

**THE ROLE OF FOCAL ADHESION KINASE IN VASCULAR SMOOTH  
MUSCLE CELL MIGRATION**

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in  
partial fulfillment of the requirements for the degree of Master of Science in the  
Department of Pathology and Laboratory Medicine

Chapel Hill  
2008

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## **ABSTRACT**

Lee Mangiante: The Role of Focal Adhesion Kinase in Vascular Smooth Muscle Cell Migration

(Under the direction of Joan M. Taylor)

Focal adhesion kinase (FAK) integrates diverse signaling pathways to regulate proliferation, migration, and differentiation. Germline deletion of FAK in mice results in embryonic lethality with pronounced defects in vascular structure and integrity, implicating FAK as critical to the development and migration of vascular smooth muscle cells (SMCs). However, the precise function of FAK in SMCs remains elusive. Primary aortic SMCs from *fak<sup>flox/flox</sup>* mice were infected with Cre-expressing adenovirus, ablating FAK production. FAK deletion inhibited migration to platelet-derived growth factor (PDGF). While PDGF-induced membrane ruffling was FAK-independent, PDGF-induced polarization was reduced fivefold in FAK-depleted cells. The RhoA effector, Dia2, localized to focal adhesions in a FAK-dependent manner, and ablation of FAK attenuated myosin light chain phosphorylation. Finally, we detected a novel interaction between Dia2 and the Arp2/3 activator, cortactin. These data suggest that FAK may promote cell contraction and polarized migration toward PDGF by regulating distinct pools of RhoA activity.

This work is dedicated to my good friend and colleague, Dean Staus, whose knowledge, insight, and generosity of spirit have been essential to its completion. May he fulfill all his diaphanous dreams of glory.

## ACKNOWLEDGMENTS

It is my pleasure to thank the following people for their contribution to this work:

*Dr. Joan Taylor*, for the opportunity to pursue a fascinating project, and to broaden my knowledge and skills beyond anything I ever imagined;

*Drs. Christopher Mack, James Bear, William Coleman, and Adrienne Cox*, for sharing time, space, resources, and scientific advice;

*The families of Dr. Robert H. Wagner and Joseph E. Pogue*, whose gracious financial support has afforded me the opportunity to attend UNC;

*The students and technicians of the Taylor and Mack labs*, for providing constant inspiration, instruction, amusement, and support;

*Dr. Robert Bagnell and Victoria Madden*, who awakened and indulged my passion for microscopy at every turn, and were my lab away from lab;

*Dr. Jennifer Sallee*, for her inexhaustible supply of Rho beads, constructs, and general wisdom;

*My Chapel Hill friends*, for welcoming me into their lives, and making life in a new place wholly enjoyable;

*Elizabeth Enlow*, my friend of 8 years and roommate for four, for sharing this crazy ride called graduate school, for taking care of my cat, and just for being there;

*Dr. Benjamin Carson*, for encouraging me and giving me the tools to take the risk;

*Drs. Stephanie Muga and Michael Wargovich, their graduate students, and staff*, without whose outstanding mentorship I would never have pursued a career in scientific research;

*Tonia Crook and Valerie Kennedy*, who introduced me to the world of lab science, and taught me to believe in myself as a scientist;

*Drs. Leslie Jones, Loren Knapp, Robert Raguso, George Khushf, Kevin Lewis, and Jan Smoak*, of the University of South Carolina, who inspired me to be a true philosopher;

*David Salter, Amelia Ligon, Art and Lisa Lader, Patrick Keating, and Joseph Laorenza*, whose outstanding teaching gave me the tools to succeed;

*Rachel Rosansky, Kieran Dawson, Andrew Frassetto, Drew Griffen, Evan Owens, Aseem Sood, David and Laura LaBoone, Kristen Buch, Michael Yehl, Cassandra Lovato, and Jennifer Hamlett*, whose constant love has guided me through MONSTER challenges; and

*My family*, trusted purveyors of love, faith, hope, truth, wisdom, utter hilarity, and the world's tastiest meatballs since 1982. I love you guys.

## **PREFACE: IF I NEVER SAW ANOTHER DORSAL RUFFLE...**

HORATIO

O day and night, but this is wondrous strange!

HAMLET

And therefore as a stranger give it welcome.

There are more things in heaven and earth, Horatio,  
Than are dreamt of in your philosophy.

*Hamlet*, Act 1, Sc. 5

Upon reflection, my time at the University of North Carolina has been a series of mind-expanding events. Before embarking upon the scientific pursuit contained herein, I had obtained some exposure to the world of science through my undergraduate research at the University of South Carolina. I grew cancer cells in a dish; I looked at human tissues through a microscope; I separated proteins on a gel; I composed a thesis based on my findings. I considered myself a fairly seasoned scientist at the ripe age of 22, and ready to take the next step into graduate school.

My ensuing arrival at UNC, however, brought me back sharply to the reality of my own inexperience. In the classroom, I hacked my way through dense forests of scientific literature, battled examinations the likes of which I had never seen before, and struggled to keep up with older, more experienced classmates. In my rotations, I had to be babysat by unfortunate senior graduate students so that I could learn to do PCR mutagenesis, yeast two-hybrid screens, and DNA methylation analysis. The academic, technical, and interpersonal challenges of my first year called for some serious adaptation on my part.

Really, though, the initial culture shock that necessarily accompanies the transition to graduate school pales in comparison to the mind-blowing things I've witnessed in the pursuit of cell biology. I knew that cells had nuclei, organelles, and a cytoskeleton, but never before had I seen them with my own eye, lit up in gorgeous colors, and brought into striking relief through the power of a fluorescence microscope. I knew cells had to "remodel" their cytoskeleton to move, but I never appreciated how intricate and dynamic that process was until I watched it happen in real time. I knew that cells were the basic units of all living things, but I never actually realized that cells are themselves *alive*, until I watched them going about their business over the space of days. They crawl around, searching for nourishment, bumping into each other and turning away. They can even take on personalities – some are big, fat, and sluggish; others are speedy little mischief-makers.

