac1 signal was used as a measure of Rac1 activity. n-phosphorylated signal divided by n-total signal was used as a measure of Akt, Rac1, LIMK½, and SSH1L phosphorylation. n-occludin signal divided by n-GAPDH signal was reported for both 65 kDa and 50 kDa molecular weight occludin. Typical western blot signals shown below groups. Data is mean ± SE from at least 3 animals/group with # P<0.05 and ## P<0.01 vs. UNS-VC and * P<0.05 and ** P<0.01 vs. 37%-VC.
Table 4.7: Summary of one, two, and three-way ANOVA results showing the effects of stretch magnitude and stretch duration for EHT-1864 treatment, and the interaction of these effects on monolayer permeability and protein phosphorylation readouts. X = P < 0.05, - = no effect, and n/a = not applicable.

Quantitative metrics corroborate well with EHT-1864 induced inhibition of Rac and LIMK½, showing reduced PJAR intensity (Pᵢ) in monolayers stretched for 10 and 60 min at 37% ∆SA ¼ Hz and treated with EHT-1864 (dark dotted bars) when compared to monolayers stretched at the same magnitude and time with VC (dark grey bars, Figure 4.15). Pᵢ in EHT-1864 treated stretched monolayers, however, was still significantly higher when compared to UNS-VC monolayers.

Qualitative F-actin images correlate well with quantitative metrics. Monolayers stretched for 10 min of 37% ∆SA ¼ Hz with VC (middle row left column) displayed actin remodeling and PJAR formation while monolayers stretched at the same magnitude and time incubated with EHT-1864 (middle row middle column) showed significantly reduced PJAR formation with discrete areas of peripherally located stress fibers and overall homogeneous F-actin staining (Figure 4.12).
Figure 4.15: PJAR intensity ($P_i$) was found by taking the ratio of peripheral annulus F-actin mean intensity to whole cell F-actin mean intensity ($P_i = A_i / W_i$) and plotted as a function of stretch time and treatment. $P_i$ was significantly higher than unstretched (UNS) monolayers in monolayers stretched for 10 and 60 min at 37% (dark grey) $\Delta$SA ¼ Hz. $P_i$ was attenuated by treatment with the Rac1 inhibitor EHT-1864 (dark spotted bars, 10 µM 60 min) in stretched monolayers when compared to stretched vehicle control (VC) monolayers. Though, stretched monolayers treated with EHT-1864 were still significantly higher than UNS-VC monolayers. Data shown is mean ± SE with ## $P<0.01$ and # $P<0.05$ compared to UNS and * $P<0.05$ compared to stretch-VC. Each data point based on an average 32 cells per animal from at least 4 different animals. The perijunctional F-actin fluorescent intensity of each cell was analyzed by tracing the peripheral ZO-1 (top inset), superimposing this ZO-1 trace onto the same cell stained for F-actin (bottom inset), and enlarging the trace line thickness radially inward and outward to create a boundary zone for further analysis approximately 1.6 µm thick (white contours). Mean F-actin fluorescent intensity in this peripheral annulus ($A_i$) was measured. Whole cell F-actin mean fluorescent intensity ($W_i$) was determined, including annulus and cell interior. PJAR intensity ($P_i$) was found by taking the ratio of peripheral annulus mean intensity to whole cell mean intensity ($P_i = A_i / W_i$). $W_i$ was found to be constant across all groups compared to unstretched (UNS) (data not shown). Bar = 10 µm.

We hypothesized that reduced Rac1 and LIMK activity, coupled with attenuated PJAR formation, would strengthen monolayer barrier properties in stretched monolayers treated with EHT-1864. As hypothesized, normalized area stained (nArea) by BODIPY-ouabain, hypothesized to be a measure of paracellular permeability, in monolayers treated
with EHT-1864 and stretched for 10 and 60 min of 37% ΔSA ¼ Hz was significantly lower when compared to stretched-VC monolayers and was not different from UNS-VC monolayers (Figure 4.13). Thus treatment with EHT-1864 prevented stretch-induced permeability increases. Occludin content (dotted bars) in stretched monolayers treated with EHT-1864 showed no change in the 50 kDa form when compared to stretched-VC monolayers (Figure 4.14). However, the 65 kDa form of occludin did increase in stretched monolayers treated with EHT-1864, becoming insignificantly different from UNS-VC monolayers. Thus, EHT-1864 rescues the 65 kDa form of occludin in stretched monolayers.

We expected that inhibition of Rac1 with EHT-1864 would decrease Akt phosphorylation and result in increased stretch-induced cell death. Akt phosphorylation data agreed with Rac1 activity data. While monolayers stretched for 10 min to 37% ΔSA ¼ Hz showed increased Akt phosphorylation (crosshatch bars, Ser473 and Thr308), monolayers stretched at the same magnitude and time treated with EHT-1864 showed Akt phosphorylation that was no different when compared to UNS-VC monolayers (Figure 4.14). Thus, EHT-1864 modulates the stretch-induced phosphorylation of Akt to unstretched levels. Similarly, the Ser71 phosphorylation site on Rac1 showed similar reduction with EHT-1864. Unstretched monolayers treated with EHT-1864 showed a reduction in Rac1 phosphorylation (striped bars) when compared to VC monolayers (Figure 4.14). Monolayers stretched at 10 min to 37% ΔSA ¼ Hz with VC increased Rac1 phosphorylation, while monolayers stretched at the same magnitude and time treated with ETH-1864 showed no difference in Rac1 phosphorylation when compared to UNS-VC monolayers.
As hypothesized, UNS monolayers treated with EHT-1864 showed increased cell death (inset, dark dotted bars) when compared to VC monolayers (Figure 4.9). In addition, monolayers stretched for 10 and 60 min of 37% ∆SA ½ Hz and treated with EHT-1864 showed increased cell death when compared to their respective stretched VC monolayers. Thus, EHT-1864 decreases Akt phosphorylation (activity) and increases stretch-induced cell death in AEC monolayers.

Rac1 agonist PDGF activates LIMK½ and coflin and forms PJAR in UNS monolayers

Platelet-derived growth factor (PDGF), previously shown to activate endogenous Rac1 (132, 134, 141), was used as a positive control for stretch in AEC monolayers. We hypothesized that exogenous PDGF would increase LIMK½ and coflin activity, and result in actin remodeling. Stretched and UNS monolayers treated with PDGF showed increased LIMK½ phosphorylation (light grey bars) when compared to VC monolayers (Figure 4.16). Likewise, while UNS monolayers show no difference in coflin phosphorylation (white bars) with PDGF treatment, monolayers stretched for 10 min to 37% ∆SA ½ Hz and treated with PDGF showed decreased coflin phosphorylation when compared to stretched-VC monolayers (Figure 4.16). Interestingly, SSH1L phosphorylation (checker bars) increased in both stretched and UNS monolayers treated with PDGF when compared to VC monolayers (Figure 4.16). Thus we find that PDGF treatment enhanced LIMK½ and coflin activity as hypothesized in unstretched and stretched monolayers.
Figure 4.16: Effect of Rac1 agonist platelet derived growth factor (PDGF, 10 ng/ml 15 min) on protein phosphorylation in stretched AEC monolayers. Akt (Ser473 and Thr308, crosshatch bars), Rac1 (Ser71, striped bar), LIMK½ (Thr508/5, light grey bar), and SSH1L (Ser978, checker bar) phosphorylation in unstretched monolayers was increased with PDGF compared to vehicle control (VC). Rac1 phosphorylation in stretched monolayers was unchanged with PDGF. LIMK½ phosphorylation in stretched monolayers was unchanged with PDGF. Cofilin phosphorylation (Ser3, white bars) in unstretched monolayers was unchanged with PDGF when compared to VC. Cofilin phosphorylation in stretched monolayers was decreased in monolayers with PDGF. SSH1L phosphorylation (Ser978, checker bars) in unstretched and stretched monolayers was increased with PDGF. Normalized (n, to UNS monolayers) phosphorylated signal divided by n-total signal was used as a measure of Akt, Rac1, LIMK½, and SSH1L phosphorylation. Typical western blot signals shown below groups. Data is mean ± SE from at least 3 animals/group with # p<0.05 vs. UNS-VC and ## p<0.01 vs. 37%-VC.
Table 4.8: Summary of one and two-way ANOVA results showing the effects of stretch magnitude and stretch duration for PDGF treatment, and the interaction of these effects on protein phosphorylation readouts. X = P < 0.05, - = no effect, and n/a = not applicable.

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<td>-</td>
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Qualitative F-actin images supported LIMK½ and cofilin data. UNS monolayers (top second column) and monolayers stretched for 10 min at 37% ∆SA ¼ Hz (bottom second column) treated with PDGF both show F-actin in a PJAR structure, similar to stretched monolayers without treatment (Figure 4.21). Thus, PDGF remolds actin into PJAR in UNS monolayers.

We hypothesized that because Akt phosphorylation increased, that exogenous PDGF would increase Akt activity. Indeed, Akt phosphorylation (both Ser473 and Thr308, crosshatch bars) in UNS monolayers treated with PDGF is increased when compared to VC monolayers (Figure 4.16). Rac1 phosphorylation increased at the Ser71 site (striped bars) with PDGF treatment in both UNS and stretched monolayers (Figure 4.16). Interestingly, while Akt phosphorylation was increased with PDGF, it did not result in a reduction of cell death. Percent death (% dead) did not change in UNS or stretched monolayers treated with PDGF (light grey bars) when compared to respective VC monolayers (white, Figure 4.9). Thus, while PDGF does increase Akt phosphorylation, it does not result in decreased cell death in stretched monolayers.
PAK-1 inhibitor IPA-3 strengthens monolayer barrier properties and attenuates PJAR formation during stretch

PAK-1 (p21-activated kinase), previously shown to activate LIMK½ by phosphorylation at Thr508/5 (40, 53, 101, 119, 145), can be inhibited with IPA-3 (16, 43). We hypothesized that inhibition of PAK-1 would decrease LIMK½ phosphorylation (inactivate) and increase cofilin phosphorylation (inactivate), resulting in decreased actin remodeling and strengthened paracellular barrier properties during stretch. Phosphorylation of LIMK½ (light grey bars) was decreased in UNS monolayers treated with IPA-3 when compared to VC monolayers (Figure 4.17). Surprisingly, monolayers stretched for 10 min at 25% ∆SA ¼ Hz with VC show decreased cofilin phosphorylation (white bars), while stretched monolayers treated with IPA-3 were not different from UNS-VC monolayers (Figure 4.17).
Figure 4.17: Effect of PAK-1 inhibitor IPA-3 (10 µM 60 min) on protein phosphorylation in stretched AEC monolayers. LIMK½ phosphorylation (Thr508/5, light grey) in unstretched monolayers was decreased with IPA-3. Cofilin phosphorylation (Ser3, white bars) in unstretched monolayers was unchanged with IPA-3 when compared to VC. Cofilin phosphorylation in stretched monolayers with IPA-3 was not different compared to UNS-VC monolayers. Normalized (n, to UNS monolayers) phosphorylated signal divided by n-total signal was used as a measure of LIMK½ and cofilin phosphorylation. Typical western blot signals shown below groups. Data is mean ± SE from at least 3 animals/group with # P<0.05 and ## P<0.01 vs. UNS-VC and * P<0.05 and ** P<0.01 vs. 37%-VC.

Table 4.9: Summary of one and two-way ANOVA results showing the effects of stretch magnitude and stretch duration for IPA-3 treatment, and the interaction of these effects on monolayer permeability and protein phosphorylation readouts. X = P < 0.05, - = no effect, and n/a = not applicable.
Quantitative PJAR intensity ($P_i$) data of monolayers treated with IPA-3 support LIMK$^{1/2}$ and cofilin data, showing significant PJAR intensity in monolayers stretched for 10 min at 25% (light grey) and 37% (dark grey) $\Delta$SA $1/4$ Hz but not in monolayers stretched at the same magnitudes and treated with 10 $\mu$M IPA-3 60 min (light crosshatch bars, Figure 4.11). Thus, treatment with IPA-3 reduces actin remodeling into PJAR. Qualitative images demonstrate that monolayers stretched for 10 min at 25% $\Delta$SA $1/4$ Hz (bottom left) show F-actin remodeling to form PJARs while monolayers stretched at the same magnitude and time treated with IPA-3 (bottom right) show significantly reduced PJAR formation while retaining perinuclear stress fibers when compared to VC monolayers (Figure 4.12). Similarly, monolayers stretched for 10 min to 37% $\Delta$SA $1/4$ Hz with VC (bottom left) show PJAR formation (red) while monolayers stretched at the same magnitude and time treated with IPA-3 (bottom right) show inhibited PJAR formation (Figure 4.18 and 4.19).
Previously we showed that phosphorylated LIMK½ (green, Figure 4.18) and phosphorylated cofilin (green, Figure 4.19) shifted away from the perijunction in monolayers that were stretched for 10 min at 37% ΔSA ¼ Hz, when compared to UNS monolayers where both proteins were found throughout the cells. Treatment with IPA-3 in stretched monolayers abolishes this polarization of phosphorylated LIMK½ (bottom
right, Figure 4.18) and phosphorylated cofilin (bottom right, Figure 4.19), resulting in a distribution of protein that resembles UNS monolayers without actin remodeling.

We hypothesized that inhibition of PJAR formation with IPA-3 in stretched monolayers would increase paracellular barrier properties and reduce the stretch-induced
increase in BODIPY-ouabain fluorescent tracer flux, hypothesized to be a measure of paracellular permeability. Monolayers stretched for 60 min at 25% ΔSA ¼ Hz and treated with IPA-3 (light grey crosshatch bar) showed reduced BODIPY-ouabain staining when compared to stretched-VC monolayers (light grey bar, Figure 4.13). Thus, IPA-3 reduced the monolayer permeability of BODIPY-ouabain in stretched monolayers.

PJAR is formed in unstretched monolayers treated with calyculin-A

Calyculin-A, an antagonist of type 1 and 2A protein phosphatases (36, 80) and previously shown to dephosphorylate cofilin (activate) in other cell types (75, 121, 175) and demonstrate no strengthening effect on monolayer permeability (Chapter 3), was used in AEC monolayers as a positive control for cofilin activation. We used calyculin-A to test the effect of increased cofilin phosphorylation on actin remodeling and PJAR formation. We hypothesized that cofilin activity would increase (phosphorylation decrease) in monolayers treated with calyculin-A and would result in PJAR formation. In accordance with our hypothesis, we found cofilin phosphorylation (white bars) greatly decreased in both UNS and stretched monolayers treated with calyculin-A when compared to respective VC monolayers (Figure 4.20).
Figure 4.20: Effect of type1 and 2A protein phosphatase (PP1 and PP2A) inhibitor calyculin-A (10 nM 30 min) on coflin protein phosphorylation in AEC monolayers. Cofilin phosphorylation (Ser3, white bars) in unstretched monolayers was decreased with calyculin-A when compared to vehicle control (VC). Cofilin phosphorylation in stretched monolayers was decreased in monolayers with calyculin-A when compared to stretched VC monolayers. Normalized (p, to UNS monolayers) phosphorylated signal divided by n-total signal was used as a measure coflin phosphorylation. Typical western blot signals shown to the right. Data is mean ± SE from at least 3 animals/group with ## P<0.01 vs. UNS-VC and ** P<0.01 vs. 37%-VC.

Table 4.10: Summary of two-way ANOVA results showing the effects of stretch magnitude for calyculin-A treatment, and the interaction of these effects on protein phosphorylation readout. X = P < 0.05, - = no effect, and n/a = not applicable.

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Qualitative F-actin staining data agrees with coflin phosphorylation data. Monolayers left UNS (top left) as well as stretched monolayers (bottom left) treated with calyculin-A showed PJAR formation (Figure 4.21). Thus, treatment with calyculin-A increased coflin activity (decreases phosphorylation) and results in actin remodeling and PJAR formation, even in UNS monolayers.
Figure 4.21: Effect of pathway inhibition on F-actin. Type I like rat AEC monolayers with phalloidin labeled F-actin left unstretched (UNS, top row) or after 10 min of 37% or 25% ΔSA ¼ Hz stretch (bottom row) with type 1 and 2A protein phosphatase antagonist calyculin-A (left column, 10 nM 2 hrs), Rac1 agonist platelet-derived growth factor (PDGF, second column, 10 ng/ml 15 min), ATP depletion (60 min of 2 mM 2-deoxy-D-glucose + 10 µM antimycin A, third column), or exogenous PIP3 (right column, 5 µM 60 min). Perijunctional F-actin ring forms in both UNS and stretched monolayers incubated with calyculin-A and PDGF. F-actin structure remains unvarying in stretched monolayers with ATP depletion, showing little or no remodeling or PJAR formation with stretch. Exogenous PIP3 does not affect F-actin in UNS monolayers and likewise does not affect stretch-induced PJAR formation. Bar = 10µm.

PJAR is inhibited in stretched monolayers with ATP depletion

Previously we have shown that the Rac1 pathway, including LIMK and coflin, are involved in stretch-induced PJAR formation. We hypothesized that ATP was required for pathway activation and subsequent PJAR formation. We used 2 mM 2-deoxy-D-glucose + 10 µM antimycin A for 60 min to deplete ATP as a negative control, in monolayers with and without 25% ΔSA ¼ Hz stretch (third column) stained for F-actin (red, Figure 4.21). In accordance with our hypothesis, ATP depletion inhibited actin remodeling and PJAR formation in stretched AEC monolayers. Thus, PJAR formation during stretch is ATP dependent.
RESULTS

Discussion

In summary, we have determined that the Rac1/Akt pathway effects actin remodeling, monolayer permeability, and cell death in stretched AEC monolayers. We have shown that actin remodeling into PJAR during stretch correlates with increased monolayer permeability. Attenuation of PJAR formation by inhibition of PI3K with wortmannin, Rac1 with EHT-1864, or PAK-1 with IPA-3 preserved AEC monolayer barrier strength during stretch. Likewise, treatment with exogenous PIP3, which activated the Rac1 pathway, resulted in further increased monolayer permeability in stretched monolayers. There is evidence that cell death correlates with PJAR formation in stretched AEC monolayers. PJAR formation is accompanied by increased cell death in stretched monolayers. Inhibition of PJAR formation with EHT-1864 resulted in increased cell death while Rac1/Akt pathway activation with exogenous PIP3 resulted in decreased cell death in both unstretched and stretched AEC monolayers. However, attenuation of PJAR with wortmannin did not effect cell death in unstretched and stretched monolayers. In conclusion, stretch produces actin remodeling into PJAR and increased monolayer permeability through the activation of the Rac1/Akt pathway. Inhibition of the Rac1/Akt pathway resulted in an attenuation of PJAR formation and preserved barrier properties but increased cell death in stretched AEC monolayers.

Methodological limitations

While we find a rapid injury response with high magnitude biaxial stretch, it is not yet known if these responses manifest at longer stretch durations (> 60 min) or if
underlying pathway attenuation, if possible at longer durations, results in similar injury attenuation. Likewise, while we have shown that short duration high magnitude stretch, which elicits a cellular response that includes rapid cytoskeletal remodeling, is deleterious to TJ mediated paracellular permeability, it is not yet known if this rapid response is beneficial to monolayers with long duration stretch. Inhibition of Rac1 pathways to inhibit actin remodeling and attenuate increased monolayer permeability in stretch monolayers, while helpful during short duration stretch, may prove disastrous for barrier function or monolayer viability past 60 min of stretch. However, we speculate that by attenuating the rapid injury mechanisms we also suppress chronically accelerating feedback injury mechanisms and promote intrinsic injury mediation and long-term survival of AEC monolayers.

Monolayer paracellular permeability quantification using fluorescent tracer BODIPY-ouabain has inherent limitations. First, this method it is not a direct measure of permeability, but a quantitative measure of tracer binding to the basolateral surface of barrier-compromised AEC monolayers. Ouabain binding to the basolateral transmembrane Na\(^+\)-K\(^+\)-ATPase of barrier compromised AECs may affect monolayers in longer duration stretch experiments. Secondly, it is not known if cell adhesion has an effect on tracer binding. Thirdly, while quantification of whole-monolayer permeability is an important indicator of AEC barrier strength, it would be helpful to have the ability to quantify the specific contribution of TJ-mediated paracellular permeability in stretched monolayer. Furthermore, Cavanaugh et al. utilized the same experimental method with slight differences and demonstrated that 60 min of 37% ∆SA ¼ Hz stretch, but not 25% or 12%, significantly increased BODIPY-ouabain staining in AEC monolayers (26) and
noted these data corroborate well with normal physiologic functionality. Our data corroborates with this data except in monolayers stretched at 25% ∆SA which we found to demonstrate a significant increase in BODIPY-ouabain staining. We speculate the differences are attributed to the slight changes made in the experimental method: We utilized a slightly higher threshold estimation by setting the threshold at maximum background intensity instead of mean background intensity and a 10× objective was used to image BODIPY-ouabain staining instead of a 20× in order to incorporate a higher cell population in the analysis. These changes were made to minimize intra-sample variance. We speculate that permeability was increased in monolayers stretched to 25% ∆SA (shown previously to be unchanged) due to differences between physiologic systems and the in vitro model, which lacks an array of alveolar cell types, comparable ECM and architecture, and other paracrine mechanisms that may facilitate barrier functionality in ATI cells.

Western blot, used to quantify the amount of phosphorylated and total protein concentrations, are limited in a few ways. Quantification is limited by antibody availability, known phosphorylation sites on the target protein, and previous data on functionality of phosphorylation sites. Furthermore, we were only able to quantify total protein lysate from whole monolayers. This represents the ensemble of chemical signaling of a few million cells and thus lacks local, cell to cell signaling. Additionally, cells can employ spatial compartmentalization as well as subcellular binding to direct biochemical signaling. Finally, numerous other signaling proteins may contribute to Rac1/Akt pathway regulation during stretch (10, 34, 46, 58, 61, 71, 96, 110, 119, 145, 160).
Rac1, Akt, LIMK½, and cofilin activity increased with stretch

Consistent with our hypothesis, stretch-induced PJAR formation was mediated by an increase in activity of Rac1, Akt, LIMK½, and cofilin in stretched AEC monolayers. Specifically, stretch increased activity of Rac1 in stretched AEC monolayers. Others have shown increased Rac1-GTP with stretch in other cell types (98, 105). Swiss 3T3 fibroblasts injected with Rac (600 µg/ml 15min) or PDGF (5 ng/ml 15 min) show peripheral accumulation of F-actin (PJAR) and peripherally located vinculin (116). Rac was found to induce focal adhesion complexes at the leading edge of the cells (116) and stimulate membrane ruffling and actin cytoskeleton reorganization (134). Counter to our findings, Desai et al. found decreased Rac1 activity in stretched cells, though demonstrated in alveolar epithelial type II cells at longer stretch durations (2 hrs) (47). In conclusion, our data showing that Rac1 is activated in stretched AEC monolayers, accompanied by actin cytoskeleton remodeling to form PJARs, agree with a majority of previous studies in other cell types.

LIMK½, downstream of Rac1, correlated well with Rac1 data showing activation in stretched AEC monolayers. Previously, activated LIMK was shown to phosphorylate cofilin, lead to an accumulation of actin filaments (8, 143), form PJAR (173), and stabilize F-actin structures (8) in a Rac dependent manner (8, 53, 173). Dominant-negative LIMK1 inhibited accumulation of F-actin in COS-7 cells (8) while inactive LIMK suppressed Rac induced lamellipodium and PJAR formation (173). Also similar to our LIMK½ immunostaining data, others have shown that LIMK2 was found in punctae that resemble endosomes (1) while it has also been shown that LIMK1 localized to focal adhesion sites in many primary cells and cell lines (1). Interestingly, whole-cell
cofilin became dephosphorylated (activated) with AEC stretch. These results agree with data showing stretch induced redistribution to form PJAR (50) which we speculate would require actin depolymerization. Similar to LIMK localization and immunostaining results in AEC monolayers, ADF/cofilin was found in small localized points that resemble endosomes (161). Previously, capsaicin and ionomycin were shown to activate (dephosphorylate) cofilin by 30 min in Caco-2 cells, resulting in actin filament accumulation at the cell-cell junction (112). Others have shown that Rho-GTPase pathway activation can inhibit cofilin depolymerization activity during stress fiber formation (101) while previously we have shown a reduction in Rho kinase (ROCK) and downstream myosin light chain (MLC) in stretched monolayers (Chapter 3). Additionally, capsaicin reduced phosphorylated MLC (113) and may be regulated through a pathway that is similar to stretch-induced cofilin activation. Alternatively however, a separate investigation reported that LIMK2 stimulated cofilin phosphorylation was mediated by Rho and Cdc42, but not Rac in COS-7 cells (146). However, Rac, but not Rho, was implicated in F-actin uncapping in resting platelets (72). Finally, cofilin activity was shown to be required for lamellipodia growth (77). In conclusion, our data showing Rac1, LIMK½, and cofilin activation in stretched AEC monolayers, accompanied by actin cytoskeleton remodeling to form PJARs, supports previously published studies in other cell types.

Stretch-induced increased monolayer permeability corroborates well with occludin data showing decreased occludin content (both 50 and 65 kDa) in stretched monolayers. Likewise, AEC monolayers stretched at longer durations (60 min) at 25% ∆SA ¼ Hz (27) or 37% ∆SA ¼ Hz (27, 38) also show loss of occludin content. An
additional study in Escherichia coli infected intestinal epithelial cells showed an increase in TJ permeability accompanied by decreased 65 kDa occludin and increased 50 kDa occludin (142). Loss of occludin has been correlated with decreased tight junction barrier function (33). Others have shown that occludin resolves as two bands with SDS-PAGE (135, 170) and that Ser/Thr phosphorylated occludin represents the higher molecular weight band (135). Furthermore, phosphorylation appears to be required for its association with the membrane and the tight junction (135, 154, 170) as it has been shown to resists detergent extraction in MDCK cells (170). We speculate that PJAR formation, accompanied by coflin activation, results in actin-occludin bond disassociation and lowered monolayer permeability in stretched AEC monolayers. Free floating occludin may be washed away or diluted in extracellular culture media. We conclude that our results, showing occludin content decreasing rapidly in stretched AEC monolayers, agree with previous studies in other cell types.

Stretch of AEC monolayers did not change the phosphorylation state of slingshot-1L (SSH1L). Others have shown that phosphatases slingshot, PP1, PP2A, PP2B, PP2C, as well as chronophin may be involved in dephosphorylation of coflin at the Ser3 site (6, 104, 149), although slingshot may have higher specificity to mediating coflin (114). Slingshot has been shown to colocalize with F-actin (54) and we speculate that SSH1L may activate coflin by Ser3 dephosphorylation in a subcellular specific manner. Thus, future investigations could focus on additional targets or the subcellular localization of SSH1L to determine the mechanism behind coflin dephosphorylation.

Previously, stretched AEC monolayers exhibited decreased Rho kinase (ROCK) and myosin light chain (MLC) activation (Chapter 3), implicated in decreased cell
adhesion to the extracellular matrix (ECM) in other cell types (12). Additionally, post-injury wound closure in Madin-Darby canine (MDCK) epithelial cell monolayers, similar to the stretch injury model and alveolar wound repair (166), was dependent on Rac- and phosphoinositide-dependent crawling and not by perimarginal actomyosin purse-string (57). Rac1 activation was essential for basement membrane-dependent survival of epiblasts (73). Furthermore, loss of fibroblast attachment led to inactivation of Rac1, a rapid decrease in Akt phosphorylation (Ser473), and activation of the proapoptotic pathway Raf-MEK-Erk (177). Defects in adhesion could be rescued with active Rac1 or PAK-1 expression in fibroblasts (95). Finally, Rac1 was implicated in anoikis, a specific type of apoptosis which is induced by detachment from the ECM in anchorage-dependent cells (177). We speculate that AEC monolayers may activate the Rac1 pathway to inhibit anoikis when stretched to high magnitudes. In the process of Rac1 activation, we hypothesize that rapid cytoskeletal reorganization to form PJAR disrupts TJ-actin bonds and results in increased paracellular permeability. Inhibition of stretch-induced Rac1 activation attenuates stretch-induced increased permeability but did not abolish cell death during stretch whereas treatment with exogenous PIP3 did reduce death in stretched monolayers. Additionally, there is a paucity of data showing if Rac1 inhibition is beneficial for long duration (> 60 min) stretch. Possible future investigations may include longer duration stretch and pharmacological intervention to strengthen monolayer adhesion to the ECM accompanied by Rac1 inhibition to attenuate cytoskeletal remodeling and increase monolayer permeability during stretch.

Akt phosphorylation supports Rac1 activation data, showing increase phosphorylation at both Ser473 and Thr308 sites with stretch of AEC monolayers.
Furthermore, Akt phosphorylation, shown to inhibit apoptosis (19, 20, 25, 59, 60, 176), is magnitude dependent, similar to stretch magnitude dependent PJAR formation and cell death. Others have shown that PI3K-Akt signals may be linked to actin remodeling through p70S6K activation or Girdin/Akt-phosphorylating enhancer (APE) (55, 130). Rac1 phosphorylation at Ser71, shown to be Akt dependent (94), increased with stretch similar to the rapid increase found in endothelial cells (98). However, Rac1 phosphorylation did not attenuate Rac1 activity, as Rac1 net activity was found to increase with AEC stretch. Others have shown that moderate physiological levels of cyclic (6-10% 1 Hz 30 min) stretch of endothelial cells activated Akt (phosphorylated) and BAD and inhibited apoptosis, while higher strain magnitudes (20% 1 Hz) stimulated apoptosis (99). Additionally, Akt activation could be inhibited with wortmannin treatment. Others, however, found decreased Akt phosphorylation (Ser473) in rat alveolar type II cells stretched for 24 hrs (70), recoverable by rapamycin, and decreased PI3K/Akt phosphorylation in cultured type II alveolar epithelial cells exposed to 18% cyclic stretch for 2 hrs (85). We conclude that our results, showing that Akt is phosphorylated rapidly in stretched AEC monolayers, supports a majority of previous studies in other cell types.

Cell death and monolayer permeability increase with increasing stretch

Stretch-induced increased permeability to BODIPY-ouabain agrees well with previous data at longer (60 min) stretch durations (26). Monolayer permeability to the fluorescent tracer increased rapidly in stretched AEC monolayers in a magnitude dependent manner, similar to the rapid formation of PJARs and the movement of actin
cytoskeleton anchored membrane bound microbeads (Chapter 2 and (50)). Previously, stretch of alveolar epithelial cells was shown to result in increased permeability in vitro (144) and speculated to be the underlying cause of pulmonary edema in high magnitude stretch pulmonary injury (157, 163). We concluded that our current results, showing monolayer permeability increases rapidly (< 10 min) in stretched AEC monolayers in a magnitude dependent manner, agree with previous data.

Cell death in stretched monolayers agrees with previous data at longer (60 min) stretch durations (152). Additionally, Tschumperlin et al. showed negligible death due to a single deformation (152) with very little change in cell death between 60 min and 5 hrs of stretch (153). We speculate that cell death is biochemically mediated in stretched AEC monolayers. We conclude that our results, showing stretch-induced cell death occurs rapidly, agree with the findings of longer duration stretch studies.

**Exogenous PIP3 decreases cell death but does not affect actin remodeling or monolayer permeability**

As hypothesized, exogenously applied PIP3, the catalytic product of PI3K (78), increased Rac1 activity and Akt phosphorylation in unstretched AEC monolayers. Previously, PIP3 ectopically implanted into the apical portion of MDCK epithelial cells increased GTP-bound Rac and phosphorylation of Akt by 5 min, both of which could be attenuated with PI3K inhibitor LY294002 (64). Others found that stimulating Akt directly with PIP3 resulted in decreased cell death (177). Furthermore, constitutively active PI3K or Akt was shown to block anoikis (88). As further support for our hypothesis, PIP3 applied exogenously to stretched AECs showed an increase in
monolayer permeability at 10 min of 37% ΔSA ¼ Hz stretch, but no change at 60 min, as measured by the flux of fluorescent tracer BODIPY-ouabain. Interestingly, PIP3 rescued stretch-induced occludin reduction to unstretched levels. We speculate this may be due to decreased cell death with PIP3 during stretch. We conclude that our results, showing Rac1 and Akt are activated with exogenous PIP3, agree with previous studies in other cell types.

PIP3 attenuated cell death in AEC monolayers stretched for 10 and 60 min to 37% ΔSA ¼ Hz, corroborating with data showing increased Akt phosphorylation (activation) and the antiapoptotic affect it has in other cell types (88, 177). While PIP3 did attenuate stretch-induced cell death, it did not result in a net decrease in monolayer permeability. This, coupled with data showing Rac1 activation and increased monolayer permeability in PIP3 treated monolayers, leads us to speculate that Rac1 mediated cytoskeletal reorganization has more of an effect in regulating monolayer barrier properties than does modest monolayer cell death in short (< 10 min) duration stretch. We conclude that our data, showing PIP3 activates the Rac1 pathway in unstretched cells and increases Akt phosphorylation which attenuates cell death during stretch, supports previous work in other cell types.

