THE RHOA GUANINE NUCLEOTIDE EXCHANGE FACTOR, LARG, MEDIATES ICAM-1-DEPENDENT MECHANOTRANSDUCTION IN ENDOTHELIAL CELLS TO STIMULATE TRANSENDOTHELIAL MIGRATION

Elizabeth Chase Lessey-Morillon

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell and Molecular Physiology (Cell and Developmental Biology).

Chapel Hill
2014

Approved by:

James Bear

Keith Burridge

Claire Doerschuk

Ben Major

Eleni Tzima

ABSTRACT

Elizabeth Chase Lessey-Morillon: The RhoA guanine nucleotide exchange factor, LARG, mediates ICAM-1-dependent mechanotransduction in endothelial cells to stimulate transendothelial migration
(Under the direction of Keith Burridge)

RhoA-mediated cytoskeletal rearrangements in endothelial cells (ECs) play an active role in leukocyte transendothelial cell migration (TEM), a normal physiological process in which leukocytes cross the endothelium to enter the underlying tissue. While much has been learned about RhoA signaling pathways downstream from ICAM-1 in ECs, little is known about the consequences of the tractional forces that leukocytes generate on ECs as they migrate over the surface before TEM. We have found that after applying mechanical forces to ICAM-1 clusters, there is an increase in cellular stiffening and enhanced RhoA signaling compared to ICAM-1 clustering alone. We have identified that the Rho GEF LARG/ARHGEF12 acts downstream of clustered ICAM-1 to increase RhoA activity and that this pathway is further enhanced by mechanical force on ICAM-1. Depletion of LARG decreases leukocyte crawling and inhibits TEM. This is the first report of endothelial LARG regulating leukocyte behavior and EC stiffening in response to tractional forces generated by leukocytes.

DEDICATION

This thesis is dedicated to my parents, Bruce and Barbara Lessey, my husband Maurice Morillon, and to all who have supported me through my life and graduate school.

ACKNOWLEDGEMENTS

First, I need to thank Keith Burridge for allowing me to join his lab and in doing so, providing me with a truly wonderful graduate school experience. The current and former Burridge Lab members make the Burridge Lab an ideal place for research to occur. A huge thank you goes out to my two postdoc mentors Tom Sampson and Christophe Guilley. During my graduate career, both have supported me greatly. Beth Benson has also been a great mentor with career advice as well as with presentations and papers. I would like to thank Erika Wittchen for her guidance both in and outside of the lab. Lisa Sharek is an amazing lab manager; the Burridge Lab could not function without her. Without her support, none of this would be possible. My time in the Burridge Lab would have been very different and much harder without my fellow graduate students, Caitlin Tolbert and Amir Aghajanian.

I could not have done this project without the aid of Richard Superfine's Laboratory in the Physics Department. I am grateful to Dr. Superfine for permitting me to use his laboratory. Luke Osborne and Tim O'Brien assisted me greatly with the physics side of my project. I've received fantastic support from the IVB training program and the former Cell and Developmental Department and current Department of Cell and Molecular Physiology.

Finally, I'd like to thank my thesis committee, James Bear, Claire Doerschuk, Ben Major, Eleni Tzima. Their guidance and mentorship has been outstanding. I am lucky to have had such an involved and supportive thesis committee during my graduate school training

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

DH	dbl homology
EC	endothelial cell
ECM	extracellular matrix
GAP	guanine nucleotide exchange factors
GEF	guanine nucleotide exchange factor
ICAM-1	inter-cellular adhesion molecule-1
HUVEC	human umbilical cord endothelial cell
HMVEC	human microvascular endothelial cells
LARG	leukemia-associated Rho guanine nucleotide exchange factor
LFA-1	leukocyte function-associated molecule-1
Mac-1	macrophage-1 antigen
mDia	mammalian homolog of Drosophila diaphanous
MHC	major histocompatibility complex
MLC	myosin light chain
MSC	mesenchymal stem cells
PH	pleckstrin homology
pMLC	phosphorylated myosin light chain
Rho	Ras homology proteins
ROCK	Rho kinase
SFK	src family kinase

TEM	transendothelial cell migration
VE-cadherin	vascular endothelial-cadherin
VLA-4	very late antigen-4

Chapter 1: BACKGROUND AND SIGNIFICANCE

Leukocyte Transendothelial cell migration

Endothelial cells (ECs) make up the lining of blood vessels and provide a protective barrier to the underlying tissue. Dynamic regulation of the ECs and cell-cell junctions is required to allow leukocyte diapedesis. During inflammation or infection, chemoattractant signaling cues the leukocytes to exit the blood stream (Figure 1). Pro-inflammatory signaling increases expression of adhesion receptors, including E-selectin, ICAM-1, and VCAM-1, on the luminal surface of the EC (1-4). These adhesion molecules bind to receptors on the leukocyte. For example, E-selectin binds to sialylated glycoproteins on leukocytes (5), whereas ICAM-1 binds to β_2 integrins such as leukocyte function-associated molecule-1 (LFA-1)($\alpha_L\beta_2$, CD11a/CD18) (6, 7), and macrophage-1 antigen (Mac-1) ($\alpha_M\beta_2$CD11b/CD18) (8, 9), and VCAM-1 binds to β_1 integrins such as very late antigen-4 (VLA-4) ($\alpha_4\beta_1$, CD49a/CD29) (10).

Initially, the leukocyte loosely binds to the surface of the endothelium (Figure 1A). The endothelial adhesion receptors that bind to the leukocyte during this initial stage are selectins, chiefly E-selectin. The leukocyte rolls over the endothelium due to the force of the blood flow and the weak adhesion between the leukocyte and the endothelial surface. The loss of E-selectin reduces leukocyte adhesion following

inflammation (11). While E-selectin binds the leukocyte to slow it down from the circulation, E-selectin also links to the endothelial cytoskeleton to alter EC signaling (12).

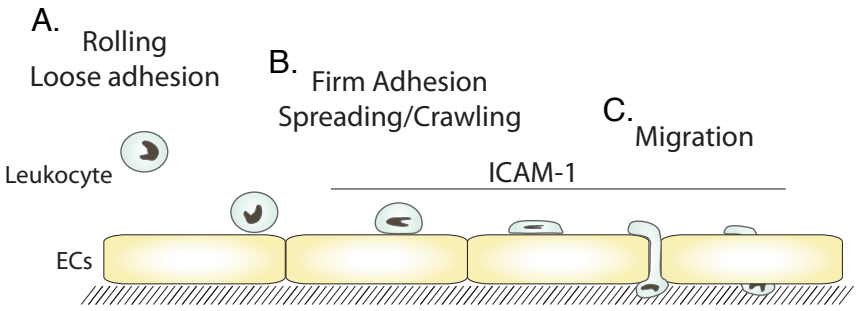


Figure 1: Steps in Leukocyte TEM
(A) The leukocyte loosely adheres to the endothelium. (B) Then, the leukocyte spreads and crawls. (C) Lastly, the leukocyte crosses the endothelium at an EC junction, paracellular migration, or though an EC, transcellular migration.

Next, the leukocyte binds to other adhesion receptors, including ICAM-1, then spreads and crawls on the endothelial surface (Figure 1B). ICAM-1 engagement initiates many signaling pathways within the EC to assist in leukocyte TEM (discussed later in this chapter) (13-21). The crawling leukocyte extends protrusions to probe the surface of the endothelium (20). Transmigration can occur by two routes, transcellular and paracellular diapedesis (Figure 1C) (22-24). Paracellular migration is the most well studied route of diapedesis and occurs by the leukocyte migrating through the junction between two ECs. For a leukocyte to be able to cross at an endothelial cell-cell junctions the junctional proteins must disengage (25-27). Leukocyte bindings to adhesion receptors on the EC surface induces the junctions to weaken (28). The alternative route is transcellular diapedesis, where a leukocyte migrates through a single EC. Like in paracellular migration, leukocyte engagement of EC receptors initiates the process. A combination of actin protrusions and vesicle

trafficking create pore in the ECs allowing the leukocyte to pass through (18, 29, 30). Both routes depend on endothelial changes in the cytoskeleton, which are controlled by Rho GTPases (14, 15, 30, 31),

The Rho Family of Small GTPase-binding proteins

Rho GTPases are a subfamily of the Ras superfamily of GTPases, which act as molecular switches. By cycling through an on/off cycle, they are able to regulate the cytoskeleton and cell contractility. There are 22 members of the Rho family in mammals (32). The most well studied family members are RhoA, Rac1 and CDC42. RhoA promotes stress fibers and actomyosin contractility (33, 34). Rac1 stimulates actin polymerization and induces the branched actin network that makes up the lamellipodia at the leading edge of a cell (33). Cdc42 also stimulates actin polymerization and regulates protrusions known as filopodia (35, 36). In addition, these GTPases regulate many other activities (37).

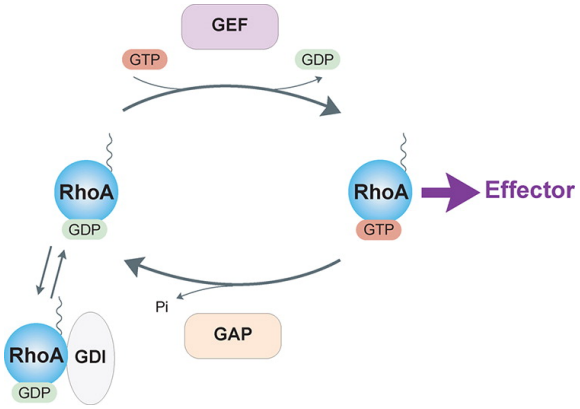


Figure 2: RhoA cycle
Like most G proteins, RhoA cycles between an inactive GDP-bound form and an active GTP-bound form. Activation is mediated by GEFS that catalyze exchange of GDP for GTP. GAPs inactivate RhoA by stimulating intrinsic GTPase activity. GDI sequesters inactive GDP-bound RhoA in the cytoplasm.

Rho GTPases are activated by guanine nucleotide exchange factors (GEFs), which induce conformational changes in Rho GTPases that promote the exchange of GDP for GTP. With GTP bound, Rho GTPases interact with downstream effectors to initiate signaling cascades, which then lead to cytoskeletal changes (Figure 2).

Rho GTPases are inactivated by GTPase-activating proteins (GAPs) that enhance the intrinsic GTPase activity by stimulating hydrolysis of GTP to GDP. It is striking that there are more GEFs or GAPs than Rho family members. This most likely reflects that many signaling pathways can converge on individual Rho proteins and that different GEFs and GAPs function within these different pathways.

Some redundancy between GEFs appears to exist and is indicated by the modest or negligible phenotype induced in mice where a single GEF has been knocked out. PDZ-RhoGEF knockout mice or LARG knockout mice have no obvious phenotype, but the double knockout is lethal suggesting compensation between the GEFs in the single knockout mice (38). Also, LARG deficient mice have a lower than expected birth rate which might be a result of GEF compensation in the surviving mice (38). A close examination of Vav deficient mice show that compensation between GEFs, even closely related isoforms, does not always occur. Only Vav1, not Vav2 or Vav3, regulates thymic selection based the phenotype of the single isoform knockout animals (39-42). When Vav1 and Vav3 are knocked out, there is further impairment of thymic selection, suggesting that Vav3 is compensating for Vav1 (39, 40). However, Vav2 is unable to do so as the double Vav1 and Vav2 knockout mice have the same thymic selection defect as the single Vav1 knockout mice (43). While single GEF knockout animals frequently lack a strong phenotype, the Trio knockout mice are not viable from a neuronal and muscle defect (44).

The hallmark of most RhoGEFs is the dbl homology (DH) region. The DH domain, located at the C-terminal, is the region responsible for the GEF activity. The

DH domain is able to bind inactive Rho and induce dissociation of GDP, temporally leaving Rho in a nucleotide-free state. However, due the high ratio of GTP to GDP within the cell, GTP quickly binds, thus transferring Rho into its active state. The neighboring pleckstrin homology (PH) domain can associate with phosphoinositides causing plasma membrane localization as well as assisting with GTPase binding. The DH-PH domain is also responsible for the specificity of GEFs for Rho GTPases. A single DH-PH domain can exchange nucleotide for a specific GTPases or can act on multiple GTPases. For example, Leukemia-associated Rho guanine nucleotide exchange factor (LARG) can only activate RhoA (45) while Vav shows less specificity and can activate RhoA, Rac, Cdc42 and RhoG (46). Some GEFs like, Trio, have multiple DH-PH domains each with different Rho family specificities (47).

GAP proteins are not as well characterized as GEFs but are just as important in understanding Rho GTPase function. The first identified GAP was Rho GAP (48). Subsequent family members have been identified by the presence of the GAP domain. The GAP domain binds to active GTP-bound Rho proteins and promotes hydrolysis of GTP to GDP, thus cycling Rho GTPases into their inactive form. Like GEFs, GAPs have specificities for Rho family members. This allows for tight regulation of the Rho GTPase family member.

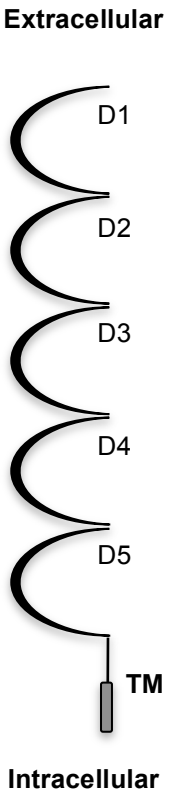
Rho GTPases control the cytoskeleton by signaling to downstream effector proteins. In their active state, Rho GTPases bind to effectors. The RhoA effector, Rho kinase (ROCK) regulates actomyosin contractility. ROCK signaling leads to phosphorylation of the myosin light chain (MLC) either directly or indirectly by

inactivating MLC phosphatase, which then causes increased actomyosin contractility (49-52). In some cells such as fibroblasts, the activation of myosin stimulates the formation of stress fibers (53). ROCK can also signal through LIM kinase to promote actin stress fiber formation. ROCK can activate LIM kinase, which then phosphorylates cofilin (54). Cofilin functions as an actin-severing protein and is inactivated after phosphorylation. Formins, including mammalian homolog of Drosophila diaphanous (mDia), are another class of Rho effectors that function to promote stress fiber formation by nucleating actin polymerization to create F-actin (55).

ICAM-1 Signaling

One of the most-well studied adhesion receptors in Rho GTPases signaling during leukocyte TEM is ICAM-1. ICAM-1 has 5 extracellular IgG like domains and a small 22 aa cytoplasmic tail (Figure 3). β2 integrins, such as LFA-1 or MAC-1, bind to specific IgG domains to induce ICAM-1 clustering. ICAM-1 is then translocated into the lipid insoluble regions (56). This clustering is then able to bring together the intracellular domains initiating downstream signaling cascades (57, 58).

Figure 3: A diagram of ICAM-1



One central consequence of ICAM-1 clustering is rearrangement of the EC cytoskeleton, which assists in leukocyte TEM. This is predominantly due to increases in RhoA signaling and changes in the actin cytoskeleton (14-16, 59, 60). After ICAM-1 clustering, F-actin and actin binding proteins, including ezrin, moesin, radixin, and α-actinin, associate with the ICAM-1 complex to induce cytoskeletal changes (21, 31, 61-64). FAK, paxillin, p130Cas, ezrin, and cortactin are phosphorylated in response to ICAM-1 crosslinking (64, 65). Src phosphorylation also occurs and is responsible for cortactin phosphorylation (64). Interestingly, Src also becomes phosphorylated after E-selectin clustering (64). Leukocyte-induced ICAM-1 clustering activates RhoA to assist in migration across the EC monolayer (14-16, 59, 60). Inhibiting RhoA signaling in ECs greatly attenuates leukocyte adhesion, spreading, and migration (14, 15, 59, 60). RhoA signaling leads to activation of the effector Rho-associated protein kinase (ROCK) (50). Rock dependent enhanced EC actomyosin contractility results in weakened cell-cell junctions allowing the formation of gaps through which leukocytes can migrate across the EC monolayer (49, 50).

Paracellular migration

Paracellular diapedesis is a well-studied route for leukocyte TEM and requires ECs to alter their cell-cell junctions to allow leukocytes to cross. Normally, adherens and tight junctions formed between neighboring ECs create a barrier to protect the

integrity of the blood vessel. Pro-inflammatory signaling can disrupt cell-cell junctions to increase permeability (23). Also, leukocyte binding to the EC induces a signaling cascade to weaken the cell-cell junctions to assist in diapedesis.

Adherens junctions are central regulators of leukocyte TEM. One of the more well studied cell adhesion molecules found in adherens junctions is vascular endothelial-cadherin (VE-cadherin). VE-cadherin regulates the strength of EC junctions and is critical in leukocyte TEM (28). Significantly, VE-cadherin is a downstream target of RhoA-dependent contractility. The extracellular domain of VE-cadherin creates a homophilic interaction with a VE-cadherin on a neighboring cell, and this interaction is calcium dependent (66). Blocking VE-cadherin increases leukocyte TEM (25, 26). Conversely, Mice expressing a mutant VE-cadherin-α-catenin fusion protein, which prevents the disassociation of VE-cadherin at cell-cell junctions, leads to decreased permeability and leukocyte TEM (27).

The dissociation and loss of VE-cadherin from adherens junctions during leukocyte TEM triggers cell-cell junctions to weaken allowing the leukocyte to cross, then VE-cadherin returns shortly after to reseal the junction (67, 68). Phosphorylation of VE-cadherin assists in the disruption of cell junctions (28, 69-71). Specifically, ICAM-1 crosslinking induces downstream signaling to phosphorylation of VE-cadherin by activating Src and pyk2 (28).

Endothelial junctions also contain tight junction proteins that regulate leukocyte TEM as well. Endothelial tight junctions typically differ from tight junctions found in other cell types like epithelial cells. In epithelial cells tight junctions are

restricted apically, whereas in ECs tight junctions extend throughout the junction interface and intermingle with the adherens junction proteins (72). The role of tight junction proteins in leukocyte TEM has not been as extensively studied as the role of adherens junction proteins. However, phosphorylation of the tight junctional proteins occludin and claudin-5 are reported during leukocyte TEM, and depend on RhoA and ROCK signaling (73). Highly regulated control of junctional proteins is required for efficient leukocyte TEM.

Transcellular migration

Transcellular diapedesis is a less anticipated route for a leukocyte to cross the endothelium. Instead of crossing that the endothelial junctions, the leukocyte passes through a single EC. Transcellular diapedesis initially was observed in electron micrographs of leukocyte TEM occurring in *in vivo* models of inflammation (74, 75). This route is less well characterized than paracellular TEM but does involve some of the same adhesion receptors, like ICAM-1 (17, 18). After leukocyte adhesion, caveolin-1 co-localizes with the transcellular pore (18). ICAM-1 rich microvilli-like structures extend from the EC around the leukocyte before it ultimately transmigrates (17, 18, 20, 28, 30).

Another Rho family member involved in leukocyte TEM is RhoG. van Buul *et al.* found the actin rich cups forming by the EC around the transmigrating leukocyte require RhoG activity (30). The RhoG GEF, SGEF, appears to be responsible for this process (30). It is worth noting the possibility of RhoA being involved in this process, as inhibiting RhoA expression prevents RhoG activation after ICAM-1 clustering (30).

It is unclear why transcellular or paracellular diapedesis becomes the chosen route for a leukocyte. However, it does appear that the EC type might play a role. Whereas in HUVECs there is very a small number of transcellular diapedesis events, less than 10%, it is as much as 30% in microvascular ECs (20). The route picked might also be dependent on the type of stimulus to induce inflammation. Under pro-inflammatory conditions, like treatment with VEGF, histamine, LPS or IL-1β, paracellular diapedesis occurs more frequently (27). While paracellular diapedesis frequently occurs at a higher rate, this does not rule out an important role for transcellular diapedesis.

Summary

Regardless of the transcellular or paracellular path the leukocyte takes, the endothelium is an active player in the process in part due to the role of Rho Family GTPases. The endothelial cytoskeleton is responding to endothelial receptors engaging with the leukocyte to assist in diapedsis. TEM illustrates the important role for Rho family GTPases in this example of cell-cell interactions, but TEM is also critical in the normal inflammatory response and in inflammatory diseases. Understanding the role of Rho GTPases in TEM may therefore provide insight into ways of regulating TEM and suggest potential therapies for controlling inflammation in disease situations.

Chapter 2: FROM MECHANICAL FORCE TO RHOA ACTIVATION[1]

Throughout their lives all cells constantly experience and respond to various mechanical forces. These frequently originate externally but can also arise internally as a result of the contractile actin cytoskeleton. Mechanical forces trigger multiple signaling pathways. Several converge and result in the activation of the GTPase RhoA. In this review we focus on the pathways by which mechanical force leads to RhoA regulation, especially when force is transmitted via cell adhesion molecules that mediate either cell-matrix or cell-cell interactions. We discuss both the upstream signaling events that lead to activation of RhoA, as well as the downstream consequences of this pathway. These include not only cytoskeletal reorganization and, in a positive feedback loop, increased myosin-generated contraction, but also profound effects on gene expression and differentiation.

---

[1] This chapter appeared as a review article in Biochemistry. Reproduced with permission from Lessey, E. C., Guilluy, C., and Burridge, K. (2012) From Mechanical Force to RhoA Activation, Biochemistry 51, 7420-7432. Copyright 2013 American Chemical Society.

Introduction

All cells are exposed to mechanical forces and to a greater or lesser degree responds to these forces. In the vertebrate body, cells experience different types of force according to their tissue location. For example, ECs lining blood vessels, as well as epithelial cells lining certain ducts or cavities, experience mechanical force from the passage of fluid over the cell surface. Cells in the skeletal system (bone and cartilage) but also many other cells are exposed to compression. Throughout most tissues, cells experience varying degrees of tension, which can arise from external forces or from within the cell as a result of actomyosin contractility. It is important to note, however, that the very high tensional forces experienced by some tissues, such as tendons and ligaments, are usually transmitted by extracellular matrix (ECM) components such as collagen fibers and the cells within these tissues are shielded from the tension by the ECM (76). Some forces on cells may be cyclical as experienced by cells in contact with the blood circulation, or as a result of rhythmic activities such as breathing or walking, whereas other cells experience sustained force for varying periods of time.

Experiments exploring how cells respond to different types of mechanical force go back a long way. For example, in early experiments stretching cells was shown to stimulate their proliferation (77). Stretching of myotube cultures induced responses equivalent to muscle hypertrophy (78). The growth cones of elongating neurites were found to exert mechanical force (79) and to respond to externally applied forces (80). Similarly, fibroblasts and other cells were observed to generate

tractional forces on the underlying substratum (81) and to be able to harness these forces to orient collagen fibers (82). Application of mechanical tension to migrating cells in culture using a microneedle inhibited extension perpendicular to the axis of tension but allowed or even promoted extension that was parallel with the force (83).

Although research in the field of mechanotransduction has been active for many years, much of it was focused on systems, tissues and cells that are very overtly affected by mechanical stimuli, such as vascular ECs and vascular smooth muscle exposed to flow and/ or stretch, or osteoblasts that experience compressive forces. However, during the past decade there has been an explosion of interest in the more universal responses of cells to mechanical forces and progress is occurring rapidly. Whether the forces are applied exogenously on cells or are generated endogenously, they are usually transmitted to the ECM or to neighboring cells via cell adhesion molecules. Consequently, considerable interest has been directed at understanding the signaling pathways that are initiated in response to mechanical forces that are applied to adhesion molecules (84). Multiple signaling pathways have been identified, including tyrosine kinases, ion channels and GTPases (85). One of the pathways that appears to be involved in many cells responding to mechanical force involves activation of Rho family GTPases, particularly RhoA. In this review we will focus primarily on the signaling pathways that lead to activation of RhoA in response to mechanical force and we will discuss the consequences of this pathway. The reader is directed to recent comprehensive reviews for information about mechanotransduction in various contexts (86-91).

The Rho pathway

In contrast to most plant cells that have rigid cell walls, the mechanical properties of animal cells are critically dependent on their cytoskeletons, consisting of microtubules, actin microfilaments, various types of intermediate filaments and also septins (92). All of these filament systems may contribute to the mechanical properties of animal cells, although with respect to how cells respond to exogenously applied forces most attention has been directed toward the actin cytoskeleton. When actin filaments are highly crosslinked they can give rise to a relatively rigid cell cortex. However, this can be rapidly remodeled to allow cell protrusion and changes in cell shape. The polymerization of actin filaments drives many types of cell extension. In conjunction with myosin, actin filaments can generate contractile forces, exerting traction on the surrounding matrix or on other cells and contributing to major changes in cell morphology. The interaction of myosin with actin not only contributes to the response of cells to exogenously applied forces but is responsible for generating endogenous forces within cells.