Truly, the things my cellular friends have shown me are beyond anything I ever dreamt of in my philosophy. They have moved me to laughter with their antics, and inspired awe with their astounding beauty. Despite their microscopic size, they have humbled me with their power, resilience, and complexity, and deepened my

appreciation for the miracle that constitutes all life on earth. Every step we take, every breath, every laugh, every gulp, every bat of an eyelash, every thought or memory or dream we ever have is the manifestation of a symphony of molecular events, refined over billions of years and executed with staggering precision.

I recall a conversation with a friend who joined a geophysics program after we left college. His fieldwork and conventions were taking him to all sorts of exotic places. He was always jaunting off to Hawaii, Romania, or some faraway paradise. I once expressed envy at all the traveling he got to do, while I was stuck in the lab with my cells. But then I realized I was just as much of a traveler as my friend was. Through the microscope, I have traveled to a universe that most people never get to see— a fast-paced, ever-changing, densely populated universe full of colorful characters. A universe with power plants, factories, shipping services, highways, banks, garbage dumps, libraries, police officers, construction workers, repairmen, and undertakers, all functioning in concert in a world only a few micrometers across. This is the universe of the cell, and I am privileged to have been its inhabitant for the past three years.

Even if I never stare down the barrel of a microscope again, the lesson the cells have taught me will still ring true as Hamlet's: *keep an open mind, welcome new things though they be strange, and you might discover miracles underneath your very nose*. In essence, this is the true credo of the scientist, to which I hope to remain faithful wherever life takes me.

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## LIST OF ABBREVIATIONS

3D	three-dimensional
ASAP	Arf GTPase activating protein
ATP	adenosine triphosphate
BSA	bovine serum albumin
Cas	Crk-associated substrate
Cdc42	cell division cycle 42
Cre	cyclization recombination recombinase
DAD	diaphanous autoinhibitory domain
DID	diaphanous inhibitory domain
DOCK180	dedicator of cytokinesis 1
DRF	diaphanous-related forming
ERK	extracellular signal-related kinase 1/2
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FFSMC	floxed-fak smooth muscle cell
FH1	formin homology 1 domain
FH2	formin homology 2 domain
FH3	formin homology 3 domain
FRNK	focal adhesion kinase-related non kinase
GAP	GTPase activating protein
GBD	GTPase binding domain
GDI	guanine nucleotide dissociation inhibitor

GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GRB2, 7	growth factor receptor-bound protein 2, 7
GST	glutathione-s-transferase
GTP	guanosine triphosphate
LacZ	$\beta$ -galactosidase
LoxP	locus of X over P1
Dia2	mouse Diaphanous 2
MLC	myosin light chain
p, phospho-	phosphorylated
PBD	p21 binding domain
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDGF-BB	platelet-derived growth factor, BB homodimer
PDGFR	platelet-derived growth factor receptor
PKL	paxillin kinase linker
PLC	phospholipase C
PYK2	protein tyrosine kinase 2
Rac	Ras-related C3 botulinum toxin substrate
RBD	Rho binding domain
Rho	Ras homolog gene family
ROCK	Rho-associated, coiled coil-containing protein kinase

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFKs	Src family kinases
SH2	Src homology 2 domain
SH3	Src homology 3 domain
SMC	smooth muscle cell
SOCS	suppressor of cytokine signaling
SRF	serum response factor
TBS	Tris-buffered saline
uL	microliter(s)
VSMC	vascular smooth muscle cell
WAVE	Wiskott-Aldrich syndrome protein family verprolin-homologous protein
Y397F	tyrosine 397 mutated to phenylalanine
YFP	yellow fluorescent protein



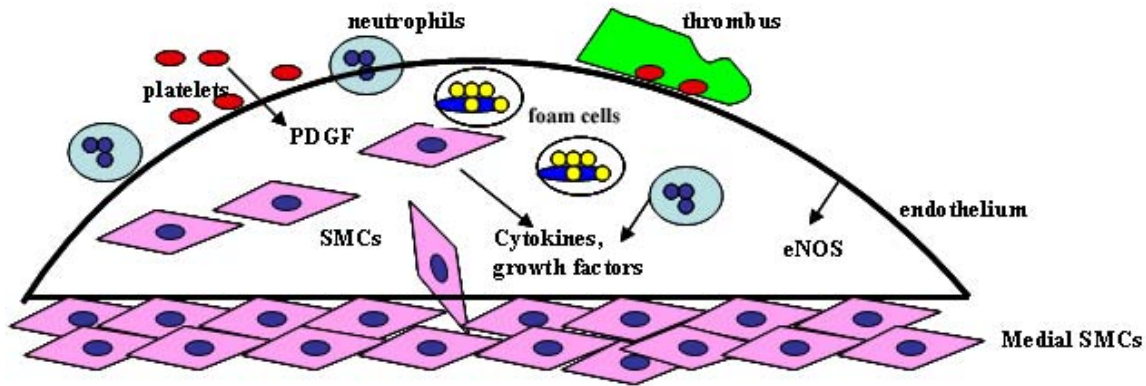
## INTRODUCTION

### *Smooth Muscle Cells in Vascular Development and Pathogenesis.*

Smooth muscle cells (SMCs) line all the hollow organs of the body. Vascular SMCs, in particular, comprise the medial layer of large arteries, where they regulate blood pressure by controlling vessel tone. Proper growth, migration, and differentiation of SMCs are critical to neovascularization, both during development and as part of the wound healing response. However, SMCs also play a pivotal role in the progression of several pathological states involving vascular occlusion, including atherosclerosis, restenosis, and hypertension.