PI3K inhibitor wortmannin strengthens monolayer barrier properties and inhibits actin remodeling during stretch

As hypothesized, PI3K inhibitor wortmannin attenuated Akt, LIMK½, and cofillin activity in unstretched AEC monolayers. Furthermore, wortmannin attenuated the stretch-induced increase in Akt, LIMK½, and cofillin activity. Previously, wortmannin
was shown to block dephosphorylation (activation) of cofilin in fMLP-stimulated human blood neutrophils (121). Similarly, wortmannin was shown to decrease cofilin activity (increase phosphorylation) in LLC-PK1 cells (79). Finally, wortmannin treatment resulted in a two fold reduction in phosphorylated Akt in peritoneal mesothelial cells (85). While PIP3 application showed an attenuation of cell death in stretched AEC monolayers, wortmannin did not effect cell death in AEC monolayers. Wortmannin did result in a recovery of occludin content in stretched monolayers. Furthermore, wortmannin showed inhibition of stretch-induced actin cytoskeleton remodeling and PJAR formation in both qualitative micrographs and quantitative PJAR intensity evaluation. Monolayer permeability studies using BODIPY-ouabain corroborated with occludin and PJAR intensity data, such that permeability was attenuated with wortmannin treatment in monolayers stretched for 10 and 60 min at both 25% and 37% ∆SA ¼ Hz stretch, though at 60 min 37% ∆SA ¼ Hz staining was attenuated only slightly by treatment with 100 nM wortmannin. Likewise, monolayers treated with 100 nM jasplakinolide for 10 min to stabilize the actin cytoskeleton exhibited a stretch-magnitude dependent decrease in BODIPY-ouabain staining and inhibited PJAR formation (Chapter 2). We conclude that wortmannin inhibits stretch induced PJAR formation by inhibiting Rac1 dependent LIMK½ and cofilin. Future studies should address if LIMK½ and cofilin activity reduces actin remodeling and actin-TJ bond rupture, as a mechanism for our observed increases in barrier properties during stretch.
Rac1 inhibitor EHT-1864 strengthens monolayer barrier properties and attenuates PJAR formation during stretch but increases cell death

As hypothesized, EHT-1864, shown previously to associate with Rac1 and cause a loss of nucleotide (141), decreased Rac1 and LIMK½ activation in both unstretched and stretched AEC monolayers. Similarly, U87-MG cells incubated with 25 µM EHT-1864 for 5 min showed greatly reduced GTP-bound Rac1 (48). Similar to wortmannin data, treatment with EHT-1864 attenuated both the stretch-induced PJAR formation and increase in monolayer permeability, corroborating with Rac1 and LIMK½ activity data. While PJAR formation was attenuated in stretched monolayers treated with EHT-1864, it was still significantly higher when compared to unstretched monolayers. Unlike treatment with wortmannin however, EHT-1864 treatment did not recover stretch-induced loss of tight junction protein occludin back to unstretched levels. Previously, treatment with PIP3 was found to increase occludin content and decrease cell death in stretched monolayers. Together, with data showing increased cell death with EHT-1864, we speculate that occludin may be decreased due to cell death in stretched monolayers. Finally, EHT-1864 did not affect SSH1L phosphorylation in stretched monolayers or monolayers left unstretched.

Monolayers treated with EHT-1864 showed a reduction in Rac1 activity in unstretched and stretched monolayers. Stretched monolayers incubated with EHT-1864 showed a concurrent reduction in Akt phosphorylation. Similarly, Rac1 phosphorylation at Ser71 was reduced in unstretched and stretched monolayers treated with EHT-1864. Interestingly, cell death was increased in both unstretched and stretched monolayers treated with EHT-1864 when compared to their respective vehicle controls, evidence that
Rac1 has a protective effect. We speculate that decreased Rac1 activation reduces Akt phosphorylation and its antiapoptotic mechanism. Likewise, others have shown increased cell death with decreased Rac1 activation by EHT-1864 (177), increased apoptosis with decreased PI3K or Akt activity (88), or increased anoikis with Rac1 inhibition (95). Similarly, Rac1 activation was shown to promote COS 7 cell survival by activation of PI3K and Akt (110). Interestingly, a mutation in ezrin, a protein that crosslinks the actin cytoskeleton to the plasma membrane, has been shown to induce cell apoptosis by impairing Akt activation (36), providing evidence that actin cytoskeletal remodeling may interact with Akt activation through Ezrin. We conclude that our results, showing inhibition of Rac1 and Akt activation with EHT-1864 results in increased cell death in unstretched and stretched AEC monolayers, agree with previous studies in other cell types.

**Rac1 agonist PDGF activates LIMK½ and cofilin and forms PJAR in UNS monolayers**

As hypothesized, unstretched monolayers treated with platelet-derived growth factor (PDGF) mimicked changes found in stretched monolayers. PDGF increased Akt phosphorylation in unstretched monolayers and likewise increased Akt dependent Rac1 phosphorylation at Ser71 in unstretched and stretched monolayers. However, increased Akt phosphorylation did not result in decreased cell death in monolayers treated with PDGF with and without stretch. Treatment with PDGF did increase LIMK½ as well as SSH1L phosphorylation in both unstretched and stretched AEC monolayers. While PDGF did not increase cofilin activation in unstretched monolayers, it did increase activation in stretched monolayers, further decreasing phosphorylation when compared to
stretched vehicle control monolayers. Finally, PDGF induced PJAR formation in both unstretched and stretched monolayers. Previously, PDGF treated Swiss 3T3 cells exhibited increased stress fiber stimulation (133, 134). Similarly, PJAR was found in unstretched cells treated with PDGF (116). Likewise, Saos2 cells (28) and smooth muscle cells (86) treated with PDGF show increased Akt phosphorylation and activation. We conclude that, similar to the stretch-induced response, exogenously applied PDGF activates Akt, LIMK½, and cofilin, and promotes actin remodeling and PJAR formation in unstretched AEC monolayers, which is consistent with findings in other cell types.

**PAK-1 inhibitor IPA-3 strengthens monolayer barrier properties and attenuates PJAR formation during stretch**

Similar to PI3K inhibition with wortmannin and Rac1 inhibition with EHT-1864, inhibition of PAK-1 with IPA-3 showed a protective effect with Rac1 pathway inhibition during stretch. Treatment with IPA-3 decreased LIMK½ phosphorylation in unstretched monolayers. Monolayers stretched for 10 min to 25% and 37% ∆SA ¼ Hz exhibited PJAR formation which could be inhibited with IPA-3 pretreatment. Similarly, IPA-3 treatment attenuated stretch-induced increase in monolayer permeability. We conclude that PAK-1 inhibition with IPA-3 resulted in decreased LIMK½ phosphorylation in unstretched monolayers. Furthermore, PAK-1 inhibition attenuates the PJAR formation and increased monolayer permeability found in stretched AEC monolayers.

While active LIMK has been shown to deactivate cofilin by phosphorylation, it is not yet understood why whole-cell LIMK activation is accompanied by whole-cell cofilin activation, shown here in stretched AEC monolayers and previously in other cell types.
Unstretched monolayers immunostained for phosphorylated-LIMK½ and phosphorylated-cofilin exhibit homogeneous punctuate distribution of both proteins, similar to previous (1, 161). With high magnitude biaxial stretch we see active (phosphorylated) LIMK½ and inactive (phosphorylated) cofilin colocalize to the perinuclear region, an increase in whole-cell LIMK½ phosphorylation and a decrease (activation) in whole-cell phosphorylated cofilin, and PJAR formation. We see the same changes in LIMK½ and cofilin phosphorylation as well as PJAR formation in unstretched monolayers treated with Rac1 agonist PDGF or PP1/PP2A phosphatase inhibitor calyculin-A. Furthermore, when whole-cell LIMK½ and cofilin become inactivated, as in unstretched monolayers or stretched monolayers incubated with IPA-3 to inhibit PAK-1, subcellular polarization of phosphorylated LIMK½ and phosphorylated cofilin is abolished, perinuclear stress fibers remain, and PJAR formation is inhibited. Similarly, stretched monolayers incubated with PI3K inhibitor wortmannin or Rac1 inhibitor EHT-1864 show whole-cell inactivation of LIMK½ and cofilin and attenuated PJAR formation. Furthermore, the actin cytoskeleton has been shown to undergo constant remodeling (Chapter 2 and (7, 21, 22, 35, 50)). Taken together, we speculate that biaxial stretch produces a shift in spatial actin (de)polymerization equilibrium from homogenous to subcellular polarization, resulting in overall perinuclear F-actin depolymerization and excess perijunctional stress fiber formation (PJAR) or accumulation. Additionally, activation of Rac1 and localization of active LIMK½ and inactive actin-depolymerizing cofilin at the perinuclear region may be an activated mechanism against passive mechanical force F-actin redistribution to the perijunction. Alternatively, others have speculated that LIMK½ and cofilin are activated independently of each other (125). We
speculate active (dephosphorylated) cofilin at perjunctonal regions, which may serve to rupture actin-TJ bonds via depolymerization. This rupture may lead to increased paracelluar permeability found in stretched monolayers which was shown to be inhibited with Rac1 pathway inhibitors wortmannin, EHT-1864, and IPA-3. In conclusion and consistent with previous findings in other cell types, stretch activates Rac1, LIMK½, and cofilin, polarizes the subcellular localization of active LIMK½ and cofilin, and results in the reduction of perinuclear stress fibers and the formation or accumulation of peripheral stress fibers (PJAR). This pathway can be inhibited with IPA-3, wortmannin, or EHT-1864, resulting in attenuated or a completely abolished formation of PJAR.

**PJAR is formed in unstretched monolayers treated with calyculin-A and inhibited in stretched monolayers with ATP depletion**

Previously, calyculin-A, an antagonist of type 1 and 2A protein phosphatases (PP1 and PP2A) (36, 80), was shown to greatly dephosphorylate cofilin (activate) (75, 121, 175). Likewise, AEC monolayers incubated with calyculin-A showed decreased cofilin phosphorylation (activity) in both unstretched and stretched monolayers, leading us to speculate that AEC monolayers contain a phosphatase other than PP1 and PP2A that was active against cofilin, similar to previous (175). Furthermore, PJAR is found in calyculin-A treated stretched and unstretched AEC monolayers. Together, these data support the hypothesis that activated cofilin is involved in actin remodeling and PJAR formation in AEC monolayers. Additionally, ATP depletion showed no evidence of PJAR formation in both unstretched and stretched monolayers. We speculate that actin remodeling and PJAR formation in stretched AEC monolayers is ATP dependent. While
ATP depletion inhibits PJAR formation in stretched monolayers, previously shown to be beneficial to paracellular barrier strength, it has been shown to breakdown tight junctions in previous studies (9, 27). Thus, consistent with previous findings in other cell types, we speculate that PJAR formation is cofilin dependent and requires energy.

Summary

We have demonstrated that Rac1, LIMK½, and cofilin activate rapidly in stretched primary AEC monolayers, and that activation is accompanied by actin remodeling, PJAR formation, occludin content reduction, and increased monolayer permeability. We have shown that inhibition of PI3K with wortmannin, Rac1 activity with EHT-1864, or PAK-1 with IPA-3, all attenuate Rac1 pathway activation, PJAR formation, and increased monolayer permeability found in stretched monolayers. Additionally, exogenous PIP3 was found to reduce cell death in stretched AEC monolayers and activate Akt while Rac1 inhibitor EHT-1864 increased cell death in both unstretched and stretched monolayers and reduced Akt activation. Finally, Rac1 agonist PDGF and PP1/PP2A antagonist calyculin-A were shown to activate cofilin, resulting in PJAR formation in unstretched AEC monolayers. These data reveal that high magnitude biaxial stretch within the physiologic range rapidly increases Rac1 pathway activity, resulting in actin cytoskeletal remodeling and increased monolayer permeability. We further speculate that cytoskeletal remodeling, including cofilin mediated actin depolymerization at the perijunction, results in actin-TJ bond rupture. Future studies may investigate the effect of actin remodeling pathway inhibitors on retaining monolayer
permeability during long duration stretch as well as in *in vivo* alveolar stretch models, to explore opportunities to prevent ventilator induced lung injury.
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CHAPTER 5: SUMMARY AND FUTURE WORK

SUMMARY OF RESULTS

The overall goal of this research was to elucidate the functional consequences and underlying pathways of actin cytoskeletal remodeling in alveolar epithelial cell (AEC) monolayers stretched to injurious physiologic relevant biaxial magnitudes. The results presented in this dissertation support the hypothesis that AEC monolayers stretched to pathological magnitudes undergo rapid actin cytoskeleton remodeling, mediated by the Rac1 pathway, which is deleterious to tight junction mediated barrier properties.

The research presented in Chapter 2 demonstrated that actin rearranges rapidly in primary AEC monolayers to form perijunctional actin ring during biaxial stretch, and that formation depends on stretch magnitude and frequency. We have shown mechanistically that perijunctional actin ring (PJAR) formation was synchronous with an increase in actin binding site movement, which was attenuated to baseline levels by 10 min. We have established that monolayer permeability increases with stretch in a magnitude dependent manner and can be modulated by cytoskeletal stabilization. These data reveal that high magnitude biaxial stretch within the physiologic range increases the fluidity of the actin cytoskeleton binding sites and actin which reorganizes to form perijunctional actin rings. Together, our current and previous studies demonstrate that similarly large stretch magnitudes and higher rates adversely affect monolayer permeability (13, 14, 18).

The research presented in Chapter 3 demonstrated that Rho kinase (ROCK) activity and myosin light chain (MLC) phosphorylation decrease with high magnitude stretch of primary AEC monolayers. This inactivation is stretch-magnitude dependent,
and we find the same stretch magnitude effect in MLC phosphorylation in HPAEC monolayers. We have shown that rescuing MLC phosphorylation during stretch did not modulate monolayer permeability or PJAR.

The research presented in Chapter 4 demonstrated that stretch activates Rac1, Akt, LIMK½, and coflin rapidly in primary AEC monolayers, and that activation is accompanied by actin remodeling, PJAR formation, occludin content reduction, decreased cell death, and increased monolayer permeability. We have shown that separate inhibition of the Rac1 pathway at several points all attenuate Rac1 pathway activation, PJAR formation, occludin loss, and significantly reduced the effect of stretch on monolayer permeability. Specifically we inhibited phosphatidylinositol-3-kinase (PI3K) with wortmannin, Rac1 activity with EHT-1864, and PAK-1 with IPA-3. Additionally, stimulation of the Rac1 pathway with exogenous PIP3 was found to activate Akt and reduce cell death in stretched AEC monolayers while specific Rac1 inhibitor EHT-1864 reduced Akt activation and increased cell death in both unstretched and stretched monolayers. Finally, Rac1 agonist PDGF and PP1/PP2A antagonist calyculin-A were shown to activate coflin, resulting in PJAR formation in unstretched AEC monolayers. These data reveal that high magnitude biaxial stretch within the physiologic range rapidly increases Rac1 pathway activity, resulting in actin cytoskeletal remodeling and increased monolayer permeability.

CLINICAL RELEVANCE

Mechanical ventilation is vital for supporting patients with pulmonary insufficiency. However, it has been implicated in the etiology of pulmonary barrier
dysfunction (55, 58, 77). Experimental data suggests that pulmonary injury during mechanical ventilation is a result of elevated regional lung volumes (24, 32) resulting in excessive AEC stretch due to basement membrane deformation and reduced barrier function (26-28, 48). However, high inflation volume is necessary to supply the patient with the required amount of oxygen. Previously, decreased ventilator tidal volumes resulted in 22% lowered patient mortality (9, 41). High frequency ventilation, speculated to reduce regional lung volumes by improved alveolar recruitment and shown to decrease air leaks in infants when compared to conventional ventilation methods (1, 4, 5), improved gas exchange and reduced patient mortality (31). The use of positive end-expiratory pressure (PEEP) with fixed end-inspiratory volumes and low tidal volumes resulted in reduced edema when compared to higher tidal volumes (78). Finally, pulmonary injury was shown to be volume dependent (23).

These clinical results support our data showing stretch magnitude dependent actin cytoskeleton remodeling rate and perijunctional F-actin ring formation as well as stretch magnitude dependent activation of Akt, cofilin, rho kinase (ROCK), and myosin light chain (MLC). In addition, the functional consequences of these changes, including monolayer permeability and cell death, also show stretch magnitude dependency and agree with clinical studies and previously published results at 60 min of stretch (13-15, 71, 72). Pathway inhibitors similarly showed magnitude dependency. As an example, treatment with jasplakinolide to stabilize the actin cytoskeleton or wortmannin to inhibit PI3K resulted in complete attenuation of monolayer permeability to unstretched levels in AEC monolayers stretched to 25% $\Delta$SA $1/4$ Hz. However, use of jasplakinolide and
wortmannin in monolayers stretched for 60 min to 37% ΔSA ¼ Hz resulted in marginal or no reduction of stretch-induced increased monolayer permeability.

Studies in mechanically ventilated animals and whole lungs show that Rac1 pathway inhibition in vivo are consistent with our in vitro findings that wortmannin (PI3K inhibitor) attenuates the stretch-induced increase in monolayer permeability and occludin loss in stretched AEC monolayers. Specifically, high ventilation peak inflation pressures were shown to increase capillary permeability in isolated mouse lungs, while inhibition of PI3K attenuated this inflation-induced increase in permeability and Akt inhibition augmented it (56). Similarly, Fanelli et al. found increased PI3Kγ, phosphorylated Akt, cAMP, and bronchoalveolar lavage protein content in high magnitude (end-inspiratory pressure = 25 cmH₂O) mechanically ventilated mouse lungs (29). In addition, PI3K inhibition was also shown to decrease NF-κβ activation and inflammatory cytokine gene activation in high positive inspiratory pressure ventilated mouse lungs (73, 74). In another study, high volume ventilation in PI3Kγ knockout mice resulted in reduced histological evidence of lung injury when compared to wild type mice (52). Furthermore, Hirsch et al. demonstrated that the PI3Kγ knockout partially protected against ventilation induced attenuation of lung elasticity and edema formation as well as decreased Akt phosphorylation (42). Finally, inhibition of Akt with wortmannin was shown to impair alveolar development in the rat in vivo lung model while forced expression in the in vitro model promoted cell survival (3). These studies also agree with our data demonstrating that active (phosphorylated) Akt has a protective effect in stretched AEC monolayers. In contrast to our results however, Peng et al. has demonstrated decreased PI3K/Akt phosphorylation in mechanically ventilated mice (61)
with increased lung edema and further diminished phosphorylation of Akt in wortmannin treated mice. Additionally, restoration of Akt activity with p38 MAPK kinase alleviated capillary leakage. Taken together, we conclude in vivo and in vitro agreement inhibition of PI3K with wortmannin or via genetic manipulation attenuates stretch-induced increase in monolayer permeability.

LIMITATIONS AND FUTURE DIRECTIONS

The use of AECs from rat tissue provides for safe and practical access to a renewable quantity of samples on which to perform injury experiments. However, use of this model presents the possibility of species specific responses to mechanical injury, responses that may not manifest in human alveolar epithelial cells or mechanically ventilated patients. Future studies might benefit from utilizing human cells to verify or build upon promising in vitro rat model findings.

The use of an in vitro cell monolayer culture preparation also presents limitations in predicting the clinical response to mechanical injury, because it lacks numerous cell types, an immune response, and an extracellular matrix. Cultured cells provide the investigator with greater control of environmental variables and increased output measurement specificity. However, cell isolation and in vitro cultures lack an array of other alveolar cell types as well as interacting mechanisms that may take place in vivo during mechanical injury, such as alveolar type II (ATII) cells, which have been shown to secrete surfactant (51), and macrophages (47). Also, differences in intercellular architecture might affect strain localization and cytoskeletal morphology during deformation. Patel et al. has shown that it is possible to isolate an alveolar type I-II co-
culture (59). Future studies might benefit from the use of a co-culture preparation as they incorporate the effects of type II cell surfactant and paracrine signaling. Type I only AEC monolayers also lack a significant immune response. Patients requiring ventilation are also often struggling with disease or virus, and it was previously shown that AECs isolated from septic rats include increased cytokine levels, changes in tight junction (TJ) protein occludin and claudin expression, and reduced transepithelial resistance (TER) (19) when compared to healthy rat AECs. Additionally, cytokine release was increased in hyperventilated mouse lungs (75). AEC monolayers also lack significant extracellular matrix (ECM) as well as fibroblasts, which in normal human lung makeup 30-40% of the total lung cell count (21). Others have shown an overall decrease in lung elasticity during fibrosis, a condition that affects the molecular makeup of the lung ECM (6, 66). AEC cultures also experience a different apical environment when compared to the alveolus in vivo during respiration. Finally, Gattinoni et al. demonstrated that specific regions of the lung are injured in acute respiratory distress syndrome due to lung heterogeneity (35). Different regions of the lung require different pressures for recruitment. Thus, while whole lung inflation may be below pathological limits (100% total lung capacity), specific regions may become overinflated and injured (44). Future studies might utilize whole-lung or in vivo models, which incorporate interactions from other cell types, the immune response, or the ECM, to verify and build upon promising in vitro findings including stretch induced Rac1 mediated cytoskeletal remodeling.

The investigations in this dissertation were limited to the immediate injury responses during short duration (10-60 min) stretch. Patients are often ventilated for durations that are no shorter than a few days, and sometimes weeks or longer (63).
However, it is important to elucidate the initial injury mechanisms that take place during mechanical injury. We have shown that injury mechanisms initiate rapidly (by 10 min) including actin cytoskeleton remodeling and PJAR formation, Rac1 pathway activation, cell death, and increases in monolayer permeability in stretched AEC monolayers. We speculate that by attenuating the initial injury insult, cells will have a greater chance of long-term survival. Also, with a lower level of environmental stress, cells may benefit from the improved conditions by activating normal physiologic mechanisms able to correct moderate-injury, including the ATII proliferation and wound healing mechanism. We speculate that attenuation of rapid injury mechanisms will also suppress chronically accelerating feedback injury mechanisms including edema formation and changes in ECM elasticity during mechanical ventilation.

While we find a rapid injury response with high magnitude biaxial stretch, it is not yet known if these responses manifest at longer stretch durations (> 60 min) or if response attenuation via pathway inhibitors results in similar injury attenuation. Similarly, while we have shown that short duration high magnitude stretch, which elicits a cellular response that includes rapid cytoskeletal remodeling, is deleterious to TJ mediated paracellular permeability, it is not yet known if this rapid response is beneficial to monolayers with long duration stretch. Inhibition of Rac1 pathways to inhibit actin remodeling and attenuate monolayer permeability in stretch monolayers, while helpful during short duration stretch, may prove disastrous for retention of barrier function or monolayer viability past 60 min of stretch. With short stretch duration we have illustrated rapid changes in cytoskeletal structure, protein phosphorylation, and barrier functionality. Others have shown changes in gene expression with longer duration
stretch (20, 80). Future studies could investigate the impact of these genetic changes on monolayer permeability at long duration stretch through genetic manipulation. Furthermore, it may be worthwhile to investigate if rapid cytoskeletal responses to stretch, shown in these studies, bring about or interact with genetic changes found at long durations as a strategy to attenuate increases in monolayer permeability during long duration stretch.

Finally, our in vitro model was used to investigate injury mechanisms in AEC monolayers that start out unstretched then immediately become stretched to high magnitudes. Patients with mechanical ventilation likely experience more gradual changes in inflation volumes and cellular deformation. Future AEC models may benefit by using preconditioning with low magnitude biaxial stretch similar to previous (30), but with longer durations.

The phenomena investigated in this dissertation are limited to very specific changes in AEC monolayers during stretch. We have demonstrated rapid actin cytoskeleton remodeling with stretch. However, there is a vast array of other cytoskeletal elements including microtubules and intermediate filaments and associated pathways that may interact with cell mechanics, signaling, and TJ maintenance of barrier properties. In addition, our investigations were limited to the tight junction protein occludin. Furthermore, we can only speculate that the loss of occludin in stretched monolayers was a result of actin-TJ bond disruption. Further investigation is needed to show that actin-TJ bond rupture occurs and results in disruption of barrier function in stretched AEC monolayers. Likewise, there are many other proteins that make up the tight junction including several isoforms of claudin (40), junction adhesion molecule (JAM), and
zonula occludens (ZO), which may be affected by stretch. Claudin has been investigated in septic rats (19) as well as healthy rats and has been shown to contribute to barrier function (76). In addition, other cell-cell adhesion structures including adherens junctions, desmosomes, and gap junctions may significantly effect paracellular permeability and the cytoskeletal response to mechanical deformation. Finally, it will be important to elucidate signaling pathways upstream of PI3K. Others have shown that focal adhesion kinase (FAK) (39) can activate PI3K. Likewise, epidermal growth factor receptor (EGFR) has the ability to signal PI3K and ERK downstream (22, 33) and has been shown to respond to mechanical deformation in airway epithelium (69).

The extracellular matrix plays an important role in cell mechanics and cytoskeletal response. An evaluation of differences between the ECM of in vitro cultured cells and the alveolus in vivo is important for understanding the limitations of these results and possible future studies. Olsen et al. showed that ATII cells differentiate based on the substrate matrix present in culture (57). Similarly, the ECM was shown to affect claudin expression and barrier function in culture (49). Dunsmore et al. demonstrated that ATII cells, while differentiating to ATI cells in culture, lay down an ECM consisting of fibronectin, laminin, type IV collagen, plasminogen activator inhibitor-1, and other unidentified proteins, with a majority of this ECM produced by the second day of culture (25). Similarly, fibronectin was laid down during alveolar reepithelialization, as ATII cells repopulate the alveolar surface and differentiate into ATI cells (17, 67). Based on these previous results, our studies and others have utilized freshly isolated primary rat AECs cultured for up to 5 days on a flexible Silastic membrane coated with fibronectin (72). The in vivo ECM is perhaps more complex, composed of collagen and elastin (62,
fibronectin and laminin (34), proteoglycans and glycosaminoglycans (12, 54, 65), and matrix metalloproteinases (38). Mechanical deformation can bring about changes in ECM composition and mechanics (2), likely affecting alveolar mechanics, cell adhesion, blood-gas permeability, and AEC mechanics. Thus in vivo ECM, which has been shown to be quite similar to the ECM produced by cultured AECs in vitro, may interact with the AEC monolayer differently during stretch due to differences in architecture and composition.

The ECM also plays an important role in cell adhesion and cytoskeletal response. Two major adhesion stratum must be taken into account when investigating cell adhesion and cytoskeletal response to deformation: epithelial cell adherence to the ECM mediated by integrins (45, 70) and other molecular adhesions; and the ECM adherence to the Silastic membrane or, in vivo, the ECM linkage to the basal lamina through collagen type IV (60). Liu et al. demonstrated that integrins can be coupled to actin through α-actinin, tensin, filamin, and talin (53), with further evidence demonstrating the importance of talin1 during force-dependent integrin-cytoskeleton reinforcement (37). Choquet et al. demonstrated strengthened integrin-cytoskeletal linkages with forces (16) while others have shown that jasplakinolide increased cell adhesion by increasing the number of focal adhesions in airway epithelial cells while latrunculin-A improved cell viability by increased cell fluidization (79). Together, these studies illustrate the importance of the ECM on cell adhesion and the cytoskeletal response to deformation. Furthermore, we have shown decreased rho kinase (ROCK) activity and increased Rac1 pathway activity in stretched AEC monolayers, previously speculated to be the initial stages of anoikis, a form of apoptosis that is induced by loss of adhesion, in other cell
types (7). Additionally, we have shown a protective effect of cytoskeletal stabilization with jasplakinolide in AEC monolayers stretched to 25% ΔSA ¼ Hz. Future studies might investigate the role of ECM and adhesion in stretched AEC monolayers. We speculate that stretch decreases AEC adhesion and could result in changes to barrier function and cell death. If so, strengthening cell adhesion may change the cytoskeletal response and attenuate stretch-induced increased paracellular permeability in stretched AEC monolayers. Additionally, it may be beneficial to investigate whether cell adhesion pathways and cell adhesion functionality are affected similarly in in vivo alveoli inflated to pathologic volumes when compared to in vitro AEC cultures.

Pharmacological inhibitors are valuable in elucidating underlying mechanistic pathways and are relatively easy to apply to in vitro cell cultures. However, inhibitor efficacy and measured cell functional output is largely dependent on treatment concentration, cell membrane permeability, cellular clearance, and other confounding variables. Furthermore, specific protein inhibitors, which are very limited in availability to begin with, could have unknown substrates or effects on other pathways. Future studies could employ siRNA or genetically impaired PI3K, Rac1, LIMK, or cofilin constructs to evaluate the specific effect on cytoskeletal remodeling and monolayer permeability in stretched AEC monolayers.

Treatment with jasplakinolide was found to attenuate monolayer permeability in monolayers stretched for 60 min at 25% ΔSA ¼ Hz but not at 37% ΔSA ¼ Hz. It may be beneficial to explore alternative treatments which mimic the effects of jasplakinolide, but do so using intrinsic physiological mechanisms. Cano et al. showed that α-actinin, an actin filament cross-linking protein, inhibited the rate of pyrenyl F-actin
depolymerization *in vitro* (10). Similarly, Jackson et al. showed increased whole-cell stiffness of human osteoblasts with overexpression of α-actinin (46). Likewise, ezrin has been shown to link actin microfilaments to the membrane and signal survival through PI3K/Akt pathway *in vitro* (36). Future studies may also investigate the effect of cytoskeletal stabilization or relaxation using dibutyryl cyclic adenosine monophosphate (DBcAMP, bucladesine), shown to tighten cell-cell barriers previously in endothelial cells (50) and pulmonary epithelial cells (8). Pretreatment with DBcAMP may result in cytoskeletal relaxation, similar to increased cytoskeletal fluidity of stretched AEC monolayers (Chapter 2), but without the speculated actin-TJ bond rupturing effects of cofilin. Solutions using these pathways could prove more effective or practical in the clinical setting.

Monolayer permeability quantification using fluorescent tracer BODIPY-ouabain (Chapters 2 and 4) has limitations. First, this method it is not a direct measure of permeability, but a quantitative measure of tracer binding to the basolateral surface of barrier-compromised AEC monolayers. Future permeability studies could benefit from the use of a stretchable, biocompatible membrane that has submicron pores which allow tracer to pass through but do not affect AECs. Using this membrane, monolayer permeability could be assessed with more accurate fluorometric techniques using a variety of tracers, which do not require AEC binding, that have diverse sizes and charges. The initial design ideas and fabrication data of a such a membrane are presented in Appendix H. Secondly, while quantification of whole-monolayer permeability is an important indicator of AEC barrier strength, it would be helpful to have the ability to
quantify the specific contribution of TJ-mediated paracellular permeability in stretched monolayer.

Western blot methods (Chapter 3 and 4), used to quantify the amount of phosphorylated and total protein concentrations, are limited in a few ways. Quantification is limited by antibody availability, known phosphorylation sites on the target protein, and previous data on functionality of phosphorylation sites. Furthermore, we were only able to quantify total protein lysate from whole monolayers. This represents the ensemble of chemical signaling of a few million cells and thus lacks local, cell to cell signaling. Additionally, cells can employ spatial compartmentalization as well as subcellular binding to direct biochemical signaling. Further investigation is needed to quantify the signaling mechanism between the LIMK½ and coflin proteins. While we were able to show spatial localization using immunocytochemistry, quantitative western blot data of subcellular specific lysate would further our understanding of the subcellular mechanistic link between these proteins. Elucidating the mechanism behind protein translocation will be important to fully understanding cytoskeleton remodeling in stretched AEC monolayers. Furthermore, future studies could investigate the phosphatase(s) or mechanism responsible for dephosphorylating coflin in stretched monolayers. Finally, it may be beneficial to investigate the significance of coflin activation at only 60 min of 12% ΔSA ¼ Hz stretch without change in permeability at this time/magnitude (Chapter 4) by measuring upstream protein activity with Western blot. We speculate that the cell is able to slowly adapt the cytoskeleton to stretch at a non-injurious rate. If this is so, can we learn from the pathways activated in this process
to engineer a biomimetic response to deal with the rapid high magnitude deformations shown to cause barrier dysfunction.

Future investigation may focus on biochemical pathways that may be responsible for actin nucleation and polymerization. Actin related protein 2/3 (Arp2/3) and capping protein (CP) have been shown to assemble actin filaments in the leading edge of a motile cell (64). Future studies might investigate whether Arp2/3 and CP drive PJAR formation in stretched AEC monolayers or if it is passive mechanically mediated accumulation? Also, Cano et al. presented a novel method to determine the relative amounts of F- and G-actin in cells (11, 43). Future studies could investigate if there is net F-actin depolymerization in stretched monolayers or if the ratio of F- and G-actin changes with cofilin activation in stretched AEC monolayers.