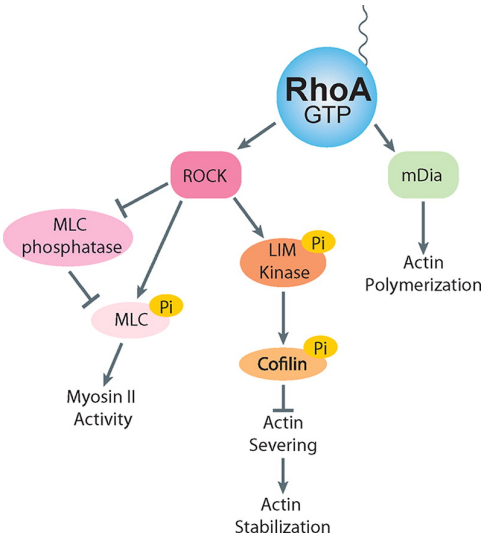


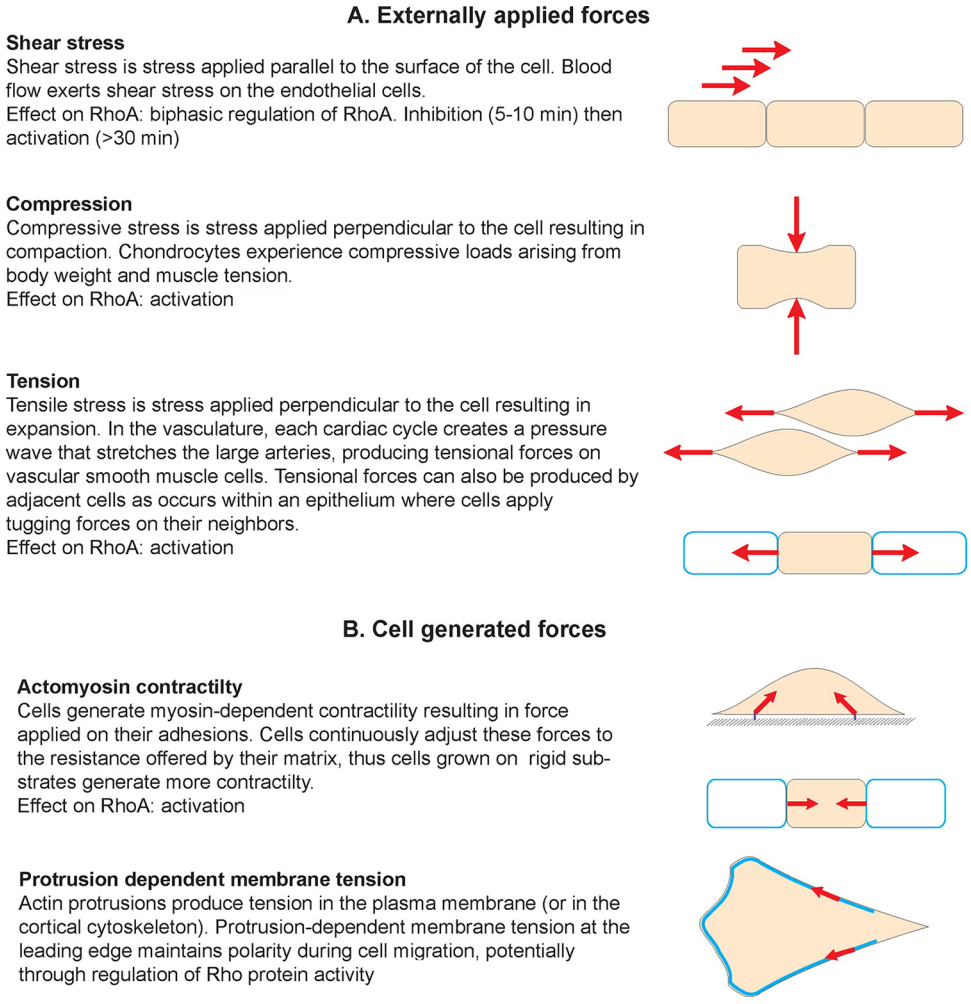
Figure 4: RhoA effector signaling
Activated RhoA interacts with effector proteins, which lead to actomyosin contractility and actin stabilization. ROCK signals by MLC phosphorylation to increase myosin II activity and LIM kinase to increase the level of actin stabilization. mDia nucleates actin polymerization.

The Rho family of GTPases are key regulators of the actin cytoskeleton. The mammalian genome encodes approximately 20 Rho GTPases, although the three ubiquitous ones, RhoA, Rac1 and Cdc42, are

the most studied and each has distinct effects on the actin cytoskeleton (33, 34). In the context of mechanotransduction, most effort has been directed at determining the role of RhoA, which is the focus of this review. In large part, this reflects the fact that RhoA regulates the activity of myosin II and consequently is responsible for much of the intracellular tension and force that is generated within cells (93). RhoA cycles between an inactive GDP state and an active GTP state (Figure 2). Three classes of proteins regulate this cycle: GEFs, GAPs and guanine nucleotide-dissociation inhibitors (GDIs) (94). GEFs activate Rho proteins by catalyzing the exchange of GDP for GTP (32) and GAPs stimulate the intrinsic GTPase activity, leading to the return to the inactive state (95). The inactive pool of RhoA is maintained in the cytosol by association with GDI (96) and it is in the active GTP-bound conformation that RhoA interacts with its effectors and performs its functions (Figure 2). With respect to regulating the activity of myosin II, the critical effector is ROCK, which exists in two isoforms, ROCK1 and ROCK2. Both isoforms promote myosin II activity by elevating the phosphorylation of the regulatory MLC. This occurs both directly by phosphorylation of the regulatory MLC (51) and indirectly by phosphorylation and consequent inhibition of the MLC phosphatase (52). The phosphorylation of the MLC promotes assembly of myosin II into bipolar filaments and enhances the ATPase activity of myosin II. Together these effects increase the contractile force generated by myosin II on actin filaments. ROCK also phosphorylates and activates another kinase, LIM kinase, which in turn phosphorylates and inhibits the actin-severing protein cofilin (54). By inhibiting

cofilin's actin severing activity, this increases the stability of actin filaments. Active RhoA also promotes actin filament polymerization. This occurs by RhoA binding different effector, mDia1, which is member of the formin family of actin nucleating factors (55) (Figure 4).

Figure 5: Mechanical force in cell biology
Diagram summarizing the different types of force that cells can experience. These can be externally applied (A) or generated by the cell itself and its own cytoskeleton (B). The effect on RhoA activity is indicated for each example. Force is a vector with magnitude and direction that causes an object with mass to change its velocity (units of newtons). Stress is force per unit of area (units of pascals).



In the context of mechanical signals, one can distinguish two types of forces experienced by cells: (1) forces which are externally applied to the cells, such as the shear stress exerted by blood flow on the surface of ECs, or (2) forces which are

generated by the cell itself with its cytoskeleton (97, 98) (Figure 5). Despite the apparent differences between these two signals, applied forces and cell-generated forces share some similarities in their transduction modalities and seem to regulate the same molecular mechanisms (89, 98). In both cases, cell surface adhesions, cytoskeleton and membrane tension cooperate to transmit forces which eventually affect the conformation of “mechanosensors” and trigger the mechanoresponse (85, 89). Interestingly, numerous GEFs and GAPs are known to associate with cytoskeletal and cell adhesion components, suggesting that mechanical forces can directly affect the activity or the localization of RhoA regulators.

GEFs and GAPs

Some GEFs specific for RhoA have been found to associate with the cytoskeleton and adhesions (Table 1). Integrin-based adhesions constitute a major site of mechanotransduction (99) and experience very diverse types of forces. For example, they are subjected to tensional forces when the ECM is stretched or when cells are grown on rigid substrates and generate more myosin-dependent contractility (Figure 5). Therefore, it’s not surprising that the GEFs associated with Cell-ECM adhesions are involved in the mechanoresponse (Figure 6A). Among them, vav2 was reported to be phosphorylated and activated in response to cyclic stretch in mesangial cells (100). Vav2 phosphorylation required EGFR

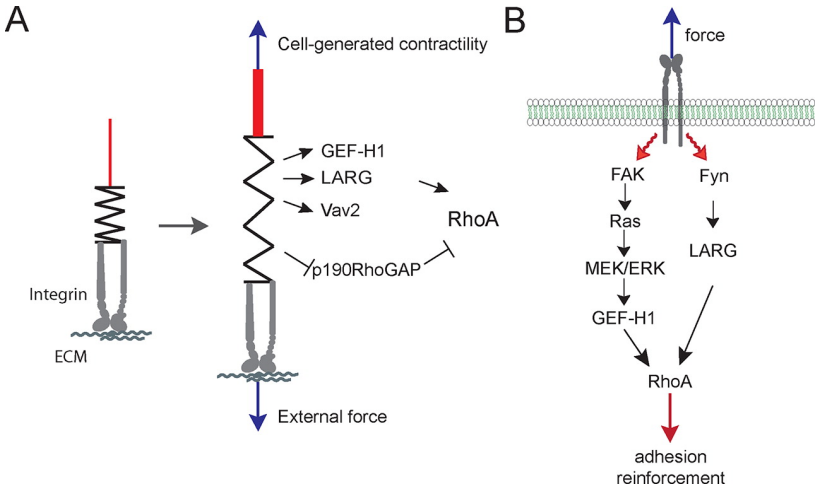
GEFs	Localization	References
p115 (ArhGEF1)	Cell-ECM adhesion	(101, 102)
GEF-H1(ArhGEF2)	Cell-ECM adhesion Cell-Cell adhesion Microtubule	(101, 102) (103, 104) (105)
LARG (ArhGEF12)	Cell-ECM adhesion	(101, 102)
Vav	Cell-ECM adhesion	(106)
p190RhoGEF	Cell-ECM adhesion	(107)
p114RhoGEF (ArhGEF18)	Cell-Cell adhesion	(107)
Trio	Intermediate filaments	(108)
PDZRhoGEF (ArhGEF11)	Cell-ECM adhesion	(109)
GAPs		
p190RhoGAP	Cell-ECM adhesion	(110)
DLC1	Cell-ECM adhesion	(111)
Myo-IXA	Cell-Cell adhesion	(113)

Table 1: RhoA GEFs and GAPs which associate with the cytoskeleton or adhesions

transactivation. Depletion of vav2, as well as EGFR inhibition, prevented stretch-induced RhoA activation (100). Applying tensional forces on fibronectin coated beads bound to fibroblasts, our group recently showed that force on integrins activates RhoA through two GEFs, GEF-H1 and LARG (101) (Figure 6B). Weobserved that mechanical forces induce the recruitment of GEF-H1 and LARG to the adhesions. We found that Fyn regulates LARG activity, whereas GEF-H1 is activated by a FAK/Ras/ERK signaling pathway. Consistent with these findings, Waterman and colleagues observed that myosin- dependent contractility promotes GEF-H1 recruitment to Cell-ECM adhesions (114). Interestingly, another group found that GEF-H1 is more active when epithelial cells are grown on rigid substrates (115). This suggests that both externally applied forces and cell- generated forces activate the same GEFs, reinforcing the idea that these two distinct mechanical signals trigger the same signaling pathways. However, on rigid substrates, Heck and

colleagues observed that microtubule stability, and not the Ras/ERK pathway, regulates GEF-H1 activity (115). This apparent discrepancy could be due to the difference of cell types that were used in these studies. Indeed, working with fibroblasts another group observed that RhoA activation in response to stretch was not affected by taxol-induced microtubule stabilization (116), whereas in ECs RhoA activation in response to stretch requires GEF-H1 and is prevented by taxol (117).

Figure 6: RhoA GEFs and GAPs regulated by force
(A) Schematic diagram showing the GEFs and GAPs whose activities are regulated by external force or cell-generated tension on cell–ECM adhesions. (B) Diagram showing the signaling pathways that regulate GEF-H1 and LARG activity in response to force on integrin (31).



Cell-cell adhesions are also subjected to tensional forces which are generated by neighboring cells or by the cell's own contractile machinery (Figure 5). It is now clear that tugging forces play an important role in intercellular junction maturation and growth (see below) (118-120). Interestingly, some RhoA GEFs have been found to localize at intercellular adhesions. GEF-H1 associates with cingulin at tight junctions (103, 104), however this interaction was reported to inhibit GEF-H1 and RhoA. More recently, p114RhoGEF was shown to localize at tight junctions and to activate RhoA locally, leading to junction assembly (121). Since mechanical tension

induces junction maturation (118), it would be interesting to determine if p114RhoGEF activity is regulated by tugging forces.

Mirroring the GEFs, some RhoA GAPs localize at adhesions (Table 1) and play a role during the mechanoresponse. DLC1 (111, 122-124) and p190RhoGAP (125-128) associate with Cell-ECM adhesion components. Shear stress regulates p190RhoGAP activity in a biphasic pattern in ECs (129). It was found that short term application of shear stress (<5 min) activates p190RhoGAP through Src Family Kinase-mediated phosphorylation, but longer application of shear stress (>30 min) induces p190RhoGAP dephosphorylation and inactivation. This biphasic regulation of p190RhoGAP leads first to RhoA inactivation followed by activation, similar to what has been observed during adhesion to matrix. Interestingly, p190RhoGAP is necessary for stress fiber alignment in response to shear stress (52). In addition, p190RhoGAP was shown to be necessary for the regulation of two transcription factors, GATAII and TFII-I, in response to increased matrix rigidity in a model of capillary tube formation (130). This suggests that cell-generated contractility may affect p190RhoGAP activity, although this remains to be determined. More recently, Myo-IXA, a single headed myosin with a GAP domain, has been shown to associate with actin at cell- cell junctions, locally restraining RhoA activity to allow proper junction formation (113). It would be interesting to analyze if application of tensional force on intercellular junctions affects Myo-IXA activity or localization.

Other Rho proteins

There is extensive crosstalk between RhoA and Rac1 that contributes to processes such as cell migration (131). In the context of mechanotransduction several pathways have been identified, particularly where high RhoA activity and mechanical tension may depress Rac1 activity. During polarized migration it is important to confine protrusion to the front of the migrating cell and to suppress inappropriate protrusions at other sites on a cell's periphery. Recent studies have implicated membrane tension generated during cell migration in the suppression of Rac1 activity at sites away from the leading edge and in the maintenance of cell polarity during migration (132). Several potential pathways are suggested by previous work. In migrating leukocytes, high RhoA/ROCK activity was shown to restrict membrane protrusion to the leading edge, in part via the LIM kinase pathway inhibiting cofilin and thereby stabilizing actin filaments at the cell periphery away from the cell front (133). In other work, inhibiting the Rac GAP FilGAP was found to increase membrane protrusions around the periphery of cells (134) suggesting that this Rac GAP confines protrusion to the cell front. Interestingly, FilGAP activity was activated by ROCK-mediated phosphorylation, providing a mechanism by which high RhoA activity can inhibit Rac1 activity. In a subsequent study using a reconstituted actin gel with several purified proteins, it was shown that FilGAP dissociates from filamin A in response to mechanical tension (135). When released it was suggested to relocate to the plasma membrane where it can act to inhibit Rac1 activity. The related Rac GAP, ArhGAP22, is activated in cells by endogenous mechanical force

to depress Rac1 activity (60). Blocking myosin activity, either directly with blebbistatin or indirectly by inhibiting ROCK activity, decreased ArhGAP22 activity(136). The Rac GEF βPIX binds to Myosin II and actomyosin contractility induces βPIX dissociation from cell substrate adhesions (114). This contributes to Rac1 inhibition during adhesion maturation. It seems likely that all of these mechanisms may synergize to confine Rac1 activity and membrane protrusion to the leading edge of migrating cells and away from regions of high mechanical tension and RhoA/ROCK activity. As a consequence of the competitive binding to RhoGDI, increasing the binding affinity of one Rho protein leads to the release and degradation and/or activation of other Rho proteins (137). Interestingly, actomyosin contractility induces GDI dissociation from cell-matrix adhesions (114). However, the mechanism of GDI recruitment to adhesions is not known.

Experimentally manipulating force

Before considering some of ways that force can be applied to cells, it is useful to consider some of the forces that cells can exert and experience. The force exerted by a single myosin motor is between 1 and 8 pN (138-140). (1 Newton = 105 dynes. Dynes were used for many of the more classical measurements of force, but today Newtons are the unit of force generally used.) The maximum tension developed by striated muscle has been calculated to be ~ 3 × 106 dynes/cm2 (= ~300 nN/μm2), which translates to ~3 × 10−5 dynes per thin filament (i.e. ~300 pN per thin filament) (141). For cells in culture, various forms of traction force microscopy have been used to measure the tension that they generate on their adhesions and the substratum.

Here we will consider just a few of the values that have been obtained. In some of the first experiments investigating the force generated on the substratum by cultured cells, Harris and colleagues calculated an approximate value of 10 nN per μm of cell length (81). Lee and coworkers concluded that the maximum force generated by fish keratocytes was ~20 nN (142). Using a cantilever device, Galbraith and Sheetz obtained a force of 0.2 to 4 nN/μm2 for migrating fibroblasts (143). Geiger's lab examined the tension developed by focal adhesions and found that the stress was proportional to the size of the focal adhesion with a value of about 5.5 nN/μm2 (144, 145). In general, the area of a focal adhesion relates to the diameter of the stress fiber attached to it. Consequently, because the number of force- generating myosin molecules will relate to the diameter of a stress fiber, intuitively one might expect there to be a constant ratio between the size of a focal adhesion and the force that is being transmitted through it to the substratum. However, an unexpected discovery was made by Beningo et al. who found that in migrating cells, more force was transmitted to the substratum by small nascent adhesions at the leading edge of cells than in larger more mature focal adhesions behind the leading edge (146). A possible resolution to the apparent discrepancy between these two sets of results comes from the work of Chen's lab, who have studied traction generated by cells plated on deformable micro-posts (micro-needles) (147). Like the Geiger lab they found that for most adhesions there was a correlation between the size of a focal adhesion and the stress exerted at the adhesion. Indeed, they found a similar value of ~4–5 nN/μm2. However, in their work they also found a subset of smaller

adhesions less that 1 μm2 that generated high levels of stress that did not correlate with the size of the adhesion (147). These latter adhesions most likely relate to the adhesions studied by Beningo et al. at the leading edge of migrating cells (146).

Manipulations of the ECM and flexible substrata can be used to mimic the tensional forces cells experience in the body. Simply plating cells on more rigid rather than on more compliant substrata increases the tension generated by cells on their underlying matrix due to increased RhoA activity (see discussion below) (148). Various devices have been developed that allow investigators to stretch cells by stretching the substratum to which the cells are adhering. The development of culture dishes with a flexible base that can be stretched by applying a vacuum facilitated subjecting cells to periods of cyclic stretch (149, 150). The period of the stretch as well as the degree of stretch imposed on cells can be readily varied and a large literature now exists describing many signaling pathways that become activated in response to cyclic stretch. Tension has also been applied to individual cellsusing glass rods or needles (83, 151). With these it is often more difficult to know the precise force that is being applied to cells, although the amount of force required to bend a needle by a certain angle can be determined experimentally.

Stretching or deforming a cell via a flexible substratum or by a glass rod or needle simultaneously affects many properties, including cell shape, the cytoskeleton, as well as a cell's adhesion to the matrix and/or its neighbors. In order to examine the effects of tension on specific adhesion molecules different approaches have been developed taking advantage of optical (laser) tweezers or

magnetic tweezers to exert forces on beads that are attached to cells via specific ligands or antibodies. Wang and colleagues used ferromagnetic beads that were attached to cells via integrin ligands (152). The beads were magnetized in one direction and then a second magnetic field was applied at 90° inducing the beads to twist and exert a shear force. This allowed them to show that there was a stiffening response as force was applied and that this depended on the cytoskeleton (152). Sheetz's group used optical tweezers to manipulate beads similarly coated with integrin ligands or antibodies(153). They used the optical tweezers to restrain individual beads against the force exerted by the cell. It was observed that cells sensed the restraining force and strengthened the cytoskeletal linkages to oppose this. One advantage of optical tweezers is that beads can be individually manipulated with great precision, allowing them to be placed at different points on a cell's surface and to be moved in different directions. Optical tweezers can generate forces up to about 500 pN, but in the higher range of forces heat generated by the laser can be detrimental and limit the use of this approach. Whereas an advantage is the ease of examining single cell responses, optical tweezers are not suitable for bulk biochemical analyses of signaling pathways.

Ingber and his group used an electromagnetic microneedle to apply force on magnetic beads coated with adhesion molecule ligands or antibodies (154, 155). With this system it is easy to apply predetermined pulses of force on beads by turning the current on for defined periods. The time between the magnet being on can also be varied so that the behavior of cells responding to the cessation of force

can also be examined. The magnitude of the force generated by magnetic tweezers can be easily varied by altering the magnetic field and bead size, resulting in forces ranging from 1 pN to 100 nN (154). This wide range of force that can be generated is a potential advantage of the technique. However, the application of force is unidirectional and the position of the beads relative to the cell surface is essentially random, reflecting where the beads have dropped. However, a significant advantage of using magnetic beads to generate force on cells is that tension can be applied to all the cells in a dish provided that sufficient beads are added and a permanent magnet is used (156). This facilitates biochemical analysis of signaling pathways induced by sustained force (101, 157). The forces generated on cells using magnetic beads and permanent magnets have been discussed in detail elsewhere (156, 158). As an example, studying the application of collagen- coated 3 μm magnetic beads to fibroblasts growing in a 60 mm dish and using a permanent ceramic magnet 2 cm above the dish, Zhao et al. calculated that they exerted 480 pN per cell or 0.65 pN/μm2 (157).

A large body of work has examined the effects of flow and shear force particularly on ECs. Because of their location lining blood vessels, these are exposed and respond to blood flow throughout their existence. Hemodynamic forces vary over a wide range within the vasculature, but most work has focused on the arterial system because the high flow within arteries is critical not only to their normal physiology but also is a major factor in the pathological development of atherosclerosis. ECs experience force perpendicular to the endothelium as a result

of blood pressure and force parallel to the blood vessel wall as a result of flow. The frictional force of blood flow generates shear stress that acts at the surface of ECs (159). This has pronounced effects on endothelial behavior (88, 90, 159-161). Straight regions of arteries result in laminar flow but this becomes disturbed when a vessel curves, bifurcates or branches. The mean wall shear stress of large arteries has been determined to be between 20 and 40 dynes/cm2 (159, 162), but much higher values (exceeding 100 dynes/cm2) have been recorded transiently at the peak of pulsatile flow resulting from the heart beat (163). Turbulent flow results in shear stress experienced by the endothelium that has been calculated to vary from negative values through zero to levels of between 40 to 50 dynes/cm2 (163). Several devices have been developed to allow the effects of flow and shear stress to be examined on cells in culture. These include the cone plate viscometer, in which flow is generated by the rotation of a cone above cells growing in a culture dish (164, 165). The shear stress and whether flow is laminar or turbulent are determined by the angle of the cone, the viscosity of the medium and speed of rotation. Parallel plate flow chambers are frequently used to study the effects of flow on cells. In these, fluid is pumped between two glass sheets, on one of which the cells of interest have been cultured (166, 167). Cells can also be grown in capillary tubes through which fluid is similarly pumped at levels determined by the investigator to mimic the desired shear forces (168, 169). With both parallel plate chambers and capillary tubes, turbulent flow can be generated by reversing the direction of flow or by stopping and starting flow. Shear stress values can be generated that cover the

full range experienced by arterial ECs in vivo (159, 162).