Platelet-derived growth factor, BB homodimer (PDGF-BB; hereafter PDGF), is a potent chemoattractant for SMCs in both a developmental and pathological context. Germline deletion of PDGF results in perinatal lethality, cardiovascular defects, and failed pericyte recruitment<sup>1-3</sup>. PDGF signaling is also required for angiogenic sprouting and vessel enlargement, suggesting that this growth factor is critical for vasculogenesis. Further, release of PDGF at sites of injury recruits vascular SMCs for the purpose of repairing the damaged vasculature. In the case of atherosclerosis, platelets respond to endothelial damage incurred by macrophages by releasing PDGF (Figure 1). Medial SMCs, in turn, migrate towards PDGF and invade into the intima, occluding the arterial lumen<sup>4</sup>. Thus, the migratory response of vascular SMCs toward PDGF can be viewed as

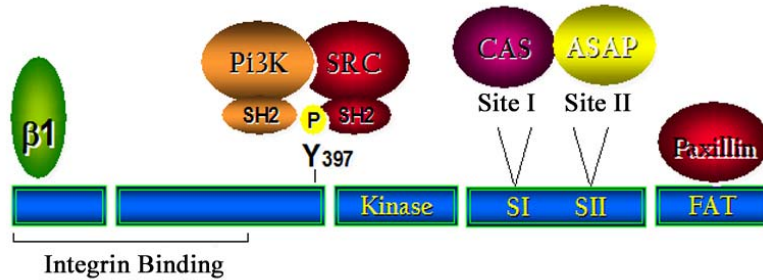
a turning point in the pathogenesis of atherosclerosis, and is of great relevance to our understanding of cardiovascular disease.



**Figure 1. SMC invasion in atherosclerotic lesion formation.** Fatty deposits along the arterial intima are phagocytosed by inflammatory cells, resulting in endothelial cell damage and platelet recruitment. Release of cytokines, eNOS, and PDGF stimulates proliferation and invasion of medial SMCs into the intimal layer, resulting in occlusion of the arterial lumen.

### ***Focal Adhesion Kinase Signaling in Vascular Smooth Muscle Cells.***

Focal adhesion kinase (FAK) is a 125 kD nonreceptor tyrosine kinase that, while ubiquitously expressed, may play a special role in SMCs. FAK can be divided into four major protein domains: an N-terminal integrin-binding domain; a central kinase domain; an SH3-binding domain; and a C-terminal focal adhesion-targeting domain<sup>5</sup> (Figure 2). Although FAK can be phosphorylated at six different residues (Table 1)<sup>6,7</sup>, FAK activation is dependent on autophosphorylation at tyrosine 397<sup>8</sup>. This autophosphorylation event enables binding to a number of SH2-containing proteins, including Src, PI3 Kinase, and Cas<sup>9</sup>.



**Figure 2. Protein domains of FAK.** Integrins bind at the N-terminus, stimulating autophosphorylation of Y397 and facilitating protein-protein interactions. FAK is localized to focal adhesions via the C-terminal FAT domain.

FAK is classically understood as a transducer of integrin signaling. Binding of integrins to extracellular matrix proteins such as fibronectin results in integrin clustering and activation of FAK via Y397 autophosphorylation<sup>10</sup>. However, more recent evidence shows that FAK is also activated by several growth factors including PDGF, and contractile agonists such as angiotensin II<sup>4,11</sup>. Autophosphorylation of FAK by these varied stimuli has been shown to activate numerous downstream signaling pathways, including the Rac-PAK-JNK and Ras-MEK-ERK pathways<sup>5,12</sup>. Thus, FAK has emerged as both a critical structural anchor and a major signaling hub, capable of cooperating with growth factor receptors, G-protein-coupled receptors, and matrix protein receptors to impact multiple downstream pathways with varied biological consequences for the cell.

Several lines of evidence suggest that FAK may play a unique role in SMCs. First, germline deletion of FAK in mice was shown to result in embryonic lethality by day E8.5<sup>13</sup>. FAK-null embryos exhibited a malformed mesoderm and anteroposterior axis, as well as poorly developed aortic and enteric arteries after day E8<sup>13,14</sup>. This vascular phenotype could reflect a defect in proper growth, differentiation, and/or migration of vascular SMCs. In support of this, FAK has been shown to be necessary for PDGF-stimulated growth and migration of vascular SMCs<sup>15,16</sup>. In addition, FAK has

<b>Tyrosine Residue</b>	<b>Significance of Phosphorylation</b>
<b>397</b>	Creates binding site for Src family kinases, PI3 kinase, PLC gamma, SOCS, GRB7, p120 Ras GAP
<b>407</b>	Required for binding of paxillin, vinculin
<b>861</b>	Associated with enhanced p130Cas binding to FAK C-terminal domain; required for paxillin binding
<b>925</b>	Creates binding site for GRB2
<b>576, 577</b>	Promotes maximal catalytic activity of FAK

**Table 1. Tyrosine phosphorylation sites on FAK.** Autophosphorylation at residue 397 is the best understood method of FAK activation. Additional phosphorylation sites at 407, 861, and 925 facilitate binding of FAK to other proteins, while 576 and 577 lie within the “activation loop” that helps maximize catalytic activity of FAK.

been implicated in the regulation of vascular SMC contractility, possibly by facilitating increases in  $\text{Ca}^{2+}$  current<sup>17</sup>. Finally, an endogenous dominant negative of FAK, termed Focal Adhesion Kinase-Related Non-Kinase (FRNK), is produced exclusively in SMCs<sup>18</sup>. FRNK is expressed at specific times during development and is upregulated following vascular injury<sup>18</sup>, indicating that FRNK expression in SMCs is significant at the organismal level, and suggesting that FAK requires a unique regulatory mechanism in SMCs. As a whole, this body of knowledge suggests that a SMC-specific role of FAK is physiologically relevant and could have important consequences for our understanding of cardiovascular development and disease.