CONCLUSION

The research in this dissertation furthers our knowledge of the etiology of pulmonary injury due to mechanical ventilation. We have demonstrated that high magnitude physiologically relevant biaxial stretch (25% and 37% ΔSA) but not low (12%) results in rapid actin cytoskeleton remodeling accompanied by activation of the Rac1 pathway and increased monolayer permeability and loss of occludin. Furthermore, inhibition of the Rac1 pathway resulted in an attenuation of actin remodeling and the increased monolayer permeability found in stretched monolayers. Activation of the Akt pathway with exogenous PIP3 helps lower death in stretched AEC monolayers, but was found to increase net monolayer permeability in stretched monolayers. Inhibition of Rac1 specifically with EHT-1864 lowered Akt and increased death in unstretched and
stretched monolayers, but resulted in a net decrease of stretch-induced increased monolayer permeability. With this data we can begin to develop treatment strategies aimed at reducing cellular injury during high magnitude biaxial deformation. We anticipate these treatments will reduce the incidence of pulmonary injury and mortality in patients that require mechanical ventilation.
REFERENCES


APPENDIX A: SURGERY AND ALVEOLAR EPITHELIAL CELL ISOLATION

AFTER WELL USE
- Immediately boil glass (NO soap)
- Disassemble wells, scrub (light soap, rinse off immediately), boil wells/o-rings
- Let dry RT (room temp)
- decon incubator about 1/month
- replace water in incubator about 1/week

DAY BEFORE (protocol optimized for 2 separate rats)
- 2 x IgG plates (6.5 ml Tris-HCl + 35 mg rat IgG (Sigma-Aldrich, I4131-100mg)), swirl until most dissolved, kept under hood (OR day of IgG works with same efficacy, prepare in morning)
- Autoclave
  - make, boil (again), autoclave wells and glass
  - forceps
  - 2 x set filters (Nitex mesh 160 µm, 15 µm, 6 µm)
  - instruments (curved kelly hemostat, long hemostat, blunt large scissors, sharp small scissors, Ochner (large-flat) forceps, spoon)
- Solution I (140 ml needed)
- Solution II (200 ml needed)
- Elastase (58 ml needed) (Worthington Biochemical, LS002292)
  - Warm sterile Solution II to 37°C
  - Note units of elastase/mg on bottle (example 3.15 u/mgP)
  - Multiply units by 100 and divide by 3 (example 3.15 u/mgP × 100 ÷ 3 = 105ml solution 2
  - add required solution 2 to separate cell culture bottle
- Dissolve elastase (powder, 4°C) into required solution 2 volume, rinse elastase bottle with solution 2
- Swirl bottle occasionally and place back into 37°C water bath for 30 - 60 min (should dissolve, and appear mostly clear, sometimes slight cloudy)
- Sterile filter (0.22µm pore) solution into 250ml tissue culture bottle
- Label and store at 4°C (sterile)

-MEM+FBS+A/G (in sterile 250ml glass bottle)
  - Sterile (0.22µm pore) filter 225 ml MEM (Invitrogen, 11095072)
  - Sterile (0.22µm pore) filter 25 ml FBS (Sigma-Aldrich, F6178-500mL)
  - Add 125µl Gentamicin (1:2000; Invitrogen, 15750060)
  - Add 250µl Fungizone (Amphotericin B, 1:1000; Invitrogen, 15290018)
  - Label and store at 4°C (sterile)

-MEM+A/G (in sterile 250ml glass bottle)
  - Sterile (0.22µm pore) filter 100 ml MEM (Invitrogen, 11095072)
  - Add 50µl Gentamicin (1:2000; Invitrogen, 15750060)
  - Add 100µl Fungizone (Amphotericin B, 1:1000; Invitrogen, 15290018)
-Label and store at 4°C (sterile)
-Fibronectin stock (order from Cell Center, 1142)
-DO NOT agitate fibronectin
-Fibronectin Human lyophilized (Invitrogen 33016015-5mg; 4°C)
-under hood, add 5 ml cool sterile water (filtered or autoclaved) to FN container (VERY SLOWLY, as not to agitate FN)
-incubate 60 min 37°C (incubator); make sure stopper does not blow off; FN will appear clear after incubation
-under hood SLOWLY pipette 50, 100, or 200 ul aliquots
-Label and store at -20°C

DAY 0 (Isolation day)
-Turn ON water-bath (37°C)
-FN wells - place in incubator (37°C, 5% CO₂)
-50ul FN stock / 1.2 ml MEM (per 4 wells)
-be gentle with FN
-2 x IgG plate (6.5 ml TrisHCl + 35 mg IgG), swirl very well until all dissolved (is not different vs. day-1 IgG, tested)

-get retrieve 2 rats

-bucket ice
-2 x 8 ml S1 in 10cc syringe
-120 ml S1 (approx)
-2 x 20 ml S2 (approx)

-RT
-2 x 10 ml S2 in Erlenmeyer flask (use pipette)
-2 x 3 ml MEM+FBS (under hood)
-60 ml S2 (with 10cc syringe, cannula, and needle)

-37C waterbath
-80 ml S2 (approx)
-58 ml Elastase (under hood)

-Anesthetize rat with 65 mg/kg Nembutal Sodium given IP.
If more anesthetic needed give in increments of 0.1cc.
(rat weight (g-rat) * 60 mg-drug / 50 kg-rat / 1000 g-rat/kg-rat = usually 0.3 ml Nembutal each rat)

[SURGERY - ISOLATION HOOD]
-Shave from pubis to chin; place on positioning board; wipe down with Wex-cide.
-INCISE from pubis to chin
-Expose the trachea (underneath muscle tissue; make low lateral cut, then cut up towards chin)
-Thread suture beneath trachea
- Start a knot
- Slide hemostat under trachea
- Cut between tracheal rings and insert cannula (with ventilator ON). Cut high (cranially) in case of trach-anchoring mistake
- Tighten suture: few quick tugs
- Open abdomen, cut up until ribs
- Continue cutting up towards chin in Y pattern (be careful of cutting lungs)
- Incise the diaphragm at the top to collapse lungs
- Use hemostat to hold sternum/cartilage tissue peeled back, exposing heart
- Displace abdominal viscera and cut the caudal vena cava/abdominal aorta
- Place paper towel over the abdomen to absorb blood
- Secure heart with hemostat (do not squeeze heart) and quickly incise right ventricle of heart
- With one hand holding a hemostat and the other holding the perfusion cannula, insert cannula into the bottom of right ventricle towards pulmonary artery, secure with a hemostat. Clamp the hemostat on to the right ventricle (muscle).
- 3 x perfuse with solution 2 using 10 cc syringe (total 30 ml)
- Lungs will begin to turn white (cut left atrium if its large to relieve systemic pressure)
- Once lung are white, shut OFF ventilator
- Inject some of 8 cc of solution 1 through trachea cannula
- Remove lungs: cut trachea and esophagus above tracheotomy (NOT UNDER), use solution 1 syringe to pull trachia/lungs up, continue cutting behind trachia and lungs while lifting; cut rest of diaphragm and descending arteries/veins
- Disconnect ventilator, from cannula and attach syringe of Solution 1
- Inject rest of 8 cc of solution 1
- Place carcass in plastic bag and freeze

[ISOLATION - BENCHTOP HOOD]
Withdraw the 8 cc of solution 1 from the lungs
Lavage lungs 8 X with 7 ml of cold solution 1 while gentle bounce up and down to aid in withdrawal of solution

Place 20 cc warm solution 2 into a new beaker and perfuse lungs 2 X with 8 cc after moving lungs into the beaker containing the remaining 20 cc of warm solution 2

Lavage once with 6ml elastase
Add 7 cc warm elastase to the lungs - incubate water bath for 30 min
Repeat 2 more times, for total of 21 cc elastase and approx 1.5 hr of incubation
- Use incubation time to return rat carcasses, clean surgical instruments, prepare tissue chopper, and clean isolation area

Add a “pinch” of DNase to Erlenmeyer flask with 10 ml of solution 2 (ground the spatula by touching it to the flask)

Keep lungs separated
Cut lung lobes from trachea into beaker with the 3 mL of MEM & 10% FBS (~1 cm lengths)

Cut each lobe into thirds for chopping. Chop with machine or scissors. Over chopping will cause an increase in debris, too little and the tissue will not go through the pipette orifice and there will be fewer cells.

- Using tissue chopper, chop 2-3 lung pieces using 2 complete AUTO-passes (offset at 90° angle) on chopper
- Add to Erlenmeyer flask with solution 2 with DNAse
- wash tissue chopper with 3 ml of MEM with FBS and place in the flask.

After lung is finished chopping, place flask in 37° C water bath and swirl by hand for 2-3 minutes to release cells from the digested tissue. Set up filters in the ice

Filter tissue through a series of 3 filters use forceps to move tissue around on all available surface of the filter. Tap sides and rub the bottom of filters to remove all possible tissue. Keep cells on ice.

(keep lungs separate) Divide the cells EQUALLY between two sterile 50 cc centrifuge tubes

- unlikely, but if two tubes significantly unbalanced, add cells from one lung to the other to equilibrate

Spin for 11 min at 1000 × g. During the spin, place MEM (NO FBS) in water bath.
(Note: Dobbs et al. uses 300 × g for spinning cells into pellet; 1000 × g works well and may maximize yeild slightly)

[CELL CULTURE - CELL CULTURE HOOD]
Take in two 10 ml pipettes, a 5 ml pipette and warm MEM without FBS.

Use suction to remove supernatant from cells and the IgG plate.

Tap tubes to loosen pellet.

Aspirate 10 ml of MEM (NO FBS) and add 5 ml to each IgG plate (1st wash)
Aspirate 10 ml of MEM (NO FBS) and add 5 ml to each centrifuge tube. Resuspend the pellets (keep separate).

Keep pipette in centrifuge tube. Suction off 5 ml from IgG plates.

Aspirate 10ml MEM (NO FBS) using a new pipette and add 5 ml to each IgG plate (2nd wash). Suction off.
Place 5 ml MEM + cells onto IgG plates. Aspirate 10 ml MEM (NO FBS, using new pipette) and wash the now empty 50 cc tube to collect the residual cells.

Divide 10 mL MEM + cells onto IgG plates - for a total of 10 cc’s on the IgG plate.

Incubate IgG plate for 90 min at 37 C and 5% CO₂

**After 90 min incubation is complete:**
Remove cells from IgG plate by gently swirling (**slight pan**) the plate and pipet into 50 ml centrifuge tube

- record exact volume (usually 10 ml)

Spin for 15 min at 1000 × g (Place MEM+FBS in water bath).
(NOTE: Dobbs et al. uses 300 × g for spinning cells into pellet; 1000 × g works well and may maximize yeild slightly)

COUNT (during spin):
1- Remove a 10 ul sample and place in a microcentrifuge tube for counting. Add 10 ul of Trypan Blue. Add 10 ul to one side of a hemocytometer. Count three/four squares; take average count of three
   NOTE: bunches of cells = 1, count only large/round cells that are white (blood cells are smaller, about 1/3 size of alveolar epithelial cells, do not count blood cells)

2- multiply average by 96% (under-estimates cell count; found to improve success rate)

3- multiple by 20,000 (million cells / mL)

4- multiply by volume (mL) from IgG (million cells)

5- Divide by 1.227 (area of small glass insert used in small wells)
   (or 1.408: area of 'thin-wall' small glass insert)

6- Multiply by 0.7 (volume of seeding; ml)
   (this seeds at a concentration of 1 million cells/ cm²)

Suction off supernatant and resuspend pellet in calculated volume of MEM+FBS for each lung; aspirate gently, enough to break apart cell pellet

Wash FN in wells gently once with MEM+FBS

Seed cells (700 ul) using ml pipette - place in incubator
DAY 1
-feed relatively early (if isolation finished at 5PM day 0, feed around 11 AM day1)
-warm media, place MEM+FBS into T25 (up to about 30 ml fits with T25 almost flat), incubate for at least 15min 37ºC 5% CO₂
-media condition (in incubator) found to be important to regulating pH and very important to keeping monolayers from differentiating during day 3, 4, and 5
-remove glass from wells, slight shake of wells, aspirate supernatent, add 800 ul MEM+FBS / well to side of well (not directly onto cells)
do not need to ethanol dishes, but it does not hurt
-slight shake after feed

DAY 2
-see day 1, except no glass removal
-have skipped this day before (by adding 1.5 ml day 1) and cells looked very nice

DAY 3
-see day 1, except no glass removal

DAY 4
-see day 1, except no glass removal
-serum deprive DMEM+Hepes 2 - 3hrs in 0% CO₂ incubator

Additional supply ordering information:
Suture material (Harvard Apparatus, 517698), hemocytometer cover glass (Fisher Scientific, 0267153), chopping blades (Electron Microscopy Sciences, 72000), DNase Deoxyribonuclease I (Sigma-Aldrich, DN25-1G), Culture dishes (Neta Scientific, Inc., 877232). Adson-Brown Forceps (500177), Dissecting Scissors 10cm straight (14393), Forceps Rochester-Oschner Hemostatic 14 cm strait (501709), Graefe Forceps 7cm long serrated strait (14142), Mosquito Hemostatic Forceps 12.5cm curved (15921), Scissors Mayo 17cm strait (501751), and Sterilizer Tray (500253) all from (World Precision Instruments Inc.), media bottle autoclavable 250 mL (Fisher Scientific 064141B), cell culture filters small mouth (Neta Scientific Inc., 976150), cell culture filters large mouth (Neta Scientific Inc., 0976152), oversleeves (Lab Safety Supply Inc., 27172) rodent restraint bag (Fisher Scientific, NC9559141), T25 flask 50mL capacity (BD Labware, 1194-353108), Trypan Blue (Cell Center, MT25-900-C1).

NOTE:  when making small wells, cut membrane 1.5" square, place into well with o-ring, cut off 4 corners so that there is a small lip of Silastic that holds the well up (this creates a space between the membrane and culture dish, prohibiting the membrane from sticking to the plate); try to cut the corners so the lip is even around the entire diameter, that way the well lays flat.
AEC cell observation: Produces high quality type I-like AEC monolayers from 2 separate lungs: Confluent cobblestone appearance, very slight/small domes, very little if any impurity and mesenchymal-like cells in culture.

Figure A.1: After 4 days in culture, primary alveolar epithelial cells isolated and seeded onto fibronectin coated Silastic membranes form a confluent monolayer (top, phase image). Monolayers stain positively for alveolar epithelial type I (ATI) cell specific protein RTI₄₀ (bottom left, green) and negatively for mesenchymal specific cell marker vimentin (bottom right). Bars = 40 µm.

(isolation protocol by Brian DiPaolo, bdipaolo@seas.upenn.edu; derivation of protocol by Michele Hawk based on Dobbs et al. (7) with slight modification (12))
ISOLATION SOLUTIONS

Stock solutions

- NaPO$_4$ (0.1 M)
  5.68g Na$_2$HPO$_4$ into 400ml ddH$_2$O (0.1 M Na$_2$HPO$_4$)
  1.38g NaH$_2$PO$_4$ into 100ml ddH$_2$O (0.1 M NaH$_2$PO$_4$)
  combine for a total of 500ml at pH 7.4

- KCl (0.15 M)
  1.12g KCl into 100ml ddH$_2$O

- CaCl$_2$ (0.1 M)
  1.47g CaCl$_2$ 2H$_2$O into 100ml ddH$_2$O

- MgSO$_4$ (0.15 M)
  3.7g MgSO$_4$ 7H$_2$O into 100ml ddH$_2$O

- HEPES Na salt (0.2 M)
  26.3g HEPES Na salt into 500ml ddH$_2$O (Sigma H7006-500g)
  pH to 7.4 with HCl

50 mM Tris-HCl ph 9.5

- 121.1 g/mol Trizma Base (Tris; Sigma T-1503) × 0.05 M × 0.5 L =
  3.03g Tris into 500 ml ddH$_2$O
- pH to 9.5 using HCl
- Sterile filter solution under hood into tissue culture bottle (0.22 μm pore
  cellulose acetate filter, Corning 430513)
- Add 50 µl Tween-20 after filtering
- Label and store at 4ºC (sterile)

Solution 1

- Thaw Pen-Strep (Invitrogen 15140122; -20ºC)
- Physiological saline:
  1 L ddH$_2$O into 1.5 L graduated cylinder with stir bar
  9g NaCl (Sigma S7653-1Kg)
  1g D-glucose (Sigma G5767-500g)

- Add 30ml NaPO$_4$ stock (4ºC)
- Add 40ml KCl stock (4ºC)
- Add 70mg EGTA (stir until dissolved; shelf)
- Add 10ml Pen-Strep
- Add 60ml HEPES stock (4ºC)
- Sterile filter solution under hood into tissue culture bottle (0.22 μm pore filter,
  Corning 430513)
- Label and store at 4ºC (sterile)
-Solution 2
- Thaw Pen-Strep (Invitrogen 15140122; -20ºC)
- Physiological saline:
  1 L ddH$_2$O into 1.5 L graduated cylinder with stir bar
  9g NaCl (Sigma S7653-1Kg)
  1g D-glucose (Sigma G5767-500g)
- Add 30ml NaPO$_4$ stock (4ºC)
- Add 40ml KCl stock (4ºC)
- Add 20ml CaCl$_2$ stock (4ºC)
- Add 10ml MgSO$_4$ stock (4ºC)
- Add 10ml Pen-Strep
- Add 60ml HEPES stock (4ºC)
- Sterile filter solution under hood into tissue culture bottle (0.22 µm pore filter, Corning 430513)
- Label and store at 4ºC (sterile)

Dulbecco's Modified Eagle Medium (DMEM) + 0.02 M Hepes (H) solution
DMEM+H is used for stretching cells in a 0% CO$_2$ incubator or outside of the incubator:
it lacks the red dye normally found in MEM that might interfere with imaging and its
buffered with Hepes rather than bicarbonate allowing correct pH maintenance outside of
a 5% CO$_2$ incubator

- acquire DMEM powder (without glutamine, phenol red, sodium pyruvate, sodium bicarbonate; Cellgro 90-013-PB; 4ºC)
- Thaw 20 ml L-Glutamine aliquot (Invitrogen 25030081; -20ºC)
- Thaw 10 ml PenStrep (Invitrogen 15140122; -20ºC)
- Add 850 ml ddH$_2$O into large beaker with spin bar
  - reserve 50 ml to rinse DMEM weigh boat
- Add 12.78 g DMEM powder to beaker while stirring, rinse empty weight boat
- Stir until all dissolved
- Add 20 ml L-Glutamine (make sure its clear before use, vortex if needed)
- Add 10 ml PenStrep
- Add 100 ml 0.2 M Hepes solution (4ºC)
- pH to 7.4 at room temp using NaOH
- Bring volume to 1000 ml using ddH$_2$O
- Sterile filter solution under hood into tissue culture bottle (0.22 µm pore cellulose acetate filter, Corning 430513)
  - filtering will bring pH up about 0.2-0.3 units; final pH will be about 7.6
- Label and store at 4ºC (sterile)

(solutions protocol by Brian DiPaolo, bdipaolo@seas.upenn.edu; derivation of protocol by Michele Hawk based on Dobbs et al. (7) with slight modification (12))
MICROSCOPE AND CELL STRETCHER CALIBRATION

MICROSCOPE SCALE CONVERSION FACTORS

<table>
<thead>
<tr>
<th>model</th>
<th>px/um</th>
<th>scale (um/px)</th>
<th>SCOPE</th>
<th>OBJECTIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.846015</td>
<td>1.182012</td>
<td>CON</td>
<td>10X 0.30 DIC L inf-0.17 WD 16.0 PlanFluor</td>
<td></td>
</tr>
<tr>
<td>1.685340</td>
<td>0.593352</td>
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<td>20X 0.50 DIC M inf-0.17 WD 2.1 PlanFluor</td>
<td></td>
</tr>
<tr>
<td>3.353350</td>
<td>0.298209</td>
<td>CON</td>
<td>40X 0.75 DIC M inf-0.17 WD 0.72 PlanFluor</td>
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</tr>
<tr>
<td>3.393350</td>
<td>0.294694</td>
<td>CON</td>
<td>40X 1.30 OIL inf-0.17 WD 0.22 SFluor</td>
<td></td>
</tr>
<tr>
<td>Fluor 1</td>
<td>1.548768</td>
<td>0.645675</td>
<td>EPI</td>
<td>10X 0.30 DIC L inf-0.17 WD 16.0 PlanFluor</td>
</tr>
<tr>
<td>3.100875</td>
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<td></td>
</tr>
<tr>
<td>6.161667</td>
<td>0.162294</td>
<td>EPI</td>
<td>40X 0.75 DIC M inf-0.17 WD 0.72 PlanFluor</td>
<td></td>
</tr>
<tr>
<td>(CON software)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.000000</td>
<td>0.2</td>
<td>CON</td>
<td>60X OIL PA</td>
<td></td>
</tr>
<tr>
<td>8.333333</td>
<td>0.12</td>
<td>CON</td>
<td>100X OIL</td>
<td></td>
</tr>
</tbody>
</table>

NOTES:
- scale slide: 0.01 mm slide, SWIFT (cat MA663, no 10)
- CON: confocal scope
- EPI: epifluor scope

CELL STRETCHER ZERO AND MAGNITUDE CALIBRATION
- check distance between top plate (well holder) and bottom plate (indentor) using micrometer
- use average distance between plates (mm) across front - left, center, right, back right, back left

HOME position: stretcher zero position, when its not in use (bottom plate almost fully down)
Unstretched: visually verify that most/all wells just touching bottom of indentor right before stretching begins
12, 25, 37%: measure when stretched at magnitude during static hold stretch

<table>
<thead>
<tr>
<th>MAGNITUDE</th>
<th>Distance (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOME</td>
<td>68.60</td>
</tr>
<tr>
<td>12%</td>
<td>17.90</td>
</tr>
<tr>
<td>25%</td>
<td>16.00</td>
</tr>
<tr>
<td>37%</td>
<td>14.65</td>
</tr>
</tbody>
</table>

NOTES:
- this calibration is for ’OLD/larger-height’ wells
- use ’NEW/small-height’ as UNS controls (they have a slightly thinner base)
- ’newstart’ program may initialize slightly high
- check stretch device each week to ensure proper calibration

(calibration by Brian DiPaolo, bdipaolo@seas.upenn.edu)
APPENDIX B: A549 AND HUMAN PULMONARY ARTERY ENDOTHELIAL CELL CULTURE PROTOCOL

MEDIA
(HOOD)
-EBM-2 media (clonetics CC-3156, 4°C) – no growth factors
+ 
-EGM-2 (clonetics CC-4176, -20°C) – supplements and growth factors
  -add each cryovial to the media bottle (EXCEPT FBS)
  -rinse each cryovial with media
  -(once singlequotes are added, use media within 1 month)

-filter 220 ml above media into a 250 ml bottle with a 0.2 µm filter

+25ml FBS (10%, -20°C)
+2.5 ml L-glutamine (1%, -20°C)
+2.5 ml Pen./Strep. (1%, -20°C, 100 U/ml)

THAWING
Bullet passage #:

-T75 + 15 ml MEDIA (or T25 + 5 ml MEDIA)
  -place in incubator for 30 min to equilibrate

-remove cryovial from liquid nitrogen
  -in HOOD, twist cap quarter turn to relive pressure, then retighten
  -quickly thaw in 37°C waterbath (do not submerge entire vial)
  -when last sliver of ice melts, remove from bath quickly
  -HOOD, dispense cells into T75
  -gently rock to evenly distribute cells

-return to incubator

-seed HPAECs at 2,500 – 5,000 cells/cm² (0.188 – 0.375 million cells in T75)

FEEDING
-change media day after passage and EVERY OTHER day after
  -under 45% confluence: feed with 1.5 ml / 5 cm² (22.5 ml T75, 7.5 ml T25)
  -over 45% confluence: feed with 2 ml / 5 cm² (30 ml T75, 10 ml T25)

SUBCULTURE
-subculture when 70-80% confluent (and contain many mitotic figures)
-thaw Trypsin/EDTA to RT (6 ml for T75)
-bring HBSS (no Ca\(^{++}\), no Mg\(^{++}\)) to RT (24 ml for T75) (OR 1xDPBS no Ca\(^{++}\), no Mg\(^{++}\))
-bring MEDIA to 37\(^\circ\)C

-aspirate media from T75
-rinse cells with 15 ml HBSS, aspirate off
-neutralizes trypsin by removing medium complex proteins and Ca\(^{++}\)
-add 6 ml Trypsin/EDTA to T75
-place on scope
-wait for \(\approx\)90\% cells to round up (2 – 6 min)
-rap flask against hand (should remove most of cells, if not wait 30s and try rap again)
-neutralize trypsin with 12 ml MEDIUM
-transfer cells to 50 ml centrifuge tube
-rinse flask with 4 ml HBSS
-T75 should have <15\% cells left in it
-pellet cells: 300 \(\times\) g for 5 min (protocol calls for 220 \(\times\) g for 5 min)
-aspirate most of supernatant (except about 100-200 ul)
-flick tube to loosen pellet
-dilute pellet into 9 ml MEDIA
-count (using Trypan Blue)

-SEED new plates at 10,000 cells/cm\(^2\)

-SEED wells at 162,932 cells/cm\(^2\) (see below, wells coated with Collagen I)
-wait 48 hrs
-change media (experiment performed in complete culture media with 10\% FBS)
-stretch

-FREEZE bullet with 1-3 million cells / ml (cryotube 1.5 ml)
-add 10\% DMSO to tube of cells
-QUICKLY place cryotube on dry ice
-transfer to -80\(^\circ\)C freezer overnight or liquid N for long term storage

SEEDING WELLS
-Birukov et al. seeded wells at 8x10\(^5\) cells/well coated with collagen I (2)
-well is 2.5 cm diameter: 4.91 cm\(^2\) area
-thus: 162,932 cells/cm\(^2\)
-after 48 hrs, medium changed and cyclic stretch (equibiaxial)

-plate wells with collagen I (4\(^\circ\)C) at 5 ug/cm\(^2\)
-collagen: 5 ug/cm\(^2\) * 1.227 cm\(^2\) = 6.135 ug / well
-solution: 6.135 ug/well ÷ 50 ug/ml = 0.1227 ml/well = 123 ul/well
  -small glass tubing needs more to cover area: add 246 ul/well

-collagen comes in acetic acid (this lot is 3.75 mg/ml)
  -dilute to 50 ug/ml in 0.02N acetic acid
    -for 8 wells: (if 3.75 mg/ml) take 13.33 ul stock and place into 970.7 ul 0.02N acetic acid (984 ul TOTAL)

-0.02N acetic acid stock:
  -must dilute acetic acid from 17.4 N (Fisher A38-500mL) to 0.02 N, a 1/870 dilution
    -1 ul 17.4 N acetic acid into 869 ul ddH₂O water
    OR
    -10 ul 17.4 N acetic acid into 8690 ul ddH₂O water
  -pipette filter into a 50 ml tube

-incubate wells with collagen I at RT for 1hr
-aspirate, rinse with serum free medium, use immediately or air dried

A549 CELL CULTURE
-Human alveolar epithelial adenocarcinoma A549 cell line (ATCC, Manassas, VA) handled similar to HPAECs (above) except that:
  -A549s thawed, subcultured, fed, and seeded using MEM+FBS (see AEC isolation protocol)

-SEED wells at 250,000 cells/cm² onto fibronectin coated (see AEC isolation protocol) Silastic membranes overnight
  -serum deprive in DMEM+HEPES (see AEC isolation protocol) overnight
  -stretch

(protocol by Brian DiPaolo, bdipaolo@seas.upenn.edu; derived from Clonetics information sheet, PrimaPure HPAEC information sheet, and (2))
APPENDIX C: IMMUNOCYTOCHEMISTRY AND ANALYSIS PROTOCOL

IMMUNOCYTOCHEMISTRY
-check STRETCHER HOME = 68.60 mm (for old/large-height wells) using micrometer
1. **SERUM DEPRIVE** (2-3 hrs) - IN 0% CO₂ INCUBATOR
2. STRETCH
3. 1 × WASH DPBS (no Mg⁺⁺, no Ca⁺⁺) – quickly RT (room temperature)
4. **(HOOD) FIX** 5 drops 1.5% Paraformaldehyde (16% diluted with DPBS) - 15 min RT
   -(note: ZO1 does not work with 4% para, Phalloidin does not work with Methanol fixation)
   -1.5% PARA stock: 12.69 ml DPBS + 1.31 ml 16%PARAformaldehyde
5. 2 × WASH DPBS – quickly RT
6. **PERMEABILIZE** 50 ul 0.1% TritonX100 in DPBS – 10 min RT
   (NOT for wells staining with RTI40)
7. 2 × WASH DPBS – quickly RT
8. (optional, for high background cases): 2 × WASH 10 mM Glycine buffer (1:10 of 100 mM stock 4ºC)
   -recipe 100mM Glycine buffer: 7.507 mg / ml
9. **BLOCK** 30 ul 5% GoatSerum-DPBS – 1 hr RT (in 50 ml TUBE to prevent evaporation)
10. **30 ul PRIMARY** in block buffer - 1 hr RT shaker OR 4°C (in TUBE on shaker)
   1:50 (5 ug/ml) Rabbit-anti-ZO1:
   1:50 Mouse-anti-Occludin:
   1:50 Rabbit-anti-phospho-LIMK1/2:
   1:50 Rabbit-anti-phospho-Cofilin:
   1:50 Rabbit-anti-phospho-MLC:
   1:50 Rabbit-anti-total-MLC:
   GS-DPBS:
11. 3 × WASH DPBS – 5 min RT
12. **35 ul SECONDARY/Phalloidin** - 90 min RT (TUBE on shaker)
   1:50 FITC-donkey-anti-RABBIT (ZO-1, LIMK, Cofilin, MLC):
   1:50 Phalloiden-633 (F-actin):
   1:50 Dylight-594-mouse-anti-MOUSE (occludin):
   GS-DPBS:
13. 3 × WASH 70 ul - 10 min RT
14. MOUNT drop RT DAPI-antifade (BLUE channel – Nuclei) OR RT DAKO mounting media (drop small amount onto top of each slide, no bubbles)
   -IMPORTANT: DO NOT press on coverslip (it will damage cells),
   -WARM mount media
NOTE DISK#:
NOTE: After fixation, holes may appear between a few cell populations in stretched monolayers; we speculate this is due to fixation lowering elasticity of the monolayer, as it is not found in live post-stretched monolayers (BODIPY-oubain, live phalloidin staining, live/dead staining, etc.). We do not expect it to influence staining patterns in monolayers.

SLIDE METHOD: add solution to top of slice (not directly onto cells) and aspirate from bottom, for incubations rock gently to get solution to cover all cells
- greatly reduces amount of required primary and secondary antibody, cutting cost by about 90%
- utilizes hydrophilic property of monolayer and hydrophobic property of glass slide
- TUBE = 15 ml centrifuge tube, with about 5 ml H2O at bottom, sealed (no parafilm needed if in ov4ºC)
- abbreviations: Room temp (RT), Overnight (Ov)

Materials:
Goat serum (GS; Sigma G9023-10ml)
16% Paraformaldehyde (Electron Microscopy Sciences RT15710)
ProLong Gold antifade reagent with DAPI (Invitrogen P36931)
Primary antibodies:
Mouse-anti-CK18 (Chemicon MAB3234)
Rabbit-anti-ZO1 (Invitrogen 61-7300)
Mouse-anti-Occludin (Invitrogen 33-1500)
Rabbit-anti-phospho-MLC (Thr18/Ser19; Cell Signaling 3674)
Rabbit-anti-total-MLC (Cell Signaling 3672)
Rabbit-anti-phospho-Cofilin (Ser3; Cell Signaling 3313)
Rabbit-anti-total-Cofilin (Cell Signaling 3318)
Rabbit-anti-phospho-LIMK1/2 (Thr508/5; Cell Signaling 3841)
Rabbit-anti-total-LIMK1 (Cell Signaling 3842)
Rabbit-anti-total-LIMK2 (Cell Signaling 3844)
Mouse-anti-F/G-ACTIN (Chemicon MAB1501R)
Rabbit-anti-proSPC (surfactant protein C) use at 1:200
Mouse-anti-RTI40 (rat type I marker) use at 1:250
Mouse-anti-vimentin (mesenchymal marker) use at 1:200
Secondary antibodies:
FITC-goat-anti-mouse (Jackson Immuno 115-095-146)
FITC-donkey-anti-rabbit (Jackson Immuno 711-095-152)
Alexa Fluor Phalloidin-633 (Invitrogen A22284)
Prolong Gold antifade reagent with DAPI (Invitrogen P36931)
DyLight 488 donkey-anti-mouse IgG (H+L; Jackson Immuno 715-485-150)
DyLight 594 donkey-anti-mouse IgG (H+L; Jackson Immuno 715-515-150)

(on slide protocol developed by Brian DiPaolo, bdipaolo@seas.upenn.edu)
(immunocytochemistry protocol by Brian DiPaolo; derived from protocol by Michele Hawk)
PERIJUNCTIONAL ACTIN RING (PIAR) QUANTIFICATION PROTOCOL

OPEN ImageJ (ver. 1.43o)

FILE – OPEN – (Sample letter/well)(Image location number)_PrjData0(dye number).tif
OR, if project –
FILE – OPEN - (Sample letter)(Image location number)_PrjData0(dye number).pic
Analyze – SetScale – no scale
IMAGE – STACKS – Z Project…. – default slice numbers (unless one image is full intensity)
-Projection Type: Max Intensity (this is what confocal software does by default, and what I have done in my images at in the past)
-SAVE (optional)

VERIFY (at top of project image) that 202.92 x 202.92 µm (1024 x 1024)

-TO GET OBJECTIVE: Image – Show Info: INFO_OBJECTIVE_NAME = ?