Not only can exogenously applied force be experimentally modulated, but the endogenous forces generated by actomyosin contractility within cells can also be controlled by the investigator. This can be achieved by directly affecting myosin activity or by modifying upstream signaling pathways. Myosin ATPase activity can be inhibited by the drug blebbistatin, which has become a valuable tool for cell biologists interested in decreasing endogenous tension (170). The major limitation using this drug is that it is photo-sensitive and therefore cannot easily be used with live cell imaging. Alternatively, the expression of myosin II isoforms (usually myosin IIA or IIB) can be knocked down using siRNA techniques. Given the key role of RhoA and ROCK in regulating myosin activity in cells, contractility is often manipulated by inhibiting the RhoA/ROCK signaling pathway. Direct inhibition of RhoA is achieved using treatment with the Botulinum exotransferase C3 which ADP-ribosylates RhoA. Several ROCK inhibitors have been developed, but the most frequently used experimentally is Y27632 (171)(95). The disadvantage of perturbing the RhoA/ ROCK pathway is that contractility is only one of many downstream signaling events that is affected, often making interpretation of results difficult. Stimulating contractility can be induced in several ways. Expression of constitutively active RhoA drives activation of the ROCK pathway and elevates myosin activity, but again there will be many other effects. The level of MLC phosphorylation can also be enhanced by inhibiting phosphatase activity pharmacologically, for example with calyculin A. This potently stimulates contractility (172), but here too there will be many side effects.

The phosphorylation state of the regulatory MLC can also be mimicked by expression of mutant MLCs in which one or both of the critical phosphorylatable residues (threonine18 and serine19) are mutated to aspartic acid. These generate constitutively active forms and have been used in several studies (see for example (173)). The difficulty with these mutants is that the dynamic nature of regulation by phosphorylation is blocked because the myosin molecules are locked into a single activated state.

Rigid substrata, stress fibers and focal adhesions

On substrata of different compliance, cells exhibit strikingly different behaviors. Compared with when they are cultured on more rigid surfaces, on more compliant substrates fibroblasts are less able to develop stress fibers and focal adhesions but migrate more rapidly (174). Culturing cells on substrates of different compliance can also have profound effects on gene expression (175, 176). The behavior of cells on relatively soft substrata relates to the general observation that in tissue culture many cells develop stress fibers and focal adhesions, although the same cells within their host tissues rarely develop these structures (177). What is it about tissue culture and rigid substrata that promote the formation of these structures that often dominate a cell's cytoskeletal appearance? In tissue culture, frequently one factor is the presence of agents in serum such as LPA and S1P that activate RhoA (34). These derive from platelet secretions during blood clot formation. In wound healing they probably contribute to the contraction of cells surrounding a wound site. Notably, tissue culture has often been likened to a wound

response. However, even in the absence of serum and these factors, many fibroblasts develop stress fibers and focal adhesions when plated on rigid substrata coated with matrix proteins. Conversely, even in the presence of serum, cells adhering to soft substrata are unable to assemble these structures (174). The rigidity of the substratum is a second factor contributing to the development of focal adhesions and stress fibers. On rigid substrata cells such as fibroblasts generate strong tractional forces to the matrix components adsorbed to the surface of the culture dish or cover glass. The resulting isometric tension was suggested many years ago as a factor in the development of these structures (97). Subsequent work has shown that culturing cells on rigid surfaces elevates RhoA activity (178, 179). The importance of tension in the development of these structures is supported by a large body of evidence, including numerous experiments showing that inhibiting the RhoA/ROCK pathway or myosin activity blocks the development of stress fibers and focal adhesions, and leads to the disassembly of these structures if they have already formed (34, 93, 180-182). The development of stress fibers and focal adhesions on rigid substrata is the quintessential example of endogenously generated tension affecting the organization of the cytoskeleton and cell behavior. Synergy between endogenously generated tension and tension applied exogenously promoting the assembly of these structures was elegantly demonstrated by Riveline and coworkers who showed that applying tension on cells adhering to rigid substrata promoted the growth of focal adhesions (151).

In addition to endogenous tension contributing to the assembly of focal

adhesions and stress fibers, a major contribution to the activation of RhoA derives from integrin engagement with the ECM. This is a complex biphasic response, in which integrin-mediated adhesion initially depresses and then elevates RhoA activity (126, 183). The RhoA GEFs, p115/Lsc, LARG and p190RhoGEF were all shown to be activated upon adhesion to fibronectin (102, 107). Both the engagement of integrins and the mechanical tension exerted on these adhesion molecules leads to the activation of RhoA (101, 102, 107).

ECM Compliance and gene expression

It has been known for a long time that the differentiated phenotype of many cells is often lost when they are grown on rigid plastic substrates as opposed to being cultured on more appropriate ECM proteins. This is particularly true when the growth and differentiation characteristics of epithelial cells are compared between cultures growing on plastic or on ECM components that recapitulate many of the characteristics of basement membrane (184). Many studies revealed that the expression of differentiated genes depends not only on the presence of appropriate growth factors but also on an appropriate ECM. For example, the morphology and gene expression exhibited by breast epithelial cells were profoundly influenced not only by the composition of the matrix but also its physical state. Thus it was shown early on that culturing breast epithelial cells on floating collagen gels, which are compliant, compared with collagen gels anchored to rigid culture dishes affected the expression of specific genes (185).

With hindsight, many of the effects of matrix rigidity or cell shape on the

differentiated phenotype can be understood in the context of RhoA/ROCK signaling. Numerous studies have led to the conclusion that the level of RhoA activity affects differentiation and gene expression (88, 186, 187). For example, Sordella and coworkers studying the phenotype of the p190-B RhoGAP null mouse discovered that mice lacking this major negative regulator of RhoA activity, not only had elevated RhoA activity, but were defective in adipogenesis and had enhanced myogenesis. They concluded that there was a Rho-dependent switch that regulated stem cells to differentiate in a myoblast direction under conditions of high RhoA activity but to differentiate into adipocytes under low RhoA activity (188). This work was extended by others. For example, McBeath and colleagues using human mesenchymal stem cells (MSCs) in culture demonstrated that their commitment into osteoblasts or adipocytes was determined by their cell shape and that MSCs that flattened and spread became osteoblasts whereas the same cells prevented from spreading became adipocytes (189). These investigators found that inhibiting the RhoA pathway drove the MSCs toward the adipocyte pathway, but activating RhoA induced the osteoblast lineage. They went on to show that this latter pathway was mediated by the RhoA effector, ROCK, and that expression of activated ROCK was sufficient to drive osteogenesis. Interestingly, this occurred even when the cells were kept in a rounded state, whereas expressing activated RhoA was not sufficient to overcome the inhibitory effect of cell rounding on osteogenesis. These results suggested that the link between RhoA and ROCK could be uncoupled by cell rounding. Pursuing this further, Chen's group showed that indeed in rounded cells

there was high RhoA activity but low ROCK activity and that the level of myosin light chain phosphorylation was similarly low. Additionally, they found that inhibiting endogenous cell tension either by disrupting the cytoskeleton with cytochalasin or by blocking myosin with blebbistatin also inhibited ROCK activity, although in the case of cytochalasin treatment this decrease in ROCK activity occurred in the presence of high RhoA activity (182). Their results suggested a positive feedback mechanism by which mechanical tension is needed to maintain high ROCK activity. In terms of the uncoupling between ROCK and RhoA activities, tyrosine phosphorylation of ROCK2 was shown to inhibit its activation by RhoA (190). This tyrosine phosphorylation was found to occur in response to adhesion and likely allows RhoA signaling to activate mDia but not ROCK, such that actin polymerization and cell spreading are promoted but contraction is inhibited. The high activity of RhoA that has been detected at the leading edge of migrating cells (191) has been difficult to explain in terms of models where RhoA drives contractility but can be easily accommodated in models where there is a regulatory bifurcation downstream from RhoA such that ROCK is inhibited while mDia is activated. However, in the case of cell rounding leading to ROCK inhibition, we suspect that this may involve other pathways because cell rounding is usually associated with decreased levels of tyrosine phosphorylation for many proteins (192).

In a detailed study in which MSCs were cultured on matrices that closely related to the compliance of their endogenous tissue environments, it was shown that soft substrata resembling the stiffness of brain induced a neurogenic pattern of

gene expression, whereas on stiffer substrata mimicking muscle the same cells were myogenic, and on the stiffest matrices resembling collagenous bone the cells were osteogenic (176). Significantly, it was found that blocking myosin II activity inhibited the effects of matrix compliance on the resulting phenotype providing further support for the importance of myosin and tension in the sensing of matrix rigidity. Exploring how transcription may be regulated by matrix rigidity, cytoskeletal tension and RhoA activity, Piccolo's group examined the transcriptional profiles of several cell types on substrates of differing compliance and identified the YAP/TAZ transcriptional regulators as key factors in controlling the enhanced expression of specific genes on more rigid substrates (193). Specifically, the distribution of these factors in the nucleus or in the cytoplasm was found to be determined by rigid versus soft matrices, respectively. Inhibiting RhoA, ROCK or myosin II activity was found to keep YAP and TAZ in the cytoplasm, whereas active RhoA drove them into the nucleus and induced the expression of genes associated with rigid matrices. It will be interesting in the future to learn how this is accomplished, but together with many of the studies mentioned above this work establishes a pathway by which a cell responds to the rigidity or compliance of its environment and alters its pattern of gene expression accordingly.

Tension at Cell-Cell junctions

The role of RhoA activity and mechanical tension in cell-cell junctions is complex. Numerous studies with agents that increase endothelial permeability, such as thrombin, have implicated both increased RhoA activity and myosin-based

contractility with opening of endothelial junctions and increased permeability (194, 195). However, other work has indicated a role for RhoA in junction assembly(121, 196-199). Not only is RhoA activity required for junction assembly, but several studies have shown that myosin-induced tension downstream from RhoA can promote junction assembly (118, 200-202). These results appear at first sight to be contradictory. We suspect that under conditions where tension is associated with junctional disruption other factors must contribute to weakening the junctions. Support for this idea comes, for example, from studies of HIV-induced encephalitis in which there is increased monocyte passage across the blood/brain barrier and disruption of endothelial tight junctions. The weakening of tight junctions was related to ROCK-mediated phosphorylation of two tight junction proteins, occludin and claudin-5 (203). It seems likely that many agents that increase permeability and open cell-cell junctions simultaneously increase tension while weakening the adhesive strength of the junctional CAMs. On the other hand in situations where RhoA and increased contractility enhance junction assembly, we assume that the signals must segregate such that the junctional CAMs maintain their adhesive strength or increase it so that increased tension does not break the adhesions and open gaps between the cells. With respect to the strengthening of junctions in response to mechanical force, it was discovered that α-catenin changes its conformation in response to tension on epithelial junctions to expose a cryptic site that can bind vinculin (121, 202). In parallel work, it was shown that vinculin is recruited to adherens junctions in response to mechanical tension and that tension

on E-cadherin leads to a stiffening response that is dependent on vinculin (120). Together these studies support a model in which RhoA-mediated tension on cell-cell junctions can have opposite effects depending on the adhesive strength of the junctional CAMs and their associated protein complexes.

Cadherin engagement has been found to either decrease (204, 205) or increase RhoA activity (206-208). Differences in these results may reflect in part the different signaling pathways initiated downstream from different cadherins. However, some of the differences may be due to the presence or absence of force on the cadherins. Working with ECs and VE-cadherin, Nelson and colleagues observed that sustained adhesion via VE- cadherin resulted in a peak of RhoA activity 6 hours following VE-cadherin engagement and they provided evidence that this was dependent on tension being transmitted to the sites of cell-cell adhesion. In contrast, the depression in RhoA activity upon E-cadherin engagement was rapid (204). Consistent with the idea that mechanical force on the cadherin may switch the signaling pathway from depressing RhoA activity to elevating it, we have found that while simple engagement of E-cadherin leads to decreased RhoA activity, applying force to the cadherins elevates RhoA activity (Marjoram and Guilluy, unpublished results). It will be interesting to identify the GEFs that become activated in response to tension on E-cadherin.

In many situations mechanical force on cells is associated with increased proliferation. Investigating the role of cell-cell adhesions versus cell-matrix adhesions in mechanical signaling to induce cell proliferation, Chen's group compared the

response of ECs and vascular smooth muscle cells to mechanical force (209). Subjecting both cell types to stretching stimulated proliferation but ECs required cell-cell adhesion and engagement of VE-cadherin for proliferation to occur, whereas smooth muscle cells responded to stretch by proliferation in the absence of cell-cell contact. Interestingly, the authors found that stretching ECs activated Rac1 and this was required for proliferation. However, upon stretching smooth muscle cells RhoA activation was needed for proliferation (209). This result is in contrast to the absence of RhoA activation found by Schwartz' group when smooth muscle cells were stretched, but differing conditions probably account for the apparent discrepancy (210).

Cancer

During development, the rigidity/compliance of different regions of embryos is thought to have a major impact on the differentiation and organization of various tissues and organs. This view is supported by the large body of work studying cells grown in culture that indicates the importance of the physical characteristics as well as the composition of the microenvironment. There are also disease situations where the rigidity of tissues alters and affects cell behavior. Examples include many solid tumors, the hardening of arterial walls that occurs with age, atherosclerosis, and fibrotic diseases where there is increased deposition of ECM. Solid tumors are often detected by physical palpation, an indication that they are less compliant than the surrounding tissues. The increased rigidity of tumors not surprisingly has been associated with increased RhoA activity and other altered signaling pathways (179).

Many epithelial cell types adopt a more normal morphology and phenotype when grown in relatively soft 3D matrices and this is lost when the same cells are cultured on rigid two dimensional surfaces (211). Working with breast epithelial cells in culture, it was found that changing the compliance of the ECM alone promoted a more malignant cancer phenotype (179). Cells grown on a stiff matrix exhibited larger colony size, increased ERK activity, elevated RhoA activity, more focal adhesions, and greater tractional force applied to the ECM compared to cells grown on a soft ECM. Blocking ROCK activity caused the cells on the stiff ECM to behave more like cells grown on a compliant ECM. Elevated RhoA-dependent signaling disrupted the normal epithelial morphology of the breast epithelial cells, which in soft matrices grow as spheroids with a cell polarity mimicking that found in the normal gland. On more rigid substrata the cells lost their polarized organization and the cell aggregates failed to develop lumens. These changes are reminiscent of the changes associated with malignancy (179, 212). Elevated rigidity has been shown to have protumorigenic effects in other cell types as well. For example, expression of activated forms of ROCK2 in skin resulted in increased stiffening associated with increased collagen deposition (213). This was associated with nuclear accumulation of β-catenin, transcriptional activation and hyperproliferation. Interestingly, when human skin squamous cell carcinomas were examined, the majority were found to have elevated ROCK expression and activity (213).

Mechanical tension in tumors is associated not only with increased cell proliferation but also with enhanced invasion (214). Tumor cells migrate along

aligned collagen fibrils and this is promoted by increased mechanical tension within the tumor (215). When tumor cells move in tissues either they migrate as cell collectives where a group of cells migrate together while maintaining their cell-cell contacts or they migrate as individual cells (216). In the latter situation they have been found to migrate in two distinct ways, which have been described as mesenchymal versus amoeboid or rounded (217, 218). These two types of migration appear to be interchangeable and the mesenchymal form can be driven to become the amoeboid type by inhibiting proteases involved in degrading the ECM or by elevating RhoA and ROCK activity. Conversely, the mesenchymal mode of migration is promoted by high Rac1 but low RhoA activity (136, 216).

Future directions

The discovery that mechanical forces exerted exogenously on cells or generated endogenously within them leads to Rho protein activation and signaling has many implications. This pathway is important in development, preferentially driving stem cell differentiation along one lineage versus another. With the increasing interest in potential stem cell therapies, recognition of the impact of the physical properties of the environment is important. However, knowing that these effects of the environment are driven by the RhoA/ ROCK pathway should permit these environmental influences to be overridden by manipulating this signaling pathway so as to direct the differentiation of stem cells along predetermined lines. Elucidating the signaling pathways from mechanical force to Rho protein activation may also impact the approach to various pathologies such as fibrosis and cancer.

However, with tumors there is a red flag in that tumor cells can switch their mode of migration from a mesenchymal type to an amoeboid type according to the relative activities of Rac1 and RhoA. Consequently, the tempting idea that decreasing tumor cell RhoA activity may be beneficial, leading to decreased cell proliferation and favoring a more normal phenotype, may have unexpected consequences converting invasive tumor cells from one migratory phenotype to another. Nevertheless, when combined with other therapies, such as inhibiting Rac1-driven migration, targeting RhoA activity in tumors may be advantageous. The identification of upstream signaling components such as GEFs promises to provide novel targets for therapeutic development, not only for certain cancers but also for other disease where mechanosensitive signaling may be involved.

Chapter 3: THE RHOA GUANINE NUCLEOTIDE EXCHANGE FACTOR, LARG, MEDIATES ICAM-1-DEPENDENT MECHANOTRANSDUCTION IN ENDOTHELIAL CELLS TO STIMULATE TRANSENDOTHELIAL MIGRATION[2]

Introduction

Leukocyte extravasation is a tightly controlled process that involves signaling in both the leukocyte and EC. Neutrophils are early responders to sites of infection. Pro-inflammatory signals prompt them to exit post-capillary venules and infiltrate tissues to ingest microbes or foreign bodies, destroying them with proteolytic enzymes and/or the release of reactive oxygen species. In response to inflammatory signals, several adhesion molecules become expressed or increased on the EC surface including ICAM-1. Leukocyte TEM starts with leukocyte rolling, mediated by leukocyte binding to selectins on the surface of ECs (219). β_2 integrins on the leukocyte then bind to ICAM-1 (13-21). The strong adhesion resulting from ICAM-1

[2] The citation is as follows: Lessey-Morillon, E. C., Osborne, O., Monaghan-Benson, E., Guilluy. G., O'Brien, T. E., Superfine. R., Burridge, K., (2014) The RhoA guanine nucleotide exchange factor, LARG, mediates ICAM-1-dependent mechanotransduction in endothelial cells to stimulate transendothelial migration. Journal of Immunology. http://www.jimmunol.org/content/early/2014/02/28/jimmunol.1302525

engagement and clustering allows leukocytes to spread and crawl on the surface of the endothelium. Finally, leukocytes cross the EC monolayer, either passing through the junctions or through the ECs themselves (20, 30, 220) to enter the underlying tissue. Without ICAM-1, leukocyte spreading, crawling and TEM are impaired (57, 59).

Engagement and clustering of ICAM-1 by leukocytes induces multiple signaling pathways within ECs (22) that promote passage of the leukocytes across the endothelium. After ICAM-1 clustering, F-actin and actin binding proteins associate with the clustered complex to assist in the cytoskeletal changes that occur during leukocyte adhesion and TEM (21, 31, 61-64). One of the pathways responsible for these changes involves the GTPase RhoA, which was shown to be activated following ICAM-1 engagement and clustering (16, 31). Inhibiting RhoA signaling in ECs reduces leukocyte adhesion, spreading, and migration (14, 15, 59, 60). RhoA is also activated by various agents, such as thrombin, that increase the permeability of EC junctions (49, 221, 222). In part, this is due to RhoA-stimulated actomyosin contraction that exerts tension on the junctions, however, there is additional evidence that the adhesive strength of the junctions is weakened by signaling downstream of active RhoA (203). Clustering of ICAM-1 also elevates tyrosine phosphorylation of multiple proteins and several studies have identified Src family kinases (SFKs) as being responsible and being activated downstream of ICAM-1 (64, 65, 223, 224). However, the relationship between SFK activity and Rho protein activation downstream from ICAM-1 has not been explored.

Cell migration requires the cell to exert tractional forces on the underlying substratum. The amount of traction force generated by migrating leukocytes has been estimated to be between 5 and 50 pN (225-227). It is unclear if EC signaling is altered in response to the tractional force applied by leukocytes to adhesion molecules expressed on the EC luminal surface. At the outset of this work, we were interested in determining whether the tractional forces exerted on ICAM-1 as leukocytes migrate affect RhoA signaling, and secondly, we were interested in identifying GEFs that activate RhoA downstream of ICAM-1. Here we identify LARG, also known as ARHGEF12, as the critical RhoA GEF activating RhoA downstream of ICAM-1, show that it is activated by SFK-dependent tyrosine phosphorylation, and demonstrate that applying mechanical force on ICAM-1 clusters equivalent to the forces generated by migrating neutrophils enhances this signaling pathway. We provide evidence that this activation of RhoA not only promotes neutrophil TEM but stiffens the endothelial surface thereby enhancing the migration of neutrophils over it.

Results

Mechanical force on ICAM-1 increases cellular stiffness around ICAM-1 clusters.

We first sought to determine if mechanical force on ICAM-1 induces a cellular response. We used beads coated with aICAM-1 mAb as a model to mimic leukocyte-induced ICAM-1 clustering (30). The beads were also magnetic, allowing us to apply force on the ICAM-1 clusters. To assess cellular stiffness, we measured displacement of attached beads during pulses of force (101, 155, 228). We applied

pulses of 160 pN force on ICAM-1 and tracked the bead location during each pull (Figure 7A). There was no statistically significant difference in the initial average displacements of the beads on cells grown on collagen (0.5 μm) or fibronectin (0.4 μm). We observed that after the first pulse of force subsequent pulses did not displace the beads as much, indicating cellular stiffening (Figure 7B). This stiffening response occurred whether the ECs had been cultured on a fibronectin or collagen ECM, revealing that the response was not affected by the integrins through which the ECs were adhering to the matrix (Figure 7B,C). Since there was little change in bead displacement between the second pulse and subsequent pulses, for most experiments we have compared the bead displacement generated by the first and second pulse.

To explore the basis for the force-induced stiffening, we examined the effects of agents that perturb the cytoskeleton. The average initial bead displacement for control cells, and cells treated with blebbistatin, cytochalasin D, Y-27632 and SU6656 were 0.4, 0.6, 0.8, 0.4, and 0.6 μm, respectively. The stiffening response was blocked by disrupting the actin cytoskeleton with cytochalasin D or by inhibiting myosin activity with blebbistatin (Figure 7D). To inhibit the RhoA/ROCK pathway we used the ROCK inhibitor Y-27632 (Figure 7D) and used adenoviral delivery ofmiRNA to knockdown RhoA expression (Figure 7E, F). We found that knockdown of RhoA as well as inhibition of ROCK both inhibited the force-induced stiffening response. The SFK inhibitor, SU6656, also was able to prevent any change in bead displacement between pulses (Figure 7D). Taken together these results suggest that

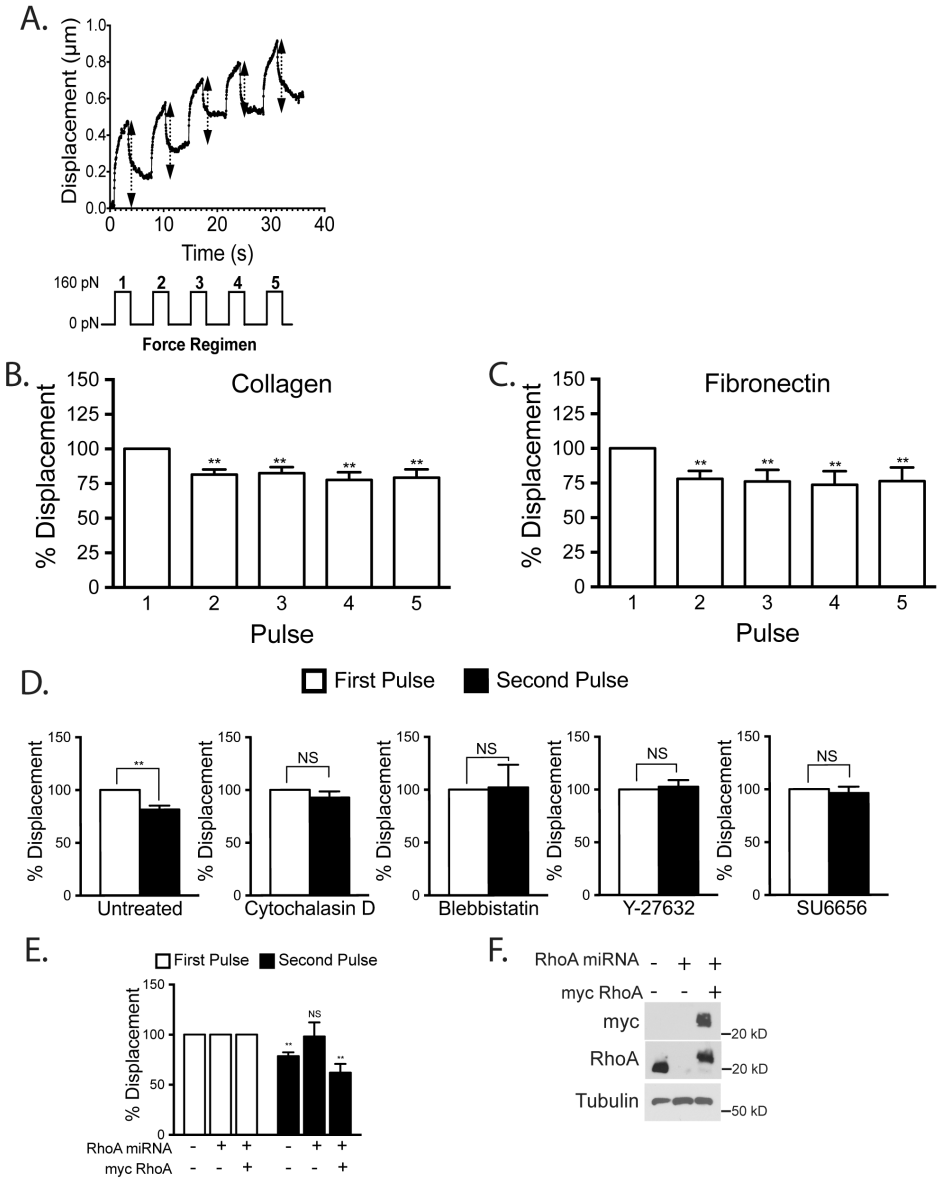


Figure 7: Mechanical force on ICAM-1 increases cellular stiffening

Magnetic beads coated with ICAM-1 mAb were added to a monolayer of TNF-treated HMVECs. Magnetic tweezers were used to apply pulses of force to individual beads and bead movement recorded with high-speed video. (A) Typical displacement of a bead bound to ICAM-1. Arrows denote displacement distance (Top). A diagram of the 160 pN force regimen used (3s of force with 5s recovery for 5 pulses) (Lower). Percentage bead displacement in response to sequential pulses of force for ECs plated on collagen (B) or fibronectin (C). For D-F, the ECs were plated on collagen. (D) Bead displacements on HMVECs treated with specified inhibitors for 30 min followed by 2 pulses of force. (E) Bead displacement on HMVECs and HMVECs treated with miRNA to inhibit RhoA expression with or without rescue with myc-RhoA. (F) Western blotting confirms RhoA knockdown and myc-RhoA re-expression. (B-E) Quantification of bead displacement with each pulse normalized to the first pulse. Asterisks shows p-value of statistical significance compared to the control (, p≤0.05; **, p≤0.01). The means ± SEM of ≥9 independent bead pulls are shown.*

ECs respond to mechanical force on ICAM-1 and the observed stiffening response is dependent on the actin cytoskeleton, myosin activity, RhoA signaling and SFK activity.