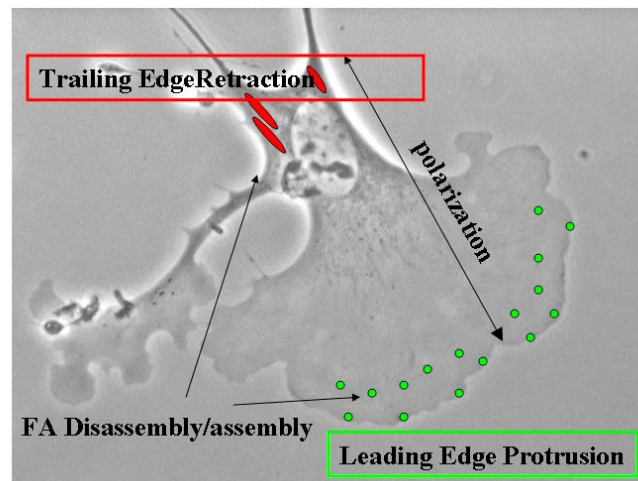
### ***FAK Signaling in Cell Migration.***

Studies using a wide variety of cell types have shown FAK to be a critical mediator of cell migration. Depletion of FAK or inhibition of FAK activity impairs the adhesion-driven motility of fibroblasts, neurons, keratinocytes, endothelial cells, and

several tumor cell lines<sup>14,19-22</sup>. Further, emerging evidence indicates that FAK is critical for growth factor-induced motility, engaging in cooperative signaling between integrins and growth factor receptors<sup>23</sup>. In SMCs, our group has shown that 3D migration towards PDGF-BB is blocked by adenoviral overexpression of FRNK. Others have shown that FAK activation and cell migration is diminished in SMCs exposed to hypoxia<sup>24</sup>, and disruption of alpha integrin-mediated FAK activation results impedes SMC migration *in vitro*<sup>25</sup>. Conversely, overexpression of FAK or enhanced FAK activity correlates with increased motility and tumor cell invasiveness<sup>26-28</sup>. However, the structural and molecular events that underlie FAK-mediated migration remain elusive and exhibit substantial variation between cell types.

Cell migration can be understood as a cyclical progression of four biomechanical events: cell polarization and membrane protrusion at the leading edge; construction and deconstruction of new focal adhesions; contraction of the cell body; and retraction of the trailing edge<sup>29</sup> (Figure 3).

Several lines of evidence suggest a role for FAK in one or more of these events. Fibronectin-stimulated 3D migration, cell spreading, wound healing, and focal adhesion turnover are inhibited in FAK-null fibroblasts<sup>14,30</sup>. FAK-depleted keratinocytes also exhibit



**Figure 3. The cycle of cell migration.** Branched actin polymerization facilitates lamellipodial protrusion at the leading edge. The cell polarizes by repositioning the nucleus at the stress fiber-rich trailing edge of the cell. Focal contacts are assembled and disassembled at the leading edge. Disassembly of existing focal adhesions and contraction of the cell body enables retraction of the trailing edge.

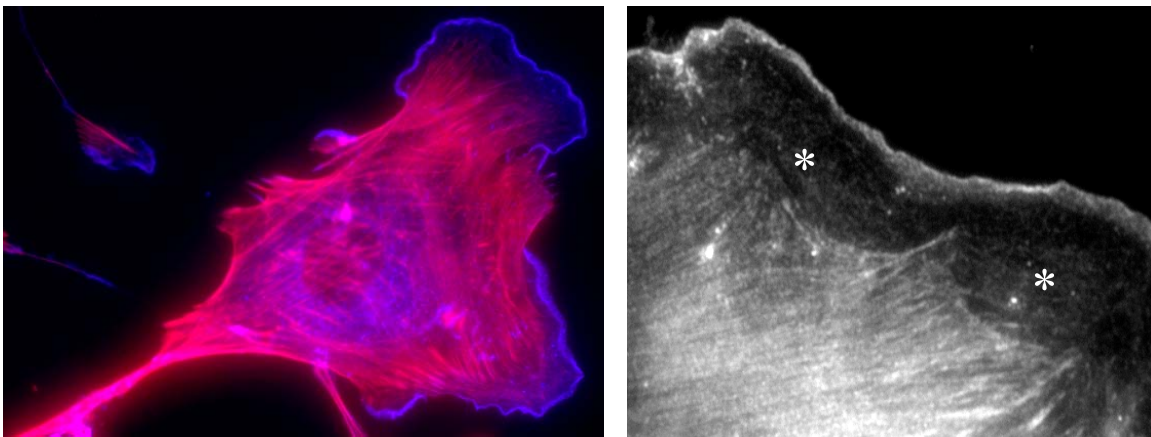
impaired focal adhesion turnover, as well as a disorganized actin network and enhanced stress fiber formation<sup>20</sup>. Randomly migrating endothelial cells, fibroblasts, and tumor cells display impaired lamellipodial extension and leading edge formation in the absence of FAK<sup>19,30</sup>. FAK has also been implicated in microtubule organization of migrating neuronal cells<sup>31</sup>, as well as microtubule stabilization at the leading edge of migrating fibroblasts<sup>32</sup>.