Import first OVERLAY by:
-ANALYZE – TOOLS – ROI manager
-More – OPEN: OVERLAY_9squares_RoiSet.zip
 (this is an overlay that simply divides image into 9 equal square parts)
-IMAGE – OVERLAY – FROM ROI MANAGER
-(ROI Manager) DELETE all ROIs to continue with image
-after this is done, you can import the overlay in subsequent images without having to first import to ROI Manager (IMAGE – OVERLAY - SHOW OVERLAY)

DUAL ACTIN-ZO1 STAINING selection
(channel 1 should be F-ACTIN)
-open 2 channel .tif
-Image – Adjust – Brightness/contrast
-channel 2 – AUTO (to see cells more clearly)
-make cell outlines on CHANNEL 2 (ZO-1), freehand selection tool
-‘JOG’ to channel 1
-THEN hit T to capture (will place ROI onto channel 1, F-ACTIN)
-NOTE (ROI manager will display first four digits as 0001 if placed in correct channel)
-go to CHANNEL 1 – RESET brightness contrast
-THEN run script/macro ('MultiCell_40X_AdinBri.txt'; program below in section 'Cell fluorescence intensity (whole, cytoplasm, annulus) calculator script for Image J')

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NOTES:

CELL SELECTION CRITERIA: any cell with at least 50% (approximate, bye eye) of its area contained in regions 1, 3, 5, 7, and 9 (that is, if we label regions from 1 – 3 on top row, 4-6 in middle row, and 7-9 in bottom row)

-FREEHAND tool (looks like a heart)
  -trace boundary of cell
  -ZOOM the image to help accuracy
  -(ROI Manager) Add [t]  (HOTKEY: t)

-repeat for all cells that meet CELL SELECTION CRITERIA
  -Note: If you wish to see the cells you’ve already selected: (ROI Manager) – Show All

-PLUGINS – MACROS – RUN: Multicell_40X_AdinBri.txt
  -NOTE: there may be a few studies that were imaged with higher/lower magnification; in which case we will run a slightly different script
    -for 60X images, use enlarge/band values of 4/8 instead of 5/10 used in the 40X program

  -NOTE: be sure to wait for journal to complete its analysis (there will be 3x measurements for every cell you’ve outlined: measure for WHOLE cell, measure for CYTOPLASM, measure for BAND)

-IF ERROR (invalid distance):
  -note ROI that is causing error
  -RESET ImageJ
  -open image, open saved temp ROIs
  -delete ROI causing error and rerun

-Import measurements into running xls sheet
  -this sheet has an extra column: disk # (add this information)

  -import data into three separate columns which line up:
    -whole cell measurements (first half of your measurement data)
    -cytoplasm measurements
    -band measurements (second half of your measurements data)

-Repeat for each image

(protocol by Brian DiPaolo, bdipaolo@seas.upenn.edu; with input from Adi Yerrapureddy)
CELL FLUORESCENT INTENSITY (WHOLE, CYTOPLASM, ANNULUS)
CALCULATOR SCRIPT FOR IMAGEJ

//---START PROGRAM---
//MultiCell_40X_AdinBri.txt
//TO RUN: open Image J (ver. 1.43o)
   //trace cell boundaries (add each to ROI manager with the key 'T')
   //Plugins - Macros - Run..., select this script file

//Cell periphery analysis script for ImageJ
//by Brian DiPaolo (bdipaolo@seas.upenn.edu) with input from Adi Yerrapureddy
// 40X EPI scope version (boundary thickness is scale dependent)

// INITIALIZE SCALE
run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel");

// WHOLE-CELL MEASUREMENT
for (i=0; i<roiManager("count"); i++) {
   roiManager("Deselect");
   roiManager("Select", i);
   run("Enlarge...", "enlarge=5");
   roiManager("Update");
}
run("Set Measurements...", "area mean standard modal min centroid center perimeter bounding fit shape
feret's integrated median skewness kurtosis area_fraction stack display redirect=None decimal=3");

roiManager("Deselect");
roiManager("Measure");

// INITIALIZE SCALE
run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel");

// CYTOPLASM MEASUREMENT
for (i=0; i<roiManager("count"); i++) {
   roiManager("Deselect");
   roiManager("Select", i);
   run("Enlarge...", "enlarge=-10");
   roiManager("Update");
}

roiManager("Deselect");
roiManager("Measure");

// INITIALIZE SCALE
run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel");

// ANNUlus MEASUREMENT
for (i=0; i<roiManager("count"); i++) {
   roiManager("Deselect");
   roiManager("Select", i);
   run("Make Band...", "band=10");
   roiManager("Update");

   // INITIALIZE SCALE
}
run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel");
}

roiManager("Deselect");
roiManager("Measure");
//---END of PROGRAM---
APPENDIX D: MICROBEAD STRETCH PROTOCOL

SOLUTIONS
PBS (pH 7.4): 400 ml stock
- 38 ml Monobasic (27.602 NaH$_2$PO$_4$.H$_2$O gm/liter ddH$_2$O)
- 162 ml Dibasic (28.392 Na$_2$HPO$_4$ gm/liter ddH$_2$O)
- 3.6 g NaCl
- 200 ml ddH$_2$O
- check pH for 7.4

50 mM Carbonate buffer: 250 ml stock
- Measure **0.3975** g of Sodium carbonate (Na$_2$CO$_3$)
- Measure **0.7325** g of Sodium bicarbonate (NaHCO$_3$)
- Mix and homogenize in a beaker with **245** ml ddH$_2$O
- Adjust pH to 9.4 (using NaOH, and HCl if go over)
- Adjust volume to **250** ml in volumetric flask using ddH$_2$O
- Filter for sterilization (HOOD) using 0.22 µm filter

MICROBEAD PREPARATION
- Vortex beads in ethanol stock solution (provided by Harvard University)
- Calculate volume needed for 1 mg beads using stock solution density
  ex: density 2.9 mg/ml => need 0.345 ml for 1 mg beads
- HOOD Remove volume and place in centrifuge tube, ADD PBS to total 1 ml
- HOOD Centrifuge (16,000 × g), stop, replace solution with fresh PBS
- HOOD Centrifuge (16,000 × g), stop, replace buffer with fresh PBS
- HOOD Centrifuge (16,000 × g), stop, replace buffer with 1 ml carbonate buffer
- Skirt sonicator with ethanol then wipe down
- Test sonicator in ddH$_2$O
- Set dial to about 1 (results in about 5 watts rms) for 9 seconds and test
- Place solution so sonicator tip is centered and press start
- Clean sonicator with ddH$_2$O
- HOOD Add 150 µg peptide (per 1 mg beads)
  (30 µl of 5 mg/ml [RGD] solution)
- FRIDGE (4ºC) Rotate labeled tubes overnight (can keep for few months)

Materials:
  RGD (Arg-Gly-Asp-Ser; Sigma A9041-2mg)
  AcLDL (acetylated low-density lipoprotein; Invitrogen L35354)
  Ferromagnetic microspheres:
    Harvard School of Public Health, Physiology Program Lab
    Emil Millet (emillet@hsph.harvard.edu)
    665 Huntington Ave., Boston, MA 02115
  (protocol by Brian DiPaolo, bdipalo@seas.upenn.edu; derived from protocol by Guillaume Lenormand at Harvard University)
MONOLAYER WITH MICROBEADS STRETCH PROTOCOL

DATE:     WELLS:
-Cells:  4 day old AECs or A549
-Microbead prep date:
-Serum deprivation time:
   1.5 ml DMEM+Hepes
-TREATMENT:
-Apply bead solution w/ RGD/AcLDL
   SMALL WELL:  100 µl in glass well
   MEDIUM WELL: 100 µl in glass well

-Incubate beads for 50 minutes, very gentle shake
-Meanwhile image glued bead slide standard for control (5 minutes – 300s)

-Wash off beads with Room Temperature solution
-wash 2× with same DMEM+Hepes (microbead supernatent)
-wash 2× with new DMEM+Hepes
-add 4.0 ml DMEM+Hepes to medium well (1.0 ml to small well)

-Metamorph: acquire time-lapse with parameters below; make sure each sample runs for 5 min (time it with separate timer)

-save each 5 min interval as SAMPLE_TIMEPOINT (ex BM_PW05) in folder w/ same name (folder and sample file designations important for bead analysis MATLAB program)

-Place on scope (RT) and setup optics and program (total step ~5 min)
   -Image for 5 min at 5, 15, 25 (post wash, PW) – so total post wash time is 30min

-Stretch (5.5 turns for 25%∆SA, 7 turns for 37%∆SA - see (11)
   fig 5.)
   -(S) Image at 1, 10, 20, 30, 40, 50, 60 min
   -(R) Release stretch and image for 5 min

   (1280x1024 .bmp 8-bit image)
   Objective: 20x N.A.: 0.5
   Frequency: 1 image / s
   Capture time: 300 s
   Exposure time: ms (usually 20 ms) with setting D

NOTE DISK:
NOTE GROUPS: sample coating stretch treat serum dep
(protocol by Brian DiPaolo, bdipalo@seas.upenn.edu; based on assistance provided by Guillaume Lenormand at Harvard University)
BEAD ANALYSIS SOFTWARE PROTOCOL
After acquiring images as above, perform the following steps using supplied MATLAB files to process bead motion data into mean squared displacement (MSD) information. Programs run in MATLAB ver. 7.6.0.324 (R2008a) with Image Processing Toolbox

1 - Run tracking.m on each folder of images
    (use multiple_tracking.m)
    -if program cannot find image folder – cut actual folder name and input it into the folder_names variable (for some reason typing sometimes does not allow program to recognize, but this is very uncommon)

    -if program quits for this reason:
        ??? Undefined function or variable 'xcorr2'.
        -Simply restart Matlab and try again (perhaps buffer flushing cures this problem)

2 - Run MSD_Bri_2 on each _ana file (output is disk mean MSD)
    -select _ana file
    -input output file names (fineName.tmed, .tcmed, and .time)
    -turn on auto_exclude_method (set = 1) to automatically exclude extreme single bead outliers
    -program will allow you to visually exclude beads (remove stuck together beads, beads tracked improperly based on red tracking trail, and other odd looking beads/tracks)
    -IMPORTANT: exclude only beads that visually appear erroneous
    -with manual exclusion turned ON, program will allow you to exclude beads based on x and y displacement values (by comparing them to x and y standard variations (1 light blue box, 2 dark blue box))
      -click in circle (next to bead number) to exclude – number will turn red
      -click beads to exclude and click to the left of Y-axis to refresh variation box calculation and removed already excluded beads
      -when finished excluding, click below X-axis to run program to completion and produce addended bead file (named diskxxx)
    -close data windows
    -OPTIONAL- copy excluded bead data from Matlab window into word file
    -run for each _ana file in a disk

3 - Run total_data_analysis on .tmed file (output is a restructured MSD file for an entire disk called .tmedX)
    -copy all RUN_FOLDER files into disk analysis folder (for program, analysis value, and structure reference and backup)

    -OUTPUT (2 files)
.tmedX: file with median MSD (of all beads) from each image epoch at each image time (1 - 300 seconds)
   - rows (1 - 300): sample time (corresponding to time in .time file)
   - columns (# of samples): median MSD for each image epoch (corresponding to sample designations in .time file)
   - for MSD_{100}, use data from row 100

.time: file with time for each
   - rows (# of samples): different samples at each image epoch
   - column 1: sample name (letter) and image epoch name (05PW, 15PW, 25PW, 01S, 10S, 20S, 30S, 40S, 01R)
   - column 2 - 301: sample times corresponding to each row in .tmedX file

OPTIONAL FOR SINGLE BEAD DATA Run single_bead_reader.m on each .mmr file (output is a total disk file with each bead MSD)

(protocol by Brian DiPaolo, bdipalo@seas.upenn.edu; based on assistance provided by Guillaume Lenormand at Harvard University)
MICROBEAD TRACKING MATLAB CODE

USER EXECUTED MAIN PROGRAMS (see above for procedure):

multiple_tracking.m:
%---START PROGRAM---
%multiple_tracking.m
%by Brian DiPaolo, bdipaolo@seas.upenn.edu

%#1: INPUT FOLDER NAMES BELOW
%#2: PLACE 000 images in each folder

%#3: change LINE 45 of (tracking.m):
%directory = strcat('C:\Documents and Settings\Head Injury Lab\My Documents\BDiPaolo\Beads\SAMPLES', input_folder_name)
%to suite proper directory name

clear;
folder_names{1} = 'CD_PW25';
folder_names{2} = 'CD_S40';
folder_names{3} = 'CD_SR01';
%''                     ADD ALL FOLDERS HERE
for i = 1:size(folder_names, 2)
    analyze = char(folder_names(:, i));
    tracking(analyze);
end
%---END PROGRAM---

MSD_Bri_2.m:
%---START PROGRAM---
%MSD_Bri_2.m
%by Brian DiPaolo with assistance from Guillaume Lenormand
%requires uigetfiles.m

%%%%% DIRECTIONS
%Run tracking.m with following correct parameters
%diameter
%mag
%max_im
%file_name_base
%dim
%Run this program (MSD_Bri.m) on the output txt file _ana

****************** BEGINNING OF PROGRAM
******************************
clear all;
colordef black;

%4-21-05
%Reads pixel placement data from tracking.m program into matrix and calculates mean squared displacement (MSD)

%VARIABLES
***************************************************************
fileName.tmed = ['disk120.tmed'];  %total disk median MSDr
fileName.tcmed = ['disk120.tcmed'];  %total disk cumulative median MSDr
fileName.time = ['disk120.time'];

objective = '20X';
%image_frequency = 1;  % (images per s)
%time_conversion = 1 ./ image_frequency;
time_conversion = 1;  %s / image#  (0.6876)

%exclusion Parameters
remove_first_frames = 0;  %1 will remove first 000 image (which is a copy of 001) (0 removes zero images - similar to GuillaumeMSD.m output)
manual_exclude_method = 1;  %Mouse Over Beads=1  Bead # Input=2  NoExclude=0
auto_exclude_method = 0;  %MEDIAN=1  MEAN=2  NoExclude=0
number_medians_to_exclude_outlier = 5;  %higher number, less beads excluded
number_means_to_exclude_outlier = 3;  %higher number, less beads excluded

home_directory = 'C:\Documents and Settings\Head Injury Lab\My Documents\BDiPaolo\Beads\RUN_FOLDER';
%**********************************************************************
***

%INITIALIZATIONS
if objective == '20X'
distance_conversion = 335.561;  %nm / pixel for 1280x1024 bmp
%distance_conversion = 415;  %nm / pixel Dr. Lanir Data
else objective == '40X'
distance_conversion = 168.541;  %nm / pixel for 1280x1024 bmp
end
remove_columns = [];  %keep 1 so bead outlier selection loop will begin
stage_move = [];
beads_removed = [];

distance_conversion = 1   %for testing purposes (pixels)

%READ _ANA FILE INTO A MATRIX
[fileName.raw,directory] = uigetfiles('*.txt','Select the DATA file for analysis');
if isempty(fileName.raw), return; else end;
if directory == []
end
cd(directory);
fileName.raw = char(fileName.raw);
%changes fileName into a string
ANA_DATA = dlmread(fileName.raw,';');
cd(home_directory);
disp(' '); disp(' '); disp('________________________________');
disp('Mouse < Y axis = renew plot')
disp('Mouse < X axis = finalize plot')
disp(date)
disp(sprintf('Objective: %s ', objective))
disp(sprintf('Data file: %s', fileName.raw))

[timepoints, beads_raw] = size(ANA_DATA);
total_beads = (beads_raw - 3 - 1).* 1 ./ 3; %uses size of array to find bead #, subtracts blank columns, time column, and other such extra columns
disp(sprintf('Total beads: %d', total_beads))

%Reading in time and initializing BeadX and BeadY
nzTIME = ANA_DATA(:,1);
BEAD_X = [];
BEAD_Y = [];

for i = 1:size(nzTIME, 1)
    TIME(i) = nzTIME(i) - nzTIME(1);
end

%Reading coordinates into BEAD_Y and BEAD_Y
for bead = 1:total_beads
    BEAD_X = [BEAD_X ANA_DATA(:, ((bead-1) * 3) + 4))];
    BEAD_Y = [BEAD_Y ANA_DATA(:, ((bead-1) * 3) + 5))];
end

%where
%BEAD_X(time point, bead #)
%BEAD_Y(time point, bead #)

%remove first x number of timepoints (based on initial variables)
remove_first_frames_vec = [1:1:remove_first_frames];
BEAD_X(remove_first_frames_vec, :) = [];
BEAD_Y(remove_first_frames_vec, :) = [];
timepoints = timepoints - remove_first_frames;
TIME(remove_first_frames_vec) = [];

%MANUAL BEAD REMOVAL
if manual_exclude_method == 1;
    [figfileName, directory] = uigetfiles('*.fig','Select the plot file for analysis');
    if isempty(figfileName), return; else end
cd(directory);
    figfileName = char(figfileName); %changes fileName into a string
    openfig(figfileName);
disp(sprintf('Figure file: %s', figfileName))
cd(home_directory);
% Placing bead numbers next too corresponding beads
set(0,'DefaultTextFontSize',8)
figure(1)
for bead = 1:total_beads
    hold on
    plot(BEAD_X(1, bead), BEAD_Y(1, bead), 'yo')
    rectangle('Position', [BEAD_X(1, bead)+6 BEAD_Y(1, bead)+7 24 17], 'FaceColor', [0 0 0])
    point = sprintf('%d', bead);
    text(BEAD_X(1, bead)+8, BEAD_Y(1, bead)+16, point, 'color', [1 0.7 0.3])
end

x_mouse = 1;
y_mouse = 1;

while x_mouse > 0
    [x_mouse, y_mouse] = ginput(1);
    for bead = 1:total_beads
        if x_mouse < BEAD_X(1, bead)+8 && x_mouse > BEAD_X(1, bead) - 8
            if y_mouse < BEAD_Y(1, bead)+8 && y_mouse > BEAD_Y(1, bead) - 8
                remove_columns = [remove_columns bead];
                point = sprintf('%d', bead);
                text(BEAD_X(1, bead)+8, BEAD_Y(1, bead)+16, point, 'color', 'red')
            end
        end
    end
end

beads_removed = remove_columns;
elseif manual_exclude_method == 2;
    remove_columns = input('Input bead numbers to exclude(matrix format): ');
else manual_exclude_method == 0;
    remove_columns = [];
end

BEAD_X(:, remove_columns) = [];
BEAD_Y(:, remove_columns) = [];
total_beads = total_beads - length(remove_columns);
disp('Visual Manual exclusion: ');
disp(sprintf('%d ', remove_columns))
beads_removed = [beads_removed remove_columns];

set(0,'DefaultTextFontSize',14) %original default value

% CONVERT FROM PIXELS (1ST PROGRAM OUTPUT) TO ACTUAL DISTANCE
BEAD_X = BEAD_X .* distance_conversion;
BEAD_Y = BEAD_Y .* distance_conversion;
TIME = TIME .* time_conversion;

%AUTOMATIC REMOVE OUTLIER BEADS
warning off MATLAB:divideByZero;
remove_columns = [];

if auto_exclude_method == 1
    % Calculate distance of each bead from timepoint 0
    for counter = 1:timepoints
        X_dist_0(counter, :) = BEAD_X(counter, :) - BEAD_X(1, :);
        Y_dist_0(counter, :) = BEAD_Y(counter, :) - BEAD_Y(1, :);
    end
    % calculates the maximum distance traveled (from 0) for each bead
    max_x = max(abs(X_dist_0(1:timepoints, :)));
    max_y = max(abs(Y_dist_0(1:timepoints, :)));%
    % calculates the median (over all beads) of each beads maximum traveled distance
    med_max_x = median(max_x);
    med_max_y = median(max_y);

    for bead = 1:total_beads,
        if (max_x(bead) > number_medians_to_exclude_outlier .* med_max_x) || (max_y(bead) > number_medians_to_exclude_outlier .* med_max_y)
            remove_columns = [remove_columns bead];
        end
    end
elseif auto_exclude_method == 2
    % Calculate distance of each bead from timepoint 0
    for counter = 1:timepoints
        X_dist_0(counter, :) = BEAD_X(counter, :) - BEAD_X(1, :);
        Y_dist_0(counter, :) = BEAD_Y(counter, :) - BEAD_Y(1, :);
    end
    % calculates the maximum distance traveled (from 0) for each bead
    max_x = max(abs(X_dist_0(TIME, :)));
    max_y = max(abs(Y_dist_0(TIME, :)));%
    % calculates the median (over all beads) of each beads maximum traveled distance
    mean_max_x = mean(max_x);
    mean_max_y = mean(max_y);

    for bead = 1:total_beads,
        if (max_x(bead) > number_means_to_exclude_outlier .* mean_max_x) || (max_y(bead) > number_means_to_exclude_outlier .* mean_max_y)
            remove_columns = [remove_columns bead];
        end
end
elseif auto_exclude_method == 0
    remove_columns = [];
end

s = size(X_dist_0);

med_x = median(X_dist_0');
med_y = median(Y_dist_0');
X_dist = X_dist_0 - med_x' * ones(1, total_beads);
Y_dist = Y_dist_0 - med_y' * ones(1, total_beads);
MSD_r = zeros(s(1) - 1, total_beads);
for i = 1:s(1),
    for j = 1:s(1) - i,
        Temp = 1/(s(1) - i)*((X_dist(j+i, :) - X_dist(j, :)).^2 +
        (Y_dist(j+i, :) - Y_dist(j, :)).^2);
        MSD_r(i, :) = Temp + MSD_r(i, :);
    end
end

BEAD_X(:, remove_columns) = [];
BEAD_Y(:, remove_columns) = [];
total_beads = total_beads - length(remove_columns);
disp('Auto exclusion:');
disp(sprintf('%d ', remove_columns));
beads_removed = [beads_removed remove_columns];

%PLACE BLUE MARK ON BEADS THAT WERE INCLUDED
if manual_exclude_method == 1;
    for bead = 1:total_beads
        plot(BEAD_X(1, bead)/distance_conversion, BEAD_Y(1, bead)/distance_conversion, 'b+');
    end
end

%MAIN PROGRAM
finish_remove = 0;
while finish_remove == 0
    [MSD_x, MSD_y, MSD_r, MSD_r0_square] = calc_MSD(BEAD_X, BEAD_Y, timepoints, total_beads);
    [remove_columns, finish_remove] = outlier_removal(MSD_x, MSD_y, total_beads);
    BEAD_X(:, remove_columns) = [];
    BEAD_Y(:, remove_columns) = [];
total_beads = total_beads - length(remove_columns);
    beads_removed = [beads_removed remove_columns];
end
\[
\text{[MSD}_x, \text{MSD}_y, \text{MSD}_r, \text{MSD}_r0\_square] = \text{calc\_MSD}(\text{BEAD}_X, \text{BEAD}_Y, \text{timepoints}, \text{total\_beads});
\]
\[
\text{[noise}_x, \text{noise}_y, \text{noise}_r] = \text{noise}(\text{BEAD}_X, \text{BEAD}_Y);
\]
\[
\text{disp('Manual+Auto exclusion:')};
\text{disp(sprintf('\%d ', beads\_removed))}
\]
\[
\text{MSDx\_avg} = \text{mean(MSD}_x');
\text{MSDy\_avg} = \text{mean(MSD}_y');
\text{MSDr\_avg} = \text{mean(MSD}_r');
\text{MSDx\_med} = \text{median(MSD}_x');
\text{MSDy\_med} = \text{median(MSD}_y');
\text{MSDr\_med} = \text{median(MSD}_r');
\text{MSDx\_std} = \text{std(MSD}_x');
\text{MSDy\_std} = \text{std(MSD}_y');
\text{MSDr\_std} = \text{std(MSD}_r');
\]
\[
\text{disp('Median Noise [x, y, r] (nm^2):')};
\text{disp(sprintf('\%f \%f \%f', median(noise}_x), median(noise}_y), median(noise}_r))}
\]
%DATA
%changes TIME into timepoints - 1 TIME due to MSD calculation is a
%change in time calculation
\[
\text{msd\_TIME} = \text{TIME};
\text{msd\_TIME(timepoints)} = [];\text{msd\_TIME(timepoints-1)} = [];\text{msd\_TIME(timepoints-1)} = [];
\]
\[
\text{figure(4)}
\text{plot(msd\_TIME, MSDr\_med)}
\text{title('Median MSDr vs. time')}
\text{xlabel('Time (s)')}
\text{ylabel('Median MSDr')}
\]
\[
\text{figure(5)}
\text{plot(msd\_TIME, cumsum(MSDr\_med))}
\text{title('Cumulative Median MSDr vs. time')}
\text{xlabel('Time (s)')}
\text{ylabel('Median MSDr')}
\]
\[
\text{for}\ i = 1:\text{total\_beads}
\text{figure(6)}
\text{hold on}
\text{\%msdr}\_\text{bead} = \text{MSD}\_r(:,\ i);
\text{semilogy(msd\_TIME, MSD}\_r(:,\ i))
\text{title('MSDr (all beads) vs. time')}
\text{xlabel('Time (s)')}
\text{ylabel('MSDr')}
\text{end}
% % % FIND BEADS IN WHICH TO EXCLUDE BASED ON GRAPHS
% % % REPLOT ALONG WITH CUMULATIVE MSD PLOT

% Writing data to files
% Writes a file with current files time, averageMSDr, medianMSDr, r0^2, and single beads MSD for each time
fileName.root = strtok(fileName.raw, '.');
fileName.mmr = [fileName.root '.mmr'];
fid = fopen(fileName.mmr, 'w');

fprintf(fid, '%s	', fileName.mmr);
fprintf(fid, '%s
', ' ');
fprintf(fid, '%s	', 'time (s)', 'MSDr_avg', 'MSDr_med', 'MSD_r0^2', 'single bead MSD');
fprintf(fid, '%s
', ' ');
fprintf(fid, '%s
', ' ');
for i = 1:length(msdTIME),
    fprintf(fid, '%g	', msdTIME(i));
    fprintf(fid, '%g	', MSDr_avg(i));
    fprintf(fid, '%g	', MSDr_med(i));
    fprintf(fid, '%g	', MSD_r0_square(i));
    for j = 1:total_beads,
        fprintf(fid, '%g	', MSD_r(i, j));
    end
    fprintf(fid, '%s
', ' ');
end
fclose(fid);

% Writes a file with all the current DISKS median MSDr
fid = fopen(fileName.tmed, 'a');
fprintf(fid, '%s	', fileName.root);
for i = 1:length(msdTIME),
    fprintf(fid, '%g	', MSDr_med(i));
end
fprintf(fid, '%s
', ' ');
fclose(fid);

% Writes a file with all the current DISKS cumulative median MSDr
fid = fopen(fileName.tcmed, 'a');
cumulative_MSDr_med = cumsum(MSDr_med);
for i = 1:length(msdTIME),
    fprintf(fid, '%g	', (cumulative_MSDr_med(i)));
end
fprintf(fid, '%s
', ' ');
fclose(fid);

% Writes a file with all the current DISKS timepoints
fid = fopen(fileName.time, 'a');
fprintf(fid, '%s\t', fileName.root);
for i = 1:length(msdTIME),
    fprintf(fid, '%g\t', msdTIME(i));
end
fprintf(fid, '%s
', ' ');
fclose(fid);

% mean of all beads displacement^2 over all times
disp(sprintf('Total beads (after exclusion): %d', total_beads))
% MSD = mean(transpose(bead_time_mean)); % - stage_MSD

% Cumulative_stagemove = sum(stage_move);
% disp(sprintf('Cumulative stage move (nm^2): %f nm^2', Cumulative_stagemove));

% Cumulative_stdev = sum(bead_bead_stdev);
% disp(sprintf('Cumulative standard deviation (nm^2): %f nm^2', Cumulative_stdev));

Cumulative_avg_MSD = sum(MSDr_avg);
disp(sprintf('Cumulative avg MSD (nm^2): %f nm^2', Cumulative_avg_MSD))
Cumulative_med_MSD = sum(MSDr_med);
disp(sprintf('Cumulative median MSD (nm^2): %f nm^2', Cumulative_med_MSD))

%figure(5)
%hist(bead_time_mean(:))
%title('Time mean MSD in each bead')
%xlabel('(nm)')
%ylabel('number')

%figure(6)
%plot(msdTIME, cumsum(bead_bead_mean))
%title('Bead mean MSD vs. time')
%xlabel('Time (s)')
%ylabel('Cumulative MSD (nm^2)')

fileName.raw = regexprep(fileName.raw, 'ana.txt', '');
mean_outfileName.raw = strcat(fileName.raw, 'OUTPUTmean.txt');
med_outfileName.raw = strcat(fileName.raw, 'OUTPUTmed.txt');
%fid = fopen(outfileName.raw, 'w');
%fprintf(fid, '%s', outfileName.raw);
%dlmwrite(mean_outfileName.raw, cumsum(bead_bead_mean), '\r');
%dlmwrite(med_outfileName.raw, cumsum(bead_bead_med), '\r');

disp('________________________________');
%*********************** END OF PROGRAM
******************************************************************************
%---END PROGRAM---
**total_data_analysis.m:**

```matlab
%---START PROGRAM---
%total_data_analysis.m
%by Brian DiPaolo
%change input disk file names fineName.data and .time

%Program sequesters all whole disk data, plots it, and transposes data into
%a separate file so it may be imported into excel

%uses output from MSD_Bri_2.m

clear all;
fileName.data = ['disk120.tmed']; %total disk median MSDr
fileName.time = ['disk120.time'];
names = textread(fileName.data, '%s*[^\n]');

%reading data from files
    data = dlmread(fileName.data, '\t', 0, 1);
    time = dlmread(fileName.time, '\t', 0, 1);

%removes end-of-line character from data
    data(:, size(data, 2)) = [];
    time(:, size(time, 2)) = [];

plot(time(1, :), data)
legend(names)
xlabel('time (s)');
ylabel('Cumulative median MSD (nm^2)');

transpose_data = data';
transpose_names = names';

fileName.root = strtok(fileName.data, '.');
fileName.dataX = [fileName.root '.tmedX'];
%fileName.dataXname = [fileName.root '.tcmedXn'];
fwrite(fileName.dataXname, transpose_names, 'schar');
dlmwrite(fileName.dataX, transpose_data, '\t');

%run STUDY_data_analysis.m for MATLAB analysis
%include best_fit_function.m
%---END PROGRAM---
```

**single_bead_reader.m:**

```matlab
%---START PROGRAM---
%single_bead_reader.m
%by Brian DiPaolo

clear;
```
fileName.data = ['AK_PW15_ana.mmr']; %total disk median MSDr

file_data = dlmread(fileName.data, '\t', 2, 4);
    file_data(:, size(file_data, 2)) = []; %removes last column of 0's, not needed

file_name = char(textread(fileName.data, '%s'));

f_name = [];
counter = 1;
i = 'O';
while not(strcmp(i, '.'))
    i = file_name(1, counter);
    if not(strcmp(i, '.')) f_name = [f_name i]; end
    counter = counter + 1;
end

%[names, times] = data_analysis_sort(file_name(1, :))

%Writes a file with all the current DISKS cumulative median MSDr
fileName = 'disk999.tmed';
for bead_number = 1:size(file_data, 2)
    fid = fopen(fileName, 'a');
    fprintf(fid, '%s	', fileName.root);
    for i = 1:size(file_data, 1),
        fprintf(fid, '%g	', transpose(file_data(:, bead_number)));
    end
    fprintf(fid, '%s
', ' ');
    fclose(fid);
end

%Writes a file with all the current DISKS timepoints
fid = fopen(fileName, 'a');
fprintf(fid, '%s	', fileName.root);
for i = 1:length(msdTIME),
    fprintf(fid, '%g	', msdTIME(i));
end
fprintf(fid, '%s
', ' ');
fclose(fid);

%---END PROGRAM---

MICROBEAD TRACKING FUNCTIONS:

average_slope.m:
%---START PROGRAM---
%average_slope.m
%by Brian DiPaolo

function [avg_intercept, avg_slope] = average_slope(MSD_data, time_data)
%~~~~~~~~~~~~~~~~~~~~ for all samples data
    slopes = [];
intercepts = [];
for i = 1:size(MSD_data, 1)-1
    slopes = [slopes; (log((MSD_data(i+1, :))) - log((MSD_data(i, :)))) ./ (log(time_data(i+1)) - log(time_data(i))));
    intercepts = [intercepts; log((MSD_data(i, :))) - (slopes(i) .* log((time_data(i))));
end

avg_slope = mean(slopes);
avg_intercept = exp(mean(intercepts));
%---END PROGRAM---

%---START PROGRAM---
% bead_mask.m
%by Ben Fabry, Harvard University

function bead = bead_mask(radius, dim)
%creates a synthetic bead
bead = [];
xi = 0;
for x = -dim:dim,
    % x = radius*(-1 + 2*(i-1)/(dim-1));
    xi = xi + 1;
    yi = 0;
    yp = sqrt(radius^2 - x^2);
    for y = -dim:dim,
        xp = sqrt(radius^2 - y^2);
        yi = yi + 1;
        if x^2 < radius^2 & y^2 < radius^2 & (x^2+y^2) < radius^2,
            Area=0.5*(xp-abs(x))*(yp-abs(y));
            Area = min(Area,1);
            Area = max(Area,0);
            bead(xi, yi) = Area;
        else
            bead(xi, yi) = 0;
        end;
    end
end
%---END PROGRAM---

%---START PROGRAM---
% calc_msd.m
%by Brian DiPaolo with assistance from Guillaume Lenormand and Ben Fabry

% CALCULATES MSD 063005
%run from MSD_Bri.m

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function [MSD_x, MSD_y, MSD_r, MSD_r0_square] = calc_msd(BEAD_X, BEAD_Y, timepoints, total_beads)

% Calculate distance of each bead from timepoint 0
for counter = 1:timepoints
    X_dist_0(counter, :) = BEAD_X(counter, :) - BEAD_X(1, :);
    Y_dist_0(counter, :) = BEAD_Y(counter, :) - BEAD_Y(1, :);
end

% Initial bead coordinates
X_0 = BEAD_X(1, :);
Y_0 = BEAD_Y(1, :);

% Calculates median 0 distance for each time
med_x = median(X_dist_0');
med_y = median(Y_dist_0');