RhoA is activated by mechanical force on ICAM-1

After we had determined that the cellular stiffening was dependent on RhoA expression and actomyosin contractility we next wanted to examine RhoA activity levels. A considerable body of work has revealed the importance of RhoA within ECs in facilitating the passage of leukocytes across the endothelium (15, 16, 31, 57). To cluster ICAM-1, we incubated cells for 15 min with magnetic beads coated with aICAM-1 mAb, and then applied force with a permanent magnet placed above the cell culture dish for 1 min to provide ~10 pN of force (Figure 8A). Consistent with previous findings (14, 15, 31), ICAM-1 clustering increased RhoA activity over untreated cells (Figure 8A). RhoA activity was further increased within 1 min of mechanical force on the ICAM-1 bead clusters (Figure 8A). To evaluate if the observed activation of RhoA was specific to ICAM-1, we clustered and applied force on MHC class I. Neither clustering, nor force application on MHC class I significantly affected RhoA activity (Figure 8B), confirming that the activation of RhoA is not a universal response to tension on the cell surface. Both MHC class I beads and the ICAM-1 mAb coated beads were able to bind to the EC monolayer as seen by phase contrast microscopy (Figure 8C). MLC phosphorylation is frequently elevated downstream from RhoA activation and this was observed paralleling the increase in RhoA activity as ICAM-1 was clustered and then subjected to force (Figure 8D).

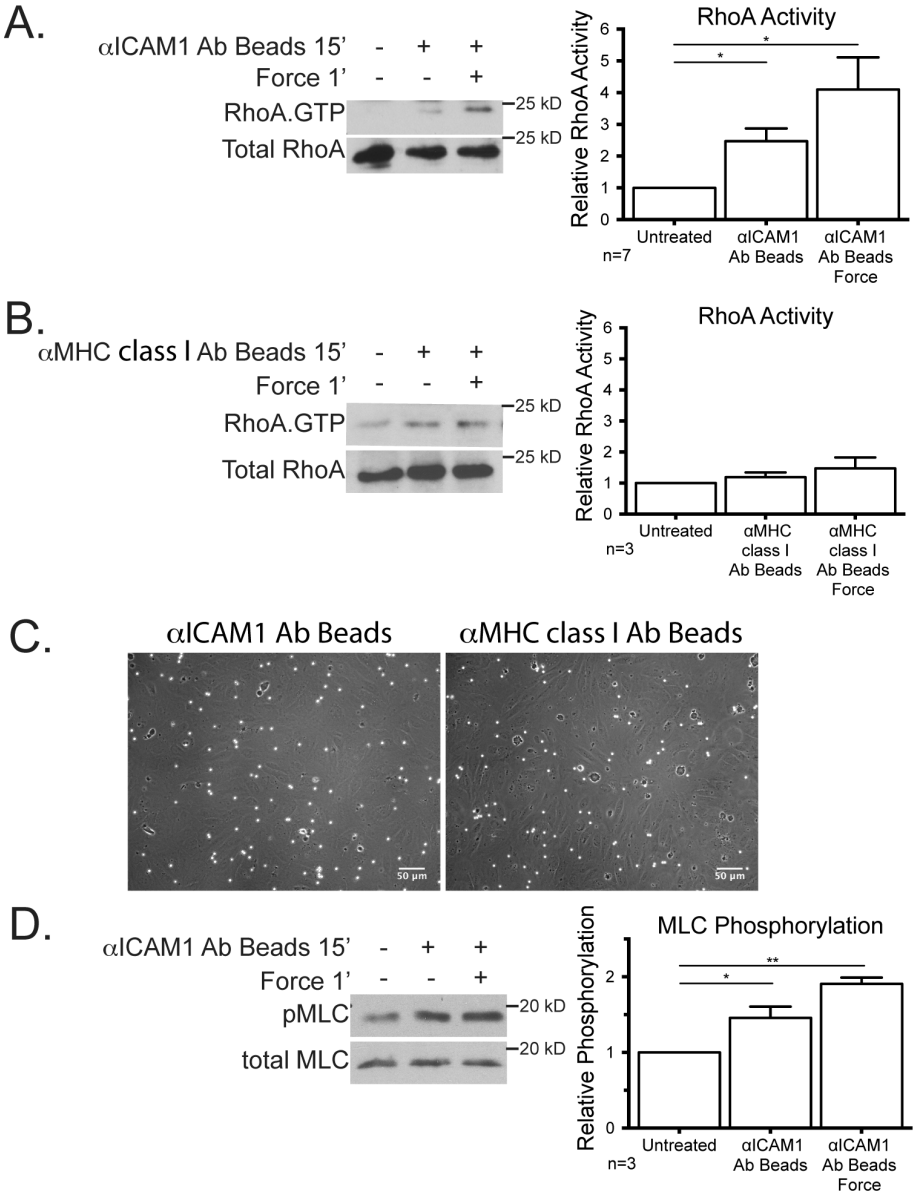


Figure 8: Mechanical force on ICAM-1 increases RhoA activity and MLC phosphorylation
Magnetic beads coated with mAb against ICAM-1 (A, C, and D) or MHC class I (B and C) were added for 15 min to a monolayer of TNF-treated HUVECs and ~10 pN force was applied with a ceramic magnet placed above the cells for 1 min. (A and B) Using GST-RBD, RhoA.GTP was isolated and detected by immunoblotting. (C) Phase contrast images of EC monolayers 15' after beads were added and washed 2x with media before fixing. (D) Lysates were immunoblotted for total MLC or MLC phosphorylated on Thr18/Ser19. Graphs show quantification of RhoA activity (A and B) or pMLC levels (D) from ≥3 independent experiments. Graphs show the means ± SEM. Asterisk shows p-value of statistical significance compared to control (, p≤0.05; **, p≤0.01).*

ICAM-1 signaling activates LARG

While a downstream role for RhoA activity after ICAM-1 engagement has long been established, the GEF mediating activation of RhoA has not been determined. Using the binding of GEFs to nucleotide-free mutant $RhoA^{G17A}$ as an indicator of GEF activation (229), we tested several candidate GEFs including LARG, p190RhoGEF p115RhoGEF, G-H1 and PDZ-RhoGEF, but only observed activation of LARG in response to ICAM-1 clustering (Figure 9A-E). There was an additional increase in LARG activity when force was applied to the clustered ICAM-1 (Figure 9A). Neither clustering MHC class I, nor applying tension on this receptor affected LARG activity (Figure 9F). ICAM-1 clustering induced LARG tyrosine phosphorylation and application of force on ICAM-1 further elevated this phosphorylation (Figure 9G). Treatment of cells with the SFK inhibitor, SU6656, inhibited LARG activation induced by ICAM-1 clustering and greatly attenuated LARG activation after ICAM-1 clustering with force (Figure 9H). These results strongly suggest a pathway in which clustering of ICAM-1 activates SFKs (64, 65, 223, 224) to phosphorylate and activate LARG.

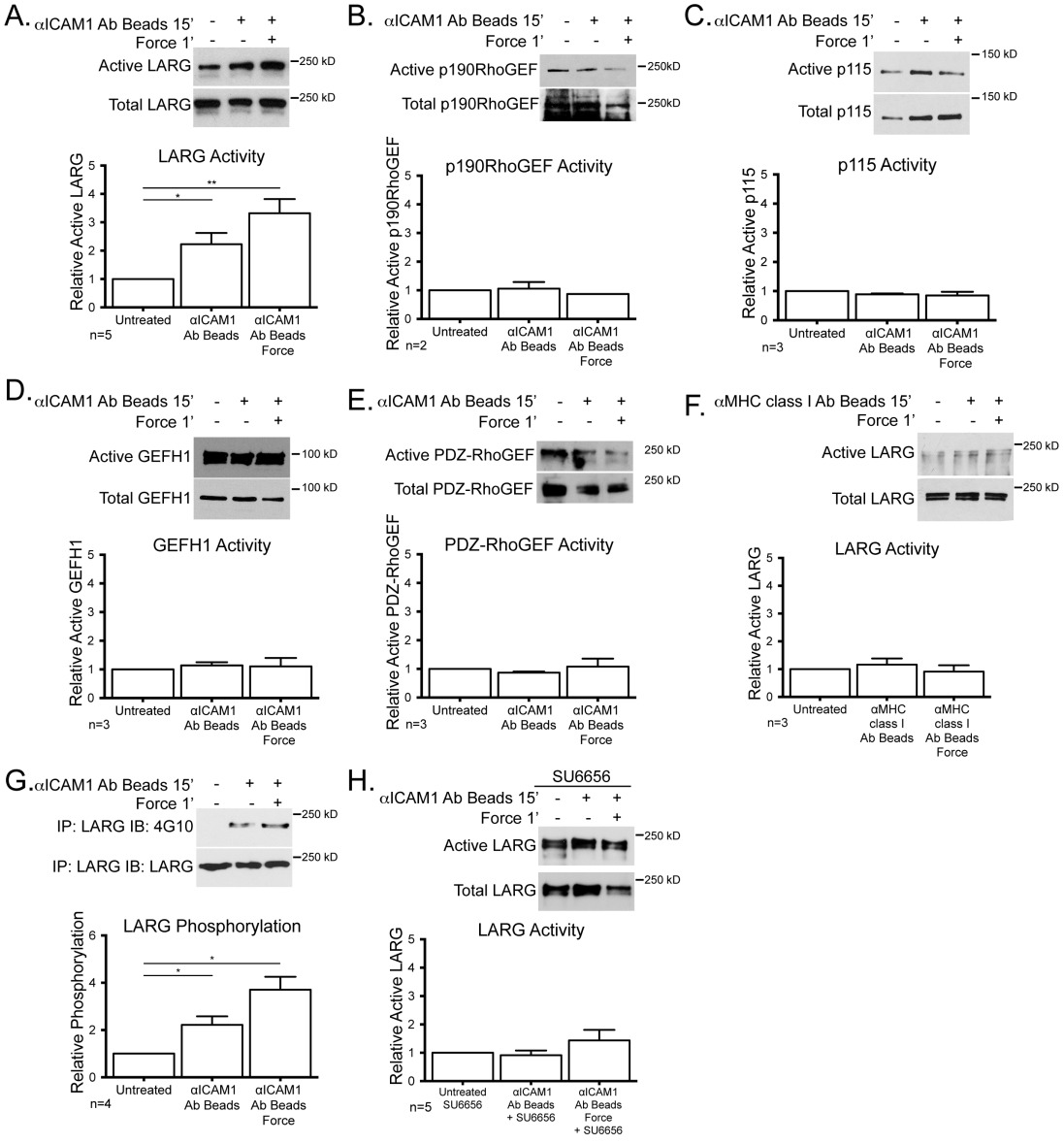
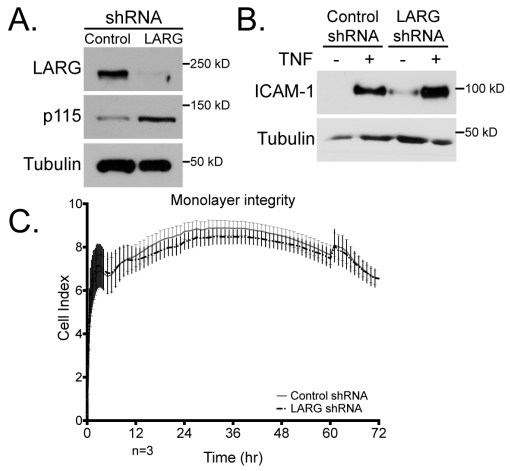


Figure 9: LARG is activated downstream of ICAM-1 clustering alone and enhanced with mechanical force

TNF-treated HUVECs were treated with mAb-coated beads. (A-F) GEF activity was determined by affinity purification via GST-RhoA^{G17A} and detected by immunoblotting for the specified GEF, LARG (A and F), p190RhoGEF (B), p115 (C), GEF-H1 (D), PDZ-RhoGEF (E). (G) LARG was immunoprecipitated and immunoblotted for phosphotyrosine and LARG. (H) Active LARG was detected by sedimentation with GST-RhoA^{G17A} in the presence of SU6656. For all experiments, a representative blot of ≥2 independent experiments is shown. Graphs show the means ± SEM. Asterisk shows p-value of statistical significance compared to control by t test (, p≤0.05; **, p≤0.01).*

Knockdown of LARG expression inhibits RhoA activation downstream of ICAM-1 clustering

To further investigate whether LARG activation is responsible for the increase in RhoA activity downstream of ICAM-1 signaling, we used lenti-shRNA technology to depress LARG expression. ECs were infected with LARG shRNA or scrambled control shRNA. We confirmed by immunoblotting that LARG protein levels were reduced by the lenti-shRNA treatment and that the levels of similar GEFs like p115 were not decreased (10A). Interestingly, p115RhoGEF showed a slight increase in expression in response to LARG knockdown. TNF induction of ICAM-1 expression was preserved with control and LARG shRNA treatment (10B). We also sought to confirm that LARG knockdown did not alter resting junctional permeability. This was examined by assaying electrical impedance (10C). After LARG knockdown there was no RhoA activation in response to ICAM-1 clustering or when force was applied to the ICAM-1 clusters (Figure 11A). The control shRNA-treated ECs exhibited ICAM-1-clustering and force-dependent RhoA activation similar to wild type ECs (Figure 11B).



10: Confirmation of LARG knockdown

HMVECs were treated with control or LARG shRNA lenti-virus for 48 h and selected for with 2.5 ng/ml puromycin for 24 h. (A) EC lysates were immunoblotted with the indicated pAb. (B) Western blotting shows that ICAM-1 expression before and after TNF-treatment is not affected by LARG knockdown. (C) Electrical impedance was used to measure monolayer integrity for HMVECs plated at high density for 72 h. No significant difference was found in impedance values after control or LARG knockdown. n=3 independent experiments preformed in triplicate wells.

To determine if LARG knockdown affected the cellular stiffness at ICAM-1 clusters, we used magnetic tweezers as in Figure 7. We measured the stiffness of the cells with a single pulse of force on ICAM-1 (Figure 11C). The stiffness measured was 50 Pa in control cells compared to 37 Pa in LARG knockdown cells. While there was a reproducible trend of ECs becoming softer after LARG knockdown, this difference was not statistically significant. However, after LARG knockdown there was a loss of the adaptive stiffening at ICAM-1 clusters in response to force after the first pulse. Compared to the control knockdown ECs or untreated cells, LARG knockdown ECs revealed no change in bead displacement between the first and second pulse (Figure 11D).

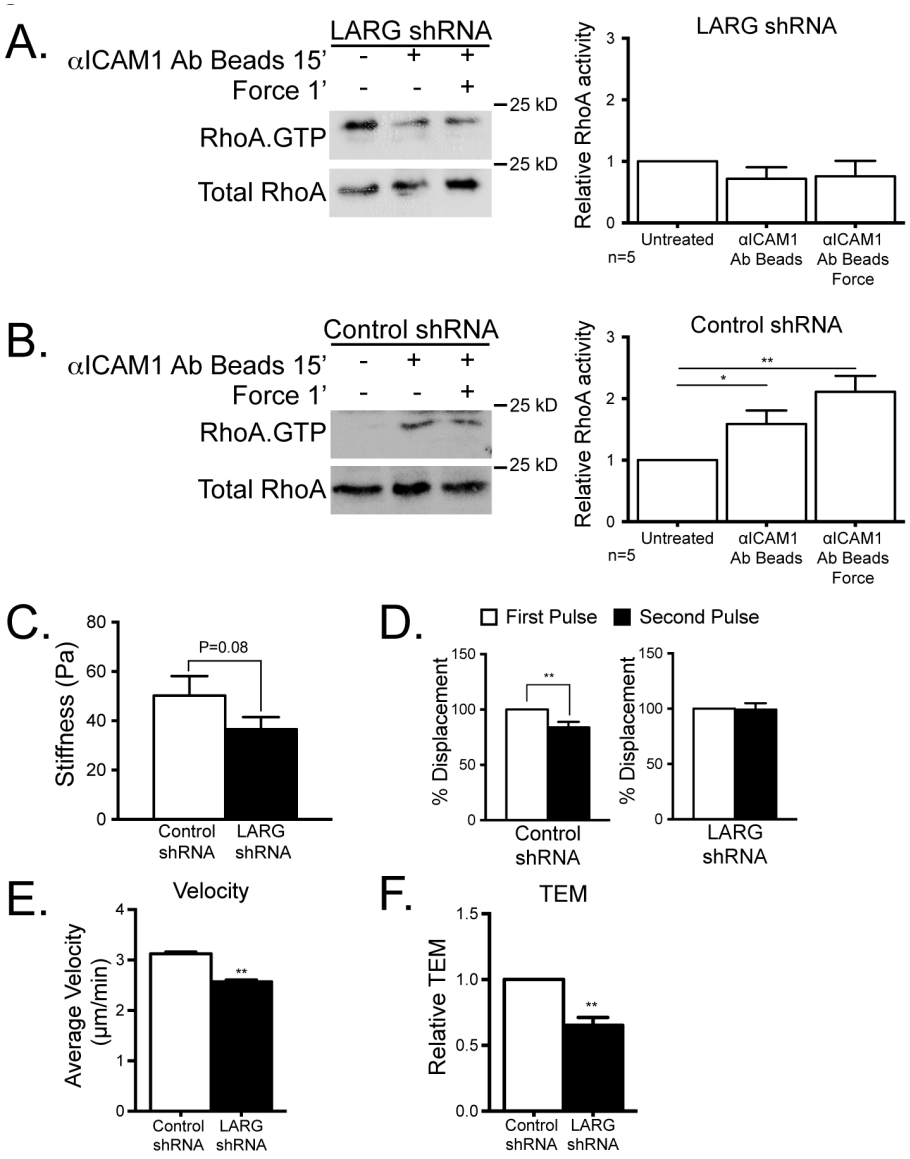


Figure 11: LARG mediates EC response to mechanical force on ICAM-1 and affects neutrophil crawling and TEM

HUVECs were treated with control (B and C) or LARG (A and C) shRNA lenti-virus for 48 h and selected with 2.5 ng/ml puromycin for 24 h, then TNF-treated overnight. (A and B) RhoA activity was determined by immunoblotting after ICAM-1 clustering with or without force in HUVECs (left) and quantified (right). The means ± SEM of ≥4 independent experiments are shown. Asterisk shows p-value of statistical significance by t test (, p≤0.05). (C) The stiffness of HMVECs was measured using magnetic tweezers and magnetic beads coated with ICAM-1 mAb. (D) Relative displacement of magnetic beads coated with ICAM-1 mAbs was measured in control HMVECs or in HMVECs in which LARG expression had been knocked down. The means ± SEM of N≥15 independent bead pulls are shown. Asterisk shows p-value of statistical significance by t test (p≤0.01). (E and F) Neutrophils were added to a monolayer of TNF-treated HMVECs after LARG expression had been knocked down. (E) Neutrophils were imaged as they migrated over the HMVEC monolayer surface and their velocity was measured using tracking software. Data are the average of 3 experiments with ≥15 neutrophils measured per experiment. (F) The passage of neutrophils across a confluent EC monolayer was measured using transwell tissue culture inserts. Data are the average of 3 experiments each performed in duplicate. The means ± SEM are graphed. Asterisk shows p-value of statistical significance (*, p≤0.05; **, p≤0.01).*

To determine whether ICAM-1-induced stiffening might contribute to increased leukocyte migration over the EC surface, we disrupted this pathway by knocking down LARG expression. Neutrophils were plated on a TNF-treated EC monolayers and live cell imaging was used to calculate the average velocity of neutrophil migration. For neutrophils crawling on control knockdown ECs, the average velocity was 3 μm/min, whereas after LARG knockdown in ECs the average velocity decreased to 2.5 μm/min (Figure 11E). Given that leukocytes migrate more rapidly over stiffer surfaces, these results are consistent with LARG-dependent stiffening of ECs induced by neutrophil traction enhancing neutrophil migration over the EC surface.

Endothelial LARG contributes to leukocyte TEM

To determine whether endothelial LARG contributes to neutrophil TEM, we counted and compared the number of neutrophils crossing a control shRNA EC monolayer with the number crossing a monolayer in which LARG expression had been decreased by shRNA. The percentage of leukocytes crossing the EC monolayer after LARG knockdown was decreased by ~35% compared with the control EC monolayer (Figure 11F). These results show that LARG activity in ECs promotes both neutrophil migration over the endothelial surface as well as neutrophil TEM.

Discussion

Leukocyte TEM is an essential step in the recruitment of leukocytes out of the blood circulation and into tissues during inflammation. In order for TEM to

occur, leukocytes must first adhere to the endothelium and this is mediated by receptors on both the leukocyte and ECs. ICAM-1 is a key endothelial receptor which functions as a ligand for β_2 integrins on the surface of leukocytes, promoting leukocyte spreading and migration (6). However, ICAM-1 is more than an adhesive ligand, its engagement and clustering by the leukocyte generates many signals in ECs that promote TEM (22). It is widely considered that increased RhoA activity downstream from ICAM-1 clustering (16, 31) contributes to leukocyte TEM both by weakening the junctions and increasing tension on them to open them (15, 22, 23, 59, 60, 230). At the outset of this work, we were interested in identifying the GEF(s) responsible for RhoA activation downstream of ICAM-1, and secondly, we were interested in determining whether the tractional forces exerted on ICAM-1 as leukocytes migrate affect RhoA signaling. Here we identify LARG as the critical RhoA GEF activating RhoA downstream of ICAM-1, show that it is activated by SFK-dependent tyrosine phosphorylation, and demonstrate that applying mechanical force on ICAM-1 clusters equivalent to the forces generated by migrating neutrophils enhances this signaling pathway. This is the first report of RhoA activation downstream of ICAM-1 being regulated by SFKs. The stiffness of the ECs at ICAM-1 clusters is of great importance as this is where the leukocyte makes contact with the EC, exerts tractional force and senses the EC stiffness. We provide evidence that this activation of RhoA not only promotes neutrophil TEM but stiffens the endothelial surface which may enhance the migration of neutrophils over it.