The proposed signaling events behind these structural phenomena are diverse, reflecting the multitude of proteins directly and indirectly impacted by FAK activity. Impaired focal adhesion turnover in FAK-deficient fibroblasts has been attributed to elevated Rho activity, possibly due to mislocalization of Src and p190 RhoGAP<sup>20,33</sup>. Conversely, it has been suggested that FAK-mediated focal adhesion turnover is dependent not on Rho, but on the GTPase dynamin<sup>34</sup>. FAK has been reported to promote activity of the Rac1 GTPase via interaction with a Src/Cas/Crk/DOCK180 complex<sup>35</sup>, but more recent evidence suggests that FAK may inhibit localized Rac1 activity<sup>36</sup>. Photoactivation of a caged FAK peptide halts lamellar extension, putatively by competitive binding of Src and PI3 Kinase<sup>37</sup>. In conclusion, while some consistencies have emerged, it is clear that the role of FAK in migration depends largely upon cell type and migratory stimulus.

### ***Remodeling of the Actin Cytoskeleton During Cell Migration.***

Cell migration represents the dynamic integration of myriad molecular events to promote the structural changes and mechanical forces necessary for movement<sup>38</sup>. The processes of polarization, lamellipodial extension, and focal adhesion formation/turnover

all require the concerted remodeling of various cytoskeletal components. Of these players, the most extensively studied is actin. Polymerized actin is important for the formation of numerous motile structures<sup>39</sup>. Long, unbranched bundles of actin form filopodia, which function as transient sensory extensions for the cell. Unbranched actin bundles also constitute large cable-like structures called stress fibers (Figure 4), which aid in structural support and contractility, and are particularly abundant in SMCs. Alternatively, actin can form as a network of branched filaments, thereby promoting lamellipodial protrusion and membrane ruffling, both of which require localized clearing of stress fibers.

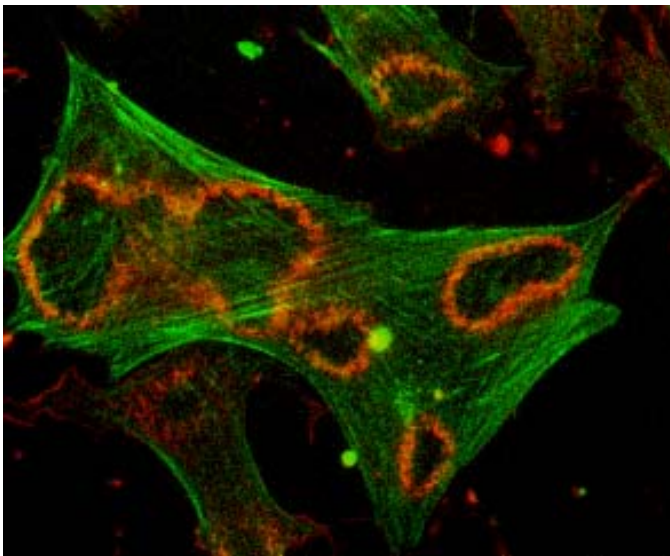


**Figure 4. Unbranched and branched actin networks in migrating SMCs.** *Left:* Actin stress fibers, stained by phalloidin (red), are bundles of unbranched actin that provide structural support and facilitate contractile force. Branched actin, highlighted by cortactin (blue), promotes protrusion at the leading edge. Note that stress fibers are cleared around sites of branched actin, and rich in the tapered trailing edge. *Right:* 100x magnification of phalloidin staining illustrates the cleared region (asterisks) between stress fibers and unbranched actin at the edge of a protruding lamellipod.

In addition to driving protrusion at the periphery of the cell, branched actin also promotes several types of specialized structures, including podosomes, invadopodia, and dorsal ruffles<sup>40</sup>. Dorsal ruffles are so called because they form on the dorsal surface of

the cell membrane. PDGF is a classic trigger of dorsal ruffle formation in multiple cell types, although epidermal and hepatocyte growth factors can also elicit this response.

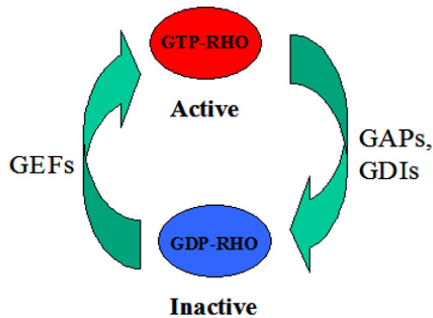
Two decades after their discovery, the biological function of dorsal ruffles remains unclear. Originally, they were thought to be important for generating force to drive cell motility. More recent evidence indicates that dorsal ruffles may facilitate lamellipodial extension by clearing stress fibers at the leading edge of the cell, or promote invasion by degrading the extracellular matrix. It has also been proposed that dorsal ruffles might function in the processes of macropinocytosis and internalization of ligand-bound growth factor receptors.



**Figure 5. Dorsal ruffles in a PDGF-treated SMC.** Cortactin (red), which drives branched actin polymerization, localizes to dorsal ruffles as soon as 2 minutes after PDGF treatment, and ruffling usually subsides by 30 minutes. Stress fibers (phalloidin, green) are generally cleared in the area immediately surrounding a dorsal ruffle.



### ***Rho Family GTPases: Master Regulators of the Cytoskeleton.***



**Figure 6. Regulation of Rho GTPases.** GEFs facilitate Rho signaling by exchanging GDP for GTP. GAPs and GDIs inactivate Rho by promoting the GDP-bound state.