% Plots median distances (of all beads) over time
figure(2)
subplot(2, 2, 1)
plot(med_x)
XLABEL('time point (#)')
YLABEL('median x 0-distance (nm^2)')
subplot(2, 2, 2)
plot(med_y);
XLABEL('time point (#)')
YLABEL('median y 0-distance (nm^2)')
subplot(2, 2, 3)
plot(sqrt((med_x.^2)+(med_y.^2)))
XLABEL('time point (#)')
YLABEL('median r 0-distance (nm^2)')

% Subtracts median (at each time) from each bead
X_dist = X_dist_0 - med_x' * ones(1, total_beads);
Y_dist = Y_dist_0 - med_y' * ones(1, total_beads);

% Uncomment to remove stage movement inclusion in calculation
% X_dist = X_dist_0;
% Y_dist = Y_dist_0;

Temp = 0;
s = size(X_dist_0); % Removes initial 000 images (which is a copy of 001 anyway)
MSD_x = zeros(s(1) - 1, total_beads);
for i = 1:s(1),
    for j = 1:s(1) - i,
        Temp = 1/(s(1) - i)*(X_dist(j+i, :) - X_dist(j, :)).^2;
        MSD_x(i, :) = Temp + MSD_x(i, :);
    end
end
clear Temp

MSD_y = zeros(s(1) - 1, total_beads);
for i = 1:s(1),
    for j = 1:s(1) - i,
        Temp = 1/(s(1) - i)*(Y_dist(j+i, :) - Y_dist(j, :)).^2;
        MSD_y(i, :) = Temp + MSD_y(i, :);
    end
end

clear Temp

MSD_r = zeros(s(1) - 1, total_beads);
for i = 1:s(1), %loop from 1 to number of timepoints
    for j = 1:s(1) - i, %loop from 1 to (number of timepoints -
current timepoint)
        Temp = 1/(s(1) - i)*((X_dist(j+i, :) - X_dist(j, :)).^2 +
        (Y_dist(j+i, :) - Y_dist(j, :)).^2);
        MSD_r(i, :) = Temp + MSD_r(i, :);
    end
end

clear Temp

%test calculates r_0 using a different method
r0 = sqrt(X_dist.^2 + Y_dist.^2);
r0_square = r0.^2;
MSD_r0_square = mean(r0_square');
std_r0_square = std(r0_square');

%---END PROGRAM---

---START PROGRAM---
center.m
%center.m
%by Ben Fabry, Harvard University

function coord = center(image,coord,dim)
% center of mass calculation

D = size(image);
for i = 1 : size(coord,1),
    total_mass=0;
    Sx=0;
    Sy=0;

    if coord(i,1)-dim<1
        xstart=1;
    else
        xstart=coord(i,1)-dim;
    end;
    if coord(i,1)+dim>D(1,2)
        xend=D(1,2);
    else
        xend=coord(i,1)+dim;

end;
end;
  if coord(i,2)-dim<1
    ystart=1;
  else
    ystart=coord(i,2)-dim;
  end;
  if coord(i,2)+dim>D(1,1)
    yend=D(1,1);
  else
    yend=coord(i,2)+dim;
  end;

clear b bb;
b=image(ystart:yend,xstart:xend);
b = (b - min(min(b)));
b = b/max(max(b));
tresh=0.5;
[y,x]=find(b>tresh);
for k = 1:length(x),
  int=b(y(k),x(k));
  total_mass=total_mass+int;
  Sx=Sx+(x(k)+xstart-1)*int;
  Sy=Sy+(y(k)+ystart-1)*int;
end;
% refine treshold
cx=round(Sx/total_mass);
cy=round(Sy/total_mass);
if cx-dim<1,
  xstart=1;
  cx=xstart+dim;
  xend=cx+dim;
else
  xstart=cx-dim;
end;
if cx+dim>D(1,2),
  xend=D(1,2);
  cx=xend-dim;
  xstart=cx-dim;
else
  xend=cx+dim;
end;
if cy-dim<1,
  ystart=1;
  cy=ystart+dim;
  yend=cy+dim;
else
  ystart=cy-dim;
end;
if cy+dim>D(1,1),
  yend=D(1,1);
  cy=yend-dim;
  ystart=cy-dim;
else
  yend=cy+dim;
end;
b = image(ystart:yend, xstart:xend);
b = (b - min(min(b)));
b = b / max(max(b));
cx = cx - xstart + 1;
cy = cy - ystart + 1;
bb = b;
bb(cy - (dim - 1):cy + (dim - 1), cx - (dim - 1):cx + (dim - 1)) = 0;
tresh = max(max(bb(:,:,:))) + 0.05;
if tresh > 0.99,
    tresh = 0.99;
end;
% re-calculate center of mass based on new treshold
total_mass = 0;
Sx = 0;
Sy = 0;
[y, x] = find(b > tresh);
for k = 1:length(x),
    int = b(y(k), x(k));
    total_mass = total_mass + int;
    Sx = Sx + (x(k) + xstart - 1) * int;
    Sy = Sy + (y(k) + ystart - 1) * int;
end;
coord(i, 1) = Sx / total_mass;
coord(i, 2) = Sy / total_mass;
coord(i, 3) = total_mass;
end;
%---END PROGRAM---

find_bead.m:
%---START PROGRAM---
%find_bead.m
% by Ben Fabry, Harvard University

function coord = find_bead(image, bead, dim)
% find beads via cross correlation
D = size(image);
v = xcorr2(image, bead);
v = (v - min(min(v)));
v = v / max(max(v));
vvv = v;
figure(3); imagesc(v); colormap(gray);
threshold = 0.4 * (max(max(v)) - mean(mean(v))) + mean(mean(v));
[yy, xx] = find(v > threshold);
coord = [];
% looking for the beads
[ymax, xmax] = size(v);
for k = 1:length(xx),
    if ((yy(k) > dim + 2) & (yy(k) < ymax - dim - 2) & (xx(k) > dim + 2) & (xx(k) < xmax - dim - 2)),
        test_image = v(yy(k) - dim:yy(k) + dim, xx(k) - dim:xx(k) + dim);
        max_int = max(max(test_image(:,:,:)));
        vvv(yy(k), xx(k)) = 0;
[y,x]=find(test_image>=max_int);
for l=1:length(x),
    if y(l)==dim+1 & x(l)==dim+1,
        center_image=test_image(2:2*dim,2:2*dim);
        test_image(2:2*dim,2:2*dim)=0;
        border=max(max(test_image));
        center=mean(mean(center_image));
        if border<0.3*(max_int-center)+center & xx(k)>2*dim & 
            xx(k)<D(1,2) & yy(k)>2*dim & yy(k)<D(1,1),
            coord = [coord; xx(k)-dim, yy(k)-dim, 0];
        end;
    end;
end;
figure(4); imagesc(vvv); colormap(gray);
%---END PROGRAM---

noise.m:
%---START PROGRAM---
%noise.m
%by Brian DiPaolo

%NOISE CALCULATION

%Measuring noise. With beads in glue, if noise is below 10nm, system
%noise is insignificant for our purposes
%clear X_dist_change Y_dist_change R_dist_change counter

function [noise_x, noise_y, noise_r] = noise(BEAD_X, BEAD_Y)
    timepoints = 20;
    for counter = 1:timepoints
        X_dist_change(counter, :) = BEAD_X(counter + 2, :) - 
        BEAD_X(counter + 1, :);
        Y_dist_change(counter, :) = BEAD_Y(counter + 2, :) - 
        BEAD_Y(counter + 1, :);
        R_dist_change(counter, :) = sqrt(X_dist_change(counter, :).^2 + 
        Y_dist_change(counter, :).^2);
    end
    noise_x = std(X_dist_change);
    noise_y = std(Y_dist_change);
    noise_r = std(R_dist_change);
    nmx = sprintf('Median x noise:  %f nm^2', median(noise_x));
    nmy = sprintf('Median y noise:  %f nm^2', median(noise_y));
    nmr = sprintf('Median r noise:  %f nm^2', median(noise_r));
    nx = sprintf('Mean x noise:  %f nm^2', mean(noise_x));
ny = sprintf('Mean y noise: %f nm^2', mean(noise_y));
nr = sprintf('Mean r noise: %f nm^2', mean(noise_r));

figure(7)
plot(noise_x, noise_y, 'r+')
title('Noise level (std of displacements) for each bead');
xlabel('std(x displacement) nm^2');
ylabel('std(y displacement) nm^2');
text(1, max(noise_y), nmx)
text(1, max(noise_y) - (1/25).*max(noise_y), nmy)
text(1, max(noise_y) - (2/25).*max(noise_y), nmr)
text(1, max(noise_y) - (4/25).*max(noise_y), nx)
text(1, max(noise_y) - (5/25).*max(noise_y), ny)
text(1, max(noise_y) - (6/25).*max(noise_y), nr)

%---END PROGRAM---

outlier_removal.m:
%---START PROGRAM---
%outlier_removal.m
%by Brian DiPaolo
%runs from MSD_Bri.m

function [remove_beads, finish_remove] = outlier_removal(MSD_x, MSD_y, total_beads)

    sum_MSD_x = sum(MSD_x);
    sum_MSD_y = sum(MSD_y);
    figure(3)
    plot(sum_MSD_x, sum_MSD_y, 'g.');
    xlabel('SUM of MSD x')
    ylabel('SUM of MSD y')

    maximum_sum_x = max(sum_MSD_x);
    maximum_sum_y = max(sum_MSD_y);

    mean_sum_x = mean(sum_MSD_x);
    mean_sum_y = mean(sum_MSD_y);
    std_sum_x = std(sum_MSD_x);
    std_sum_y = std(sum_MSD_y);

    set(0,'DefaultTextFontSize',8)

    hold on
    %rectangle position (bottom_left_corner width height)
    %width and height 1 stdev
    rectangle('Position', [mean_sum_x-1.*std_sum_x mean_sum_y-1.*std_sum_y 2.*std_sum_x 2.*std_sum_y], 'Facecolor', [0.0 0.0 1])
    %width and height 2 stdevs
    rectangle('Position', [mean_sum_x-0.5.*std_sum_x mean_sum_y-0.5.*std_sum_y std_sum_x std_sum_y], 'Facecolor', [0.3 0.6 1])

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text(mean_sum_x-0.5*std_sum_x, mean_sum_y+0.5*std_sum_y, '1 std', 'color', [1 1 1]);

text(mean_sum_x-1*std_sum_x, mean_sum_y+1*std_sum_y, '2 std', 'color', [1 1 1]);

plot(mean_sum_x, mean_sum_y, 'cx');

%line([mean_sum_x+(1/2).*std_sum_x mean_sum_x+(1/2).*std_sum_y],[0-(1/4).*maximum_sum_y maximum_sum_y+(1/4).*maximum_sum_y]);

%Placing bead numbers next too corresponding beads
figure(3)
for bead = 1:total_beads
    hold on
    plot(sum_MSD_x(bead), sum_MSD_y(bead), 'yo')
    rectangle('Position', [sum_MSD_x(bead)+maximum_sum_x./110 sum_MSD_y(bead)+maximum_sum_y./100 maximum_sum_x./17 maximum_sum_y./25], 'FaceColor', [1 1 1])
    point = sprintf('%d', bead);
    text(sum_MSD_x(bead)+maximum_sum_x./65, sum_MSD_y(bead)+maximum_sum_y./35, point, 'color', [0.2 0.5 0.3])
end

x_mouse = 1;
y_mouse = 1;
remove_columns = [];

square_xlen = maximum_sum_x./100;
square_ylen = maximum_sum_y./100;

while x_mouse(mouse_count) > 0 && y_mouse(mouse_count) > 0 && x_mouse(mouse_count) < 1281 && y_mouse(mouse_count) < 1025
    [x_mouse, y_mouse] = ginput(1);
    if y_mouse < 0,
        finish_remove = 1;
    else
        finish_remove = 0;
    end
end

for bead = 1:total_beads
    if x_mouse < sum_MSD_x(bead)+square_xlen && x_mouse > sum_MSD_x(bead) - square_xlen
        if y_mouse < sum_MSD_y(bead)+square_ylen && y_mouse > sum_MSD_y(bead) - square_ylen
            remove_columns = [remove_columns bead];
            point = sprintf('%d', bead);
            text(sum_MSD_x(bead)+maximum_sum_x./65, sum_MSD_y(bead)+maximum_sum_y./35, point, 'color', 'red')
        end
    end
end
end
hold off

    remove_beads = remove_columns;
%---END PROGRAM---

tracking.m:
%---START PROGRAM---
$tracking.m$
%by Ben Fabry at Harvard University with modifications by Brian DiPaolo
(bdipaolo@seas.upenn.edu)

function [] = tracking(input_folder_name);
% calculation of point of mass
P = path;
P = [P 'C:\Documents and Settings\Head Injury Lab\My
Documents\BDiPaolo\Beads\RUN_FOLDER'];
path(P);
cd('C:\Documents and Settings\Head Injury Lab\My
Documents\BDiPaolo\Beads\RUN_FOLDER');
%clear all;
colormap(gray);
home_directory = 'C:\Documents and Settings\Head Injury Lab\My
Documents\BDiPaolo\Beads\RUN_FOLDER';

% create the bead mask for use to crosscorrelate against a real image
% at present, the radius defined from observations of the image by the
user

diameter = 16;            %diameter of the bead in pixels
%diameter = 12
    %use diameter 16 for normal runs
    %use diameter 12 if you get many warning:divide by zero then errors

mag=20;                  %pixel magnification for red tracking (does not
effect displacement measurements)
%mag=40;

max_im=302;              % number of image

time_interval = 1;       % time interval between two images (KEEP AT 1 -
HANDLED IN MSD_Bri.m)

file_name_base = input_folder_name;
%file_name_base='AO_SN01';

radius = diameter/2;    
% mask dimensions, must be greater than radius
%dim = 22;

bead = bead_mask(radius,dim);
bead = (bead - min(min(bead)));
bead = bead/max(max(bead));
figure(1); imagesc(bead); colormap(gray); drawnow;

%load first image
[fileName.raw, directory] = uigetfiles("*.bmp','Select the first file for analysis");
fileName.raw = strcat(input_folder_name, '000', '.bmp')
directory = strcat('C:\Documents and Settings\Head Injury Lab\My Documents\BDiPaolo\Beads\SAMPLES\', input_folder_name)

if isempty(fileName.raw), return; else end;

%use this directory if image name is too long and program auto-quits
%directory = 'C:\Documents and Settings\Head Injury Lab\My Documents\BDiPaolo\LEFTOVERS - PUT ON CD\ON CD BeadTest070805\glue20X_0.75NA';
cd(directory);

file_no=1;
fileName1 = strcat(file_name_base, num2str(file_no-1, '%03d'));
[b2 map] = imread(fileName1, 'bmp');
cd(home_directory);

b2=ind2gray(b2,map);
%b2=b2(30:600,50:500);
b2 = double(b2);
b2 = (b2 - min(min(b2)));
b2 = 1-b2/max(max(b2));
figure(1); imagesc(b2); title('first image'); colormap(gray); drawnow;
coord = find_bead(b2, bead, dim);
figure(2); imagesc(b2); title('first image'); colormap(gray); drawnow;
figure(2); hold on;
plot(coord(:,1), coord(:,2), 'ro');
hold off;
[nb_of_beads,1]=size(coord);

figure(1) %added in to only print bead movement on last image (saves time)

for file_no = 1:max_im,
%load second image
clear b2 b3 v xx yy coord1 q test mx mxindex prod prod1 prod2;
fileName2 = strcat(file_name_base, num2str(file_no-1, '%03d'));

cd(directory);
[b2 map] = imread(fileName2, 'bmp');
cd(home_directory);

b2=ind2gray(b2,map);
%b2=b2(30:500,30:500);
b2 = double(b2);
b2 = (b2 - min(min(b2)));
b2 = 1-b2/max(max(b2));
% center of mass calculation
coord1(:,1)=round(coord(:,1));
coord1(:,2)=round(coord(:,2));
coord1=center(b2,coord1,dim);
coord1(:,1)=round(coord1(:,1));
coord1(:,2)=round(coord1(:,2));
coord=center(b2,coord1,dim);
xy(file_no).coord = coord;

%removed following to save time of processing (only prints final
image)

%figure(1); %imagesc(b2);
%title(fileName2); colormap(gray); drawnow;
if file_no==2,
  for i=1:length(xy(file_no).coord(:,1)),
    xy(max_im+3).coord(i,1)=(xy(file_no).coord(i,1)-xy(file_no-1).coord(i,1))*mag+xy(file_no-1).coord(i,1);
    xy(max_im+3).coord(i,2)=(xy(file_no).coord(i,2)-xy(file_no-1).coord(i,2))*mag+xy(file_no-1).coord(i,2);
    h=line([xy(file_no-1).coord(i,1) xy(max_im+3).coord(i,1)],[xy(file_no-1).coord(i,2) xy(max_im+3).coord(i,2)]);
    set(h,'color','red');
  end;
end;
if file_no>2,
  for i=1:length(xy(file_no).coord(:,1)),
    xy(max_im+4).coord(i,1)=(xy(file_no).coord(i,1)-xy(file_no-1).coord(i,1))*mag+xy(max_im+3).coord(i,1);
    xy(max_im+4).coord(i,2)=(xy(file_no).coord(i,2)-xy(file_no-1).coord(i,2))*mag+xy(max_im+3).coord(i,2);
    h=line([xy(max_im+3).coord(i,1) xy(max_im+4).coord(i,1)],[xy(max_im+3).coord(i,2) xy(max_im+4).coord(i,2)]);
    set(h,'color','red');
    xy(max_im+3).coord(i,:)=xy(max_im+4).coord(i,:);
  end;
end;
end

%prints final bead movement image only (saves time during processing)
figure(1); %imagesc(b2);
title(fileName2); colormap(gray); drawnow;

fid1=fopen(strcat(file_name_base,'.txt'),'wt'); %for overwrite:
    fid2=fopen('weg.txt','wt'); or 'at' for append
for file_no=1:max_im,
  fileName2 = strcat(file_name_base,num2str(file_no-1,'%02d'));
  count=fprintf(fid1,'%s',fileName2);
  for i = 1:no_of_beads,
    count=fprintf(fid1,'%9.4f,%9.4f,%9.4f,,',xy(file_no).coord(i,:));
  end;
  count=fprintf(fid1,'');
end;
status=fclose(fid1);

%%% added 23 Feb 2005 to be used by omtcana by Guillaume
%%% time at which each image is taken
for file_no=1:max_im,
    time(file_no) = (file_no-1)*time_interval;
end;

fid2=fopen(strcat(file_name_base,'_ana','.txt'),'wt'); %for overwrite:
fid=fopen('weg.txt','wt'); or 'at' for append
for file_no=1:max_im,
    %fileName2 = strcat(file_name_base,num2str(file_no-1,'%02d'));
    %count=fprintf(fid2,'%s;',fileName2);
    % add time and torque
    count=fprintf(fid2,'%9.4f;%9.4f;',time(file_no), 0.0);
    %count=fprintf(fid2,'
');
    count=fprintf(fid2,'%9.4f;', 0.0);
    for i = 1:no_of_beads,
        count=fprintf(fid2,'%9.4f;%9.4f;;',xy(file_no).coord(i,1:2));
    end;
    count=fprintf(fid2,'
');
end;
status=fclose(fid2);

%msd_test_posttrack_BRI
return
%---END PROGRAM---
APPENDIX E: WESTERN BLOT AND ANALYSIS PROTOCOL

REDUCING LYSIS BUFFER WITH PROTEASE INHIBITORS RECIPE
- CHECK: STRETCHER HOME = 68.60 mm (for old/large-height wells)
- MAKE FRESH LYSIS BUFFER (100 ul TOTAL)
  20.0 µl 5X LoadingBuffer (without blue)
  45.25 µl ddH2O
  12.5 µl β-mercaptoethanol
  5 µl NaF 1M stock (for final conc 50 mM)
  1.00 µl PMSF 100 mM stock (for final conc 1.0 mM)
  14.3 µl 7X stock PI cocktail
  0.65 µl 0.5M EDTA pH 8 (for final conc 3-5 mM)
  1.3 µl 125 mM Na orthovanadate (Na3VO5) activated (2 mM final conc)

- 1X Final composition: 30 mM TrisHCL, 1.25% SDS, 12.5% B-mercaptoethanol, 10% glycerol

STOCKS:
  - add 1xPI cocktail (Roche 04 693 124 001)
    - 7X stock: 1 tablet into 1.5 ml ddH2O (stable 1-2 wks 4ºC, 12 wks -20ºC)
  - Na orthovanadate (Biomedicals 159664) in ddH2O
    - to activate, boil and pH to 10
  - SDS will permeabilized cells, mercaptoethanol will inhibit proteases by unfolding/crosslinking all proteins (Cell Signaling)

(protocol by Brian DiPaolo, bdipaolo@seas.upenn.edu; derived from protocol by Michele Hawk, Johnathan Lakins, and Matt Fry at Cell Signaling)
RIPA LYSIS BUFFER WITH PROTEASE INHIBITORS RECIPE

-NOTE: make fresh day of lysing, especially important for protease inhibitors; thaw on ice (slowly, might take 1 hr)

0.510 ml Ripa Buffer pH 7.4 (see Ripa Buffer modified for use with Roche Complete Mini Tablet Protocol)
4 µl of 0.5M EDTA pH 8 (3-5 mM final concentration)
1.2 µl of 1mg/ml Pepstatin (1-2 ug/ml final concentration)
1.2 µl 1 M NaF (cover with aluminum foil) (2 mM final concentration)
8 µl 125 mM Na orthovanadate activated (2 mM final concentration)
90 µl of 7 X stock inhibitor cocktail Roche Complete Mini Tablet (1 tablet in 1.5 ml in ddH2O)
(total = 614.4 µl)

-1X Final composition: Ripa buffer (50 mM Tris-HCl, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% SDS, 1.0%, IGEPAL CA-630, pH 7.4) with 5 mM EDTA, 2 µg/ml Pepstatin, 2 mM sodium fluoride, 2 mM sodium orthovanadate, complete mini

STOCKS:
- Na orthovanadate (Biomedicals 159664) in ddH2O
  -to activate, boil and pH to 10; solution will turn yellow then clear; cool to RT
- Pepstatin (Roche 1358053) dissolved in methanol at 1 or 2 mg/ml
- NaF and Na-orthovanadate are all dissolved in ddH2O

(protocol by Brian DiPaolo, bdipalo@seas.upenn.edu; derived from protocol by Michele Hawk and Johnathan Lakins)
LYSING CELLS PROTOCOL

Serum deprive IN 0% CO₂ INCUBATOR, STRETCH

Place petri dishes containing seeded wells on ice.

Aspirate all the media from the wells. The media used for rat alveolar cells contains serum which can cause difficulty in assaying protein content of the lysate.

Rinse wells thoroughly with ice-cold, isotonic, DPBS (without Ca²⁺ or Mg²⁺) solution. Suggested number of rinses are 2 for Westerns.

Lyse with 25 ul / well lysis buffer (RIPA or Reducing, see above) with a minimum of 3 wells = 75 ul.

Sonicate the lysate 5 s; turn knob slowly to about 1 (about 5 watts rms) and will hear whine sound, test on water as not to create bubbles/foam in actual samples. Keep samples on ice while sonicating (important, because sonication can raise temp of sample). Rinse probe between different groups.

(RIPA lysate: spin samples in centrifuge 4°C for 10 min at 10,621×g; save supernatent)

Quick freeze aliquots on dry ice and store at -80°C

Keep 10 µl aliquot for protein assay; the rest of the lysate can go into 2 or more aliquats of about 30 µl each (to minimize thaw/freeze cycles needed)

<table>
<thead>
<tr>
<th>Lysate#</th>
<th>box</th>
<th>wells</th>
<th>stretch</th>
<th>treat</th>
<th>cell type</th>
<th>serumdep</th>
<th>lysisbuffer</th>
</tr>
</thead>
</table>

(protocol by Brian DiPaolo, bdipalo@seas.upenn.edu; derived from protocol by Michele Hawk, Johnathan Lakins, and Matt Fry at Cell Signaling)
STOCK SOLUTIONS FOR LYSING AND WESTERN BLOT

Na Orthovanadate (MP Biomedicals 159664; stock 125 mM in ddH2O; MW 183.91 Da)
   - a tyrosine phosphatase inhibitor
   - NOTE: toxic
   - 183.91 g/mol x 0.125 M x 0.020 liters = 0.4598 grams/liter = 460 mg/20 ml ddH2O

   - Place 15 ml ddH2O in a 100 ml beaker
   - Add Na Orthovanadate while rapidly stirring
   - Adjust pH to 10 with 12 N HCl; solution turns a bright yellow
   - Heat solution until boiling in a fume hood; solution should turn clear
   - Adjust pH to 10
   - If solution remains clear, aliquot and freeze on dry ice; store at -20ºC

0.5M EDTA pH 8
   EDTA = Ethylenediaminetetraacetic Acid Tetrasodium salt (Sigma E6511; MW 416.2 g/mol)
   - 416.2 g/mol x 0.5 M x 0.010 L = 2.081 g in 10 ml ddH2O
   - Add EDTA to approximately 5 ml water.
   - Add ddH2O to 9 ml, adjust pH, then top off at 10 ml; store RT.

Pepstatin
   - potent inhibitor of various aspartic proteinases.
   - Dissolve in methanol at 1-2 mg/ml. It is stable at -20ºC.

Sodium Flouride (stock 1M; MP Biomedicals 194864; MW 41.99 g/mol)
   - general Ser/Thr phosphatase inhibitor
   - 41.99 g/mol x 1 M X 0.001 L = 42 mg / 1 ml
   - Do not aliquot
   - Wrap microcentrifuge tube in aluminium. Store RT or 4ºC (vortex before using)

Complete Mini protease Inhibitor Cocktail (7X stock solution; Roche 1836153)
   - Dissolve 1 tablet in 1.5 ml ddH2O; aliquot and freeze

   - Add 90 ul to 510 ml of concentrated Ripa Buffer
   - Aspartic protease inhibitors are lacking in this cocktail and therefore it should be used with Pepstatin (tech service)
   - Cocktail contains 1 mM EDTA (final concentration)
   - 7X stock is stable for at least 12 weeks frozen at -20ºC

Ripa Buffer
   2.25 ml of 1 M Tris HCL (pH 8.8 at RT)
   112.5 mg of sodium deoxycholate (Sigma D6750; FW 414.6 g/mol)
   (respiratory irritant; wear a dust mask)
450 µl of 10% SDS (Sodium dodecyl sulfate)
1.35 ml of 5 M NaCl
450 µl of IGEPAL CA-630 (ICN Biomedical Inc.19856)

Bring to 35 ml with ddH₂O in a 50 ml polypropylene centrifuge tube
Adjust pH to 7.4 at RT with HCl
Bring volume up to 45 ml with ddH₂O
Adjust pH if necessary at RT

Final composition: 50 mM Tris-HCl, 150 mM NaCl, 0.25% Na-deoxycholate, 0.1% SDS, 1.0 % IGPAL (ca-630), pH 7.4 at Room Temp

1.5 M Tris HCL pH 8.8 (at RT) (MP Biomedicals 816116)
stock for use in acrylamide Gels
-Tris HCl or Tris Base may be used to make this stock solution, easier to use the Tris Base (BioRad 161-0719; MW 121.14 g/mol)

(stock 150 ml)
-121.14 g/mol x 1.5 M x 0.15 L = 27.26 g Tris
-Add 80 ml of ddH₂O into a small beaker with a stir bar
-Add 27.26 g of Tris while stirring, until into solution
-pH using 12N HCl; starting pH about 11.27 (will require approximately 4.5 full glass Pasteur pipettes worth of concentrated HCl to get the pH down to 8.8
-place solution into graduated cylinder and fill up to 150 ml with ddH₂O
-no need to pH the solution again as the pH will stay at 8.8

-IF using Tris HCl; MW is 157.61 g/mol;
-157.61 g/mol x 1.5 M x 0.05 liters = 11.82 grams for 50 ml (requires a lot of 10 M NaOH; pH starts out around 3.8)

1.0 M Tris HCL pH 6.8 (at RT) (MP Biomedicals 816115)
-Stock for Acrylamide Gels and for loading buffer
-MW 157.61 g/mol

(stock 100 ml)
-157.61 g/mol x 1 M x 0.1 L = 15.761 g of Tris HCL in 100 ml ddH₂O
-Measure out 100 ml ddH₂O into a graduated cylinder
-Put approximately 70 ml of water into a beaker with a stir bar
-Add 15.761 g of Tris HCl and use some of the reserved water to rinse the weigh boat
-pH to 6.8 by adding 10 M NaOH
-Top off to 100 ml in a graduated cylinder by adding ddH₂O

0.4% stock bromophenol blue (Sigma B-8026)
40 mg bromophenol blue in 10 ml ddH₂O
10% APS (BioRat 161-0700; MW 228.2 g/mol)
- for making gel
- Place 100 mg into 900 ul ddH2O
- Freeze -20ºC

Water – Saturated N-butynol
- 4 ml N-butynol (flammables cabinet)
- Add a little water; vortex
- Add additional water, shaking between additions until you see the phase separation

5X Loading (sample) buffer
- 5 mL glycerol (MP Biomedicals 800687)
- 0.625 g SDS (MP Biomedicals 811030) (use mask to weigh out)
- 400 ul 0.4% stock Bromophenol Blue
- 1.56 mL 1 M Tris HCL pH 6.8
- Cook at 40ºC a little while and let dissolve overnight on a shaker
- Add enough ddH2O to make 10 ml (very lumpy looking)
- Cover with aluminum foil, store at RT

Reducing buffer (Loading buffer with 12.5 % β-mercaptoethanol)
- 875 ul blue loading buffer + 125 ul β-mercaptoethanol
- Final composition: 6.25% SDS w/v, 0.15 M Tris HC at pH 6.8, 0.04% Bromophenol Blue, 50% glycerol

Coomassie Blue staining solution
- 50% methanol (v/v)
- 0.05% (v/v) Coomassie brilliant blue R-250 (BioRad or Pierce)
- 10% (v/v) acetic acid
- 40% H2O
- Dissolve the Coomassie brilliant blue in methanol before adding acetic acid and water. Solution can be stored for 6 months. If precipitate is observed following prolonged storage, filter to obtain a homogeneous solution.

Destaining solution
- 5 % methanol
- 7% acetic acid
- 88% H2O
- Can be stored 1 month at RT

10X TBS pH 7.6
- 500 ml ddH2O in a 2L beaker with a stir bar
- add 24.2 g Tris base (Biorad 161-0719)
- Add 80g NaCl (Sigma S7653)
- Add 500 mL ddH₂O
- Stir approx 10 min
- Adjust pH to 7.6 with 10 N HCl (starting pH 10.82 approx; add about 17 full Pasture pipette w/ blue bulb worth)

10X Running Buffer
- 1% SDS (10 grams SDS)
  (wear a dust mask)
- 250 mM Tris (30.275 g) Tris ultra pure (BioRad 161-0719; MW 121.4 g/mol)
- 1.92 M glycine = 144.1 g Glycine (Fisher Biotech BP381)
- Add to one Erlenmyer flask or 1 liter graduated cylinder, bring up to one liter with ddH₂O, let foam settle.