Our first goal in this work was to identify the GEF(s) downstream from ICAM-1 responsible for activating RhoA. Several RhoA GEFs have been identified in signaling pathways initiated by other cell adhesion molecules. For example, in response to integrin-mediated adhesion on fibronectin, p115 RhoGEF and LARG were found in one study (102) and p190RhoGEF was implicated in another (107). Tension on fibronectin-based adhesions further activated RhoA through LARG and GEF-H1 (101). LARG has also been identified in association with CD44 (231), whereas p114RhoGEF and GEF-H1 have been associated with tight junctions (104, 121). Together with p115RhoGEF and PDZ-RhoGEF, LARG belongs to the RGS family of RhoA GEFs, implying that it can be activated downstream of G protein-coupled receptors via binding to Gα12/13. However, it can also be activated downstream from integrin engagement as well as following mechanical force on integrins (101, 102). In the latter case, activation of LARG was induced by tyrosine phosphorylation, either directly or indirectly by the SFK Fyn (101). Downstream from ICAM-1 clustering and tension on ICAM-1, we observed that LARG was activated and tyrosine phosphorylated in a time course that paralleled RhoA activation and that a SFK inhibitor blocked this response. SFKs not only have been shown to be activated downstream of ICAM-1 signaling (28, 64, 224) but LARG has also been shown to be a substrate of SFKs (101, 232). Knockdown of LARG expression in ECs blocked ICAM-1-mediated activation of RhoA, confirming that LARG is critical for RhoA activation downstream of ICAM-1. We found that p115RhoGEF expression does increase after LARG knockdown. This increase in p115RhoGEF expression is

not sufficient to restore RhoA activity downstream of ICAM-1, however it may well be that p115RhoGEF activity compensates for other signaling pathways in the LARG knockdown cells. Our results strongly suggest a pathway in which clustering of ICAM-1 activates SFKs that phosphorylate and activate LARG. Our results also indicate that mechanical tension on ICAM-1 clusters enhances this pathway leading to higher levels of LARG tyrosine phosphorylation, increased activation and elevated levels of GTP-loaded RhoA. These findings point to LARG as the major regulator of RhoA activity downstream of ICAM-1 signaling.

Previous studies examining endothelial compliance have obtained conflicting results in response to leukocyte adhesion. Initially, using magnetic twisting cytometry to pull on integrins, it was found that clustering of ICAM-1 or adhesion of neutrophils to ECs induced a stiffening response (233, 234). This is of great interest as it is the region of the cell the leukocyte would be in contact with and sensing. In contrast, subsequent work by the same group using atomic force microscopy found transient and localized softening of the endothelial surface in a zone around where neutrophils adhered but an increased stiffening of adjacent cells (235). These differences likely result in part from the different techniques used to measure stiffness, but they may also reflect slight differences in culture conditions with the former favoring paracellular transmigration (i.e. passage through the junctions) and the latter favoring transcellular migration (i.e. passage through the EC body). Atomic force microscopy has also been used to show that treatments, such as TNF, or plating ECs on stiffer substrata, increase EC stiffness (236, 237). While there have been

many studies looking at the role of EC behavior on substrata of different stiffness (237-241), here we observe that ECs alter the stiffness of their points of contact with leukocytes in response to tractional force generated by the leukocyte. The EC response we observe is independent of the type of ECM protein to which the ECs adhere. In the context of leukocyte migration, stiffer substrata have been linked to enhanced leukocyte crawling, adhesion and the generation of stronger tractional forces (226, 227, 242, 243). The finding that mechanical force on ICAM-1 induces a LARG-dependent endothelial stiffening provides a novel mechanism by which leukocytes can manipulate ECs to facilitate leukocyte TEM.

The recruitment of leukocytes from the blood circulation and into tissues is critical in the inflammatory response that contributes to defense of the host organism against invasion by infectious or other foreign agents. However, inappropriate recruitment and activation of leukocytes underlies many acute or chronic inflammatory diseases. In the search for new therapeutic targets to combat inflammatory diseases, strategies to inhibit leukocyte TEM continue to be investigated. Our finding here that inhibiting LARG in ECs decreases TEM, suggests that LARG may be a suitable anti-inflammatory target that is more specific than targeting RhoA activity itself which has diverse functions in many cells. Several studies have recently aimed at developing inhibitors of GEFs including LARG (244, 245), making this an exciting direction to pursue in the future.

Methods and materials

Reagents and antibodies

RhoA mAb and ICAM-1 mAb (western blotting) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mAb against MHC class I (HLA-A, -B, and -C) was purchased from BD Biosciences (Franklin Lakes, NJ). The pAb for LARG (for immunoprecipitation) was purchased from Abcam (Cambridge, MA). Phosphotyrosine mAb, clone 4G10, Y-27632, SU6656, and blebbistatin were purchased from Millipore (Billerica, MA). pAb for p115, and phosphorylated myosin light chain (pMLC) (Thr18/Ser19) were purchased from Cell Signaling (Danvers, MA). pAb for LARG, and PDZ-RhoGEF were made against the c-terminal tail of the proteins (Pocono Rabbit Farm and Laboratory, Canadensis, PA). Recombinant TNF and stromal cell–derived factor-1 (CXCL12) was purchased from R&D Systems (Minneapolis, MN). aICAM-1 R6.5.D6 hybridoma was purchased from ATCC (Manassas, VA). mAb for tubulin, mAb for myosin light chain (MLC), and Cytochalasin D, were purchased from Sigma-Aldrich (St. Louis, MO). pAb for GEFH1was purchased from Bethyl (Montgomery, TX). mAb for myc was purchased from Invitrogen (Grand Island, NY). Bovine Collagen Solution was purchased from Advanced BioMatrix (San Diego, CA). Fibronectin was isolated from human plasma as previously described (102). pAb for p190RhoGEF (Rgnef) was a generous gift from Dr. David D. Schlaepfer (University of California San Diego).

Cell cultures and treatments

Neonatal human dermal blood microvascular ECs (HMVEC), pooled HUVEC, growth medium and supplements were purchased from Lonza (Walkersville, MD). ECs were cultured at 37°C and 5% CO_2. For all experiments, unless otherwise noted, HMVEC were grown on 10 μg/ml collagen until confluent for at least 24 h. For all biochemical experiments at least 10^4 mAb-coated beads were added per cm^2. For all magnetic tweezer experiments at least 10^3 beads were added per cm^2. Static force was applied by placing a ceramic magnet above the tissue culture dish for the specified length of time. Primary human neutrophils were isolated from donor blood drawn by BD Vacutainer ® CPT Cell Preparation Tubes (BD Biosciences) following the manufacture's protocol. Briefly, whole blood was spun for 15 min at 15,000 g. The granulocyte and red blood cell fractions were recovered and the RBCs lysed. The remaining neutrophils were re-suspended in complete EC medium containing 1% Human serum albumin and 1 mM HEPES. Institutional Review Board for University of North Carolina at Chapel Hill has approved all human subject protocols.

mAb-coated beads and force with the permanent magnet

aICAM-1 R6.5.D6 hybridomas were grown in Cell mAb Serum Free Media (BD Biosciences) in CELLine CL-1000 Flasks (BD Biosciences) and the Ig was purified from the hybridoma culture supernatant using a Protein AG UltraLink Resin (Thermo Fisher Scientific, Waltham, MA). Purified mAb was dialyzed in 0.1 M borate

buffer, pH 9.5. Tosyl-activated Dynabeads M-450 Beads (Invitrogen, Carlsbad, CA) were prepared following the manufacturer's protocol. Briefly, beads were washed twice with 0.1 M borate buffer pH 9.5 and incubated with 1 μg/ml mAb per 10^6 beads in 0.1 M borate buffer pH 9.5 at 37^{o}C. After 30 min fatty acid-free BSA was added for a final concentration of 0.01% and rotation continued overnight. For all biochemical experiments, a continuous force (~10 pN) was applied to mAb-coated beads using a permanent ceramic magnetic (K&J Magnetics, Jamison, PA) 1 cm above and parallel to the monolayer of ECs in the tissue culture dish.

Magnetic tweezer force assay

mAb-coated beads were added to ECs for approximately 10-20 min and bead tracking was initiated. Pulses of force (~160 pN) were applied on the beads using the UNC three-dimensional force microscope. The magnetic tweezers were positioned ~25 microns above the monolayer (so as to avoid scraping the underlying monolayer of cells). Force was applied to individual beads at an acute angle. Cells were imaged using an 40x objective (Olympus UplanLN 40x/.75) on an Olympus IX81®-ZDC2 inverted microscope (Olympus) equipped with a high-speed Rolera EM-C2 camera (QImaging) to record bead movement using MetaMorph software at 30 frames per second. Bead movements above 70 nm were tracked by Video Spot Tracker (Center for Computer Integrated Systems for Microscopy and manipulation, http:// http://cismm.cs.unc.edu). Before experiments began, the magnetic tweezer system was calibrated by applying a force ramp to magnetic beads in a Newtonian

fluid of a known viscosity. By recording bead trajectories and computing bead velocities, Stokes law, $F = 6\pi a\eta v$, was used to determine the force, where a is the bead radius, η is the fluid viscosity, and v is the bead velocity. Knowledge of the bead displacement r(t) and the applied force F(t) allowed for computing the compliance signature, $J(t) = 6\pi ar(t)/F(t)$,which was then fit to a modified Kelvin-Voigt mechanical circuit model for viscoelastic liquids. The spring constant was reported as the local stiffness in pascals (Pa).

Preparation of recombinant proteins

pGEX GST-RBD and pGEX GST-RhoA^{G17A} fusion proteins were prepared from lysates from Bl21 *Escherichia coli* cells induced with 100 μM IPTG for 16 h at RT. For GST-RBD, bacterial cells were lysed in 20 mM Tris pH 7.8, 1% Triton 100, 10 mM $MgCl_2$ 1 mM DTT, 1mM PMSF, and 10 μg/ml aprotinin and leupeptin. For GST-RhoA^{G17A} bacterial cells were lysed in 20 mM HEPES pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 1mM PMSF, and 10 μg/ml aprotinin and leupeptin. The recombinant proteins were isolated from the bacterial lysates by incubating with glutathione-Sepharose 4B beads (GE Healthcare) at 4°C for 4 h. The beads were sedimented and washed 3 times in 20 mM HEPES, pH 7.5; 150 mM NaCl, 1 mM DTT.

GST-RBD and GST-RhoA^{G17A} Pull-down Assay

RhoA activation assays were preformed as described (183). HUVECs were lysed in 300 μL of 10 mM $MgCl_2$, 500 mM NaCl, 50 mM Tris, pH 7.8, 1% Triton X-

100, 0.1% SDS, 0.5% deoxycholate, 1mM PMSF, and 10 μg/ml aprotinin and leupeptin, cleared at 14,000g at 4^{o}C for 3 min and incubated with at least 20 μg of GST-RBD for 20 min at 4^{o}C. Beads were then washed 3x in 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% Triton X-100, 1mM PMSF, and 10 μg/ml aprotinin and leupeptin. Active GEFs were assayed by binding to GST-RhoA^{G17A} as described (229). In short it was performed as the RhoA activation assays with the following changes. HUVECs were lysed in 150 mM NaCl, 20 mM HEPES, pH 7.6, 10 mM $MgCl_2$, 1% Triton X-100 1mM PMSF, and 10 μg/ml aprotinin and leupeptin, and incubated with GST-RhoA^{G17A} beads for 60 min at 4^{o}C and washed in the same lysis buffer. Samples were then analyzed by western blotting.

Western Blotting

Samples were run on SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were blocked and incubated with the specified primary antibodies followed by species-specific secondary antibodies conjugated with horseradish peroxidase. Blots were developed with a chemiluminescent HRP substrate, and visualized on x-ray film. For quantification, blots were scanned and the intensity values determined using Image J software (NIH) and protein levels were normalized to control protein levels. All quantification graphs include ≥3 independent experiments. Error bars represent SEM.

Phase contrast microscopy

HUVECs were grown on collagen-coated coverslips for at least 72 h before imaging. HUVECs were treated with 5 ng/ml TNF overnight. Beads were added for 15 min then unbound beads were washed off. Cells were fixed with 3.7% paraformaldehyde and mounted on coverslips. Coverslips were then imaged using a 20x objective (Zeiss plan-Apochromat 20x/ 0.8) with a Zeiss axiovert 200 M microscope with a Hamamatsu ORCA-ERAG digital camera and MetaMorph software.

Immunoprecipitation

HUVECs were lysed on ice for 30 min in lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, containing 1mM PMSF, and 10 μg/ml aprotinin and leupeptin, and 10 μg/ml orthovanadate). Lysates were precleared with protein A/G-agarose beads, and then incubated with LARG pAb overnight at 4 °C. A/G-agarose beads added for 1 h at 4 °C, then were sedimented and washed in lysis buffer at 4°C, resuspended in sample buffer, boiled for 10 min, and analyzed by Western blotting.

Viral shRNA Knockdown of protein expression

The targeted sequence for LARG was GCGAGTATCCAGAGAAGGAAT and prepared by the UNC lenti-shRNA core. ECs were grown to 80% confluency and infected with the lowest amount of viral particles to ensure sufficient knockdown at 48 h. Infected cells were then selected with 2.5 μg/ml puromycin for 24 h. For

biochemical experiments, ECs were allowed to grow until confluent. For imaging experiments ECs were re-plated at confluence. RhoA knockdown re-expression was achieved using adenovirus miRNA and WT RhoA re-expression as previously described (246). After knockdown and RhoA rescue, cells were re-plated at a high density for experimental assays.

Electrical impedance measurement of monolayer integrity

After puromycin selection, high density HMVECs were plated on SIM plates (Roche Applied Science) coated with 6 μg/ml collagen. Electrical impedance was measured using the xCELLigence Real-Time Cell Analyzer (RTCA) system (Roche Applied Science). Each experiment was performed in triplicate and repeated 3 times.

Live-cell imaging

HMVECs were grown on collagen-coated glass dishes (MatTek Corporation) for at least 72 h before imaging. HMVECs were treated with 5 ng/ml TNF overnight. Neutrophils were added for 10 min then unbound cells were washed off. Cells were then imaged at 37°C and 5% CO_2 using a 20x objective (Zeiss plan-Apochromat 20x/ 0.8) with a Zeiss axiovert 200 M microscope with a Hamamatsu ORCA-ERAG digital camera and MetaMorph software for 30 min in EC growth medium. A manual tracking plug-in (http://rsbweb.nih.gov/ij/plugins/track/ track.html) for Image J software (NIH) was used to determine cell migration velocities.

TEM assay

TEM assays were performed in transwell plates (Corning, Corning, New York, U.S.) of 6.5-mm diameter with 8-µm pore filters. HMVECs were plated to generate a confluent monolayer on collagen-coated transwell filters and treated with 5 ng/ml TNF overnight. Neutrophils were added to the upper chamber and allowed to migrate across the monolayer to 25 ng/ml CXCL12 for 1 h at 37°C and 5% CO_2. The filters were fixed, stained with DAPI and neutrophils that had migrated to the lower chamber were counted by fluorescence microscopy using a 20x objective (Zeiss plan-Apochromat 20x/ 0.8) with a Zeiss axiovert 200 M microscope with a Hamamatsu ORCA-ERAG digital camera and MetaMorph software. The experiment was preformed four times in duplicate.

Statistical analysis

Statistical significance of data was determined using a two-tailed unpaired *t* test.

Chapter 4: CONCLUSIONS AND FUTURE DIRECTIONS

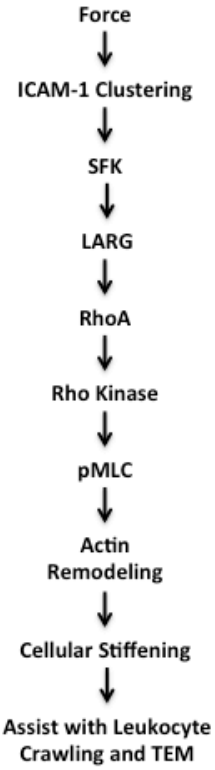
Summary

At the outset of this research, we had two goals. First, we sought to identify a role for mechanical force in ICAM-1 signaling, and second to identify the GEF responsible for the RhoA activation. There is a growing body of research looking the role for mechanical force on cells leading to RhoA activation (reviewed in Chapter 2). The results presented in Chapter 3 illustrate that mechanical force on ICAM-1 alters EC stiffness via the RhoA pathway (Figure 12). Mechanical force on ICAM-1 leads to an increase in RhoA activity and actomyosin contraction via the ROCK pathway causing cells to become stiffer. The increase in cellular stiffness at ICAM-1 clusters increases leukocyte crawling velocity and TEM. We also identified LARG as the upstream GEF regulating RhoA activity after ICAM-1 clustering. Applying mechanical force on ICAM-1 clusters leads to a further increase in LARG activation and phosphorylation. After depleting cells of LARG, there was a decrease in leukocyte crawling velocity and TEM, as well as a loss of the adaptive cellular stiffening after pulses of force on ICAM-1.

These findings are valuable as they look at a role of ICAM-1 signaling in ECs exposed to physiological forces. Previous studies looking at ICAM-1 signaling used static clustering of ICAM-1 and neglected the role of tractional forces generated by

the crawling leukocyte. We document that these forces are important for EC signaling during leukocyte TEM.

Figure 12:The Pathway downstream from mechanical force on ICAM-1



Substratum Stiffness

Pulling on ICAM-1, like a leukocyte would, induces EC stiffening as presented in chapter 3. Knocking down LARG prevents the adaptive stiffening response. Also, we observe that the leukocyte crawling velocity is reduced as well as leukocyte TEM (Figure 11). This is assumed to be a result of a suboptimal compliance of the EC for the crawling leukocyte. While substratum compliance has previously been shown to alter cell migration (226, 227, 242, 243), the effect of the compliance of an EC on crawling leukocytes has not been examined. Even measuring the EC compliance during leukocyte TEM or ICAM-1 clustering is difficult. Conflicting observations about how EC compliance changes are likely due to varying methods and locations on the EC surface used to determine the compliance (233-235), as discussed in Chapter 3. Based on the work documented in Chapter 3, we would predict that leukocytes crawling on ICAM-1 prefer a stiffer substratum. Addressing this question, proved to be more difficult than expected. Plates coated with a polyacrylamide hydrogel of different compliances ranging from 0.2-50 kPa are commercially available and easily coated with recombinant extracellular ICAM-1.

However, leukocyte adhesion and spreading is greatly affected by stiffness and concentration of ICAM-1 coating. I found that leukocytes did not adhere or spread similarly with a constant concentration of ICAM-1 on substrates of different compliance. Leukocytes either did not adhere or if they adhered did not crawl despite being in the presence of a chemoattractant CXCL12. Various conditions were tested included plates with a compliance of 8, 12, 25, or 50 kPa and ICAM-1 concentration ranging between 10-100 ng/ml. Lowering the coating concentration of ICAM-1 was able to induce some leukocyte crawling on the stiffer plates (12-50 kPa) but using the same concentration on a softer plate was insufficient for leukocytes to adhere and spread. However due to the cost of the dishes, a limited number of attempts at this experiment were made. Notably, this *in vitro* system to mimic the surface of an EC lacks other adhesion receptors that are normally present. It is likely that there is cross-talk among the adhesion receptors. Adding other adhesion molecules or more extensive testing of conditions could improve leukocyte initial adhesion, allowing the subsequent crawling velocities to be compared. Although, it is possible that regardless of the conditions, a softer substratum will always impair leukocyte adhesion. The results in Figure 11 show the initial stiffness between control and LARG knockdown was not statistically different (Figure 11E). There is a change in stiffness with the second pulse (Figure 11F). There is not a leukocyte adhesion defect to ECs lacking LARG. Perhaps this is because LARG knockdown does not change the initial compliance. To accurately address this question using an artificial substratum we need to have dishes with an adjustable compliance.

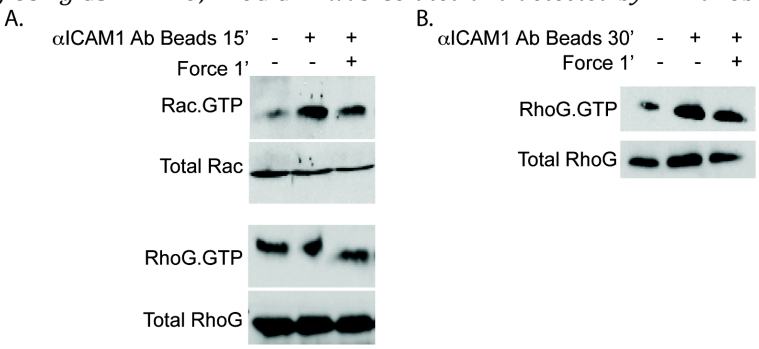
Although, these results do show that substratum stiffness regulates leukocyte adhesion to ICAM-1 coated surfaces. Without being able to keep the coating concentration of ICAM-1 constant and have similar initial leukocyte adhesion and spreading, it is impossible to look at the later stage of leukocyte crawling and to compare velocities in a meaningful way.

Other Rho Family GTPase

While RhoA is the most-well studied Rho GTPase downstream of ICAM-1, Rac and RhoG are also involved in ICAM-1 signaling. van Buul *et al*. documented that Rac and RhoG are activated after ICAM-1 clustering (30). Rac activation occurs 10 min after clustering whereas RhoG activity peaks later at 30 min. Looking at the time points in Figure 8A, Rac and RhoG activation are unchanged with mechanical force compared to clustering alone (Figure 13A). Clustering alone did increase Rac activation. Since peak RhoG activation is delayed after ICAM-1 clustering, a longer time point of 30 min of ICAM-1 clustering is also considered (Figure 13B). While RhoG activity is stimulated after longer periods of ICAM-1 clustering, there is no further increase with the application of force for 1 min. These results suggest that the force response affects RhoA and does not involve Rac or RhoG. This is intriguing because RhoA appears to be upstream of ICAM-1-dependent RhoG activity (30). RhoA could be a central regulator in directing leukocyte diapedesis via paracellular or transcellular migration. Additionally, it is possible that Rac or RhoG might be critical in the recovery response. While not studied here, recovery and junction

reformation is vital during acute damage or inflammation. It is possible for Rac, and maybe RhoG, to be involved in this later process.

Figure 13: Mechanical force on ICAM-1 does not alter other Rho family GTPases
Magnetic beads coated with mAb against ICAM-1 (A, B) were added for 15 min (A) or 30 min (B) to a monolayer of TNF-treated HUVECs and ~10 pN force was applied with a ceramic magnet placed above the cells for 1 min. (A) Using GST-PBD or GST-ELMO, Rac.GTP or RhoG.GTP, were isolated and detected by immunoblotting. (B) Using GST-ELMO, RhoG.GTP was isolated and detected by immunoblotting.



LARG Signaling

We showed that endothelial LARG mediates ICAM-1 signaling leading to RhoA activation, and that this contributes to leukocyte crawling and TEM. Traditionally, GPCR signaling activates LARG (45). Our research is among a growing body of work showing LARG regulating RhoA activity downstream of adhesion molecules (102) and mechanical force (101, 228). However, the exact mechanism leading to LARG dependent cellular stiffening appears to be different depending on the adhesion receptor. Guilluy *et al*. show that LARG is downstream of integrin signaling mediated by the src family kinase Fyn to induce cellular stiffening (101). Collins *et al.* show that LARG is involved in PECAM-1 signaling. Hemodynamic forces on PECAM-1 lead to phosphatidylinositol 3-kinase-dependent signaling to integrins, RhoA activation, new focal adhesion formation and cellular stiffening (228). LARG activity downstream of ICAM-1 is not dependent on engagement of specific integrins as the same response is seen with ECs adhering to

fibronectin and collagen (Figure 7 B and C), instead it is likely a more local response at the site of ICAM-1 clustering. Also, GEF-H1 does not appear to be involved in ICAM-1 signaling (Figure 9D). These differences suggest that adhesion receptors use different signaling pathways to activate RhoA but many of these may converge on LARG to increase RhoA activity. This is expanding the role of LARG beyond GPCR signaling. However, much about LARG signaling remains unknown.