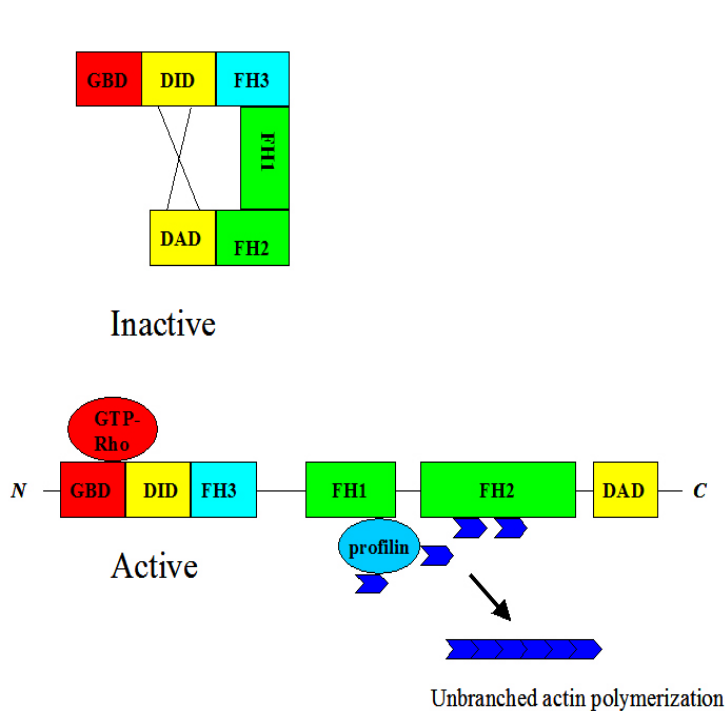
The Rho family of small GTPases consists of ten members, the best characterized of which are Rho, Rac, and Cdc42. Like other small GTPases, the Rho GTPases act as dynamic molecular switches within the cell, oscillating between a GDP-bound, or inactive, state, and a GTP-bound, or active, state (Figure 6). These enzymes are tightly regulated by three classes of molecules. GTPase Activating Proteins (GAPs), which enhance GTP hydrolysis, and Guanosine Nucleotide Dissociation Inhibitors (GDIs), which sequester GDP-Rho, act as negative regulators of Rho GTPase signaling. Conversely, Guanosine Nucleotide Exchange Factors (GEFs) promote the transition of GDP-Rho to GTP-Rho, facilitating effector binding and downstream signaling<sup>41</sup>.

A wealth of evidence has implicated the Rho GTPases as master regulators of cytoskeletal remodeling, as they impact multiple biological processes including migration, cytokinesis, phagocytosis, and axonal guidance. Rho activity has been shown to promote stress fiber formation, focal adhesion formation, and cell contractility. Rac signals to several activators of the Arp2/3 complex, including WAVE1/2, PI3 Kinase, and cortactin, thereby promoting branched actin polymerization, membrane ruffling, and lamellipodial extension. Finally, Cdc42 is associated with polarity establishment and filopodia formation<sup>41</sup>.

### *The Diaphanous-Related Formins in Cell Migration.*

The Diaphanous-Related Formins (DRFs) are the best-characterized group of the formin family of proteins, and consist of three members in mouse, termed mDia1, mDia2, and mDia3. Like all formins, the DRFs actively promote polymerization of unbranched actin via a conserved formin homology 2 (FH2) domain, and all three DRFs are effectors of the small GTPase RhoA<sup>42</sup>.

The DRFs can be divided into a regulatory N-terminal half and an actin-polymerizing C-terminal half<sup>42</sup> (Figure 7). The N-terminal portion contains the GTPase binding domain (GBD), the Diaphanous Inhibitory Domain (DID), and the formin homology 3 (FH3) domain, which contains a dimerization domain. The C-terminal



**Figure 7. Schematic of the DRF Dia2.** Dia2 is kept in an inactive conformation via an intramolecular DID-DAD interaction. Binding of GTP-Rho to the GBD disrupts this interaction, allowing Dia2 to assume an open conformation and to promote actin polymerization.

portion contains the FH1 domain, which binds profilin, the FH2 domain, which binds monomeric actin, and the Diaphanous Autoinhibitory Domain (DAD). It is thought that DRF activity is regulated via an intramolecular interaction between the DID and the DAD, whereby the DRF is kept in

a closed (inactive) conformation. Binding of active Rho to the GBD disrupts the DID-DAD interaction, whereupon the DRF assumes an open (active) conformation. In the active conformation, the FH1 and FH2 domains are exposed, allowing the DRF to complex with profilin, an abundant cytosolic protein that binds ATP-actin, and subsequently to nucleate actin filament assembly<sup>43</sup>.

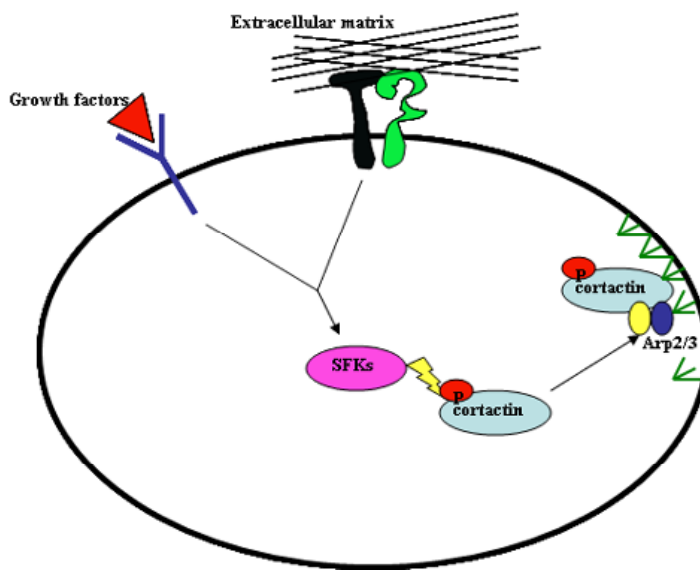
Through their actin remodeling activities, the DRFs impact a variety of cellular processes, such as cytokinesis, polarity establishment, endocytosis, and differentiation<sup>42,44</sup>. Not surprisingly, the DRFs also play a substantial role in cell adhesion and migration. Dia has been shown to localize to the tips of filopodia in multiple cell types<sup>45,46</sup>. Dial has also been implicated in stress fiber formation<sup>47</sup>, focal adhesion formation, and focal complex maturation<sup>48,49</sup>. It has also been shown that Dia targets microtubules to focal adhesions, thereby limiting focal adhesion growth and promoting turnover<sup>50</sup>. While very little is known about a connection between Dia and FAK, it was previously shown that Dial is required for microtubule stabilization at the leading edge of migrating fibroblasts, and that this process is dependent on FAK activity<sup>32</sup>. It has also been proposed that Dia promotes focal adhesion turnover by targeting Src to focal adhesions.<sup>51</sup> Existing data indicates that Src promotes focal adhesion turnover by interacting with FAK<sup>52,53</sup>, suggesting possible interplay between Src and Dia in this process.