10X Transfer Buffer
- Same as Running Buffer but do not add the SDS

- 1X Transfer Buffer
  - 100 ml 10X Transfer Buffer
  - Add 200 ml methanol
  - Add 700 ml ddH₂O

(lysing protocols by Brian DiPaolo, bdipaolo@seas.upenn.edu; derived from protocols by Michele Hawk, Johnathan Lakins, Matt Fry at Cell Signaling, and (2))
REDUCING/DETERGENT COMPATABLE PROTEIN ASSAY
-Ice bucket: Reducing buffer samples (without Bromophenyl blue), standard curve samples (250, 500, 1000, 1500 ug/ml in reducing buffer w/out Bromophenyl blue), blank
-Turn on microplate machine (to warm)
-NOTE: 24 samples max in centrifuge

-Add 25 ul BLANK and gamma-BSA STANDARDS into microfuge (in triplicate)
-For each SAMPLE:
   -Add 10 ul SAMPLE + 15 ul ddH2O (2/5 dilution) = 25 ul

WASH 1
-Add 125 ul RC Reagent I, vortex, incubate 1mRT (min room temp)
-Add 125 ul RC Reagent II, vortex
-Centrifuge 15,000xg 4mRT
   -Make A’
     (____ # standards/blank * 3) + (____ # samples * 3) x 127 ul = ___ul A
     _______ ul A x 0.020 S/A = _______ ul S
     = A’
   -Aspirate out supernatant (do not hit pellet)

WASH 2
-Add 125 ul RC Reagent I, incubate 5mRT
-Add 40 ul RC Reagent II, and incubate 30m
-Centrifuge 15,000xg 10mRT
   -Finish protocol
   -Read background from microtiter plate

-Aspirate out supernatant (do not hit pellet – may be loose)

-Add 127 ul A’, vortex AT LEAST 15s, incubate 5mRT on shaker

-into microwells in double:
   -load 26 ul sample, 0 ul A’ (2/5 dilution)
   -load 13 ul sample, 13 ul A’ (1/5 dilution)
   -load 6.5 ul sample, 19.5 ul A’ (1/10 dilution)

-Add 204 ul DC reagent B to each well, incubate 15mRT

-read at 750 nm before 1 hr passes

SAMPLES:

(protocol by Brian DiPaolo, bdipaolo@seas.upenn.edu; derived from RC DC protein assay kit BioRad 500-0121)
RIPA COMPATABLE PROTEIN ASSAY
-Ice bucket: samples, standard curve samples (250, 500, 1000, 1500 ug/ml in RIPA buffer), blank
-Turn on microplate machine for WARM UP
-STANDARD CURVE SAMPLES PREP DATE:

-Make A’
\[
\{____ \text{# standards/blank} + (____ \text{# samples x 3})\} \times 3 \times 25 \text{ ul} = _____ \text{ul A} \\
+ _____ \text{ul A} \times 0.020 \text{ S/A} = _____ \text{ul S} \\
= A’
\]

-Read background from microtiter plate

-remove 10ul Ripa buffer and place in centrifuge tube
-add 10ul sample and mix
  -1/2 dilution: add 2 x 5 ul to 96well plate
-add additional 10ul Ripa buffer to remaining 10ul ½ dilution
  -1/4 dilution: add 3 x 5 ul to 96well plate

OR
-For each SAMPLE (in 96 well plate):
  -prepare 3 aliquats of 20 ul RIPA buffer
  -add 20 ul SAMPLE to first aliquat, mix (1/2 dilution)
  -remove 20 ul and add to second aliquat, mix (1/4 dilution)
  -remove 20 ul and add to third aliquat, mix (1/8 dilution)

  -Add 5 ul each aliquat in triplicate into microtiter plate

-Add 5 ul BLANK and STANDARDS in triplicate into microtiter plate

-Add 25 ul A’ into each well

-Add 200 ul B into each well
  -pipette air to pop bubbles
  -gently agitate plate 15mRT (min room temp)

-Finalize protocol for read

-Read at 750 nm before 1 hr passes

SAMPLES #’s:

(protocol by Brian DiPaolo, bdipaolo@seas.upenn.edu; derived from protocol by Michele Hawk)
RAC1 ACTIVITY ASSAY
(Cell Biolabs STA-401-1)
- uses pink PAK1 PBD agarose beads for pull-down
- Rac1, 21 kDa; active form GTP-Rac1, inactive GDP-Rac1
- Rac1 binds specifically to p21-binding domain (PBD) of p21-activated protein kinase (PAK) (cell biolabs product sheet)

NOTE: this protocol is 1/4 volumes used in product sheet

PREP
- 30°C heat block
- Cold block
- Centrifuge at 4°C
- 1X Assay/Lysis buffer: (1000 ul TOTAL; ~585 ul per sample)
  200 ul 5X stock (240102)
  637 ul ddH₂O
  10 ul PMSF 100mM stock (final conc. 1.0 mM)
  10 ul Leupeptin 1mg/ml (final conc. 10ug/mL; Sigma L2884)
  143 ul 7X stock PI cocktail (Roche 04 693 124 001)
  (does not call for PI cocktail, but does call for 10 ug/ml aprotinin)

- 2X reducing SDS-PAGE sample buffer (100 ul TOTAL; 10 ul per sample)
  25 ul NuPage LDS load buffer 4X (NP0007) (RT)
  10 ul NuPage Sample Reducing agent 10X (NP0009) (4°C)
  65 ul ddH₂O

- 1M MgCl₂
  95.21 mg / ml (Sigma M-8266, 100g; FW 95.21)
  -weigh first, then add required ddH₂O

- lysate on ice
  - our cells: avg conc 2900 ug/ml = 2.9 mg/ml; 2 small wells typically 85 ul total (or 0.25 mg); typically 75ul / (2x 1.227 cm²) = 0.306 ul/mm²
  OR 75ul / (2x 1.227 million cells) = 30.6 ul / million-cells
  - our protocol loads about 1 million-cells
  - assay calls for typical >0.5 mg protein OR 0.5-1ml lysis / 100mm plate = 1000 ul / (5810 mm²) = 0.172 ul/mm²
  OR 1000 ul / 10 million cells = 100 ul / million-cells
  - their protocol loads between 5-10 million-cells

- store all components at -20°C

- PERFORM steps at 4°C or on ICE (reduces hydrolysis of GTP-Rac1 to GDP-Rac1)
- Lyse cells (use fresh, or use -70°C stored lysate)
-if nuclear lysis occurs (lysate very viscous), pass through 27.5-gauge syringe needle x4
-clear lysate by centrifugation 14,000×g 4ºC 10min
-collect supernatant

**GTPγS/GDP assay (+/- assay controls)**
- aliquat 40 ul lysate
- add 210 ul 1X Assay/Lysis buffer
- add 5 ul 0.5 M EDTA

- add 2.5 ul of 100X GTPγS (POSITIVE control; 240103) OR
- add 2.5 ul of 100X GDP (NEGATIVE control; 240104)

- incubate 30min 30ºC agitation
- add 16.25 ul 1M MgCl₂; mix and place on ice

**Rac1 Pull-Down Assay (samples and positive/negative controls)**
- aliquat 40 ul lysate (~0.125 mg protein)
- add 210 ul 1X Assay/Lysis buffer
- add 10 ul PAK-PBD bead-slurry (thoroughly vortexed for each sample; 240101)
- incubate 4ºC, gentle agitation, 60 min
- centrifuge 14,000×g 10s 4ºC (pellet out beads)
  - discard supernatent (very careful of beads)
- wash 3X with 125 ul 1X Assay/Lysis buffer (centrifuging and aspirating each time)
- resuspend in 10 ul 2X reducing SDS-PAGE sample buffer
- boil 5min 95ºC
- centrifuge 14,000×g 10s, 4ºC
- load 8 ul supernatent to gel

- also add 10ul Rac1 Immunoblot Positive control (240110) to gel; already in pre-boiled 1X reducing sample buffer

(protocol by Brian C. DiPaolo, bdipaolo@seas.upenn.edu; derived from Rac1 activity assay information sheet)
ROCK ACTIVITY ASSAY

(possible to run protocol at 1/5 volumes shown below; load 10 ug protein into assay then load 100% post-assay lysate (10 ug) into western gel)

Prepare

1X Kinase Buffer DTT (47 ul / SAMPLE + additional 23 ul / POSITIVE control)
- 4.7 ul 10X Kinase Buffer (-20ºC cell culture)
- 41.83 ul ddH₂O
- 0.47 ul of 100 mM DTT stock (add just prior to use; for 1 mM)

- 100 mM (15.425 mg/ml) DTT stock (make fresh):
  -(HOOD) 1.5425 mg DTT
   [4ºC, sigma D0632-1g, MW 154.25 g/mol, soluble up to 50 mg/ml in water]
- 100 ul ddH₂O
  - OR measure out DTT, then add required ddH₂O to be more accurate with concentration

1X Kinase/ATP/Substrate Solution (50 ul / SAMPLE)
- 1 ul 10mM ATP solution (-20ºC cell culture)
- 2 ul ROCK substrate (-20ºC cell culture)
- 47 ul 1X Kinase Buffer DTT (above)

4X SDS-PAGE sample buffer + blue
- 4 ml 5X SDS-PAGE sample buffer + 1 ml ddH₂O = 5 ml 4X SDS-PAGE
- 40 ul/ml 0.4% Bromophenol Blue stock

Activity Assay

1) 25 ul SAMPLE microcentrifuge tube (-80ºC)
  - load specific-constant across group protein amount to reach 40 ug protein
  - SAMPLE may be diluted with 1X Kinase Buffer

POSITIVE CONTROL:  2 ul Active-ROCK II (-20ºC cell culture) + 23 ul 1X Kinase Buffer

2) Add 50 ul 1X Kinase/ATP/Substrate Solution to SAMPLE to initiate kinase rxn

NEGATIVE CONTROL:  SAMPLE without kinase

3) Incubate 30ºC for 30 min (gentle agitation)

4) - add 25 ul 4X SDS-PAGE sample buffer to stop rxn
  - freeze on dry ice, store in -80ºC for long term storage, or move directly to next step
5) Boil 95°C - 5 min

6) Centrifuge 12,000×g 10 sec

7) Load 25 ul supernatant to gel (10 ug protein)

(ROCK activity immunoblot kit, Cell Biolabs STA-415, 20 assays)

(protocol by Brian DiPaolo, bdipaolo@seas.upenn.edu, derived from ROCK activity assay information sheet)
WESTERN BLOT PROTOCOL

POURING GEL

- Assemble polyacrylamide gel casting frame (BioRad Mini-PROTEAN 3)
- Use the 15 well 1.5 mm spacer plate.
- Check carefully to ensure the bottom and sides of the glass plates are even.
- Insert well plate into the green holder and push green handles back to close.
- Insert 15 well comb and place a mark with a Sharpie pen one cm below the bottom of the wells.
- Remove comb.
- Place the casting frame and plate sandwich on a gray foam pad in the casting stand.

Stacking and separating gel solutions prep:

- Each **stacking** gel is 5 ml and is poured on top of the separating gel.
- Each **separating** gel is 10mls. It is poured first and allowed to polymerize prior to pouring the stacking gel. (*Note: 2 gels can be poured using 15 ml, but there is very little extra)*

- Each **stacking** gel is 4% acrylamide.
- The separating gel acrylamide concentration varies depending on the weight of the protein. The smaller the protein, the higher the concentration of separating gel needed. A 15% gel was used to resolve a 22 kDa protein, while a 7.5% gel was used for the beta-4 integrin at 200 kDa.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Separating Gel - 10 ml 10%</th>
<th>Stacking Gel - 5 ml 4%</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide Stock *</td>
<td>3.33 ml</td>
<td>0.667 ml</td>
<td>5ml x 0.04/.3 = 0.667 ml</td>
</tr>
<tr>
<td>4 X 1.5M Tris pH 8.8 Stock</td>
<td>2.5 ml</td>
<td></td>
<td>10ml/4 = 2.5 ml</td>
</tr>
<tr>
<td>4X 1.0 M Tris pH 6.8 stock</td>
<td>1.25 ml</td>
<td>1.25 ml</td>
<td>5ml/4 = 1.25 ml</td>
</tr>
<tr>
<td>10% SDS stock</td>
<td>100 ul</td>
<td>50 ul</td>
<td>Final Conc. = 0.1%</td>
</tr>
<tr>
<td>10% APS** Stock</td>
<td>50 ul</td>
<td>50 ul</td>
<td>Set amount/gel</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>4.02</td>
<td>2.983 ml</td>
<td>Bring up to 5 or 10 ml</td>
</tr>
</tbody>
</table>
*WARNING: ACRYLAMIDE IS NEUROTOXIC ESPECIALLY WHEN UNPOLYMERIZED.
WEAR GLOVES!

** APS = ammonium persulfate (polymerization catalyst) should be made fresh or kept frozen at -20°C. It looses activity rather rapidly, and then the gel will not polymerize.

- Make up solutions for the gels in 50 cc conical centrifuge tubes, adding everything except the TEMED. TEMED will cause the acrylamide to polymerize.

- Assemble, 5 µl pipetman, 200 µl pipetman, 1 ml pipetman, TEMED, water-saturated N-butynol, and container with separating gel.

- Water-saturated N-butynol is biphasic with water on the bottom (6 mL of N-butynol and add ddwater until you see the separation of the two layers). Draw N-butynol from the top layer.

- Add 4-5 µl of TEMED (per gel) to acrylamide solution and mix using the pipetman. Avoid bubbles. Use the pipetman to fill the gel mold. Work rapidly to avoid polymerization. Fill until just above the mark on the glass that was placed 1 cm below the wells. The gel will contract slightly as it polymerizes.

- Add 100-200 µl of water saturated N-butynol to form a thin layer over the separating gel. This will cause the acrylamide to become level.

- Polymerization will require approximately 20 minutes. Do not leave the gel to polymerize more than an hour or the gel may dry excessively. Save the remainder of the gel in the centrifuge tube to use as an indicator of polymerization.

- Once the separating gel has polymerized, pour off the N-butynol (down sink) and gently rinse with ddwater several times using a squirt bottle. Dry by putting a piece of Whatman paper between the glass. Try to avoid touching the paper to the gel while still absorbing all the water off the gel.

- Add 5 ul per gel of TEMED to the stacking gel. Mix and immediately pipette on top of separating gel. AVOID BUBBLES.

- Pour the stacking gel so that the acrylamide is just below the top of the well plate. Ease the comb in slowly, at an angle. AVOID splashing yourself with the acrylamide as you seat the comb. Double check to ensure that the comb is inserted properly so that the lanes will be straight.

- Allow the stacking gel to polymerize for 20-30 min.
-Cover the gel in the casting frame with wet paper towels and plastic wrap and continue polymerization in the refrigerator overnight, if possible. Otherwise, 30 - 60 min is sufficient for polymerization.

(protocol by Michele Hawk)
RUNNING SAMPLES ON GEL
(ALSO SEE QUICK REFERENCE GUIDE FOR RUNNING NUPAGE)

-Determine protein concentration of your sample. See Protein quantification protocol. In addition to various concentrations of the samples of interest, always run:
1) a positive control, 2) a negative control, 3) non-specific-background (NSB) control, and 4) molecular weight markers. (The markers are kept in the -20 freezer in the box labeled “Molecular Markers”)

-Heat block to 95°C.

-Obtain ice in bucket.

-Obtain Markers, samples, controls, and place on ice. If necessary, heat samples to 37°C to dissolve SDS.

-Obtain beaker of non-sterile ddwater.

-Obtain 1.5 ml microcentrifuge tubes, label, and place in a rack.

-Obtain sample loading buffer (5 X sample loading buffer is stored in -20 freezer). Place loading buffer on ice. Note: loading buffer can be reducing (with beta-mercaptoethanol) or non-reducing. I used reducing buffer for MAPkinase samples.

-Calculate the amount of all samples needed (see below) and place in labeled centrifuge tubes with water and sample loading buffer. 5 µl is the amount needed for the markers.

-Make up extra buffer and water for blank lanes.
It is helpful to ensure your blot layout is asymmetrical so that your gel and blot orientation can be easily recognized.

Calculations:
1) Calculate the volume of sample lysate necessary to load the required protein amount (generally 20-35 µg). The volume will be limited by the well size of the gel you are running.
2) Calculate the volume of 5X loading buffer needed.
   Determine the largest loading volume you have of the samples and divide this volume by 4. Sample aliquote/4 = volume of 5 X sample loading buffer.
3) Calculate load volume.
   Load volume is the total amount that will be loaded in a well. It is usually limited to about 40 µl for a 10 well comb (80 µl is a lot to load in a well). For the MAPkinase westerns, 15 well, 1.5mm combs were used. 25 µl can be easily loaded using this comb. Tech service says 40 µl may be added but this requires a very heavy loading buffer. Load volume = largest sample volume + buffer OR sample aliquot + buffer + ddwater.

Sample calculation:
Suppose largest aliquot of lysate to be loaded is 20µl. 20µl ÷ 4 = 5 µl. 5µl is the amount of 5 X loading buffer that will be loaded in each lane. Total loading volume =5 X buffer + largest sample aliquot =20 +5 =25µl. So all lanes should have the constant loading factors of 25µl total and 5 µl of the sample buffer. The difference should be made up in ddwater. For example, if the smallest amount of protein lysate to be used is 17µl. Then, 17µl of lysate + 5µl of loading buffer = 22µl. 25µl loading volume minus 22µl = 3 µl of water needed to be added to the 17µl sample aliquot.

I calculate a 10% increase in the amount of protein I will load so that I will have extra for a pipetting loss. I actually load roughly 10% less that the calculated load volume.

-HEAT SAMPLES IN 1.5 ML MICROCENTRIFUGE TUBES FOR 10 MINUTES AT 95° C IN THE HEATING BLOCK. Before heating, keep samples on ice as much as possible. AFTER HEATING DO NOT place the samples on ice or the protein will precipitate. After heating samples will be stable for some time.

-Spin samples briefly after heating to bring down condensation on the cap.

-In order to better visualize the wells, mark the base of the wells with a Sharpie before removing the comb. Place the marks on the long glass plate, not the short plate.

-Remove the comb - very important to go straight up with the comb so the lanes are even.

-Assemble the eletrophoresis apparatus by putting the gel cassette in the hanging electrode assembly with the short plate facing inward!!!
If running only one gel, use the plastic insert (Buffer Dam) instead of a second gel. Avoid running gels of two different acrylamide percentages together.

-Place the electrode assembly into the clamping frame. Press down on the electrode assembly WHILE closing the two cam levers of the clamping frame.

-Lower the assembly into the mini eletrophoresis tank.

-Test for leakage of the electrode assembly, which forms the inner chamber of the eletrophoresis set up.
Partially fill the inner chamber (between the two plates) with Running buffer and watch for buffer to leak into the outer chamber. If there is no leakage, continue to fill the inner chamber until the glass plates are almost covered. (If loading a large amount of material, fill until just below the short plate – load wells, then gently add more buffer.) Bob Siman’s loads the gel dry and put the samples under approximately 10-20 ul of buffer. Then he uses a 25ml pipet to finish filling the inner chamber with buffer.

-Fill outside chamber until the electrode (wire) is covered (approx 200 ml).
-Load samples into designated wells. Pay attention to equalize the ion and detergent levels in each lane.
If comparing lanes of reduced and non-reduced samples, leave a blank lane in between
Load blanks in any unused wells (buffer solution + water).

- AFTER LOADING WELLS DO NOT ADD MORE BUFFER TO THE INSIDE CHAMBER UNLESS YOU DO SO GENTLY WITH A PIPET.

-Run gel. See reference chart for run times/voltage/current. Alternately, use a rainbow ladder marker to determine when to stop gel. If you run the gel too fast you will see distortions.

NOTE: The reason proteins migrate toward the positively charged anode is that the SDS makes them negatively charged. The phosphorylated proteins have a lower affinity for SDS. Therefore they migrate more slowly. They have less attraction for the anode than the non-phosphorylated proteins. You would not see a shift based on molecular weight alone between ERK and p-ERK.

-Approximately 30 minutes prior to the end of the PAGE (polyacrylamide gel electrophoresis) wet the membrane and equilibrate the gel in the transfer buffer. See below under “transferring the gel” for these procedures.

-Remove gel from glass casting frame (use fingernails to lift up on smaller sheet of glass) being careful not to rip the gel. Use a razor blade to pull away the stacking gel and discard it. Use razor blade to loosen edges of the remaining separating gel. Biorad makes a green, plastic, beveled tool for removing the gel. After loosening the gel, the gel can be floated off the plate in transfer buffer. The gel can also be flipped onto wet piece of blotting paper. Be careful to maintain the orientation of the gel. Bob Siman cuts off the lower left-hand corner for gel orientation.

-Place the gel into transfer buffer. Equilibrate the gel in transfer buffer for at least 15 min on an orbital shaker.

(protocol by Michele Hawk)
TRANSFERRING GEL

- The transfer buffer can be used 3 times. The transfer buffer has a final concentration of 20% methanol. Use 700 ml of ddwater, 100 ml of 10X transfer buffer, and 200 ml of methanol. The transfer buffer should not contain SDS. The purpose of the methanol in the buffer is to strip the SDS from the proteins to allow them to stick to the membrane. You would only use SDS if the proteins are extremely large because the SDS allows protein to unfold and move into the membrane.

- Cut out 6 Whatman paper leaves the size of the gel.
- Ensure you are wearing gloves prior to handling PVDF membrane.
- Cut out a gel size piece of polyvinylidene difluoride (PVDF) membrane 9 X 6 cm.
- Place PVDF membrane in a pipet box bottom and move to the hood.
- Wet with 100% methanol (located in specially marked container) and swirl approximately 20 seconds. Pour methanol back in the marked container for reuse.
- Cover the membrane with ddwater and incubate on the rocker for 2-10 minutes
- Pour off water and submerge membrane in appropriate transfer buffer. Bob Siman allows at least 15 minutes to equilibrate the membrane in transfer buffer.

- Gather the following:
glass pyrex dish ½ filled with transfer buffer,
White “brillo” pads
Plastic transfer compressor plates
the 6 sheets of Whatman paper
transfer apparatus
forceps
something to roll the bubbles out from between the membrane and gel.

- Assemble in the following order – AVOID TRAPPING AIR BUBBLES BETWEEN ALL SHEETS
KEEP ALL COMPONENTS WITHIN THE CONFINES OF THE COMPRESSOR PLATES.
Black compressor plate on bottom
White brillo pad (hold down with hand )
Whatman paper leaves
The gel
The wetted PVDF membrane Apply by firmly rolling across the gel, use broken pipette or a pencil
3 Whatman leaves of paper
White brillo pad
Clear compressor plate on top.
Close by pushing white plastic latch forward and then pulling back.
- Assemble the transfer tank.
- Insert iced cooling pack (from –20°C freezer).
- Insert magnetic stirrer.
- Insert soaked compressor pack containing gel and PVDF membrane.

ENSURE THAT THE WHITE PLASTIC CLOSURE IS ON TOP, THE CLEAR SIDE IS FACING THE RED ELECTRODE, AND THE BLACK SIDE IS FACING THE BLACK ELECTRODE. THE PROTEIN IS TRANSFERRED FROM THE GEL ON THE DARK SIDE TO THE MEMBRANE ON THE CLEAR SIDE. IF YOU REVERSE THE ORIENTATION THE PROTEIN IS LOST INTO THE TRANSFER BUFFER.

Place transfer tank into a rectangular container filled with ice and place container on a stir plate. Turn stir plate on low.

- See reference chart for running times/voltage/current
- Make up blocking buffer while the protein is transferring (see below).

After transfer is finished:
- Turn off Power Pac.
- Turn off the stirrer.
- Retrieve 2 pipette containers and a SHARP pencil and razor blade.
- Disassemble transfer apparatus.
- Remove cold pack. Place back in freezer.
- Recycle transfer buffer or discard in chemical waste area.
- Disassemble cassette. Discard Whatman Paper
- Remove membrane REMEMBER WHICH SIDE HAS THE PROTEIN ON IT!!!.
  - Do not allow the membrane to dry out.
  - Place membrane in container and label with pencil. Markers may fade, so use pencil to indicate location.
  - Cover membrane with TBS.
  - Stain gel if desired (See Gel Staining and drying procedure)
  - Rinse membrane 4 times with TBST

Optional step:
- Add Ponceau S protein stain to evaluate transfer and to locate lanes. Allow to rock for 5-10 minutes to stain.
- Remove Ponceau S stain and recycle.
- If necessary, destain Ponceau S slightly in water (PBS will remove too much of the dye) so that the membrane will photo copy well.
- Wrap in Saran Wrap and photocopy
- Mark lanes, and cut off excess membrane and cut off NSB (nonspecific control lanes).
- Remove stain as much as possible by rinsing 4 X in TBS.

- Cut off excess membrane using a razor blade.

- Place membrane in Blocking Buffer for at least 1 hour with rocking (can store over night at 4°C if desired). Blocking buffer for MAP kinase antibodies consisted of 3% Carnation non-fat dry milk in TBST (0.05% Tween 20)

- Dilute primary antibody in blocking buffer. **KEEP ON ICE.** Use Cell Signaling Antibodies at 1:1000 in the blocking buffer above (3% Dry milk in TBST).

- Pour off the blocking buffer from membrane and SAVE for blocking NSB bands
- Allow membrane to drain on side of container.
- Using forceps remove membrane and place in primary antibody (see above).
- Continue to allow the membrane with NSB lanes to incubate in blocking buffer.
- Incubate with antibodies on rocker overnight at 4°C.

- Rinse the NSB membrane and the primary membrane separately - 5 X 10 minutes with 0.05% Tween in 1 X TBS while on a rocker.

- Make up 20-30 ml per membrane of the secondary antibody solution. Dilute antibody [goat anti-rabbit IgG horseradish peroxidase (HRP) Santa Cruz Biotechnologies, Inc. cat# SC2004] 1:5,000 in 0.05% Tween and TBS.

- Incubate both the specific and nonspecific membranes separately in the secondary antibody for 1 hour on with rocking or agitation.

- Rinse the membrane 6 X 10 minutes in 0.05% Tween in 1 X TBS on the orbital shaker. During the washes turn on the automatic developer to warm up. After the washes are complete, proceed as described below.

(protocol by Michele Hawk)
DEVELOPING
-Simultaneously turn on the water and developer in the dark (takes 20-30 minutes to warm up).
-Turn off the monitor and camera and throw a tarp over the computer to prevent exposure to film

Materials:
-5 ml of oxidizing reagent and 5 ml of lighting solution in two separate 15 ml centrifuge tubes (can get away with 4 ml of each per membrane).
-Film Cassette
-Film (DO NOT OPEN THE FILM IN LIGHT)
-Get out 2 glow in the dark stickers (small a circle and a star – so you can orient the gel)
-Saran wrap or sheet protector
-Pipet box bottom, scissors, timer, paper towels, sharpie markers, tape, forceps, gloves, lab coat.

-In the dark room mix the oxidizing reagent into the tube with the lighting solution and pour into the pipet box bottom.
-Transfer the blot from the wash solution into the chemiluminescence solutions.
-Incubate for 1 minute with gentle agitation.
-Drain excess solution from the membrane on paper towel, but keep the membrane moist.
-Put membrane with protein side up in plastic wrap or sheet protector inside the film cassette.
-Roll out wrinkles and air bubbles.
-Place glow-in-the-dark star and circle on opposite corners of the membrane (on top of the saran wrap) in order to orient the membrane
-Turn off all lights except red lights
-Remove one or two pieces of film and then close the box. Ensure the bag opening is at the bottom of the box.
-Expose film to blot. Ensure that the film is placed on the blot in such away, that your results will not be affected. For instance, if your lanes run left to right. Apply the film top to bottom so that one side will not be exposed longer than the other. Vary time depending on amount of exposure needed.

(protocol by Michele Hawk)
STRIPPING MEMBRANE
This procedure removes antibodies so that the protein blot may be reprobed with a different antibody

Materials:
-2% SDS – 50 ml = 1 g of SDS in 50 ml of dd water
-Mercaptoethanol
-50 cc Centrifuge tube
-Oven set at 50 C
-Membrane to be stripped

-Add 30 ml of 2% SDS to a 50 CC centrifuge tube (Note that Nas uses 10 ml of 2% SDS and 140 ul of beta mercaptoethanol). I used 10 ml and 70 ul of beta-mercaptoethanol). Need roughly 100 mM beta-mercaptoethanol.

-Place membrane in tube with the protein side to the center of the tube, not against the wall of the tube.
  Do this in a hood and wear nitrile gloves.

-Place in a tube within the oven at 50°C.

-Press rotate.

-Incubate for 30 minutes.

-Wash few times in water and then PBS until odor of beta-mercaptoethanol is gone.

-Begin blocking step.

(protocol by Michele Hawk)
**QUICK REFERENCE**

**GELELS:**

<table>
<thead>
<tr>
<th>gel</th>
<th>gel %</th>
<th>type</th>
<th>thickness (mm)</th>
<th>wells</th>
<th>vol max (µl)</th>
<th>MOPS (kDa)</th>
<th>MES (kDa)</th>
<th>mol Weight (kDa)</th>
<th>gel %</th>
<th>PVDF xfer</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP0322 4-12 BisTris 1 12</td>
<td>20 15 - 260 3.5 - 160</td>
<td>&gt; 250</td>
<td>4 - 5</td>
<td>1 A 1 hr 45 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP0321 4-12 BisTris 1 10</td>
<td>25 15 - 260 3.5 - 160</td>
<td>250 - 120</td>
<td>7.5</td>
<td>1 A 90 min (250 mA 4 hrs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP0335 4-12 BisTris 1.5 10</td>
<td>37 15 - 260 3.5 - 160</td>
<td>120 - 40</td>
<td>10</td>
<td>1 A 1 hr (250 mA 4 hrs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP0388 4-12 BisTris 1.5 15</td>
<td>25 15 - 260 3.5 - 160</td>
<td>40 - 50</td>
<td>13</td>
<td>1 A 90 min (250 mA 6 hrs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**GEL run (ICE):**

- peel off white strip
- MES buffer in and out

**POST RUN:**

- cracking: gel face up (lane 1 on left)
- crack right side against box
- push gel through current slit
- peel away slowly, finger thru bottom
- cut away lanes, slit
- face down on paper, then face up on paper
- membrane on top, wet paper, ROLL flat

**XFER ASSEMBLY:**

**TOP:**
- top plate cover (+)
- 2 brillo pad
- Whatman paper leaves
- MEMbrane (1m methanol, 10m Xfer Buf)
- GEL
- 3 Whatman paper leaves
- 2 brillo pad

**BOTTOM:**
- Base plate core (-)
- 3 Whatman paper leaves
- 2 brillo pad

**XFER run (ICE):**

- XFER buffer inside cassette
- ddHO outside cassette
- press out bubbles

Quick reference by Brian DiPaolo (bdipaolo@seas.upenn.edu)

**RUNNING CONDITIONS:**

<table>
<thead>
<tr>
<th>gel/mem</th>
<th>power</th>
<th>GEL RUN (min)</th>
<th>current</th>
<th>limit V</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-12 % precast</td>
<td>25 W / GEL</td>
<td>65</td>
<td>100 mA / GEL</td>
<td>[200 V]</td>
</tr>
<tr>
<td>4-12 % precast</td>
<td>25 W / GEL</td>
<td>50</td>
<td>100 mA / GEL</td>
<td>[200 V]</td>
</tr>
</tbody>
</table>

**XFER CONDITIONS:**

<table>
<thead>
<tr>
<th>XFER</th>
<th>current</th>
<th>limit V</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5 x 10.0</td>
<td>90 min</td>
<td>100 mA / GEL</td>
</tr>
<tr>
<td>BIORAD 0.2um PVDF 25 W / GEL</td>
<td>for 250 - 50 kDa</td>
<td>2 membranes</td>
</tr>
<tr>
<td>BIORAD 0.2um PVDF 25 W / GEL</td>
<td>for 100 - 10 kDa</td>
<td>2 membranes</td>
</tr>
</tbody>
</table>

unles noted: block 1hrRT, PRIME ov4C rocker, WASH TBS/T 3x5min, SEC1 75m RT rocker, WASH TBS/T 5x5min, develop, strip 20mRT, 6x5m TBS/T, block

**SOLUTIONS:**

- TBS/T (0.01%): 100mL 10xTBS, 900mL ddH2O, 100L TWEEN20 (low MW proteins)
- TBS/T (0.1%): 100mL 10xTBS, 900mL ddH2O, 1mL TWEEN20 (medium MW proteins)
- TBS/T-milk (5%): 40mL TBS/T, 20g nonfat dry milk
- TBS/T-BSA (5%): 40mL TBS/T, 2g BSA
- 1xRunning Buffer: 100mL 10xRunning Buff (pH 8.5), 900mL ddH2O
- 1xTransfer Buffer: 100mL 10xTransBuff (pH 8.5), 200mL Methanol, 700mL ddH2O
- 5x Reducing buffer: (pH of 10x Run/Trans buffer stayed constant for at least 6 months)
- NPAGE Xfer: 50mL 20X (NP0006-1), 250methanol, 750ddH2O
- NPAGEMOPS/MES SDS Run: 50 mL 20X (NP0001/2), 950 ddH2O (MOPS and MES can use twice)

**Typical Volumes:**

- 6mL block in blue container
- 5mL primary in blue container
- 3mL primary in small clear container

**NON-dye markers:**

- 30m37C OR 1hrRT OR 04C
- secondary 30m37C OR 1hrRT

**Full range rainbow (GE Healthcare Biosciences, RRN8003): 45C 1min**

**http://www.gehealthcarebiosciences.com/rrn8003.pdf**

**CC73714C12503601D427/S198e1PRN8003PS+RecA+2007.pdf**
<table>
<thead>
<tr>
<th>Antibody</th>
<th>clone</th>
<th>isotype</th>
<th>species</th>
<th>conjugate</th>
<th>concentration</th>
<th>use</th>
<th>dilution</th>
<th>source</th>
<th>comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>269</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes:***
- dilution: 1:1000
- source: BD Biosciences
- comment: mouse anti-FLAG

**Antibody Code:**
- 269
- dilution: 1:1000
- source: BD Biosciences
- comment: mouse anti-FLAG

**References:**
QUICK REFERENCE NOTES

GEL RUN:
- ZOOM Dual Power Supply (Invitrogen ZP10001)
- NuPAGE LDS load buffer (NP0007) 4X (RT)
- NuPAGE Sample Reducing Agent (500 mM DTT; NP0009) 10X (4°C) - use when NOT using already reduced lysate
- for multiple gels: keep gels in a 30V 'holding pattern' until all gels loaded (and after while XFER setup)
  - this application of slight current keeps signals tight while waiting for other gels to begin run
- do not let gel front fall below 'current' strip (this part gets cut off before XFER)

-sample recipe (for gel well 25 ul max):
  (20.4 ul total)
  + sample volume (25 ug)
  + 5 ul 4X LBS sample buffer (reducing or non-reducing)
  + ddH2O remainder volume
  + safety volume: 0.4 ul
  = load 20 ul into gel well (25 ul max)
- heat samples 95°C 5 min
- RUN with cassettes in a bin of ice (cassettes become warm)

XFER:
- lower chamber gel xfers more completely
- note lane positions after transfer by punching 4 holes in corners of membrane (over gel)
  - mark lane 1 and gel letter with pencil on membrane
- do not let gel sit with loaded wells for more than a few minutes (protein will begin to migrate)
- do not stop xfer midway
- 0.2µm PVDF membrane
- XFER with cassettes in a bin of ice (cassettes become warm)

ANTIBODY/WASHING:
- load overnight incubation (primary) in tilter so primary runs across MW and not across lanes
- always keep wash between primary and secondary same (3x5 min)
- primary over 2 or 3 nights (4°C) is just fine (3 nights, bands a little stronger than normal)
- TBS/T 0.1% pH 7.4-7.35 (at 1X) works well; made from 20X TBS USB 75892 5L
- membrane storage
  - few days: TBS/T 4°C no shake or TBS/T+NaAzide 4°C no shake
-long term between labeled whatman filter paper in TBS/T+NaAzide 4°C
(have stained membrane after 14 days in TBS/T+NaAzide: very little background increase, works very well)
-use 7.5 ml primary/secondary/block if staining multiple membranes (4-6) in one chamber/well
-pipette wash to sides of dish, NOT directly onto blot
-if 5% milk TBS/T solution for block/antibody dilution does not work for phospho-antibodies, also try:
  -0.25% w/v porcine gelatin (Sigma 9000-70-8) in TBS (no T for block) as block/antibody dilution (because milk might contain phosphatases that break down phospho binding)
  -or 5% BSA TBS/T (BSA contains less phosphatases??)
  -lower tween concentration (0.05% or 0.01%)

FILM DEVELOPMENT:
-if exposure signal too bright, wait 2 hrs with blots in laminate to keep moist and signal will fade to normal-exposure, linear region
-if need longer incubation (waiting for darkroom), sit (do no shake) in TBS/T
-place blots in laminate, add A+B mix, 1 min while dabbing and raising/lowering laminate top sheet, then suck off excess AB at laminate interface
-take scanner pic of membrane while still in laminate (allows future line up of Rainbow with bands at specific MWs)
-quick development technique (for high intensity blots): place blot/laminate on left side of cassette and film in bottom and quickly shut and open cassette
-Amersham ECL Plus WEST blotting (RPN 2132, RPN 2133): 1ml A + 25ul B per 2x blot
  -Developer 3min, H₂O 30s, Fixer 5min
    -Developer: 103ml dev, 370ml H₂O
    -Fixer: 103ml fix, 370ml H₂O

(Western blot quick reference by Brian DiPaolo, bdipalo@seas.upenn.edu)
WESTERN BLOT QUANTIFICATION

SCAN BLOT FILM:
- OPEN EPSON SCAN (Desktop; EPSON PERFECTION V500 PHOTO)
- document type: reflective
- auto exposure type: photo
- image type: 16bit grey
- scanning quality: best
- resolution: 800
- trimming on
- no adjustments - no buttons pressed; no backlight correction
- scan as high res jpeg
- check settings in folder button
- MAKE SURE signal is within 0-255 (invert image, using line through all signals in Image J)

-NOTES:
- β-Actin not the best equivalent loading probe, GAPDH is better (6)
- Densitometry tips and standardization (8)

DENSITOMETRY:
- OPEN ImageJ
- 8 bit image (greyscale)

- CHOSE exposure where signals do not look over or under exposed, AND where difference between highest and lowest signal is greatest

- analyze - set measurements: area, integrated density (INTDEN)
  - use integrated density as SUM INTENSITY (checked for one blot - same as sum intensity in Kodak 1D)

- edit - invert
  - black background - white (intense) blot signals

- ROI manager (analyze - tools - ROI manager)

- add roi (press t)
  - make a rectangle (higher than wide) that is 1/3 the width of the signal
    - height that encompasses whole height of signal, but does not hit other signals that may be on blot (or odd background signal)
  - position middle blocks in middle (height and width) of signal

  - add same size rectangle for background measurement - above or below signal measurement
- Measure button in ROI manager

- LINE UP MW markers
  - when using visual (colored) MW marker, to line up to film use photoshop layers and reduce exposure size (blot data) to 18.74% to line up with scanned image of membranes in laminate

- STAT ANALYSIS
  - assuming normal distributions and that variances are equal
  - prove this using a Levene's test in JMP (run ANOVA, tab pulldown un-equal variances) on Akt data (both ser and thr) comparing experimental (37% 1/4hz 10min) vs control (UNS) and found no difference (cannot reject the null that variances are equal, p 0.3226)

![Figure E.1: Illustrative densitometry in ImageJ based on standardization methods in (8). Blot intensity was measured using a rectangular region (bottom row, blue) centered over the signal with a width of about 1/3 the size of the blot signal (white). Each region was quantified by measuring integrated density (sum intensity of all pixels contained in the region). Each blot signal was normalized to its respective lane background by subtracting the integrated density of a same-size rectangular region placed above the signal region (top row, blue).](image)

NOTES:
- present entire blot
- give reasons for excluding bands detected by the antibody
- important SCAN parameters
  - NO auto-gain
- background correction: baseline subtraction algorithm applied to each lane individually
  - integral of entire OD profile
- measure integral
  - width of sample: 1/3 width of lane and centered
- from ImageJ website help: Integrated Density - The sum of the values of the pixels in the image or selection. This is equivalent to the product of Area and Mean Gray Value.