LARG as a Mechanosensor

The repeated finding of LARG being activated by mechanical force is intriguing. Is LARG itself a mechanosensor? Mechanical force on the ICAM-1 complex, or on other adhesion complexes, might physically alter LARG and expose new binding sites within this GEF. Given that LARG is activated downstream of force application on many adhesion receptors, this suggests that LARG might be a mechanotransducer. The exact signaling pathway leading to LARG activity after mechanical force varies depending on the surface receptor. Applying force on LARG might expose new phosphorylation sites or binding sites allowing it to become more active. Although, it is equally possible that an intermediate mechanosenstive protein in the ICAM-1 complex becomes more activated, increasing Src or a SFK activity which then increases LARG phosphorylation.

Knockout mice

The results presented in Chapter 3 show a new role of LARG in leukocyte TEM, however these findings have not been confirmed *in vivo.* The first publication examining mice lacking LARG found a conditional knockdown of LARG in smooth

muscle cells were deficient in MLC signaling in smooth muscle cells leading to an prevention of ca+ included hypertension (247). This RhoA signaling pathway is most likely downstream of G_{12}-G_{13}, as mice deficient in G_{12}-G_{13} have a similar phenotype(247). Despite LARG having distinct expression during development and widespread expression in adult tissues (248), the initial report of LARG deficient mice reported no obvious phenotype (247). A further analysis found while mice lacking LARG appear to be normal, crossing *Larg*$^{+/-}$ mice with *Larg*$^{+/-}$ mice produce fewer *Larg*$^{-/-}$ births than expected suggesting that LARG deficiency might be lethal but with compensation from other GEFs or via another mechanism a subset of mice can still develop normally (38). To further support this, mice lacking both LARG and its close family member PDZ-RhoGEF are not viable beyond E10.5, likely due to a developmental defect in blood vessel formation (38). To date, there have been no reports examining leukocyte TEM in the LARG deficient mice or a conditional double LARG and PDZ-RhoGEF deficient mice.

The *in vivo* role of ICAM-1 in leukocyte TEM has been measured by injecting pro-inflammatory reagents and measuring leukocyte migration into tissues using ICAM-1 null mice (249). Alternatively, there are more physiological models, like pathogenic infections (89, 250, 251) or atherosclerosis models (252-254), using mice with or without ICAM-1 to measure ICAM-1-dependent leukocyte TEM. A similar approach could be used with the LARG-deficient mice to confirm *in vivo* the *in vitro* results in chapter 2.

However, using the LARG-deficient mice with any of these models could be problematic and perhaps provide conflicting results for several reasons. One reason is that while LARG appears to be involved in vascular biology it is not EC specific (38, 247, 248). The role of LARG in leukocytes has been well establish as LARG is named Leukemia-associated Rho guanine nucleotide exchange for its role in acute myelogenous leukemia by genetic rearrangement between *LARG* and *mixed lineage leukemia* (MLL) genes to express a LARG-MLL fusion protein (255). Therefore, to study LARG *in vivo* either an EC-specific LARG knockout mouse needs to be developed or the LARG null mouse needs to be reconstituted with wildtype leukocytes. There is a chance that the remaining LARG-deficient leukocytes could confound the results.

The other complicating factor with looking at the role of LARG *in vivo*, is the compensation from other GEFs. PDZ-RhoGEF and LARG deficient mice have a more dramatic phenotype than the single knockdown (38). Leukocyte TEM is a conserved process for both the innate and adaptive immune response, which can be critical for survival. Therefore, there is a high chance of compensation with other GEFs like PDZ-RhoGEF, p115RhoGEF or other RhoA GEFs. It is assumed the LARG deficient mice that make it to adulthood already have a compensatory mechanism occurring. Therefore it is reasonable to predict that the LARG deficient mice will lack a noticeable defect in leukocyte TEM. An EC specific single or double GEF knockout mouse would be a better model. There is still a chance the EC specific double GEF knockout mouse might not be viable since the global knockout

has a lethal vascular defect (38). These experiments would be the next step for this project to confirm a role for LARG *in vivo* for leukocyte TEM. However, this is not a trivial undertaking and could be a lengthy endeavor, which is why I was unable to address the matter.

Role in Cell Spreading

The regulation of cell shape by LARG is a finding not discussed in Chapter 3. After LARG knockdown, the overall spreading area of ECs increased (Figure 14A, B). To date, these observations have not been methodically studied. Preliminary results point to a spreading defect in ECs lacking LARG. After LARG knockdown, there is no observed change in junction formation (Figure 9). Also, we saw normal endothelial cell-cell junctions measured by β-Catenin staining (Figure 14A). ECs after control or LARG knockdown have on average the same diameter in suspension (Figure 14C). Normally when ECs become a monolayer they continue to divide for a short period of time to allow a tightly packed monolayer to develop. Based on my observations, I suspect that after LARG knockdown cells become confluent they stop proliferating. In subconfluent cells there does not appear to be an initial proliferation defect (Figure 14D and E). However, there is a published report that LARG knockdown in Hela cells results in a cell division defect (256). All of the observations presented in Chapter 3 and 4 use ECs right after LARG knockdown. Typically, cells were treated with lenti-shRNA virus in the experimental dish at near confluency or trypsized a single time then plated at high density in the experimental dishes. After, long-term knockdown of LARG, ECs did stop growing. However, since Hela cells

grow faster and continue to divide after confluency, this might explain why we did not see cell death and apoptosis in our experiments.

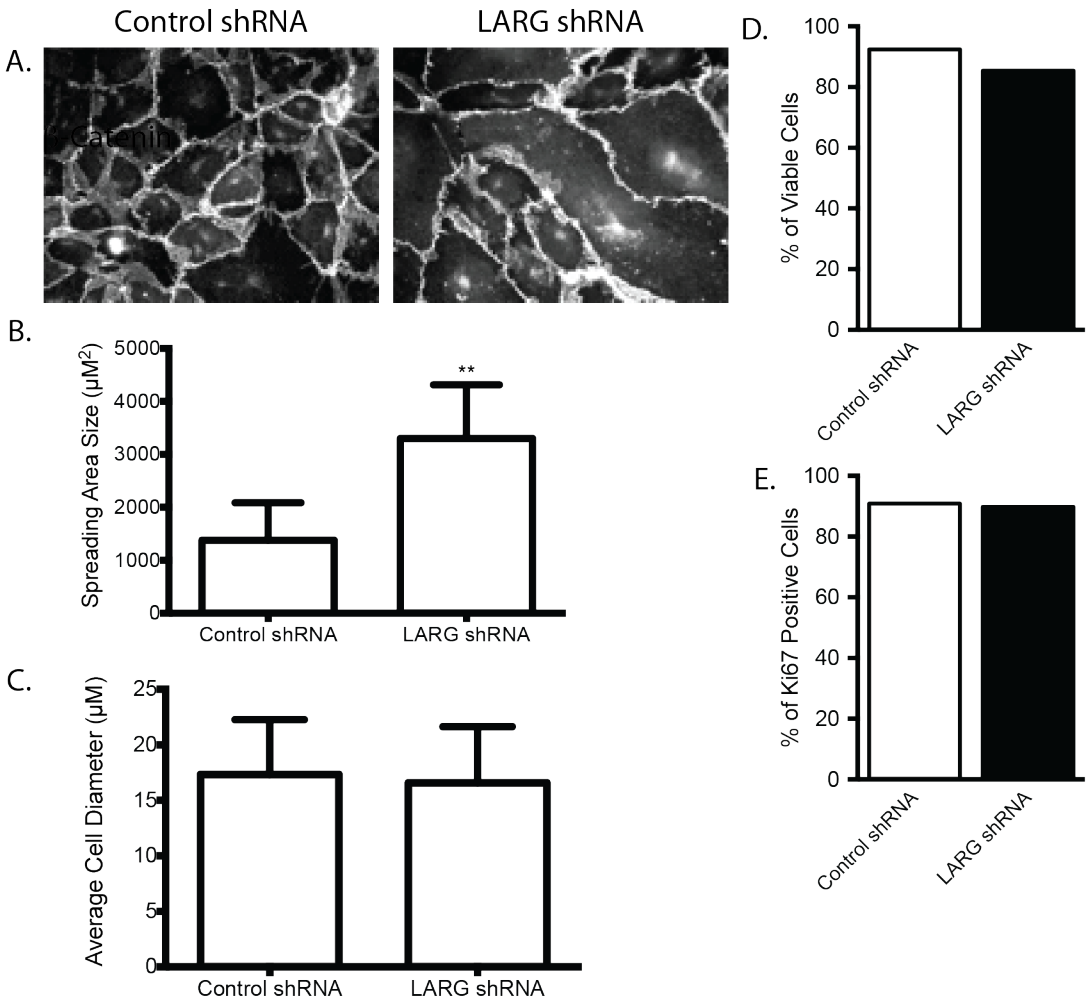


Figure 14: LARG knockdown alters cells spreading
HUVECs were treated with control or LARG shRNA lenti-virus for 48 h and selected with 2.5 ng/ml puromycin for 24 h. (A) ECs were than plated on fibronectin coated coverslips for 48 hoursCells were fixed then stain for β-Catenin. (B) The average area was calculated and graphed. The means ± SEM of ≥6 fields of view. Asterisk shows p-value of statistical significance by t test (, p≤0.05). C) ECs were trypsinized and the mean diameter was calculated. The means ± SEM of ≥4 fields of view. (D and E).*

Conclusions

We have confirmed that mechanical force on ICAM-1, like that of a crawling leukocyte, leads to changes in ECs via the RhoA pathway. Specifically, we see that RhoA activity is increased in response to force on ICAM-1 clusters, as is cellular

stiffening. We have determined that the GEF responsible is LARG. This is the first report of endothelial LARG regulating leukocyte crawling and TEM.

The work presented in this thesis has focused on signaling downstream of ICAM-1 and mechanical force. During leukocyte TEM, multiple adhesion receptors are engaged and receptor cross-talk occurs. Do other adhesion receptors respond to leukocyte generated mechanical force? It is likely that other adhesion receptors which bind the moving leukocyte will response to the physical force generated by the leukocyte. While E-selectin and V-CAM-1 are obvious candidates, there are also less well studied receptors like Thy1 (CD90) that should be considered. Thy1 is expressed on the surface of ECs during inflammation (257). Thy-1 is also implicated in leukocyte TEM (257-259). and Rho GTPase signaling (260-262). Also, does force on one of these receptors, like ICAM-1, alter the signaling pathways of other adhesion receptors. By expanding on this work to include other EC adhesion receptors we can start to fully understand the signaling that occurs during leukocyte TEM.

Historically, when we try to work out the signaling mechanism in an *in vitro* system we frequently neglect how the cell's 3D environment might be contributing to the signaling pathways. Cells *in vivo* are constantly experiencing physical forces generated by cell tension, neighboring cells, or other external cues like hemodynamic forces. It is important not to overlook the role these forces will have on the cellular behavior.

REFERENCES

1. Bevilacqua, M. P., S. Stengelin, M. A. Gimbrone, Jr., and B. Seed. 1989. Endothelial leukocyte adhesion molecule 1: an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science* 243: 1160-1165.

2. Pober, J. S., M. A. Gimbrone, Jr., L. A. Lapierre, D. L. Mendrick, W. Fiers, R. Rothlein, and T. A. Springer. 1986. Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon. *J Immunol* 137: 1893-1896.

3. Pober, J. S., L. A. Lapierre, A. H. Stolpen, T. A. Brock, T. A. Springer, W. Fiers, M. P. Bevilacqua, D. L. Mendrick, and M. A. Gimbrone, Jr. 1987. Activation of cultured human endothelial cells by recombinant lymphotoxin: comparison with tumor necrosis factor and interleukin 1 species. *J Immunol* 138: 3319-3324.

4. Dustin, M. L., R. Rothlein, A. K. Bhan, C. A. Dinarello, and T. A. Springer. 1986. Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). *J Immunol* 137: 245-254.

5. Foxall, C., S. R. Watson, D. Dowbenko, C. Fennie, L. A. Lasky, M. Kiso, A. Hasegawa, D. Asa, and B. K. Brandley. 1992. The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis(x) oligosaccharide. *J Cell Biol* 117: 895-902.

6. Marlin, S. D., and T. A. Springer. 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). *Cell* 51: 813-819.

7. Staunton, D. E., M. L. Dustin, H. P. Erickson, and T. A. Springer. 1990. The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. *Cell* 61: 243-254.

8. Diamond, M. S., D. E. Staunton, S. D. Marlin, and T. A. Springer. 1991. Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell* 65: 961-971.

9. Smith, C. W., R. Rothlein, B. J. Hughes, M. M. Mariscalco, H. E. Rudloff, F. C. Schmalstieg, and D. C. Anderson. 1988. Recognition of an endothelial determinant for CD 18-dependent human neutrophil adherence and transendothelial migration. *The Journal of clinical investigation* 82: 1746-1756.

10. Elices, M. J., L. Osborn, Y. Takada, C. Crouse, S. Luhowskyj, M. E. Hemler, and R. R. Lobb. 1990. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* 60: 577-584.

11. Milstone, D. S., D. Fukumura, R. C. Padgett, P. E. O'Donnell, V. M. Davis, O. J. Benavidez, W. L. Monsky, R. J. Melder, R. K. Jain, and M. A. Gimbrone, Jr. 1998. Mice lacking E-selectin show normal numbers of rolling leukocytes but reduced leukocyte stable arrest on cytokine-activated microvascular endothelium. *Microcirculation* 5: 153-171.

12. Yoshida, M., W. F. Westlin, N. Wang, D. E. Ingber, A. Rosenzweig, N. Resnick, and M. A. Gimbrone, Jr. 1996. Leukocyte adhesion to vascular endothelium induces E-selectin linkage to the actin cytoskeleton. *J Cell Biol* 133: 445-455.

13. Dustin, M. L., and T. A. Springer. 1988. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. *J Cell Biol* 107: 321-331.

14. Wojciak-Stothard, B., L. Williams, and A. J. Ridley. 1999. Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. *J Cell Biol* 145: 1293-1307.

15. Adamson, P., S. Etienne, P. O. Couraud, V. Calder, and J. Greenwood. 1999. Lymphocyte migration through brain endothelial cell monolayers involves signaling through endothelial ICAM-1 via a rho-dependent pathway. *J Immunol* 162: 2964-2973.

16. Thompson, P. W., A. M. Randi, and A. J. Ridley. 2002. Intercellular adhesion molecule (ICAM)-1, but not ICAM-2, activates RhoA and stimulates c-fos and rhoA transcription in endothelial cells. *J Immunol* 169: 1007-1013.

17. Carman, C. V., C. D. Jun, A. Salas, and T. A. Springer. 2003. Endothelial cells proactively form microvilli-like membrane projections upon intercellular adhesion molecule 1 engagement of leukocyte LFA-1. *J Immunol* 171: 6135-6144.

18. Carman, C. V., and T. A. Springer. 2004. A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. *J Cell Biol* 167: 377-388.

19. Yang, L., R. M. Froio, T. E. Sciuto, A. M. Dvorak, R. Alon, and F. W. Luscinskas. 2005. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood* 106: 584-592.

20. Carman, C. V., P. T. Sage, T. E. Sciuto, M. A. de la Fuente, R. S. Geha, H. D. Ochs, H. F. Dvorak, A. M. Dvorak, and T. A. Springer. 2007. Transcellular diapedesis is initiated by invasive podosomes. *Immunity* 26: 784-797.

21. Oh, H. M., S. Lee, B. R. Na, H. Wee, S. H. Kim, S. C. Choi, K. M. Lee, and C. D. Jun. 2007. RKIKK motif in the intracellular domain is critical for spatial and dynamic organization of ICAM-1: functional implication for the leukocyte adhesion and transmigration. *Mol Biol Cell* 18: 2322-2335.

22. Wittchen, E. S. 2009. Endothelial signaling in paracellular and transcellular leukocyte transmigration. *Frontiers in bioscience : a journal and virtual library* 14: 2522-2545.

23. Aghajanian, A., E. S. Wittchen, M. J. Allingham, T. A. Garrett, and K. Burridge. 2008. Endothelial cell junctions and the regulation of vascular permeability and leukocyte transmigration. *Journal of thrombosis and haemostasis : JTH* 6: 1453-1460.

24. Muller, W. A. 2011. Mechanisms of leukocyte transendothelial migration. *Annual review of pathology* 6: 323-344.

25. Gotsch, U., E. Borges, R. Bosse, E. Boggemeyer, M. Simon, H. Mossmann, and D. Vestweber. 1997. VE-cadherin antibody accelerates neutrophil recruitment in vivo. *J Cell Sci* 110 (Pt 5): 583-588.

26. Corada, M., M. Mariotti, G. Thurston, K. Smith, R. Kunkel, M. Brockhaus, M. G. Lampugnani, I. Martin-Padura, A. Stoppacciaro, L. Ruco, D. M. McDonald, P. A.

Ward, and E. Dejana. 1999. Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. *Proc Natl Acad Sci U S A* 96: 9815-9820.

27. Schulte, D., V. Kuppers, N. Dartsch, A. Broermann, H. Li, A. Zarbock, O. Kamenyeva, F. Kiefer, A. Khandoga, S. Massberg, and D. Vestweber. 2011. Stabilizing the VE-cadherin-catenin complex blocks leukocyte extravasation and vascular permeability. *EMBO J* 30: 4157-4170.

28. Allingham, M. J., J. D. van Buul, and K. Burridge. 2007. ICAM-1-mediated, Src- and Pyk2-dependent vascular endothelial cadherin tyrosine phosphorylation is required for leukocyte transendothelial migration. *J Immunol* 179: 4053-4064.

29. Mamdouh, Z., A. Mikhailov, and W. A. Muller. 2009. Transcellular migration of leukocytes is mediated by the endothelial lateral border recycling compartment. *J Exp Med* 206: 2795-2808.

30. van Buul, J. D., M. J. Allingham, T. Samson, J. Meller, E. Boulter, R. Garcia-Mata, and K. Burridge. 2007. RhoG regulates endothelial apical cup assembly downstream from ICAM1 engagement and is involved in leukocyte trans-endothelial migration. *J Cell Biol* 178: 1279-1293.

31. Etienne, S., P. Adamson, J. Greenwood, A. D. Strosberg, S. Cazaubon, and P. O. Couraud. 1998. ICAM-1 signaling pathways associated with Rho activation in microvascular brain endothelial cells. *J Immunol* 161: 5755-5761.

32. Rossman, K. L., C. J. Der, and J. Sondek. 2005. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat Rev Mol Cell Biol* 6: 167-180.

33. Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 70: 401-410.

34. Ridley, A. J., and A. Hall. 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* 70: 389-399.

35. Nobes, C. D., and A. Hall. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81: 53-62.

36. Nobes, C. D., and A. Hall. 1995. Rho, rac and cdc42 GTPases: regulators of actin structures, cell adhesion and motility. *Biochemical Society transactions* 23: 456-459.

37. Arthur, W. T., S. M. Ellerbroek, C. J. Der, K. Burridge, and K. Wennerberg. 2002. XPLN, a guanine nucleotide exchange factor for RhoA and RhoB, but not RhoC. *J Biol Chem* 277: 42964-42972.

38. Mikelis, C., T. Palmby, M. Simaan, W. Li, R. Szabo, R. Lyons, D. Martin, H. Yagi, S. Fukuhara, H. Chikumi, R. Galisteo, Y.-S. Mukouyama, T. Bugge, and J. Gutkind. 2013. PDZ-RhoGEF and LARG are essential for embryonic development and provide a link between thrombin and LPA receptors and Rho activation. *The Journal of biological chemistry* 288: 12232-12243.

39. Fujikawa, K., A. V. Miletic, F. W. Alt, R. Faccio, T. Brown, J. Hoog, J. Fredericks, S. Nishi, S. Mildiner, S. L. Moores, J. Brugge, F. S. Rosen, and W. Swat. 2003. Vav1/2/3-null mice define an essential role for Vav family proteins in lymphocyte development and activation but a differential requirement in MAPK signaling in T and B cells. *J Exp Med* 198: 1595-1608.

40. Turner, M., P. J. Mee, A. E. Walters, M. E. Quinn, A. L. Mellor, R. Zamoyska, and V. L. Tybulewicz. 1997. A requirement for the Rho-family GTP exchange factor Vav in positive and negative selection of thymocytes. *Immunity* 7: 451-460.

41. Tedford, K., L. Nitschke, I. Girkontaite, A. Charlesworth, G. Chan, V. Sakk, M. Barbacid, and K. D. Fischer. 2001. Compensation between Vav-1 and Vav-2 in B cell development and antigen receptor signaling. *Nat Immunol* 2: 548-555.

42. Tarakhovsky, A., M. Turner, S. Schaal, P. J. Mee, L. P. Duddy, K. Rajewsky, and V. L. Tybulewicz. 1995. Defective antigen receptor-mediated proliferation of B and T cells in the absence of Vav. *Nature* 374: 467-470.

43. Doody, G. M., S. E. Bell, E. Vigorito, E. Clayton, S. McAdam, R. Tooze, C. Fernandez, I. J. Lee, and M. Turner. 2001. Signal transduction through Vav-2 participates in humoral immune responses and B cell maturation. *Nat Immunol* 2: 542-547.

44. O'Brien, S. P., K. Seipel, Q. G. Medley, R. Bronson, R. Segal, and M. Streuli. 2000. Skeletal muscle deformity and neuronal disorder in Trio exchange factor-deficient mouse embryos. *Proc Natl Acad Sci U S A* 97: 12074-12078.

45. Fukuhara, S., H. Chikumi, and J. S. Gutkind. 2000. Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho. *FEBS Lett* 485: 183-188.

46. Abe, K., K. L. Rossman, B. Liu, K. D. Ritola, D. Chiang, S. L. Campbell, K. Burridge, and C. J. Der. 2000. Vav2 is an activator of Cdc42, Rac1, and RhoA. *J Biol Chem* 275: 10141-10149.

47. Debant, A., C. Serra-Pages, K. Seipel, S. O'Brien, M. Tang, S. H. Park, and M. Streuli. 1996. The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac-specific and rho-specific guanine nucleotide exchange factor domains. *Proc Natl Acad Sci U S A* 93: 5466-5471.

48. Garrett, M. D., A. J. Self, C. van Oers, and A. Hall. 1989. Identification of distinct cytoplasmic targets for ras/R-ras and rho regulatory proteins. *J Biol Chem* 264: 10-13.

49. Wojciak-Stothard, B., A. Entwistle, R. Garg, and A. J. Ridley. 1998. Regulation of TNF-alpha-induced reorganization of the actin cytoskeleton and cell-cell junctions by Rho, Rac, and Cdc42 in human endothelial cells. *J Cell Physiol* 176: 150-165.

50. Saito, H., Y. Minamiya, S. Saito, and J. Ogawa. 2002. Endothelial Rho and Rho kinase regulate neutrophil migration via endothelial myosin light chain phosphorylation. *J Leukoc Biol* 72: 829-836.

51. Amano, M., M. Ito, K. Kimura, Y. Fukata, K. Chihara, T. Nakano, Y. Matsuura, and K. Kaibuchi. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* 271: 20246-20249.

52. Kimura, K., M. Ito, M. Amano, K. Chihara, Y. Fukata, M. Nakafuku, B. Yamamori, J. Feng, T. Nakano, K. Okawa, A. Iwamatsu, and K. Kaibuchi. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 273: 245-248.

53. Burridge, K., and M. Chrzanowska-Wodnicka. 1996. Focal adhesions, contractility, and signaling. *Annual review of cell and developmental biology* 12: 463-518.

54. Maekawa, M., T. Ishizaki, S. Boku, N. Watanabe, A. Fujita, A. Iwamatsu, T. Obinata, K. Ohashi, K. Mizuno, and S. Narumiya. 1999. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 285: 895-898.

55. Watanabe, N., T. Kato, A. Fujita, T. Ishizaki, and S. Narumiya. 1999. Cooperation between mDia1 and ROCK in Rho-induced actin reorganization. *Nat Cell Biol* 1: 136-143.

56. Tilghman, R. W., and R. L. Hoover. 2002. E-selectin and ICAM-1 are incorporated into detergent-insoluble membrane domains following clustering in endothelial cells. *FEBS Lett* 525: 83-87.

57. Lyck, R., Y. Reiss, N. Gerwin, J. Greenwood, P. Adamson, and B. Engelhardt. 2003. T-cell interaction with ICAM-1/ICAM-2 double-deficient brain endothelium in vitro: the cytoplasmic tail of endothelial ICAM-1 is necessary for transendothelial migration of T cells. *Blood* 102: 3675-3683.