Our group has found that ablation of FAK promotes transcription of SMC-specific genes (unpublished data). Coupled with the requirement of FAK for SMC migration, this suggests that, in SMCs, enhanced FAK activity correlates with a less differentiated, pro-migratory phenotype, while dampened FAK activity promotes a more

differentiated, anti-migratory phenotype. Since Dia2 activity has been shown to promote expression of SMC marker genes<sup>44,54</sup>, as well as migration of other cell types, it will be interesting to investigate whether FAK might determine the biological consequence of Dia activity in SMCs.

### ***Cortactin in Actin Polymerization and Cell Migration.***

Cortactin is an SH3 domain-containing protein that directly activates the Arp2/3 complex<sup>55</sup> and stabilizes the branch points of the resultant branched actin filaments (Figure 8)<sup>56</sup>. Cortactin was originally identified as a Src substrate in transformed cells and shown to localize to peripheral membrane ruffles, lamellipodia, and podosomes<sup>57,58</sup>. Recently, the roles of cortactin as a promoter of branched actin polymerization and as a Src target have been unified by the discovery that phosphorylation of cortactin by Src enhances branched actin assembly<sup>59</sup>. In response to activation of growth factors and integrins, cortactin is robustly recruited to peripheral and/or dorsal ruffles, where it drives branched actin polymerization.<sup>60</sup>



**Figure 8. Regulation and biological activity of cortactin.** Activation of growth factor receptors and integrins leads to activation of SFKs and phosphorylation of cortactin. In its activated state, cortactin is recruited to the membrane, where it cooperates with Arp2/3 to polymerize branched actin.

Rac1 activity is necessary and sufficient for cortical translocation of cortactin, and is also required for the phosphorylation of the major Src phosphorylation sites<sup>61,62</sup>. As one might expect, evidence suggests that cortactin plays an important role in cell migration. Knockdown of cortactin by siRNA inhibits transwell migration, invasion, and random motility in fibrosarcoma cells. Additionally, cortactin silencing results in lamellipodial instability and impaired focal adhesion assembly at areas of protrusion<sup>63</sup>. Conversely, overexpression of cortactin correlates with enhanced motility and metastatic potential in transformed cells<sup>64,65</sup>. Although cortactin has not been shown to localize to focal adhesions, it is phosphorylated upon integrin engagement<sup>66</sup>, and regulates spreading and intercellular adhesion of cancer cells<sup>67</sup>. While a connection between FAK and cortactin has not been extensively explored, it was recently shown that FAK is required for phosphorylation of cortactin by Src during internalization of integrin-binding *S. aureus*<sup>68</sup>. Thus, it appears that cortactin, like FAK, can transduce integrin and growth factor signals and in some contexts, may depend on FAK to mediate cytoskeletal rearrangement.

### ***Summary.***

The process of cell migration represents a precisely coordinated cycle of structural events involving a host of molecular players. A substantial body of evidence has implicated FAK as a key facilitator of several of these events. The goal of the following work was to elucidate the specific role of FAK in SMC migration. In undertaking this project, I focused on intracellular signaling by the Rho subfamily

GTPases. Cortactin and mDia2, which function downstream of Rac1 and RhoA respectively, emerged as components of particular interest in this work.

## EXPERIMENTAL PROCEDURES

***Cell Culture and Adenoviral Infection.*** Aortic smooth muscle cells were isolated from thoracic aortas of 8-week FAK<sup>flox/flox</sup> mice by enzymatic digestion as described previously<sup>69</sup>. Cells were used from passages 7–21 and were maintained in Dulbecco's modified Eagle's medium-F-12 (1:1) plus 10% fetal bovine serum and 1% penicillin-streptomycin. LacZ- and Cre-expressing adenoviruses were obtained from the University of Iowa Gene Transfer Vector Core, and were expanded and purified using the Adenopure kit (Puresyn). Cells were infected with adenovirus 24 hours after plating. All PDGF treatments occurred 72 hours after adenoviral infection. Latrunculin B was purchased from Calbiochem, and administered for 1 hour at 0.5  $\mu$ M.

***Antibodies.*** Antibodies to the FAK N-terminus, ERK1/2, cortactin, pAKT1 (Ser473), and Rac1 were obtained from Upstate. Anti-phospho-PDGF receptor  $\beta$  was also purchased from Upstate, and used at 1:500 for IHC. All antibodies were used at a dilution of 1:1000 for western blot. Anti-FAK and anti-cortactin were used at 1:500 for immunofluorescent staining. Anti-RhoA was obtained from Santa Cruz Biotechnology and used at a dilution of 1:250 for western blot. The antibody to phosphorylated myosin light chain was purchased from Cell Signaling and used at a dilution of 1:1000 for western blot. The vinculin antibody was from Sigma, and used at a dilution of 1:500. The antibody to detyrosinated tubulin was purchased from Millipore, and used at 1:1000.

for western and 1:500 for IHC. Texas Red- conjugated anti-mouse IgG was purchased from Jackson ImmunoResearch, and FITC-conjugated phalloidin was purchased from Molecular Probes. Polyclonal anti-GFP and anti-Flag M2 were purchased from Clontech and Sigma, respectively, and were both used at 1:1000 for western blot.