(scanning protocol by Brian DiPaolo, bdipalo@seas.upenn.edu; based on (8))

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APPENDIX F: BODIPY-OUABAIN & ETHIDIUM HOMODIMER STAINING
AND IMAGING PROTOCOL

BODIPY-OUABAIN STAINING & IMAGING PROTOCOL

DATE:    DISK#:     CELLS:  
-Notes:
  -STRETCHER HOME = 68.60 mm (for old/large-height wells)
  -SHIELD WELLS FROM LIGHT (especially fluorescent shutter)
  -DMSO effects permeability of bodipy-ouabain (see vehicle control data):
    attempt to dissolve treatment into stock concentration so treatment in a
    small well (1 ml DMEM+H) does not require more than 3-4 ul DMSO (1
    ul bodipy-ouabain treatment + 2-3 ul treatment)

-Serum deprive cells TIME:
-check stretch device
  -test with 'run stretch-s' to 0%, and make sure indenter is touching bottom of well
  -if not, switch of stretch controller, open indenter and pull belt until indenter to
    bottom
    -then switch on controller, 'run newstart' and indenter should be at home
    position (retest)

-Setup scope:
  -1 turn on camera controller (hamamatsu)
  -2 turn on shutter controller (LuDL)
  -3 turn on fluorescent unit (a few minutes later)
    -fluorescence setting to 25%
  -4 turn on metamorph
    -open settings BDiPaolo_BodipyOubain for acquisition
    -NO AUTOSCALE (scale 0 - 4095: full window scale)
    -10x fluorescent objective
    -setting D (80% to cam, 20% to eyepiece)
    -filter setting G (for both eyepiece and camera)
    -room lights OFF
    -use multi-channel aquire (FITC, phase, TX for dead) - (BODIPY CHANNEL 1)

-Add Bodipy-Oubain (1 ul stock per ml stretch media: for 2 uM) TO DMEM+HEPES
  (then add to well)
  (Bodipy-Ouabain 2 mM stock: 100 mg powder + 58.2 ul DMSO)
  -shake slightly to help diffusion
  -keep bodipy-ouabain on cells for same time between samples (stretch and
    unstretched)
    -total 1 hr BODIPY-ouabain incubation 37°C
  -keep aliquot of Bodipy-Ouabain in fridge during experiment
-NOTE: use same volume of BODIPY-ouabain and treatment vehicle (DMSO) in vehicle control and treated groups
  -also, keep volume limited as DMSO affects monolayer permeability (less than 3 µl)

-Stretch (or Unstretch) wells
  -shield from light

-Wash 3 × warm DMEM+Hepes (pour out media, add, shake, repeat; need about 4 ml DMEM+H per well)
  -add 1.0 ml DMEM+Hepes
  -keep shielded from light

-Place well on scope
  -image FIRST STRETCHED well using AUTOEXPOSE
  -record exposure time: (ms) (typically 1000 ms for other experiments)
  -make sure stretched samples (usually 37% 60/10 min) light up properly (cells should be slightly green)
  -for rest of samples, use AQUIRE (and thus same exposure time)
    -take 4 images of different areas in each well
    -place UNstretch well on scope, image same

-HELPFUL: if also using ethidium homodimer for dead cells, focus on dead stain (last stain in multi-acquire), then focus "down/counter-clock" 5 steps to properly focus on bodipy/phase; IMAGE using multi-acquire
-NOTE: for analysis, will establish THRESHOLD and NORMALIZE (nArea) to lung and time specific UNS-UNT wells
-NOTE: image naming convention (compatible with MATLAB analysis program):
  -(sample designation, uppercase letter)(well #)(image #)
  -ex: A12: group A, well 1, image 2

NOTES:
-BODIPY-Oubain (Invitrogen B23461)
-it is advantageous to establish the exposure time so that cells that you expect to stain most intensely for BODIPY-ouabain (positive control wells) are on the upper end of the intensity window (red, orange, yellow range in the false color intensity display): keep in mind that monolayers can contain a slight population of unwanted dead cells or 'junk' structures that might stain heavily for BODIPY-ouabain; its ok if these structures are over-exposed because these erroneous structures will then add less to the image intensity measurement AND it will allow a better approximation of BODIPY-ouabain staining in viable monolayer areas accross the experiment because these 'good' areas will have a larger dynamic range with which to compare between stretch/treatment groups
- to fix filter blocks: open right hub and push cubes to left (will click over tracks)
  - this will allow cubes to be pushed (via thumb slider) over to the first and final positions properly

- 2 mM 2-deoxy-D-glucose + 10 µM antimycin A (Sigma-Aldrich D6134 and A8674)
  - staining with BODIPY-ouabain does NOT effect PJAR formation (visual observation in post-BODIPY-ouabain treated fixed monolayers stained for F-actin using phalloidin preliminary study)

Figure F.1: Primary rat alveolar epithelial cell (AECs) monolayer incubated with 2 µM BODIPY-ouabain (green) and stretched for 10 min at 37% ∆SA ¼ Hz. Monolayers were then fixed and stained for F-actin with phalloidin (red). Image is an x-axis projection (profile view) taken using a confocal microscope (40× objective, Bio-Rad Radiance 2000 MP laser scanner on Nikon Eclipse TE 300 epifluorescent scope). BODIPY-ouabain permeates and binds to basal side (bottom) of the monolayer after stretch. Image width = 304.4 µm, height = 17.5 µm

Figure F.2: Unstretched primary rat alveolar epithelial cell (AEC) monolayers incubated with BODIPY-ouabain (green) and left untreated (left) or treated with 2 mM 2-deoxy-D-glucose + 10 µM antimycin A for 60 min (right) to deplete ATP and break down tight junctions as positive control. ATP depletion used previously in AECs to disrupt TJ protein (4). Images also shown in phase (grey). Bar = 40 µm.

(protocol by Brian DiPaolo, bdipaolo@seas.upenn.edu; derived from Ken Cavanaugh protocol and (3) with slight changes: Used 10× objective to image, used novel MATLAB program for analysis, and utilize max background intensity instead of mean of max background to calculate normalized % Area)
BODIPY-OUABAIN ANALYSIS PROGRAM

USER EXECUTED MAIN PROGRAM:

BodipyOuabain_Thresh_BCD.m:
%---START PROGRAM---
%BodipyOuabain_Thresh_BCD.m
%by Brian C. DiPaolo (bdipaolo@seas.upenn.edu) with input from Nurit
%Davidovich

%BODIPY-OUABAIN percent-area calculator

%USER GUIDE:
%1) Run program with manual_method = 1, initialize = 1, and sample =
%UNS_NT; select background points from each image then hit enter.
%Program
%will display maximum background point. Enter this value into
%threshold_UNSNT, switch manual_method to 0, and sample to experimental
%group, and run for rest of experiment; Program will calculate %Area
%above
%UNSNT threshold

%PROGRAM will output data contained in variable DATA_both
%PROGRAM will also display a figure of all images, with RED=counted
%pxls
%above threshold, and blue=pxls lower than thresh

%SELECTING BACKGROUND:
%select a 5-8 points/areas from each corner and from the center of each
%image to
%record a homogeneous population spread

%MATLAB NOTES
%clear/add COMMENT ctrl-t/r
%(row, col)
%RUNNING MULTIPLE SAMPLES - COMMENT OUT ABOVE
%doc command (brings up help)
%I(:) (bins r x c array into a single column)
%header = ['disk' ',' 'well' ',' 'image' ',' 'max_back' ','
'avg_back' ',' 'OTSU_total_area' ',' 'OTSU_ON_pixels' ',' 'OTSU-%-area'
',' 'above-thresh' ',' 'above-thresh-percent'];
%copy and paste DATA and group (and header) into excel

%--------INITIALIZE----------
wells_per_group = 3;
images_per_well = 4;
bodipy_channel = 1;%disk153 ONLY disk where DEAD/BODIPY stain is
different channel
dead_channel = 3;%disk153 ONLY disk where DEAD/BODIPY stain is
different channel
%disk153: D,E,F FITC/FR/Phase, all else FITC/Phase/FR
%threshold = 500;
std_above_mode = 0.5;
    %program sets threshold at mode + given number of standard
deviations
manual_method = 1;
    %set to 1 to switch program to manual mode
threshold_UNSNT = 176;
initialize = 1;

%NOTE: (after 'A', COMMENT OUT; 153, change dead/bodipy; 156/157
4images/well)
disk = 160;
sample = 'A';
man_scale = [1 700];

%Add location of BODIPY-ouabain images here:
location = 'C:\Users\bdipaolo\Documents\DATA
ANALYSIS\BODIPY_Ouabain_100513_All_Analysis_thresholdUNS-
NT\GM6001_MMPinhibitor\DISK160\lung1_10MIN';
%------------------
if initialize == 1
%clear all;
%close all;
DATA_image_mode = [];
DATA_image_UNSNT = [];
DATA_both = [];
group_image = [];
above_thresh = [];
above_thresh_px = [];
supra_thresh_intensity = [];
mean_supra_thresh = [];
median_supra_thresh = [];
total_supra_thresh_intensity = [];
max_mean_background = [0 0];
max_mean_backgroung = [];
end

%-------BACKGROUND-----------
    %This portion of the program subtracts, from each image, a zero-
image;
    %the zero-image is a scope specific image, an image taken
    %when there is no sample on the scope, or an average of such images.
    %In other words, its the constant-intrinsic scope background
    %that can be subtracted off to reduce variability in intensity of
    %each sample image

    %creates an average background (no cells) background intensity
matrix

    %background does not seem to reach above 400 or 450
    %use this to remove any possible stray signals from images
(small
%bits that may be lit up in all images due to scope dust

%zero-image locations:
B1=imread('C:\Users\bdipaolo\Documents\DATA ANALYSIS\BODIPY_Ouabain_100513_All_Analysis_thresholdUNS-NT\AVERAGE background images\37 1_4hz 60m NT 10x 7 bare membrane.tif', bodipy_channel);
B2=imread('C:\Users\bdipaolo\Documents\DATA ANALYSIS\BODIPY_Ouabain_100513_All_Analysis_thresholdUNS-NT\AVERAGE background images\37 1_4hz 60m NT 10x 10 bare membrane.tif', bodipy_channel);
B3=imread('C:\Users\bdipaolo\Documents\DATA ANALYSIS\BODIPY_Ouabain_100513_All_Analysis_thresholdUNS-NT\AVERAGE background images\UNS NT 10x 5 bare membrane near 4.tif', bodipy_channel);
B4=imread('C:\Users\bdipaolo\Documents\DATA ANALYSIS\BODIPY_Ouabain_100513_All_Analysis_thresholdUNS-NT\AVERAGE background images\UNS NT 10x 6 bare membrane.tif', bodipy_channel);
figure(10)
subplot(2,1,1)
imagesc(B1)
title('back');
figure(11)
subplot(2,1,1)
imagesc(B2)
title('back');
figure(12)
subplot(2,1,1)
imagesc(B3)
title('back');
figure(13)
subplot(2,1,1)
imagesc(B4)
title('back');

%--------- removing stray signals  -----------
thresh1 = B1>400;
thresh2 = B2>410;
thresh3 = B3>420;
thresh4 = B4>400;
%thresh is an array of image size, with 1's where condition is
%true
B1(thresh1) = mean(mean(B1));
B2(thresh2) = mean(mean(B2));
B3(thresh3) = mean(mean(B3));
B4(thresh4) = mean(mean(B4));
%wherever condition is met, array gets changed to mean %statistic
%anything above 450 is signal - see example hist(single(B1));

%replaces 'stray' signals with average intensity
%mean(mean(B1));

%B1 = imerode(B1, strel('ball', 5, 5));
  %filters image based on rolling circle

figure(10)
s subplot(2, 1, 2)
imagesc(B1)
title('filtered');

figure(11)
s subplot(2, 1, 2)
imagesc(B2)
title('filtered');

figure(12)
s subplot(2, 1, 2)
imagesc(B3)
title('filtered');

figure(13)
s subplot(2, 1, 2)
imagesc(B4)
title('filtered');

BACKGROUND = double((B1 + B2 + B3 + B4) ./ 4);
%testB = uint16(zeros(size(B)));  
%testB = testB + 900;
%BACKGROUND = BACKGROUND + testB;

%BACKGROUND = uint16(zeros(size(B))); 
%BACKGROUND = BACKGROUND + 50;
figure(20)
imagesc(BACKGROUND);
title('average constant scope background');

%---------------------------------------------

i = 1;
j = 1;

for well=1:wells_per_group
  for image=1:images_per_well
    Im = imread(strcat(location, sample, num2str(well), num2str(image), '.tif'), bodipy_channel);
    %D = imread(strcat(location, sample, num2str(well), num2str(image), '.tif'), dead_channel);
    %read in tiff image; 1 for channel 1 which is usually
    %bodipy-ouabain
%figure_limits = [1 450];

figure(image)
subplot(2, 2, 1)
imagesc(Im)
title('image');

%-------------------- SUBTRACT avgBACKGROUND---------
I = double(Im) - BACKGROUND;
%i_minus_back = double(Im) - BACKGROUND; %check
 %subtracts average background intensity values (due to
 %non-homogeneous scope intensity)

subplot(2, 2, 2)
imagesc(I) %image - BACKGROUND
title('image - back');
%------------------------------------------------

%-------------------- SETS min to 0 (translation, no scaling)----
if min(min(I)) < 1
ORIGIN = min(min(I))*double(uint16(ones(size(I))));
 %makes an array (size of image) of lowest intensity values

I = I - ORIGIN;
%i_to_origin = i_minus_back - ORIGIN; %check
 %centers lowest point of image at 0
 %making sure there are no negative intensity values
%checks:
min(min(I)); %if min was negative, this should now = 0;
subplot(2, 2, 3) %image - BACKGROUND normalized to origin
(0)
imagesc(I) %should show a scaled image (scaled to full
color
%bar)
title('origin to 0');
end
%------------------------------------------------

%I1 = medfilt2(I, [13 13]);
%background = imopen(I1,strel('disk',45));
%I2 = imsubtract(I1,background);
%BW = edge(I2,0.005);
%sel = strel('disk',3);
%BW1=imdilate(BW,sel);
%total1(i) = bwarea(BW1);

%---------------------- GRAYTHRESH, OTSU METHOD -----------------------
%level1 = graythresh(I);
%level2 = graythresh(D);
%converts to binary image using a specific threshold that
minimizes %intraclass variance of thresholded black and white pixels.
USES 'OTSU'S' method

%BW = im2bw(I, level1);
%BW_2 = im2bw(D, level2);
%converts to black-white
%ON = bwarea(BW);
%ON_2 = bwarea(BW_2); %calculates the total 'white' area or stained area

[x,y] = size(BW);
%size of the image
%AREA = x .* y;

%subplot(2, 2, 4) %image - BACKGROUND normalized to origin (0)
%imagesc(BW);
%title('analyzed');

Percent_AREA = ON ./ AREA .* 100;
Percent_AREA_dead = ON_2 ./ AREA .* 100;

figure(30);
%subplot(wells_per_group, (images_per_well.*2), i) %image - BACKGROUND normalized to origin (0)
%imagesc(Im);
%title(strcat('well (',num2str(well), '), ', 'img (',
num2str(image), ')'));
%i = i + 1;
%subplot(wells_per_group, (images_per_well.*2), i) %image - BACKGROUND normalized to origin (0)
%imagesc(BW);
%title('OTSU analyzed');
%i = i + 1;

%----------------------------------------------
[x,y] = size(I);
AREA = x .* y;

-----------MANUAL BACKGROUND SELECTION -----------
if manual_method == 1;
screen_size = get(0, 'ScreenSize');
f1 = figure(40);
%set(f1, 'Position', [0 0 screen_size(3) screen_size(4)]);
%for full screen size
set(f1, 'Position', [0 0 size(I, 2) size(I, 1)]);
%for image screen size (1:1 pixels)
imagesc(I, man_scale);
% manually establishing image color thresholds (ex [1 300])
% NOT effect the pixel reading; tested using figure pointer as
% well as impixel readings

title('image - back, origin to 0; select BACKGROUND; back/del to remove; hit return to END');

% single pixel method
% pix = impixel;
% records mouse clicked data from array I
% max_mean_background = [max_mean_background; max(pix(:, 3))
mean(pix(:, 3))];

% rectangular region method
pix = 1;
while pix >= 1
    max_mean_background = [max_mean_background; max(pix(:))
mean(pix(:))];
title('place rectangle, drag or double-click it to submit, delete to END image');
pix = imcrop;
    % records intensities in a mouse-defined rectangular region
end

% threshold=max(pix(:,1));
% ANY PIXEL ABOVE MAX BACKGROUND
close(40);

else

% USING UNS-NT THRESHOLD to calculate -------------------------------

% CALCULATIONS
above_thresh = I > threshold_UNSNT;
% figure(40);
% imagesc(above_thresh);
% shows pixels above thresh

supra_thresh_intensity = above_thresh .* I;

mean_supra_thresh = mean(mean(supra_thresh_intensity(supra_thresh_intensity~=0)));
median_supra_thresh = median(median(supra_thresh_intensity(supra_thresh_intensity~=0)));
total_supra_thresh_intensity = sum(sum(supra_thresh_intensity));
above_thresh_px = sum(sum(above_thresh));
above_thresh_px_percent = above_thresh_px ./ AREA .* 100;

figure(30);
subplot(wells_per_group, (images_per_well.*2), j) %image -
BACKGROUND normalized to origin (0)
imagesc(I);
title(strcat('well (',num2str(well), '), ', 'img (',
num2str(image), ')'));
j = j + 1;
subplot(wells_per_group, (images_per_well.*2), j) %image -
BACKGROUND normalized to origin (0)
imagesc(above_thresh);
title('UNS-NT analyzed');
j = j + 1;

DATA_image_UNSNT = [DATA_image_UNSNT; disk well image
threshold_UNSNT AREA above_thresh_px_percent mean_supra_thresh
median_supra_thresh total_supra_thresh_intensity];

%--------------------------------------------------------------
%USING MODE TO CALCULATE THRESH -------------------------------
%--------------------------------------------------------------
threshold_mode = mode(I(:)) + (std_above_mode .* std(I(:)));
    %uses mode because in many images, background will be the
    %mode
    %of the image
    %for histogram, set number of bins to cube root of number
    %of data points
    %hist(I, [1:10:600])
    %P = thresh_tool(Im)

%CALCULATIONS
above_thresh = I > threshold_mode;
figure(40);
imagesc(above_thresh);
    %shows pixels above thresh

supra_thresh_intensity = above_thresh .* I;

mean_supra_thresh =
mean(mean(supra_thresh_intensity(supra_thresh_intensity~=0)));
median_supra_thresh =
median(median(supra_thresh_intensity(supra_thresh_intensity~=0)));
total_supra_thresh_intensity = sum(sum(supra_thresh_intensity));
above_thresh_px = sum(sum(above_thresh));
above_thresh_px_percent = above_thresh_px ./ AREA .* 100;

figure(31);
subplot(wells_per_group, (images_per_well.*2), i)  % image -
BACKGROUND normalized to origin (0)
imagesc(I);
title(strcat('well (', num2str(well), '), ', 'img (',
um2str(image), '))');
i = i + 1;
subplot(wells_per_group, (images_per_well.*2), i)  % image -
BACKGROUND normalized to origin (0)
imagesc(above_thresh);
title('MODE analyzed');
i = i + 1;

DATA_image_mode = [DATA_image_mode; disk well image mode(I(:))
( std_above_mode.*std(I(:)) ) AREA above_thresh_px_percent
mean_supra_thresh median_supra_thresh total_supra_thresh_intensity]

%-------------------------------------------------------
end
end
end

DATA_both = [DATA_image_UNSNT DATA_image_mode];
disp('DONE')
sample
disp('MAX, average');
max_mean_background
disp('MAX, average');
max_mean_background_all = [max(max_mean_background(:, 1))
mean(max_mean_background(:, 2))]
%---END PROGRAM---
LIVE/DEAD CELL STAINING PROTOCOL

NOTE DATE:    DISK#:    CELLS:

-STRETCHER HOME = 68.60 mm (for old/large-height wells)
-SHIELD WELLS FROM LIGHT as much as possible (especially fluorescent shutter)

-Serum deprive TIME:
-check stretch device
  -test with 'run stretch-s' to 0%, and make sure indenter is touching bottom of well
  -if not, switch of stretch controller, open indenter and pull belt until indenter to bottom
  -then switch on controller, 'run newstart' and indenter should be at home position (retest)

-Setup scope
  -1 turn on camera controller (hamamatsu)
  -2 turn on shutter controller (LuDL)
  -3 turn on fluorescent unit (a few minutes later)
    -fluorescence setting to 25%
  -4 turn on metamorph
-NO AUTOSCALE (scale 0 - 4095: full window scale)
-10x fluorescent objective
-setting D (80% to cam, 20% to eyepiece)
-filter setting G (for both eyepiece and camera)
-room lights OFF
-use multi-channel acquire (phase, TX red for dead, FITC green for live)

-Add DEAD stain (1 ul stock per 1 ml stretch media: for 2 µM), then add to well
  -shake slightly to help diffusion
  -keep on for 1 hr
  -note: ethidium homodimer-1 (Invitrogen, E1169), stock 2 mM in 1:4 DMSO/ddH₂O
-Add LIVE stain (1 ul stock per 1 ml stretch media: for 4 µM), then add to well
  -note: calcein acetoxymethyl (AM) (Invitrogen, C1430), stock 4 mM in 1:4 DMSO/ddH₂O

-Stretch (or Unstretch)
  -remove well (stretched and unstretched)
  -place in black box

-Wash 3 x 37°C DMEM+HEPES (pour out media, add, shake, repeat), need about 4ml DMEM / well
  -add 1.5 ml DMEM+Hepes
  -place back into black box
-Place well on scope
  -image **FIRST STRETCHED** well using AUTOEXPOSE
  -record exposure time: _____ (ms)
  -make sure stretched samples (usually 37% 60/10 min) light up properly
  -for rest of samples, use AQUIRE (and thus same exposure time)
    -take 3 images of different areas
  -place UNstretch well on scope, image same

Notes: to fix filter blocks: open right hub and push cubes to left (will click over tracks) - this will allow cubes to be pushed (via thumb slider) over to the first and final positions properly

---

Figure F.3: Live primary rat type I like alveolar epithelial cells (AECs) after 60 min of 37% ΔSA ¼ Hz stretch (right) or left unstretched (left) stained with dead cell specific ethidium homodimer (red) and imaged in phase (grey) on a 10× epifluorescent microscope. Stretch increases cell death (Chapter 4). Each image is 868 µm in width.

(protocol by Brian DiPaolo, bdipaolo@seas.upenn.edu; based on (11) with slight modification)
DEAD CELL ANALYSIS PROGRAM

USER EXECUTED MAIN PROGRAM:

Dead_count_BCD.m:
%---START PROGRAM---
%Dead_count_BCD.m
%Main program by Brian DiPaolo, with calls to functions developed for
%microbead tracking by Ben Fabry and Brian DiPaolo

%dead cell counter (ethidium homodimer - nucleus)
%images from epiflourescent scope - 10X magnification; stained for
%ethidium
%homodimer
%written by Brian DiPaolo (bdipaolo@seas.upenn.edu)
%program will display original image (fig 2) and interactive image (fig
%1)
%figure 1 shows AUTO located dead cells with large red O (should
%be
%little larger than a dead cell nucleus and same intensity (ie red)
and
%number of cell
%figure 1 allows you to click on cells you think are NOT dead
cells
%(but incorrectly found junk) or cells you think ARE dead cells
%AUTO found cell number is 2nd to last column in DATA_image
%manual clicks number is last column
%THUS: take AUTO +/- number of clicks to find the # DEAD
% if anything, program over estimates dead cells (finds non-cells),
so
% you will have to SUBTRACT manual counts
%this mode is benificial for images with many cells (which you
%would not want to count)

%RUN F5
%COMMENTS ctrl-t/r
%ctrl-C

%this program evokes (requires)
 %bead_mask: creates a synthetic bead
 %find_bead: finds locations of beads based on intensity
 %InvertIm: inverts image

%PROGRAM WILL OUTPUT variable (DATA_image) with the following
information
%DISK#   WELL#   IMAGE#    AUTO_COUNTED_DEAD     MANUAL_COUNTED_DEAD

%----------------------- INITIALIZE-------------------
thresh_coef = 0.07;
%thresh_coef = 0.03;  %0.12 for disk154, 0.08 for disk155, 0.07 for
%disk156
    %increase multiplyer (0.4) to select more strictly in INTENSITY
(less beads found)
    %threshold = 0.4*(max(max(v))-mean(mean(v)))+mean(mean(v));
%ORIGINAL

dead_channel = 3;%disk153 ONLY disk where DEAD/BODIPY stain is
different channel
%disk153: AT 10min, D,E,F FITC1/FR2/Phase3, all else FITC1/Phase2/FR3
%bodipy_channel = 1;%disk153 ONLY disk where DEAD/BODIPY stain is
different channel
%phase_channel = 2;

disk = 156;
sample = 'A';
wells_per_group = 3;
images_per_well = 4;

%add image location here:
location = 'C:\Users\bdipaolo\Documents\DATA
ANALYSIS\BODIPY_Ouabain_100513_All_Analysis_thresholdUNS-
NT\wortmannin_PIP3\disk152\disk152 bodipy images and misc
calc\lung1\at_10min\';

%---------------------------------------------------------------------
%------------------ HARD INITIALIZATIONS -------------------
diameter = 21;
    %diameter of the bead in pixels    %21 works well
for stretch
    %diameter = 12
    %diameter of the bead in pixels
    %use diameter 16 for normal runs
    %use diameter 12 if you get many warning:divide by zero then errors
%dead cells between 10-17 px diameter
radius = diameter/2;

dim =12;   %mask radius (synthetic bead), must be greater than radius
%12 good for stretch
%dim =7;    %mask radius (synthetic bead), must be greater than radius
%dim = 22;
%---------------------------------------------------------------------

for well=1:wells_per_group
    for image=1:images_per_well

% create the bead mask for use to crosscorrelate against a real image
% at present, the radius are defined from observations of the image by the user
bead = bead_mask(radius,dim);
bead = (bead - min(min(bead)));
bead = bead/max(max(bead));
%figure(1); imagesc(bead); colormap(gray); drawnow;

%b2=ind2gray(b2,map);
b2 = imread(strcat(location, sample,num2str(well),num2str(image),'.tif'), dead_channel);
b2 = InvertIm(b2);
%inverts image - so that dead cells are white in color
%use InvertIm.m function found at
%http://www.mathworks.com/matlabcentral/fileexchange/14609-image-inversion
%by Stead Kiger, Ph.D.,
%OR any function that inverts an image because microbead functions used to locate dead cells looks for white circles

%b2=b2(30:600,50:500);
b2 = double(b2);
b2 = (b2 - min(min(b2)));
b2 = 1-b2/max(max(b2));
%figure(1); imagesc(b2); title('first image'); colormap(gray); drawnow;

screen_size = get(0, 'ScreenSize');
f1 = figure(1);
set(f1, 'Position', [0 0 screen_size(3) screen_size(4)]);
%for full screen size

f2 = figure(2);
set(f2, 'Position', [-1.*1344./2 400 1344./2 1024./2]);

figure(2)
%subplot(2,2, 2)
imagesc(b2)
title('original image')

figure(1)
%subplot(2, 2, 1)
%imagesc(b2, [0 0.2])
%title('scaled')

coord = find_bead(b2,bead,dim, thresh_coef);

%figure(2); imagesc(b2); title('first image'); colormap(gray); drawnow;
%figure(2); hold on;
%plot(coord(:,1), coord(:,2), 'ro');
%hold off;
%------ MANUAL COUNT FOR CHECK ------
%CAN ALSO USE TO MANUALLY COUNT FALSE IDENTIFIED CELLS (subtract from AUTO
%count)

%screen_size = get(0, 'ScreenSize');
%f1 = figure(10);
%set(f1, 'Position', [0 0 screen_size(3) screen_size(4)]);
%for full screen size
%set(f1, 'Position', [0 0 size(b2, 2) size(b2, 1)]);

figure(1)
%subplot(2,2,3)
manual_count = 0;
%imagesc(b2)
title(strcat('well(',num2str(well), '), ', 'img(', num2str(image), '); MANUAL; delete or ENTER'))
pix = impixel;
%COMMENT OUT TO REMOVE MANUAL SELECTION
%average background (65420)
%average dead stain (64471)
manual_count = size(pix, 1);
%COMMENT OUT TO REMOVE MANUAL SELECTION
cla;
%-------------------------------------

disp('program count, manual count');
bead_count=[size(coord, 1) manual_count]

DATA_image = [DATA_image; disk well image bead_count];

end
end

close(1);
close(2);

disp('DONE')
sample
%clear all;
%---END PROGRAM---

DEAD CELL ANALYSIS FUNCTIONS:

bead_mask.m:
Please see Appendix D, MICROBEAD TRACKING MATLAB CODE, functions section.
find_bead.m:
Please see Appendix D, MICROBEAD TRACKING MATLAB CODE, functions section.