58. Greenwood, J., C. L. Amos, C. E. Walters, P. O. Couraud, R. Lyck, B. Engelhardt, and P. Adamson. 2003. Intracellular domain of brain endothelial intercellular adhesion molecule-1 is essential for T lymphocyte-mediated signaling and migration. *J Immunol* 171: 2099-2108.

59. Sans, E., E. Delachanal, and A. Duperray. 2001. Analysis of the roles of ICAM-1 in neutrophil transmigration using a reconstituted mammalian cell expression model: implication of ICAM-1 cytoplasmic domain and Rho-dependent signaling pathway. *J Immunol* 166: 544-551.

60. Strey, A., A. Janning, H. Barth, and V. Gerke. 2002. Endothelial Rho signaling is required for monocyte transendothelial migration. *FEBS Lett* 517: 261-266.

61. Carpen, O., P. Pallai, D. E. Staunton, and T. A. Springer. 1992. Association of intercellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and alpha-actinin. *J Cell Biol* 118: 1223-1234.

62. Yang, L., J. R. Kowalski, P. Yacono, M. Bajmoczi, S. K. Shaw, R. M. Froio, D. E. Golan, S. M. Thomas, and F. W. Luscinskas. 2006. Endothelial cell cortactin coordinates intercellular adhesion molecule-1 clustering and actin cytoskeleton remodeling during polymorphonuclear leukocyte adhesion and transmigration. *J Immunol* 177: 6440-6449.

63. Barreiro, O., M. Yanez-Mo, J. M. Serrador, M. C. Montoya, M. Vicente-Manzanares, R. Tejedor, H. Furthmayr, and F. Sanchez-Madrid. 2002. Dynamic interaction of VCAM-1 and ICAM-1 with moesin and ezrin in a novel endothelial docking structure for adherent leukocytes. *J Cell Biol* 157: 1233-1245.

64. Tilghman, R. W., and R. L. Hoover. 2002. The Src-cortactin pathway is required for clustering of E-selectin and ICAM-1 in endothelial cells. *FASEB J* 16: 1257-1259.

65. Yang, L., J. R. Kowalski, X. Zhan, S. M. Thomas, and F. W. Luscinskas. 2006. Endothelial cell cortactin phosphorylation by Src contributes to polymorphonuclear leukocyte transmigration in vitro. *Circ Res* 98: 394-402.

66. Kemler, R., M. Ozawa, and M. Ringwald. 1989. Calcium-dependent cell adhesion molecules. *Current opinion in cell biology* 1: 892-897.

67. Dejana, E. 2004. Endothelial cell-cell junctions: happy together. *Nat Rev Mol Cell Biol* 5: 261-270.

68. Allport, J. R., H. Ding, T. Collins, M. E. Gerritsen, and F. W. Luscinskas. 1997. Endothelial-dependent mechanisms regulate leukocyte transmigration: a process involving the proteasome and disruption of the vascular endothelial-cadherin complex at endothelial cell-to-cell junctions. *J Exp Med* 186: 517-527.

69. Lampugnani, M. G., M. Corada, P. Andriopoulou, S. Esser, W. Risau, and E. Dejana. 1997. Cell confluence regulates tyrosine phosphorylation of adherens junction components in endothelial cells. *J Cell Sci* 110 (Pt 17): 2065-2077.

70. Turowski, P., R. Martinelli, R. Crawford, D. Wateridge, A. P. Papageorgiou, M. G. Lampugnani, A. C. Gamp, D. Vestweber, P. Adamson, E. Dejana, and J. Greenwood. 2008. Phosphorylation of vascular endothelial cadherin controls lymphocyte emigration. *J Cell Sci* 121: 29-37.

71. Potter, M. D., S. Barbero, and D. A. Cheresh. 2005. Tyrosine phosphorylation of VE-cadherin prevents binding of p120- and beta-catenin and maintains the cellular mesenchymal state. *J Biol Chem* 280: 31906-31912.

72. Schnittler, H. J. 1998. Structural and functional aspects of intercellular junctions in vascular endothelium. *Basic research in cardiology* 93 Suppl 3: 30-39.

73. Persidsky, Y., D. Heilman, J. Haorah, M. Zelivyanskaya, R. Persidsky, G. A. Weber, H. Shimokawa, K. Kaibuchi, and T. Ikezu. 2006. Rho-mediated regulation of tight junctions during monocyte migration across the blood-brain barrier in HIV-1 encephalitis (HIVE). *Blood* 107: 4770-4780.

74. Faustmann, P. M., and R. Dermietzel. 1985. Extravasation of polymorphonuclear leukocytes from the cerebral microvasculature. Inflammatory response induced by alpha-bungarotoxin. *Cell and tissue research* 242: 399-407.

75. Marchesi, V. T. 1961. The site of leucocyte emigration during inflammation. *Quarterly journal of experimental physiology and cognate medical sciences* 46: 115-118.

76. Tomasek, J. J., G. Gabbiani, B. Hinz, C. Chaponnier, and R. A. Brown. 2002. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 3: 349-363.

77. Curtis, A. S., and G. M. Seehar. 1978. The control of cell division by tension or diffusion. *Nature* 274: 52-53.

78. Vandenburgh, H., and S. Kaufman. 1979. In vitro model for stretch-induced hypertrophy of skeletal muscle. *Science* 203: 265-268.

79. Bray, D. 1979. Mechanical tension produced by nerve cells in tissue culture. *J Cell Sci* 37: 391-410.

80. Bray, D. 1984. Axonal growth in response to experimentally applied mechanical tension. *Developmental biology* 102: 379-389.

81. Harris, A. K., P. Wild, and D. Stopak. 1980. Silicone rubber substrata: a new wrinkle in the study of cell locomotion. *Science* 208: 177-179.

82. Harris, A. K., D. Stopak, and P. Wild. 1981. Fibroblast traction as a mechanism for collagen morphogenesis. *Nature* 290: 249-251.

83. Kolega, J. 1986. Effects of mechanical tension on protrusive activity and microfilament and intermediate filament organization in an epidermal epithelium moving in culture. *J Cell Biol* 102: 1400-1411.

84. Bershadsky, A., M. Kozlov, and B. Geiger. 2006. Adhesion-mediated mechanosensitivity: a time to experiment, and a time to theorize. *Current opinion in cell biology* 18: 472-481.

85. Vogel, V., and M. Sheetz. 2006. Local force and geometry sensing regulate cell functions. *Nat Rev Mol Cell Biol* 7: 265-275.

86. Mammoto, A., T. Mammoto, and D. E. Ingber. 2008. Rho signaling and mechanical control of vascular development. *Current opinion in hematology* 15: 228-234.

87. Eyckmans, J., T. Boudou, X. Yu, and C. S. Chen. 2011. A hitchhiker's guide to mechanobiology. *Developmental cell* 21: 35-47.

88. Tzima, E. 2006. Role of small GTPases in endothelial cytoskeletal dynamics and the shear stress response. *Circ Res* 98: 176-185.

89. Hoffman, B. D., C. Grashoff, and M. A. Schwartz. 2011. Dynamic molecular processes mediate cellular mechanotransduction. *Nature* 475: 316-323.

90. Hahn, C., and M. A. Schwartz. 2009. Mechanotransduction in vascular physiology and atherogenesis. *Nat Rev Mol Cell Biol* 10: 53-62.

91. Thompson, W. R., C. T. Rubin, and J. Rubin. 2012. Mechanical regulation of signaling pathways in bone. *Gene* 503: 179-193.

92. Alberts, B. L., J.; Raff, M.; Roberts, K.; Walter, P. 2007. *Molecular Biology of the Cell. 5.* Garland Science.

93. Chrzanowska-Wodnicka, M., and K. Burridge. 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol* 133: 1403-1415.

94. Jaffe, A. B., and A. Hall. 2005. Rho GTPases: biochemistry and biology. *Annual review of cell and developmental biology* 21: 247-269.

95. Bos, J. L., H. Rehmann, and A. Wittinghofer. 2007. GEFs and GAPs: critical elements in the control of small G proteins. *Cell* 129: 865-877.

96. Garcia-Mata, R., E. Boulter, and K. Burridge. 2011. The 'invisible hand': regulation of RHO GTPases by RHOGDIs. *Nat Rev Mol Cell Biol* 12: 493-504.

97. Burridge, K. 1981. Are stress fibres contractile? *Nature* 294: 691-692.

98. Chen, C. S. 2008. Mechanotransduction - a field pulling together? *J Cell Sci* 121: 3285-3292.

99. Geiger, B., J. P. Spatz, and A. D. Bershadsky. 2009. Environmental sensing through focal adhesions. *Nat Rev Mol Cell Biol* 10: 21-33.

100. Peng, F., B. Zhang, A. J. Ingram, B. Gao, Y. Zhang, and J. C. Krepinsky. 2010. Mechanical stretch-induced RhoA activation is mediated by the RhoGEF Vav2 in mesangial cells. *Cellular signalling* 22: 34-40.

101. Guilluy, C., V. Swaminathan, R. Garcia-Mata, E. T. O'Brien, R. Superfine, and K. Burridge. 2011. The Rho GEFs LARG and GEF-H1 regulate the mechanical response to force on integrins. *Nat Cell Biol* 13: 722-727.

102. Dubash, A. D., K. Wennerberg, R. Garcia-Mata, M. M. Menold, W. T. Arthur, and K. Burridge. 2007. A novel role for Lsc/p115 RhoGEF and LARG in regulating RhoA activity downstream of adhesion to fibronectin. *J Cell Sci* 120: 3989-3998.

103. Aijaz, S., F. D'Atri, S. Citi, M. S. Balda, and K. Matter. 2005. Binding of GEF-H1 to the tight junction-associated adaptor cingulin results in inhibition of Rho signaling and G1/S phase transition. *Developmental cell* 8: 777-786.

104. Benais-Pont, G., A. Punn, C. Flores-Maldonado, J. Eckert, G. Raposo, T. P. Fleming, M. Cereijido, M. S. Balda, and K. Matter. 2003. Identification of a tight junction-associated guanine nucleotide exchange factor that activates Rho and regulates paracellular permeability. *J Cell Biol* 160: 729-740.

105. Ren, Y., R. Li, Y. Zheng, and H. Busch. 1998. Cloning and characterization of GEF-H1, a microtubule-associated guanine nucleotide exchange factor for Rac and Rho GTPases. *J Biol Chem* 273: 34954-34960.

106. Hobert, O., J. W. Schilling, M. C. Beckerle, A. Ullrich, and B. Jallal. 1996. SH3 domain-dependent interaction of the proto-oncogene product Vav with the focal contact protein zyxin. *Oncogene* 12: 1577-1581.

107. Lim, Y., S. T. Lim, A. Tomar, M. Gardel, J. A. Bernard-Trifilo, X. L. Chen, S. A. Uryu, R. Canete-Soler, J. Zhai, H. Lin, W. W. Schlaepfer, P. Nalbant, G. Bokoch, D. Ilic, C. Waterman-Storer, and D. D. Schlaepfer. 2008. PyK2 and FAK connections to p190Rho guanine nucleotide exchange factor regulate RhoA activity, focal adhesion formation, and cell motility. *J Cell Biol* 180: 187-203.

108. Bellanger, J. M., C. Astier, C. Sardet, Y. Ohta, T. P. Stossel, and A. Debant. 2000. The Rac1- and RhoG-specific GEF domain of Trio targets filamin to remodel cytoskeletal actin. *Nat Cell Biol* 2: 888-892.

109. Iwanicki, M. P., T. Vomastek, R. W. Tilghman, K. H. Martin, J. Banerjee, P. B. Wedegaertner, and J. T. Parsons. 2008. FAK, PDZ-RhoGEF and ROCKII cooperate to regulate adhesion movement and trailing-edge retraction in fibroblasts. *J Cell Sci* 121: 895-905.

110. Sharma, S. V. 1998. Rapid recruitment of p120RasGAP and its associated protein, p190RhoGAP, to the cytoskeleton during integrin mediated cell-substrate interaction. *Oncogene* 17: 271-281.

111. Li, G., X. Du, W. C. Vass, A. G. Papageorge, D. R. Lowy, and X. Qian. 2011. Full activity of the deleted in liver cancer 1 (DLC1) tumor suppressor depends on an LD-like motif that binds talin and focal adhesion kinase (FAK). *Proc Natl Acad Sci U S A* 108: 17129-17134.

112. Tripathi, V., N. C. Popescu, and D. B. Zimonjic. 2012. DLC1 interaction with alpha-catenin stabilizes adherens junctions and enhances DLC1 antioncogenic activity. *Molecular and cellular biology* 32: 2145-2159.

113. Omelchenko, T., and A. Hall. 2012. Myosin-IXA regulates collective epithelial cell migration by targeting RhoGAP activity to cell-cell junctions. *Curr Biol* 22: 278-288.

114. Kuo, J. C., X. Han, C. T. Hsiao, J. R. Yates, 3rd, and C. M. Waterman. 2011. Analysis of the myosin-II-responsive focal adhesion proteome reveals a role for beta-Pix in negative regulation of focal adhesion maturation. *Nat Cell Biol* 13: 383-393.

115. Heck, J. N., S. M. Ponik, M. G. Garcia-Mendoza, C. A. Pehlke, D. R. Inman, K. W. Eliceiri, and P. J. Keely. 2012. Microtubules regulate GEF-H1 in response to extracellular matrix stiffness. *Mol Biol Cell* 23: 2583-2592.

116. Goldyn, A. M., B. A. Rioja, J. P. Spatz, C. Ballestrem, and R. Kemkemer. 2009. Force-induced cell polarisation is linked to RhoA-driven microtubule-independent focal-adhesion sliding. *J Cell Sci* 122: 3644-3651.

117. Birukova, A. A., P. Fu, J. Xing, B. Yakubov, I. Cokic, and K. G. Birukov. 2010. Mechanotransduction by GEF-H1 as a novel mechanism of ventilator-induced vascular endothelial permeability. *American journal of physiology. Lung cellular and molecular physiology* 298: L837-848.

118. Liu, Z., J. L. Tan, D. M. Cohen, M. T. Yang, N. J. Sniadecki, S. A. Ruiz, C. M. Nelson, and C. S. Chen. 2010. Mechanical tugging force regulates the size of cell-cell junctions. *Proc Natl Acad Sci U S A* 107: 9944-9949.

119. Brevier, J., D. Montero, T. Svitkina, and D. Riveline. 2008. The asymmetric self-assembly mechanism of adherens junctions: a cellular push-pull unit. *Physical biology* 5: 016005.

120. le Duc, Q., Q. Shi, I. Blonk, A. Sonnenberg, N. Wang, D. Leckband, and J. de Rooij. 2010. Vinculin potentiates E-cadherin mechanosensing and is recruited to actin-anchored sites within adherens junctions in a myosin II-dependent manner. *J Cell Biol* 189: 1107-1115.

121. Terry, S. J., C. Zihni, A. Elbediwy, E. Vitiello, I. V. Leefa Chong San, M. S. Balda, and K. Matter. 2011. Spatially restricted activation of RhoA signalling at epithelial junctions by p114RhoGEF drives junction formation and morphogenesis. *Nat Cell Biol* 13: 159-166.

122. Liao, Y. C., Y. P. Shih, and S. H. Lo. 2008. Mutations in the focal adhesion targeting region of deleted in liver cancer-1 attenuate their expression and function. *Cancer research* 68: 7718-7722.

123. Clark, K., J. D. Howe, C. E. Pullar, J. A. Green, V. V. Artym, K. M. Yamada, and D. R. Critchley. 2010. Tensin 2 modulates cell contractility in 3D collagen gels through the RhoGAP DLC1. *Journal of cellular biochemistry* 109: 808-817.

124. Kawai, K., Y. Iwamae, M. Yamaga, M. Kiyota, H. Ishii, H. Hirata, Y. Homma, and H. Yagisawa. 2009. Focal adhesion-localization of START-GAP1/DLC1 is essential for cell motility and morphology. *Genes to cells : devoted to molecular & cellular mechanisms* 14: 227-241.

125. Tomar, A., S. T. Lim, Y. Lim, and D. D. Schlaepfer. 2009. A FAK-p120RasGAP-p190RhoGAP complex regulates polarity in migrating cells. *J Cell Sci* 122: 1852-1862.

126. Arthur, W. T., L. A. Petch, and K. Burridge. 2000. Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism. *Curr Biol* 10: 719-722.

127. Noren, N. K., W. T. Arthur, and K. Burridge. 2003. Cadherin engagement inhibits RhoA via p190RhoGAP. *J Biol Chem* 278: 13615-13618.

128. Arthur, W. T., and K. Burridge. 2001. RhoA inactivation by p190RhoGAP regulates cell spreading and migration by promoting membrane protrusion and polarity. *Mol Biol Cell* 12: 2711-2720.

129. Yang, B., C. Radel, D. Hughes, S. Kelemen, and V. Rizzo. 2011. p190 RhoGTPase-activating protein links the beta1 integrin/caveolin-1 mechanosignaling complex to RhoA and actin remodeling. *Arteriosclerosis, thrombosis, and vascular biology* 31: 376-383.

130. Mammoto, A., K. M. Connor, T. Mammoto, C. W. Yung, D. Huh, C. M. Aderman, G. Mostoslavsky, L. E. Smith, and D. E. Ingber. 2009. A mechanosensitive transcriptional mechanism that controls angiogenesis. *Nature* 457: 1103-1108.

131. Guilluy, C., R. Garcia-Mata, and K. Burridge. 2011. Rho protein crosstalk: another social network? *Trends in cell biology* 21: 718-726.

132. Houk, A. R., A. Jilkine, C. O. Mejean, R. Boltyanskiy, E. R. Dufresne, S. B. Angenent, S. J. Altschuler, L. F. Wu, and O. D. Weiner. 2012. Membrane tension maintains cell polarity by confining signals to the leading edge during neutrophil migration. *Cell* 148: 175-188.

133. Worthylake, R. A., and K. Burridge. 2003. RhoA and ROCK promote migration by limiting membrane protrusions. *J Biol Chem* 278: 13578-13584.

134. Ohta, Y., J. H. Hartwig, and T. P. Stossel. 2006. FilGAP, a Rho- and ROCK-regulated GAP for Rac binds filamin A to control actin remodelling. *Nat Cell Biol* 8: 803-814.

135. Ehrlicher, A. J., F. Nakamura, J. H. Hartwig, D. A. Weitz, and T. P. Stossel. 2011. Mechanical strain in actin networks regulates FilGAP and integrin binding to filamin A. *Nature* 478: 260-263.

136. Sanz-Moreno, V., G. Gadea, J. Ahn, H. Paterson, P. Marra, S. Pinner, E. Sahai, and C. J. Marshall. 2008. Rac activation and inactivation control plasticity of tumor cell movement. *Cell* 135: 510-523.

137. Boulter, E., R. Garcia-Mata, C. Guilluy, A. Dubash, G. Rossi, P. J. Brennwald, and K. Burridge. 2010. Regulation of Rho GTPase crosstalk, degradation and activity by RhoGDI1. *Nat Cell Biol* 12: 477-483.

138. Ishijima, A., T. Doi, K. Sakurada, and T. Yanagida. 1991. Sub-piconewton force fluctuations of actomyosin in vitro. *Nature* 352: 301-306.

139. Molloy, J. E., J. E. Burns, J. Kendrick-Jones, R. T. Tregear, and D. C. White. 1995. Movement and force produced by a single myosin head. *Nature* 378: 209-212.

140. Finer, J. T., R. M. Simmons, and J. A. Spudich. 1994. Single myosin molecule mechanics: piconewton forces and nanometre steps. *Nature* 368: 113-119.

141. Tonomura, Y., and F. Oosawa. 1972. Molecular mechanism of contraction. *Annual review of biophysics and bioengineering* 1: 159-190.

142. Lee, J., M. Leonard, T. Oliver, A. Ishihara, and K. Jacobson. 1994. Traction forces generated by locomoting keratocytes. *J Cell Biol* 127: 1957-1964.

143. Galbraith, C. G., and M. P. Sheetz. 1997. A micromachined device provides a new bend on fibroblast traction forces. *Proc Natl Acad Sci U S A* 94: 9114-9118.

144. Balaban, N. Q., U. S. Schwarz, D. Riveline, P. Goichberg, G. Tzur, I. Sabanay, D. Mahalu, S. Safran, A. Bershadsky, L. Addadi, and B. Geiger. 2001. Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. *Nat Cell Biol* 3: 466-472.

145. Schwarz, U. S., N. Q. Balaban, D. Riveline, A. Bershadsky, B. Geiger, and S. A. Safran. 2002. Calculation of forces at focal adhesions from elastic substrate data: the effect of localized force and the need for regularization. *Biophys J* 83: 1380-1394.

146. Beningo, K. A., M. Dembo, I. Kaverina, J. V. Small, and Y. L. Wang. 2001. Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. *J Cell Biol* 153: 881-888.

147. Tan, J. L., J. Tien, D. M. Pirone, D. S. Gray, K. Bhadriraju, and C. S. Chen. 2003. Cells lying on a bed of microneedles: an approach to isolate mechanical force. *Proc Natl Acad Sci U S A* 100: 1484-1489.

148. Provenzano, P. P., and P. J. Keely. 2011. Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling. *J Cell Sci* 124: 1195-1205.

149. Banes, A. J., J. Gilbert, D. Taylor, and O. Monbureau. 1985. A new vacuum-operated stress-providing instrument that applies static or variable duration cyclic tension or compression to cells in vitro. *J Cell Sci* 75: 35-42.

150. Sumpio, B. E., A. J. Banes, L. G. Levin, and G. Johnson, Jr. 1987. Mechanical stress stimulates aortic endothelial cells to proliferate. *Journal of vascular surgery : official publication, the Society for Vascular Surgery [and] International Society for Cardiovascular Surgery, North American Chapter* 6: 252-256.

151. Riveline, D., E. Zamir, N. Q. Balaban, U. S. Schwarz, T. Ishizaki, S. Narumiya, Z. Kam, B. Geiger, and A. D. Bershadsky. 2001. Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. *J Cell Biol* 153: 1175-1186.

152. Wang, N., J. P. Butler, and D. E. Ingber. 1993. Mechanotransduction across the cell surface and through the cytoskeleton. *Science* 260: 1124-1127.

153. Choquet, D., D. P. Felsenfeld, and M. P. Sheetz. 1997. Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages. *Cell* 88: 39-48.

154. Matthews, B. D., D. R. Overby, F. J. Alenghat, J. Karavitis, Y. Numaguchi, P. G. Allen, and D. E. Ingber. 2004. Mechanical properties of individual focal adhesions probed with a magnetic microneedle. *Biochemical and biophysical research communications* 313: 758-764.

155. Matthews, B. D., D. R. Overby, R. Mannix, and D. E. Ingber. 2006. Cellular adaptation to mechanical stress: role of integrins, Rho, cytoskeletal tension and mechanosensitive ion channels. *J Cell Sci* 119: 508-518.

156. Glogauer, M., and J. Ferrier. 1998. A new method for application of force to cells via ferric oxide beads. *Pflugers Archiv : European journal of physiology* 435: 320-327.

157. Zhao, X. H., C. Laschinger, P. Arora, K. Szaszi, A. Kapus, and C. A. McCulloch. 2007. Force activates smooth muscle alpha-actin promoter activity through the Rho signaling pathway. *J Cell Sci* 120: 1801-1809.

158. Tim O'Brien, E., J. Cribb, D. Marshburn, R. M. Taylor, 2nd, and R. Superfine. 2008. Chapter 16: Magnetic manipulation for force measurements in cell biology. *Methods Cell Biol* 89: 433-450.

159. Davies, P. F. 1995. Flow-mediated endothelial mechanotransduction. *Physiological reviews* 75: 519-560.

160. Wojciak-Stothard, B., and A. J. Ridley. 2003. Shear stress-induced endothelial cell polarization is mediated by Rho and Rac but not Cdc42 or PI 3-kinases. *J Cell Biol* 161: 429-439.

161. Davies, P. F. 2009. Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. *Nature clinical practice. Cardiovascular medicine* 6: 16-26.

162. Malek, A. M., and S. Izumo. 1994. Molecular aspects of signal transduction of shear stress in the endothelial cell. *Journal of hypertension* 12: 989-999.

163. Ku, D. N., D. P. Giddens, C. K. Zarins, and S. Glagov. 1985. Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. *Arteriosclerosis* 5: 293-302.