InvertIM.m:
Please see: InvertIM.m by Stead Kiger, Ph.D.,

OR use a function that will invert the image intensity
APPENDIX G: RAW DATA

NOTES:
- Animal average: the average of all wells/monolayers from an animal for each experimental group
- Passage average: the average of all wells/monolayers of a passage for each experimental group
- Abbreviations:
  - AEC: alveolar epithelial cells
  - HPAEC: human pulmonary artery endothelial cells
  - UNS: unstretched
  - UNT: untreated
  - VC: vehicle control
  - n: normalized = stretched value ÷ unstretched value
  - SE: standard error
## CHAPTER 2 DATA

### PJAR Intensity (Pi) data

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<th>mag (ΔSA)</th>
<th>UNS</th>
<th>12%</th>
<th>12%</th>
<th>12%</th>
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<th>25%</th>
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<td>¼</td>
<td>¼</td>
<td>¼</td>
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<td>¼</td>
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**average:** 1.000000 1.023010 1.018279 0.992500 0.991429 1.210714 1.217333

**SE:** 0.000000 0.020310 0.019339 0.026101 0.036478 0.048135 0.029122

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| treatment:                | 100nM JAS 100nM JAS 100nM JAS 100nM JAS |                |
| (10 min)                  | 0.380917 0.915034 1.288155 2.423191 |                        |
|                           | 1.410008 2.272129 1.691834 2.515793 |                        |
|                           | 0.751384 1.568622 2.871697 3.020186 |                        |
|                           | 0.700814 1.119914 1.593271 3.218604 |                        |
|                           | 1.345790 |                        |
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### ZO-1 Intensity (Zi) data

peripheral annulus (A1) / whole cell (W1)

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### AECs

animal average

whole cell (W1) / average (whole cell (W1) UNS-UNT)

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# CHAPTER 3 DATA

## ROCK activity assay data

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| mode (Hz): | UNS | UNS | UNS |
| time (min): | UNS | UNS | UNS |
| treat: | UNS | 10 μM | 5 μM |
| | | 60 min | 60 min |</p>
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| mode (Hz): | ¼ | ¼ |
| time (min): | 10 | 10 |
| treat: | 10 μM | 60 min |</p>
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**average:** VEGF 0.978874 0.628313 0.204061 2.216897 0.875542
**SE:** VEGF 0.086405 0.119220 0.102144 0.875542

| mag (ΔSA): | 25% 37% 37% 37% 37% |
| mode (Hz): | ¼ ¼ ¼ ¼ ¼ |
| time (min): | 10 10 10 10 10 |
| treat: | VEGF 1.000000 0.742556 0.312212 0.203208 0.808456 |
| | VEGF 2.586721 1.167673 2.964396 2.118989 0.688145 |
| | VEGF 0.308050 1.369549 0.804065 0.040002 2.772009 |
| | VEGF 1.227547 1.539352 0.925003 0.173254 1.412039 |
| | VEGF 2.370725 0.773757 1.265913 0.043399 0.641143 |

**average:** VEGF 1.447120 1.695470 1.378534 0.596828 1.057981
**SE:** VEGF 0.446869 0.465810 0.338779 0.338227 0.387334
### Akt phosphorylation (Thr308)

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**AECs**

| mag (ΔSA): | 25% | 37% | 37% | 37% | 37% | 37% |
| mode (Hz): | ¼   | ¼   | ¼   | ¼   | ¼   | ¼   |
| time (min): | 10  | 10  | 10  | 10  | 10  | 10  |
| treat:     | VC  | VC  | Wortmann | Wortmann | EHT1864 |
|           | 0.518640 | 2.910706 | 0.519727 | 0.200648 | 0.753994 |
|           | 0.462404 | 1.411638 | 2.243258 | 1.079210 | 0.529510 |
|           | 2.117904 | 0.641902 | 0.712057 | 0.104786 | 4.958271 |
|           | 0.941552 | 1.573099 | 1.090312 | 0.081088 | 3.544314 |
|           | 3.742061 | 1.871070 | 0.321782 | 0.028734 | 0.541881 |
|           | 6.308321 | 5.113164 | 1.144190 | 0.069171 | 1.051310 |

**Average:**

| mag (ΔSA): | 25% | 37% | 37% | 37% | 37% | 37% |
| mode (Hz): | ¼   | ¼   | ¼   | ¼   | ¼   | ¼   |
| time (min): | 10  | 10  | 10  | 10  | 10  | 10  |
| treat:     | VC  | VC  | Wortmann | Wortmann | EHT1864 |
|           | 0.518640 | 2.910706 | 0.519727 | 0.200648 | 0.753994 |
|           | 0.462404 | 1.411638 | 2.243258 | 1.079210 | 0.529510 |
|           | 2.117904 | 0.641902 | 0.712057 | 0.104786 | 4.958271 |
|           | 0.941552 | 1.573099 | 1.090312 | 0.081088 | 3.544314 |
|           | 3.742061 | 1.871070 | 0.321782 | 0.028734 | 0.541881 |
|           | 6.308321 | 5.113164 | 1.144190 | 0.069171 | 1.051310 |

**SE:**

| mag (ΔSA): | 0.000000 | 0.409328 | 0.187716 | 0.121315 | 3.096524 |
| mode (Hz): | UNT     | UNT     | UNT     | UNT     | UNT     |
| time (min): | UNT     | UNT     | UNT     | UNT     | UNT     |
| treat:     | UNT     | UNT     | UNT     | UNT     | UNT     |
|           | 1.000000 | 1.676058 | 0.958920 | 0.328565 | 4.620540 |

**Average:**

| mag (ΔSA): | 25% | 37% | 37% | 37% | 37% | 37% |
| mode (Hz): | ¼   | ¼   | ¼   | ¼   | ¼   | ¼   |
| time (min): | 10  | 10  | 10  | 10  | 10  | 10  |
| treat:     | VC  | VC  | Wortmann | Wortmann | EHT1864 |
|           | 0.518640 | 2.910706 | 0.519727 | 0.200648 | 0.753994 |
|           | 0.462404 | 1.411638 | 2.243258 | 1.079210 | 0.529510 |
|           | 2.117904 | 0.641902 | 0.712057 | 0.104786 | 4.958271 |
|           | 0.941552 | 1.573099 | 1.090312 | 0.081088 | 3.544314 |
|           | 3.742061 | 1.871070 | 0.321782 | 0.028734 | 0.541881 |
|           | 6.308321 | 5.113164 | 1.144190 | 0.069171 | 1.051310 |

**SE:**

| mag (ΔSA): | 0.622263 | 0.827655 | 0.634393 | 0.213860 | 0.822135 |
| mode (Hz): | UNT     | UNT     | UNT     | UNT     | UNT     |
| time (min): | UNT     | UNT     | UNT     | UNT     | UNT     |
| treat:     | UNT     | UNT     | UNT     | UNT     | UNT     |
|           | 1.556512 | 2.452789 | 1.578802 | 0.439776 | 1.732857 |

**SE:**
### Rac1 phosphorylation (Ser71)

n-phospho-Rac1 (Ser71) / n-total-Rac

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| average | 1.000000 | 1.675915 |
| SE      | 0.000000 | 0.361542 |

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<td>10 ng/μl</td>
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| average | 1.000000 | 1.967891 | 0.560328 | 0.772540 | 2.355004 | 0.656849 |
| SE      | 0.000000 | 0.562968 | 0.156997 | 0.122445 | 0.324999 | 0.167957 |

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<td>100 nM</td>
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<td>10 μM</td>
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| average | 1.530085 | 0.830886 | 0.719365 | 2.095734 | 1.016019 |
| SE      | 0.238996 | 0.214738 | 0.192090 | 0.482718 | 0.289088 |
### LIMK1/2 phosphorylation (Thr508/5)

**n-phospho-LIMK1/2 (Thr508/5) / n-total-LIMK1/2**

| **mag (ΔSA):** | UNS | 37% |
| **mode (Hz):** | UNS | ¼ |
| **time (min):** | 0 | 10 |

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**average:** 1.000000  2.388496  
**SE:** 0.000000  0.510356

| mag (ΔSA): | UNS | UNS | UNS | UNS | UNS | UNS | UNS |
| mode (Hz): | UNS | UNS | UNS | UNS | UNS | UNS | UNS |
| time (min): | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

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**average:** 1.000000  0.724404  0.406591  0.510919  3.977265  0.767055  
**SE:** 0.000000  0.248556  0.117218  0.133686  1.164020  0.368059

| mag (ΔSA): | 37% | 37% | 37% | 37% | 37% |
| mode (Hz): | ¼ | ¼ | ¼ | ¼ | ¼ |
| time (min): | 10 | 10 | 10 | 10 | 10 |

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**average:** 2.168967  0.525676  0.973130  3.359492  1.651726  
**SE:** 0.491124  0.229905  0.504734  0.823728  0.455585

---

309
### Cofilin phosphorylation (Ser3)

#### n-phospho-Cofilin (Ser3) / n-total-Cofilin

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<th>time (min)</th>
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| mag (ΔSA): | UNS | 25% | 25% | 37% |
| mode (Hz): | UNS | ¼ | ¼ | ¼ |
| time (min): | 0 | 10 | 10 | 10 |
| treat: | 10 μM | 10 μM | 10 μM | 10 μM |
| IPA-3 | 0.863087 | 1.3018557 | 0.5568516 | 1.031534 |
| VC | 3.985016 | 1.2721067 | 0.8070128 | 0.370079 |
| IPA-3 | 0.836776 | 0.9228923 | 0.877413 | 0.903271 |
| VC | 0.4888453 | 1.206204 | 0.922721 | 0.293919 |
| IPA-3 | 1.1816687 | 0.293919 | 0.293919 | 0.293919 |
| IPA-3 | 1.0876362 | 1.088368 | 1.088368 | 1.088368 |
| IPA-3 | 0.5433906 | 0.523384 | 0.523384 | 0.523384 |
| IPA-3 | 0.43512 | 0.291465 | 0.291465 | 0.291465 |
| IPA-3 | 0.2073411 | 1.120313 | 1.120313 | 1.120313 |
| IPA-3 | 0.4239101 | 0.454910 | 0.454910 | 0.454910 |
| IPA-3 | 0.9427334 | 1.234901 | 1.234901 | 1.234901 |
| average: | 1.894959 | 0.800682 | 0.861870 | 0.748624 |
| SE: | 1.045056 | 0.117825 | 0.133807 | 0.109559 |

| mag (ΔSA): | 37% | 37% | 37% | 37% | 37% |
| mode (Hz): | ¼ | ¼ | ¼ | ¼ | ¼ |
| time (min): | 10 | 10 | 10 | 10 | 10 |
| treat: | 5 μM | 10 nM | 100 nM | 10 ng/ml | 100 nM |
| PIP3 | 0.404270 | 0.269529 | 2.648825 | 0.8947108 | 0.1004106 |
| Wortmann | 0.063924 | 1.572290 | 0.025754 | 0.5334753 | 0.5011743 |
| Wortmann | 0.776874 | 0.013271 | 1.386261 | 0.221175 | 0.3904999 |
| PDGF | 0.202889 | 1.638795 | 0.717464 | 0.2495625 | 0.2535 |
| Calyculin A | 0.915968 | 0.404748 | 1.764200 | 0.2438639 | 0.2535 |
| average: | 0.472788 | 0.780982 | 1.308501 | 0.428557 | 0.311396 |
| SE: | 0.163338 | 0.280153 | 0.447490 | 0.129897 | 0.086670 |
## Occludin

Occludin (65 kDa) / n-GAPDH

| mag (ΔSA): | UNS | 37% |
| mode (Hz): | UNS | ¼ |
| time (min): | 0 | 10 |
| treat: | | |

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average: 1.000000 0.772471
SE: 0.000000 0.065416

| mag (ΔSA): | UNS | UNS | UNS |
| mode (Hz): | UNS | UNS | UNS |
| time (min): | 0 | 0 | 0 |
| treat: | 5 µM | 10 µM |
| | 60 min | 60 min |

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average: 1.000000 0.972336 1.276272
SE: 0.000000 0.309488 0.375584

| mag (ΔSA): | 37% | 37% | 37% | 37% |
| mode (Hz): | ¼ | ¼ | ¼ | ¼ |
| time (min): | 10 | 10 | 10 | 10 |
| treat: | 5 µM | 10 nM | 10 µM |
| | 60 min | 60 min | 60 min |

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<th>EHT1864</th>
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average: 0.512900 1.300775 1.504047 0.853447
SE: 0.228261 0.582782 0.629042 0.198816
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<td>EHT1864</td>
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### SSH1L phosphorylation (Ser978)

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**n-phospho-SSH1L (Ser978) / n-total-SSH1L**

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**Average:**

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**Average:**

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| mode (Hz):   | UNS ¼ ¼ |
| time (min):  | UNS 10 60 |
| treatment:   | VC VC VC |
|              | 4.8165138 12.958716 18.663991 |
|              | 2.2362385 10.636468 20.355505 |
|              | 3.956422 5.0458716 8.1422018 |
|              | 1.911315 6.8520642 10.980505 |
|              | 0.8792049 9.8623853 16.284404 |
|              | 3.0198777 7.0336391 8.931804 |
|              | 0.4969419 10.282875 25.7263 |
|              | 1.529052 13.073395 17.813456 |
|              | 1.0703364 10.626911 20.030581 |
|              | 0.764526 8.4862385 8.9449541 |
|              | 3.1727829 21.444954 |
|              | 4.7400612 26.185015 |
| average:     | 2.068043 8.564284 16.962920 |
| SE:          | 0.458811 0.930066 1.843269 |</p>
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### PJAR Intensity (Pi) data

| mag (ΔSA): | UNS | 25% | 37% | 37% | 25% |
| mode (Hz): | UNS | ¼   | ¼   | ¼   | ¼   |
| time (min): | UNS | 10  | 10  | 60  | 10  |
| treatment: |     |     |     |     |     |
| VC         |     |     |     |     |     |
| 1.020000   | 1.400000 | 1.300000 | 1.220000 | 1.007777 |
| 1.040000   | 1.380000 | 1.290000 | 1.180000 | 1.058093 |
| 0.990000   | 1.370000 | 1.390000 | 1.350000 | 0.994683 |
| 1.020000   | 1.390000 | 1.320000 | 1.080000 | 0.978354 |
| 1.060000   | 1.280000 | 1.260000 | 1.080000 | 0.962847 |
| 1.010000   | 1.310000 | 1.320000 | 1.110000 | 1.000813 |
| 1.030000   | 1.300000 | 0.970000 | 1.080000 | 0.981709 |
| 1.110000   | 1.360000 | 0.980000 | 1.350000 | 1.031069 |
| 1.070000   | 0.950000 | 1.030000 | 1.240000 | 1.084027 |
| 0.920000   | 1.030000 | 1.020000 | 1.120000 | 0.881390 |
| 0.940000   | 0.940000 | 1.174214 | 1.240000 |     |
| 0.860000   | 0.940000 | 1.184273 | 1.220000 |     |
| 0.940000   | 1.160000 | 1.189377 | 1.160000 |     |
| 0.950000   | 1.140000 | 1.195487 | 1.210000 |     |
| 0.970000   |     |       | 1.230000 |     |
| average:   | 0.995333 | 1.210714 | 1.187382 | 1.198125 | 0.998076 |
| SE:        | 0.016956 | 0.048135 | 0.036915 | 0.022327 | 0.017545 |

| mag (ΔSA): | 25% | 37% | 37% | 37% |
| mode (Hz): | ¼   | ¼   | ¼   | ¼   |
| time (min): | 10  | 10  | 60  | 60  |
| treatment: | 10 μM | 10 μM | 10 μM | 10 μM |
| IPA-3      |       |     |     |     |
| 1.032689   | 1.041500 | 1.104827 | 1.244377 |     |
| 1.006362   | 0.966993 | 1.146501 | 1.082235 |     |
| 1.011654   | 1.033278 | 1.150205 | 1.047817 |     |
| 1.105960   | 0.998065 | 1.041526 | 0.991302 |     |
| 1.030099   | 0.998842 | 1.057435 | 1.042929 |     |
| 1.021669   | 1.059648 | 0.975820 |     |     |
| 0.983100   |     |     |     |     |
| average:   | 1.037353 | 1.010492 | 1.093357 | 1.102856 |
| SE:        | 0.017892 | 0.008206 | 0.019408 | 0.038964 |
Figure G.1: Type I-like rat AEC monolayers stained for phosphorylated-LIM kinase (pLIMK) left unstretched (UNS, top row) or after 10 min of 37% ΔSA ¼ Hz cyclic stretch (bottom row). Active LIMK (pLIMK) is excluded from the perijunction in vehicle control (VC, left column) stretched monolayers while in UNS monolayers and monolayers stretched and treated with 10 µM IPA-3 (right column) for 60 min to inhibit PAK stained homogeneously. Figure represents the single channel version of triple stained images presented in Chapter 4. Bar = 10 µm.
Figure G.2: Type I like rat AEC monolayers stained for F-actin with phalloidin left unstretched (UNS, top row) or after 10 min of 37% ΔSA ¼ Hz cyclic stretch (bottom row). Vehicle control monolayers (VC, left column) stretched at 37% ΔSA produce actin stress fibers on the cell periphery (PJAR) by 10 min while monolayers stretched at the same magnitude and duration treated with 10 µM IPA-3 (right column) for 60 min to inhibit PAK showed no PJAR formation. Figure represents the single channel version of triple stained images presented in Chapter 4. Bar = 10 µm.
Figure G.3: Type I like rat AEC monolayers stained for phosphorylated-Cofilin (pCof) left unstretched (UNS, top row) or after 10 min of 37% ΔSA ¼ Hz cyclic stretch (bottom row). Inactive cofilin (pCof) is excluded from the perijunction in vehicle control (VC, left column) stretched monolayers while in UNS monolayers and monolayers stretched and treated with 10 µM IPA-3 (right column) for 60 min to inhibit PAK stained homogeneously. Figure represents the single channel version of triple stained images presented in Chapter 4. Bar = 10 µm.
APPENDIX H: PERMEABLE PDMS MEMBRANE DESIGN, PROTOCOLS, AND PRELIMINARY DATA

Quantification of alveolar epithelial cell (AEC) paracellular permeability is a vital physiologic functional readout in the exploration of deformation-induced alveolar injury. However, the methods used to quantify paracellular permeability are currently limited. Measurement of fluorescent tracer BODIPY-ouabain binding to the basolateral side of stretched AECs allows for a critical estimate of monolayer permeability, however this method is limited to the binding properties of ouabain. There are numerous cell culture substrates that allow measurement of transepithelial resistance as well as the paracellular transport of various tracers by providing nano- and micron-sized pores through the substrate. However, these substrates lack the ability to deform. While the Silastic membrane provides an excellent substrate on which to test deformation in AEC monolayers, it is impermeable (Figure H.3). Thus, our goal is to design a membrane that possesses qualities of both Silastic membrane (highly deformable, biocompatible, and inert) and permeable substrates (permeable and biocompatible). A stretchable-permeable membrane would allow deeper elucidation of the physiology of the tight junction in stretched AECs by allowing quantification of paracellular permeability with a variety of tracer sizes and charges.

This appendix contains preliminary protocols and the results of an attempt to create a stretchable-permeable membrane by replica molding (5) polydimethylsiloxane (PDMS) onto a carbon nanotube (CNT) array, resulting in a Silastic membrane with nano-sized pores (Figure H.1). The CNT array was designed with nanotubes that had spacing and diameters similar to the spacing and diameters of pores in a permeable
Millicell cell culture insert. By using a 0.4 µm pore polyethylene terephthalate (PET) Millicell cell culture insert (Millipore, Billerica, MA) as a model (Figures H.4 and H.5), we calculated a target 18% total hole percentage (total pore area). With a nanotube array of 450 - 550 nm diameter nanotubes, we calculated that we would require a CNT array with 12 - 18 % total post percentage (total post area) and a 40 µm nanotube height to mimic the pore properties of the Millicell cell culture insert. NanoLab (Waltham, MA) was able to produce a square nanotube array 10×10 mm in area with 150-200 nm diameter, 40 µm tall nanotubes at a site density of $10^9$ tubes / cm$^2$ on a 300 nm chromium-coated, 25×25 mm quartz substrate (Figures H.6 and H.7).

Figure H.1: Two hypothesized design methods used to produce PDMS (Polydimethylsiloxane) porous membranes with nanometer scale pores.
PDMS MEMBRANE SPIN COATING PROTOCOL

We used spin coating in order to create a precise PDMS membrane thickness that was less than the height of the CNT array tubes in order to allow full CNT penetration (Figure H.2).

![Silastic membrane thickness graph](image)

**Figure H.2:** Cured Silastic membrane thickness vs. spin coating rotations per minute (RPM) for a spin time of 30 s.

**PREPARING SILASTIC**

1. Mix SILASTIC in beaker at least 5X volume of polymers
   - +70 g 186 sylgard (very sticky, cure 30/15 min at 100/150°C, high tear strength)
   - +40 g 184 sylgard (slight sticky, cure 45/20/10 min at 100/125/150°C)
   - +7 g 186-xlinker (very sticky, cure agent)
   - +4 g 184-xlinker (not sticky, cure agent)
   - cures 48 hrs at RT

2. 5 min stir very well

3. 30 min place in vacuum chamber (degass silastic)
   - no heat needed
   - make sure oil between ADD and FULL
   - seal top right valve
   - hold down oil pipette and flip switch in back ON
     - will notice slight smoke from filter
- make sure pressure goes to 30 in-Hg vacuum
- when cycle (30 min) finished, turn OFF
- open top right valve SLOWLY just a little

3.1: While silastic in vacuum, prepare substrate
- scrape off old silastic (previously used glass)
- scrub w/ acetone
- HOOD: put dab (quarter size) of 1:10 silane/chloroform to coat glass (makes glass hydrophobic so membrane peels easily)
  - wipe onto all glass surfaces or let drip into nanotube areas
  - allow time to evaporate off surface

SPIN COATING
location #1: 305 Moore bldg., Wolf Nanofab facility – large SPINNER in hood on left; Brewer Science CEE100 Spinner (note: CLEAN ROOM; all skin/hair covered, bring only clean objects - polymer is ok)

location #2: spinner in 214/212 LRSM in hood, Brewer Science CB100

-President
- open top and remove large metallic O-ring
- place 2 Al foil sheets so they cover basin (X shape, fold in half to make hole)
  - make hole in middle for spinner and allow for drainage (DO NOT LET POLYMER SPILL INTO SPINNER AXLE)
- replace O-ring
- place sample chuck onto axle, make sure chuck lines up with axle notch
  - make sure vacuum lines will be covered by sample
  - clean sample with N gas and place on chuck

-Nore: for LRSM spinner, turn ON vac, switch on pump outside hood on floor left

- Program
- turn on unit
- press program and select desired program number (usually use 1), hit enter
- unit will display current program and spin division (for multiple phases)
  - VEL is velocity in rpm
  - RMP is ramp up acceleration speed in rpm/s
  - this unit accounts for ramp up time and does NOT include it in the programmed time
  - TIME in seconds

- LRSM spinner has 5s of 100 rpm/s ramp to 500 rpm in beginning
- LRSM spinner: DO NOT EXCEED 4,000 rpm
- Run program
  - clean top and bottom of sample with air (or wipe only bottom with kim wipe)
  - place dab of polymer on middle of sample (see STANDARD DROP VOLUME)

- clean top of vac chuck (if chuck is not providing enough vac, just use no chuck)

- select program to use, hit enter
- press start spin
  - unit will spin slowly so you can verify sample is lined up
  - adjust sample and press 0 for another slow spin
  - press start spin to begin spin protocol

- press STOP/RESET if you want to quit a spin during
- press STOP to stop beeping at end of run

- press reset to return to main menu (can load another program)

-LRSM spinner: leave ON unless 5-6 PM, then can shut off
-LRSM spinner: shut OFF vac

- Note: Iulian recommends slow initial spin to spread out sample
- Note: (Nanofab) for 2nd largest SPINNER in middle (Laurell technologies WS-650S-6NPP-Lite)
  - MAKE SURE N (nitrogen) IS FLOWING
- Note: (Nanofab) for 3rd SPINNER on right (Headway spinner)
  - must use face shield
  - spin parameters on outside of hood (underneath sash)
  - foot pedal

CURING
- set slide or cast with spun polymer onto hot plate
  - time = curing time at desired temperature (based on material)

-LRSM spinner: bake at 100°C on desktop, or higher 150°C in hood (do not bake above 100°C unless under hood, may release vapor, see MSDS)
  - set hood heat plate to 150°C at least 30 min in advance (take a while to heat up)
  - when finished, set back to 95°C

PEELING AND WELL MAKING
- remove side silastic (small amount of polymer sticks to 1 mm thick side substrate wall) so it does not adhere to main top surface membrane
-unstick all 4 sides of top surface by rubbing thumb across 1mm thick side in the up direction
- now only center area of membrane is adhered, but all is still laying flat
- take a long forceps, with smooth grippers, lift one whole side of a membrane and place 1 arm of forceps along the edge – then let membrane fall onto forcept edge
- close forcepts and pull ENTIRE SIDE upwards (do not let corner slip out – could easily damage membrane)

MEMBRANE THICKNESS MEASUREMENT
Device: Profiliometer (Tencor Alpha Step 200) in Nanofab
– in microfab clean room (walk into microfab, go into clean room ready room get suit on, go into clean room, turn right through set of doors, alpha-step is on left bench)
(aka ellipsometer or interferometer)

- MOST IMPORTANT – do not translate x or y while needle is on sample (will break needle) unless you know it’s a very flat surface

- NOTE: cut membrane sample with razor blade so that one side is glass and the other is membrane
- do this near the middle of the membrane where spin is most level and accurate
- profilometer is able to measure a distance relative to some surface (glass) and works best at shorter distances

- switch ON (sometimes you have to hard reset by unplugging if start screen does not come on)
- place sample, have x and y knobs of left and rotation mount
- eyeball where tip will come down on sample (get it to a level surface, next to profile you want to measure)

- TABLE up to get sample up to needle tip (video will come on, or just hit VIDEO button)
- to go down, hit TABLE down and video will go off

- establish scan parameters by hitting ENT
- will display table with ‘length of scan’ on left and ‘samples/um’ on right
- length of scan from 10,000 – 80 um, however, use less than 400 um length for accuracy

- to change length and sample density hit RANGE
- to change scan direction hit DIR and the direction you want to scan (will display in scan properties)

- scan time in (s) is automatically calculated and displayed

- make sure MODE (in scan properties) is in um
  - if set on kilo-angstroms, hit CUR to change

- hit ENT to exit scan parameters (press VID)

- hit START/STOP to begin (or end before scan is finished)

- screen will display scan profile
  - must level this measurement to flat glass surface

- hit LEVEL
  - L and R cursors will be at top of profile plot (two little triangles)
  - hit CUR to select either L or R or both
  - hit left or right arrow to move L or R or both
  - select profile plot of glass ONLY (this is a known flat surface)
  - hit ENT when finished leveling

- plot now displays proper leveled profile of sample

- screen displays VERT measurement in microns, difference between L and R current cursors
  - hit CUR to select and move these cursors over the profile plot

- to zoom
  - select area to zoom using L and R cursors
  - hit PLOT

- when measurement finished, TABLE down and remove sample

- measure another sample or switch OFF

**CUTTING GLASS**
- use glass knife, score glass using flat edge (another piece of glass)
  - run score across a few times
  - place one tweezer head below score groove and press down on far edges to break

**STANDARD DROP VOLUME CALCULATION**
- standardize drop volume to spin 1.5” by 1.5” glass plate
  - minimum amount of polymer calculation
  - 1.5” = 38.1 mm
-target membrane thickness = 40 um = 0.040 mm
-volume: 38.1 mm x 38.1 mm x 0.040 mm = 58.0644 mm$^3$
  = 0.0580644 ml = 58.06 ul

-for circle 2.264” diameter = 57.5 mm
-target membrane thickness = 40 um = 0.040 mm
-volume: PI * (57.5/2)$^2$ *0.040 = 103.87 mm$^3$
  = 0.103 ml = 103.87 ul

NOTES
Refractive indices:
  Sylgard 184, n = 1.43 (9)

-Before entering Nanofab
  -check meters on wall outside door for no dangerous particulate matter
  -card should work from 9-5 until I take class (then you must enter with a buddy)
  -sign in for charging time (will also compare with card entrance)
  -at station – place clean napkin with name, chemical, date, and time
  -do not leave hot place on when away (except bathroom)

-Emergency
  -call Ron or Iulina if problem or emergency (8-5PM)
  -must be with buddy after 5PM
  -safety manual on web
  -spill – clean up something
    -use soaker snakes for large spills, and place in bag and leave in hood –
      tell Ron or Iulian
    -use small wipes for small spills

  -DO NOT clean up nitro-flourine Waste
    -if get onto skin or inhale vapors – use kit on wall near shower
      with calcium gel (apply with glove, it soaks up NF) and call
      emergency

-waste bottles
  -found under hoods – divided into bases, acids, and general
  -3 times wash bottles are reused plastic bottles that have been washed with city
    water three times – wash, dry, and place 3x wash label
  -then apply a waste disposal decal of the waste type you want on top of the
    chemical label
  -or type up your own using the sticker maker (chemical waste name and date
    made)
  -place into cart when ¾ full of waste

(protocols by Brian C. DiPaolo, bdipaloo@seas.upenn.edu; based on assistance from
Katie von Reyn of the Injury Lab, Yongan Xu and Dinesh Chandra of the Shu Yang
group in Materials Science and Engineering, and Iulian Codreanu of the Penn NanoFab)
Figure H.3: Cell seeding surface detail of a bare flexible Silastic membrane (Specialty Manufacturing, Saginaw, MI) imaged on a transmission electron microscope (TEM, DualBeam FEI Strata DB 235 FIB, Hillsboro, OR). Samples were evaporated gold coated (sputtered) prior to imaging.
Figure H.4: Cell seeding surface detail of a permeable Millicell Cell Culture Insert (0.4 µm pore size; Millipore, Temecula, CA) imaged on a transmission electron microscope (TEM, DualBeam FEI Strata DB 235 FIB, Hillsboro, OR). Samples were evaporated gold coated (sputtered) prior to imaging.
Figure H.5: Cell seeding surface detail of a permeable Millicell Cell Culture Insert (0.4 µm pore size; Millipore, Temecula, CA) imaged on a transmission electron microscope (TEM, DualBeam FEI Strata DB 235 FIB, Hillsboro, OR). Samples were evaporated gold coated (sputtered) prior to imaging.
Figure H.6: Carbon nanotube array imaged on a transmission electron microscope (TEM, DualBeam FEI Strata DB 235 FIB, Hillsboro, OR). Samples were conductive and did not require gold coating.
Figure H.7: Carbon nanotube array detail imaged on a transmission electron microscope (TEM, DualBeam FEI Strata DB 235 FIB, Hillsboro, OR). Samples were conductive and did not require gold coating.
APPENDIX I: PRELIMINARY DATA & METHODS

**Figure I.1:** Effect of biaxial stretch on F-actin in human pulmonary artery endothelial cells (HPAECs). HPAEC monolayers with phalloidin labeled F-actin left unstretched (UNS) or after 30 min of 12%, 25%, or 37% ∆SA ¼ Hz biaxial stretch. UNS monolayers exhibited thin F-actin filaments throughout the cells. Monolayers stretched at 12% ∆SA formed heavy F-actin filaments concentrated near the cell perijunction. Monolayers stretched at 25% ∆SA exhibited homogeneous F-actin staining with slight perijunctional staining. Finally, monolayers stretched at 37% ∆SA exhibit thin parallel filaments. Previously, phosphorylated (Thr18/Ser19) myosin light chain was shown to be increased in HPAEC monolayers stretched to 12% ∆SA and decreased in monolayers stretched to 37% ∆SA (Chapter 3).
Figure I.2: Effect of biaxial stretch on F-actin arrangement in type I like rat AEC monolayers before and after 40 and 60 minutes of stretch (time on x-axis). Monolayers left unstretched (UNS) show no perijunctional F-actin ring (PJAR). Monolayers stretched for 40 or 60 min to 25% change in surface area (ΔSA) sustained tonic (0 Hz) and cyclic (¼ Hz) or 37% ΔSA ¼ Hz produce PJAR. Images comparable to those presented in Chapter 2. Bar = 10 µm.
Figure I.3: Live (non-fixed) primary rat alveolar epithelial cells (AECs) stained for F-actin using phalloidin in unstretched (UNS) monolayers (left) and monolayers stretched for 10 min to 25% ΔSA ¼ Hz (right column). Monolayers stretched then treated with phalloidin (top) showed perijunctional F-actin ring formation (PJAR). Monolayers treated with phalloidin before stretch (bottom) showed inhibition of PJAR formation. Data identical to monolayers fixed after stretch (Chapter 2). Phalloidin delivered with 0.05% Triton X-100 for 10 min as vehicle. Bar = 10 µm.
Figure I.4: Primary rat alveolar epithelial cells (AECs) immunostained for tight junction proteins claudin-5, and -7 in unstretched (UNS) monolayers and monolayers stretched for 60 min to 25% and 37% ΔSA ¼ Hz. Bar = 10 μm.
REFERENCES