164. Bussolari, S. R., C. F. Dewey, Jr., and M. A. Gimbrone, Jr. 1982. Apparatus for subjecting living cells to fluid shear stress. *Rev Sci Instrum* 53: 1851-1854.

165. Schnittler, H. J., R. P. Franke, U. Akbay, C. Mrowietz, and D. Drenckhahn. 1993. Improved in vitro rheological system for studying the effect of fluid shear stress on cultured cells. *Am J Physiol* 265: C289-298.

166. Frangos, J. A., S. G. Eskin, L. V. McIntire, and C. L. Ives. 1985. Flow effects on prostacyclin production by cultured human endothelial cells. *Science* 227: 1477-1479.

167. Levesque, M. J., and R. M. Nerem. 1985. The elongation and orientation of cultured endothelial cells in response to shear stress. *Journal of biomechanical engineering* 107: 341-347.

168. Olesen, S. P., D. E. Clapham, and P. F. Davies. 1988. Haemodynamic shear stress activates a K+ current in vascular endothelial cells. *Nature* 331: 168-170.

169. Ziegelstein, R. C., L. Cheng, and M. C. Capogrossi. 1992. Flow-dependent cytosolic acidification of vascular endothelial cells. *Science* 258: 656-659.

170. Straight, A. F., A. Cheung, J. Limouze, I. Chen, N. J. Westwood, J. R. Sellers, and T. J. Mitchison. 2003. Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. *Science* 299: 1743-1747.

171. Uehata, M., T. Ishizaki, H. Satoh, T. Ono, T. Kawahara, T. Morishita, H. Tamakawa, K. Yamagami, J. Inui, M. Maekawa, and S. Narumiya. 1997. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* 389: 990-994.

172. Peterson, L. J., Z. Rajfur, A. S. Maddox, C. D. Freel, Y. Chen, M. Edlund, C. Otey, and K. Burridge. 2004. Simultaneous stretching and contraction of stress fibers in vivo. *Mol Biol Cell* 15: 3497-3508.

173. Vicente-Manzanares, M., M. A. Koach, L. Whitmore, M. L. Lamers, and A. F. Horwitz. 2008. Segregation and activation of myosin IIB creates a rear in migrating cells. *J Cell Biol* 183: 543-554.

174. Pelham, R. J., Jr., and Y. Wang. 1997. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc Natl Acad Sci U S A* 94: 13661-13665.

175. Discher, D. E., P. Janmey, and Y. L. Wang. 2005. Tissue cells feel and respond to the stiffness of their substrate. *Science* 310: 1139-1143.

176. Engler, A. J., S. Sen, H. L. Sweeney, and D. E. Discher. 2006. Matrix elasticity directs stem cell lineage specification. *Cell* 126: 677-689.

177. Wong, A. J., T. D. Pollard, and I. M. Herman. 1983. Actin filament stress fibers in vascular endothelial cells in vivo. *Science* 219: 867-869.

178. Wozniak, M. A., R. Desai, P. A. Solski, C. J. Der, and P. J. Keely. 2003. ROCK-generated contractility regulates breast epithelial cell differentiation in response to the physical properties of a three-dimensional collagen matrix. *J Cell Biol* 163: 583-595.

179. Paszek, M. J., N. Zahir, K. R. Johnson, J. N. Lakins, G. I. Rozenberg, A. Gefen, C. A. Reinhart-King, S. S. Margulies, M. Dembo, D. Boettiger, D. A. Hammer, and V. M. Weaver. 2005. Tensional homeostasis and the malignant phenotype. *Cancer cell* 8: 241-254.

180. Pasapera, A. M., I. C. Schneider, E. Rericha, D. D. Schlaepfer, and C. M. Waterman. 2010. Myosin II activity regulates vinculin recruitment to focal adhesions through FAK-mediated paxillin phosphorylation. *J Cell Biol* 188: 877-890.

181. Hotulainen, P., and P. Lappalainen. 2006. Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. *J Cell Biol* 173: 383-394.

182. Bhadriraju, K., M. Yang, S. Alom Ruiz, D. Pirone, J. Tan, and C. S. Chen. 2007. Activation of ROCK by RhoA is regulated by cell adhesion, shape, and cytoskeletal tension. *Experimental cell research* 313: 3616-3623.

183. Ren, X. D., W. B. Kiosses, and M. A. Schwartz. 1999. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J* 18: 578-585.

184. Bissell, M. J., H. G. Hall, and G. Parry. 1982. How does the extracellular matrix direct gene expression? *Journal of theoretical biology* 99: 31-68.

185. Streuli, C. H., and M. J. Bissell. 1990. Expression of extracellular matrix components is regulated by substratum. *J Cell Biol* 110: 1405-1415.

186. Nie, M., S. Aijaz, I. V. Leefa Chong San, M. S. Balda, and K. Matter. 2009. The Y-box factor ZONAB/DbpA associates with GEF-H1/Lfc and mediates Rho-stimulated transcription. *EMBO reports* 10: 1125-1131.

187. Hill, C. S., J. Wynne, and R. Treisman. 1995. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. *Cell* 81: 1159-1170.

188. Sordella, R., W. Jiang, G. C. Chen, M. Curto, and J. Settleman. 2003. Modulation of Rho GTPase signaling regulates a switch between adipogenesis and myogenesis. *Cell* 113: 147-158.

189. McBeath, R., D. M. Pirone, C. M. Nelson, K. Bhadriraju, and C. S. Chen. 2004. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Developmental cell* 6: 483-495.

190. Lee, H. H., and Z. F. Chang. 2008. Regulation of RhoA-dependent ROCKII activation by Shp2. *J Cell Biol* 181: 999-1012.

191. Pertz, O., L. Hodgson, R. L. Klemke, and K. M. Hahn. 2006. Spatiotemporal dynamics of RhoA activity in migrating cells. *Nature* 440: 1069-1072.

192. Burridge, K., C. E. Turner, and L. H. Romer. 1992. Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J Cell Biol* 119: 893-903.

193. Dupont, S., L. Morsut, M. Aragona, E. Enzo, S. Giulitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato, S. Bicciato, N. Elvassore, and S. Piccolo. 2011. Role of YAP/TAZ in mechanotransduction. *Nature* 474: 179-183.

194. Moy, A. B., S. S. Shasby, B. D. Scott, and D. M. Shasby. 1993. The effect of histamine and cyclic adenosine monophosphate on myosin light chain phosphorylation in human umbilical vein endothelial cells. *The Journal of clinical investigation* 92: 1198-1206.

195. Dudek, S. M., and J. G. Garcia. 2001. Cytoskeletal regulation of pulmonary vascular permeability. *Journal of applied physiology* 91: 1487-1500.

196. Zhong, C., M. S. Kinch, and K. Burridge. 1997. Rho-stimulated contractility contributes to the fibroblastic phenotype of Ras-transformed epithelial cells. *Mol Biol Cell* 8: 2329-2344.

197. Braga, V. M., L. M. Machesky, A. Hall, and N. A. Hotchin. 1997. The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J Cell Biol* 137: 1421-1431.

198. Jou, T. S., E. E. Schneeberger, and W. J. Nelson. 1998. Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. *J Cell Biol* 142: 101-115.

199. Yamada, S., and W. J. Nelson. 2007. Localized zones of Rho and Rac activities drive initiation and expansion of epithelial cell-cell adhesion. *J Cell Biol* 178: 517-527.

200. Shewan, A. M., M. Maddugoda, A. Kraemer, S. J. Stehbens, S. Verma, E. M. Kovacs, and A. S. Yap. 2005. Myosin 2 is a key Rho kinase target necessary for the local concentration of E-cadherin at cell-cell contacts. *Mol Biol Cell* 16: 4531-4542.

201. Miyake, Y., N. Inoue, K. Nishimura, N. Kinoshita, H. Hosoya, and S. Yonemura. 2006. Actomyosin tension is required for correct recruitment of adherens junction components and zonula occludens formation. *Experimental cell research* 312: 1637-1650.

202. Yonemura, S., Y. Wada, T. Watanabe, A. Nagafuchi, and M. Shibata. 2010. alpha-Catenin as a tension transducer that induces adherens junction development. *Nat Cell Biol* 12: 533-542.

203. Yamamoto, M., S. H. Ramirez, S. Sato, T. Kiyota, R. L. Cerny, K. Kaibuchi, Y. Persidsky, and T. Ikezu. 2008. Phosphorylation of claudin-5 and occludin by rho kinase in brain endothelial cells. *Am.J Pathol.* 172: 521-533.

204. Noren, N. K., C. M. Niessen, B. M. Gumbiner, and K. Burridge. 2001. Cadherin engagement regulates Rho family GTPases. *J Biol Chem* 276: 33305-33308.

205. Lampugnani, M. G., A. Zanetti, F. Breviario, G. Balconi, F. Orsenigo, M. Corada, R. Spagnuolo, M. Betson, V. Braga, and E. Dejana. 2002. VE-cadherin regulates endothelial actin activating Rac and increasing membrane association of Tiam. *Mol Biol Cell* 13: 1175-1189.

206. Calautti, E., M. Grossi, C. Mammucari, Y. Aoyama, M. Pirro, Y. Ono, J. Li, and G. P. Dotto. 2002. Fyn tyrosine kinase is a downstream mediator of Rho/PRK2 function in keratinocyte cell-cell adhesion. *J Cell Biol* 156: 137-148.

207. Charrasse, S., M. Meriane, F. Comunale, A. Blangy, and C. Gauthier-Rouviere. 2002. N-cadherin-dependent cell-cell contact regulates Rho GTPases and beta-catenin localization in mouse C2C12 myoblasts. *J Cell Biol* 158: 953-965.

208. Nelson, C. M., D. M. Pirone, J. L. Tan, and C. S. Chen. 2004. Vascular endothelial-cadherin regulates cytoskeletal tension, cell spreading, and focal adhesions by stimulating RhoA. *Mol Biol Cell* 15: 2943-2953.

209. Liu, W. F., C. M. Nelson, J. L. Tan, and C. S. Chen. 2007. Cadherins, RhoA, and Rac1 are differentially required for stretch-mediated proliferation in endothelial versus smooth muscle cells. *Circ Res* 101: e44-52.

210. Katsumi, A., J. Milanini, W. B. Kiosses, M. A. del Pozo, R. Kaunas, S. Chien, K. M. Hahn, and M. A. Schwartz. 2002. Effects of cell tension on the small GTPase Rac. *J Cell Biol* 158: 153-164.

211. DuFort, C. C., M. J. Paszek, and V. M. Weaver. 2011. Balancing forces: architectural control of mechanotransduction. *Nat Rev Mol Cell Biol* 12: 308-319.

212. Provenzano, P. P., D. R. Inman, K. W. Eliceiri, and P. J. Keely. 2009. Matrix density-induced mechanoregulation of breast cell phenotype, signaling and gene expression through a FAK-ERK linkage. *Oncogene* 28: 4326-4343.

213. Samuel, M. S., J. I. Lopez, E. J. McGhee, D. R. Croft, D. Strachan, P. Timpson, J. Munro, E. Schroder, J. Zhou, V. G. Brunton, N. Barker, H. Clevers, O. J. Sansom, K. I. Anderson, V. M. Weaver, and M. F. Olson. 2011. Actomyosin-mediated cellular tension drives increased tissue stiffness and beta-catenin activation to induce epidermal hyperplasia and tumor growth. *Cancer cell* 19: 776-791.

214. Olson, M. F., and E. Sahai. 2009. The actin cytoskeleton in cancer cell motility. *Clinical & experimental metastasis* 26: 273-287.

215. Provenzano, P. P., K. W. Eliceiri, J. M. Campbell, D. R. Inman, J. G. White, and P. J. Keely. 2006. Collagen reorganization at the tumor-stromal interface facilitates local invasion. *BMC medicine* 4: 38.

216. Friedl, P., and K. Wolf. 2010. Plasticity of cell migration: a multiscale tuning model. *J Cell Biol* 188: 11-19.

217. Wolf, K., I. Mazo, H. Leung, K. Engelke, U. H. von Andrian, E. I. Deryugina, A. Y. Strongin, E. B. Brocker, and P. Friedl. 2003. Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *J Cell Biol* 160: 267-277.

218. Sahai, E., and C. J. Marshall. 2003. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat Cell Biol* 5: 711-719.

219. Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell* 67: 1033-1036.

220. Muller, W. A. 2009. Mechanisms of transendothelial migration of leukocytes. *Circ Res* 105: 223-230.

221. Essler, M., K. Hermann, M. Amano, K. Kaibuchi, J. Heesemann, P. C. Weber, and M. Aepfelbacher. 1998. Pasteurella multocida toxin increases endothelial permeability via Rho kinase and myosin light chain phosphatase. *J Immunol* 161: 5640-5646.

222. Carbajal, J. M., and R. C. Schaeffer, Jr. 1999. RhoA inactivation enhances endothelial barrier function. *Am J Physiol* 277: C955-964.

223. Etienne-Manneville, S., J. B. Manneville, P. Adamson, B. Wilbourn, J. Greenwood, and P. O. Couraud. 2000. ICAM-1-coupled cytoskeletal rearrangements and transendothelial lymphocyte migration involve intracellular calcium signaling in brain endothelial cell lines. *J Immunol* 165: 3375-3383.

224. Wang, Q., G. R. Pfeiffer, 2nd, and W. A. Gaarde. 2003. Activation of SRC tyrosine kinases in response to ICAM-1 ligation in pulmonary microvascular endothelial cells. *J Biol Chem* 278: 47731-47743.

225. Guilford, W. H., R. C. Lantz, and R. W. Gore. 1995. Locomotive forces produced by single leukocytes in vivo and in vitro. *Am J Physiol* 268: C1308-1312.

226. Smith, L. A., H. Aranda-Espinoza, J. B. Haun, M. Dembo, and D. A. Hammer. 2007. Neutrophil traction stresses are concentrated in the uropod during migration. *Biophys J* 92: L58-60.

227. Rabodzey, A., P. Alcaide, F. W. Luscinskas, and B. Ladoux. 2008. Mechanical forces induced by the transendothelial migration of human neutrophils. *Biophys J* 95: 1428-1438.

228. Collins, C., C. Guilluy, C. Welch, E. T. O'Brien, K. Hahn, R. Superfine, K. Burridge, and E. Tzima. 2012. Localized tensional forces on PECAM-1 elicit a global mechanotransduction response via the integrin-RhoA pathway. *Curr Biol* 22: 2087-2094.

229. Garcia-Mata, R., K. Wennerberg, W. T. Arthur, N. K. Noren, S. M. Ellerbroek, and K. Burridge. 2006. Analysis of activated GAPs and GEFs in cell lysates. *Methods Enzymol* 406: 425-437.

230. WojciakStothard, B., L. Williams, and A. J. Ridley. 1999. Monocyte adhesion and spreading on human endothelial cells is dependent on Rho-regulated receptor clustering. *Journal of Cell Biology* 145: 1293-1307.

231. Bourguignon, L. Y., E. Gilad, A. Brightman, F. Diedrich, and P. Singleton. 2006. Hyaluronan-CD44 interaction with leukemia-associated RhoGEF and epidermal growth factor receptor promotes Rho/Ras co-activation, phospholipase C epsilon-Ca2+ signaling, and cytoskeleton modification in head and neck squamous cell carcinoma cells. *J Biol Chem* 281: 14026-14040.

232. Chikumi, H., S. Fukuhara, and J. S. Gutkind. 2002. Regulation of G protein-linked guanine nucleotide exchange factors for Rho, PDZ-RhoGEF, and LARG by tyrosine phosphorylation: evidence of a role for focal adhesion kinase. *J Biol Chem* 277: 12463-12473.

233. Wang, Q., and C. M. Doerschuk. 2000. Neutrophil-induced changes in the biomechanical properties of endothelial cells: roles of ICAM-1 and reactive oxygen species. *J Immunol* 164: 6487-6494.

234. Wang, Q., E. T. Chiang, M. Lim, J. Lai, R. Rogers, P. A. Janmey, D. Shepro, and C. M. Doerschuk. 2001. Changes in the biomechanical properties of neutrophils and endothelial cells during adhesion. *Blood* 97: 660-668.

235. Kang, I., Q. Wang, S. J. Eppell, R. E. Marchant, and C. M. Doerschuk. 2010. Effect of neutrophil adhesion on the mechanical properties of lung microvascular endothelial cells. *Am J Respir Cell Mol Biol* 43: 591-598.

236. Stroka, K. M., J. A. Vaitkus, and H. Aranda-Espinoza. 2012. Endothelial cells undergo morphological, biomechanical, and dynamic changes in response to tumor necrosis factor-alpha. *European biophysics journal : EBJ* 41: 939-947.

237. Stroka, K. M., and H. Aranda-Espinoza. 2011. Endothelial cell substrate stiffness influences neutrophil transmigration via myosin light chain kinase-dependent cell contraction. *Blood* 118: 1632-1640.

238. Huynh, J., N. Nishimura, K. Rana, J. M. Peloquin, J. P. Califano, C. R. Montague, M. R. King, C. B. Schaffer, and C. A. Reinhart-King. 2011. Age-related intimal stiffening enhances endothelial permeability and leukocyte transmigration. *Science translational medicine* 3: 112ra122.

239. Birukova, A. A., X. Tian, I. Cokic, Y. Beckham, M. L. Gardel, and K. G. Birukov. 2013. Endothelial barrier disruption and recovery is controlled by substrate stiffness. *Microvascular research*.

240. Byfield, F. J., R. K. Reen, T. P. Shentu, I. Levitan, and K. J. Gooch. 2009. Endothelial actin and cell stiffness is modulated by substrate stiffness in 2D and 3D. *Journal of biomechanics* 42: 1114-1119.

241. Krishnan, R., D. D. Klumpers, C. Y. Park, K. Rajendran, X. Trepat, J. van Bezu, V. W. van Hinsbergh, C. V. Carman, J. D. Brain, J. J. Fredberg, J. P. Butler, and G. P. van Nieuw Amerongen. 2011. Substrate stiffening promotes endothelial monolayer disruption through enhanced physical forces. *American journal of physiology. Cell physiology* 300: C146-154.

242. Jannat, R. A., G. P. Robbins, B. G. Ricart, M. Dembo, and D. A. Hammer. 2010. Neutrophil adhesion and chemotaxis depend on substrate mechanics. *Journal of physics. Condensed matter : an Institute of Physics journal* 22: 194117.

243. Jannat, R. A., M. Dembo, and D. A. Hammer. 2011. Traction forces of neutrophils migrating on compliant substrates. *Biophys J* 101: 575-584.

244. Evelyn, C. R., T. Ferng, R. J. Rojas, M. J. Larsen, J. Sondek, and R. R. Neubig. 2009. High-throughput screening for small-molecule inhibitors of LARG-stimulated RhoA nucleotide binding via a novel fluorescence polarization assay. *J Biomol Screen* 14: 161-172.

245. Shang, X., F. Marchioni, C. R. Evelyn, N. Sipes, X. Zhou, W. Seibel, M. Wortman, and Y. Zheng. 2013. Small-molecule inhibitors targeting G-protein-coupled Rho guanine nucleotide exchange factors. *Proc Natl Acad Sci U S A* 110: 3155-3160.

246. Aghajanian, A., E. S. Wittchen, S. L. Campbell, and K. Burridge. 2009. Direct activation of RhoA by reactive oxygen species requires a redox-sensitive motif. *PloS one* 4: e8045.

247. Wirth, A., Z. Benyó, M. Lukasova, B. Leutgeb, N. Wettschureck, S. Gorbey, P. Orsy, B. Horváth, C. Maser-Gluth, E. Greiner, B. Lemmer, G. Schütz, J. Gutkind, and S. Offermanns. 2008. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nature medicine* 14: 64-68.

248. Becknell, B., T. Shen, E. Maghraby, S. Taya, K. Kaibuchi, M. Caligiuri, and G. Marcucci. 2003. Characterization of leukemia-associated Rho guanine nucleotide exchange factor (LARG) expression during murine development. *Cell and tissue research* 314: 361-366.

249. Sligh, J. E., Jr., C. M. Ballantyne, S. S. Rich, H. K. Hawkins, C. W. Smith, A. Bradley, and A. L. Beaudet. 1993. Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. *Proc Natl Acad Sci U S A* 90: 8529-8533.

250. Collins, R. G., R. Velji, N. V. Guevara, M. J. Hicks, L. Chan, and A. L. Beaudet. 2000. P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. *J Exp Med* 191: 189-194.

251. Qin, L., W. M. Quinlan, N. A. Doyle, L. Graham, J. E. Sligh, F. Takei, A. L. Beaudet, and C. M. Doerschuk. 1996. The roles of CD11/CD18 and ICAM-1 in acute Pseudomonas aeruginosa-induced pneumonia in mice. *J Immunol* 157: 5016-5021.

252. Bourdillon, M. C., R. N. Poston, C. Covacho, E. Chignier, G. Bricca, and J. L. McGregor. 2000. ICAM-1 deficiency reduces atherosclerotic lesions in double-knockout mice (ApoE(-/-)/ICAM-1(-/-)) fed a fat or a chow diet. *Arteriosclerosis, thrombosis, and vascular biology* 20: 2630-2635.

253. Collins, R., R. Velji, N. Guevara, M. Hicks, L. Chan, and A. Beaudet. 2000. P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice. *The Journal of experimental medicine* 191: 189-194.

254. Nageh, M. F., E. T. Sandberg, K. R. Marotti, A. H. Lin, E. P. Melchior, D. C. Bullard, and A. L. Beaudet. 1997. Deficiency of inflammatory cell adhesion molecules protects against atherosclerosis in mice. *Arteriosclerosis, thrombosis, and vascular biology* 17: 1517-1520.

255. Kourlas, P., M. Strout, B. Becknell, M. Veronese, C. Croce, K. Theil, R. Krahe, T. Ruutu, S. Knuutila, C. Bloomfield, and M. Caligiuri. 2000. Identification of a gene at 11q23 encoding a guanine nucleotide exchange factor: evidence for its fusion with MLL in acute myeloid leukemia. *Proceedings of the National Academy of Sciences of the United States of America* 97: 2145-2150.

256. Martz, M. K., E. Grabocka, N. Beeharry, T. J. Yen, and P. B. Wedegaertner. 2013. Leukemia-associated RhoGEF (LARG) is a novel RhoGEF in cytokinesis and required for the proper completion of abscission. *Mol Biol Cell* 24: 2785-2794.

257. Saalbach, A., U. Haustein, and U. Anderegg. 2000. A ligand of human thy-1 is localized on polymorphonuclear leukocytes and monocytes and mediates the binding to activated thy-1-positive microvascular endothelial cells and fibroblasts. *The Journal of investigative dermatology* 115: 882-888.

258. Schubert, K., T. Polte, U. Bonisch, S. Schader, R. Holtappels, G. Hildebrandt, J. Lehmann, J. C. Simon, U. Anderegg, and A. Saalbach. 2011. Thy-1 (CD90) regulates the extravasation of leukocytes during inflammation. *European journal of immunology* 41: 645-656.

259. Wetzel, A., T. Wetzig, U. F. Haustein, M. Sticherling, U. Anderegg, J. C. Simon, and A. Saalbach. 2006. Increased neutrophil adherence in psoriasis: role of the human endothelial cell receptor Thy-1 (CD90). *J Invest Dermatol* 126: 441-452.

260. Avalos, A. M., W. T. Arthur, P. Schneider, A. F. Quest, K. Burridge, and L. Leyton. 2004. Aggregation of integrins and RhoA activation are required for Thy-1-induced morphological changes in astrocytes. *J Biol Chem* 279: 39139-39145.

261. Kong, M., N. Munoz, A. Valdivia, A. Alvarez, R. Herrera-Molina, A. Cardenas, P. Schneider, K. Burridge, A. F. Quest, and L. Leyton. 2013. Thy-1-mediated cell-cell contact induces astrocyte migration through the engagement of alphaVbeta3 integrin and syndecan-4. *Biochimica et biophysica acta* 1833: 1409-1420.

262. Barker, T. H., H. E. Grenett, M. W. MacEwen, S. G. Tilden, G. M. Fuller, J. Settleman, A. Woods, J. Murphy-Ullrich, and J. S. Hagood. 2004. Thy-1 regulates fibroblast focal adhesions, cytoskeletal organization and migration through modulation of p190 RhoGAP and Rho GTPase activity. *Experimental cell research* 295: 488-496.