**3D Migration Assays.** FAK<sup>flox/flox</sup> mouse aortic SMCs were infected with LacZ or Cre adenovirus as described above. Cells were washed in PBS (calcium- and magnesium-free), trypsinized, and collected in PBS containing soybean trypsin inhibitor (1 mg/ml). Cells were pelleted at 2500 rpm and resuspended in serum-free DMEM-F12 containing 0.1% fatty acid-free BSA (Sigma). Cells were plated on 8 uM transwell inserts (BD Biosciences), which were coated with fibronectin (10 ug/mL, Sigma). The bottom well was filled with serum-free DMEM-F12 containing 0.1% BSA and vehicle or PDGF-BB (20 ng/ml, Calbiochem). Chambers were incubated at 37°C for 7 h. Chambers were rinsed with PBS and fixed in 4% paraformaldehyde for 20 min. Cells were removed from the top chamber by scraping with a cotton swab, and the remaining cells (in the bottom chamber) were stained with 1% crystal violet and counted.

**Immunofluorescent Staining.** Cells were plated on Lab Tek II chamber slides (Nunc) and infected with LacZ- or Cre-expressing adenovirus as described above. Prior to PDGF treatment, cells were starved overnight, then re-starved for 4 hours the following morning. Slides were fixed in 4% paraformaldehyde for 20 minutes (or 100% methanol for 5 minutes for microtubule staining), washed thrice in PBS, then permeabilized for 3 minutes with 0.4% Triton X-100 in PBS. Following permeabilization, nonspecific staining was blocked via 30 minute incubation in PBS containing 20% goat serum and 3% BSA. Cells were incubated with primary antibody in



blocking solution for 1 hour, washed thrice with PBS, then incubated for 1 hour in PBS containing Texas Red goat anti-mouse IgG (1:1000) and FITC-conjugated phalloidin (1:400). Secondary antibody was removed via three washes in PBS and one wash in distilled water prior to coverslipping. All steps were performed at room temperature.

***Microscopy and Kymographic Analysis.*** Fluorescence images were generated using an Olympus BX51 for fixed cells and an Olympus IX70 for live cells. Images were acquired at 40x magnification by a CoolSnap FX CCD camera (Photometrics) using iSee software (Isee Imaging Systems).

***Rho Family GTPase Activity Assays.*** LacZ- and Cre-infected SMCs were starved for 48 hours, then treated with PDGF and lysed in Rho Buffer A (50 mM Tris pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 10 mM MgCl<sub>2</sub>) containing 100 μM leupeptin, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 20 ug/mL soybean trypsin inhibitor, and 0.05 trypsin inhibitory units/ml aprotinin. Lysates (600 ug-1mg for RhoA; 200-500 ug for Rac1) were rotated with 40 ug of immobilized GST-Rho binding domain (RBD) or 30 ug GST-p21 binding domain (PBD) for 30 minutes at 4°C, then washed three times with Rho Buffer B (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM MgCl<sub>2</sub> containing the protease inhibitors listed above. Complexes were pelleted by centrifugation, boiled in SDS-PAGE buffer, electrophoresed (15% SDS-PAGE) and transferred to PVDF membrane. Western Blots were performed for Rac1 or RhoA.

***GST Pull-Down Assay.*** GST-FH2 (amino acids 596-1030 of Dia2) and GST-FH1FH2 (amino acids 532-1030 of Dia2) was purified from bacterial lysates using glutathione-agarose beads (Amersham Biosciences) as described previously (16). Cells

were lysed in Buffer A (50 mM Tris, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 0.5 mM MgCl<sub>2</sub>) containing 10 mM beta glycerol phosphate, 40 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 1 ug/mL microcystin, and protease inhibitors described above. Lysates were cleared by centrifugation for 10 min at 14000 rpm at 4°C and protein concentrations determined. 1 mg of SMC lysate was combined with 10 ug of GST-FH2 or GST-FH1FH2, and samples were rotated at 4 °C for 2 hrs. The complexes were pelleted by centrifugation and washed thrice in Buffer A containing protease and phosphatase inhibitors, then once in 1X TBS. Samples were boiled in SDS-PAGE buffer, electrophoresed (10% SDS-PAGE gel), and transferred to nitrocellulose membrane. Western blotting was performed using an anti-cortactin primary antibody as described above.

***Coimmunoprecipitation.*** COS-7 cells were cotransfected with GFP and Flag empty vectors, or with GFP-cortactin and Flag-Dia2, then lysed in NP-40 buffer (1% NP-40, 0.5% Triton-X100, 200 mM EDTA, 0.15 M NaCl, and 50 mM Tris-Cl, pH 7.2) containing protease and phosphatase inhibitors as described above. Lysates were cleared by centrifugation and protein concentrations determined. 1 mg of lysate was rotated with 35 uL of Flag-M2 agarose beads (Sigma) overnight at 4C. Complexes were pelleted by centrifugation, then washed twice in NP-40 buffer containing protease and phosphatase inhibitors, and once in 1X TBS. Complexes were resuspended and boiled in SDS-PAGE sample buffer, then electrophoresed on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane. Membrane was blocked in 5% milk for 1 hour, and western blot for GFP and Flag was performed as indicated above.

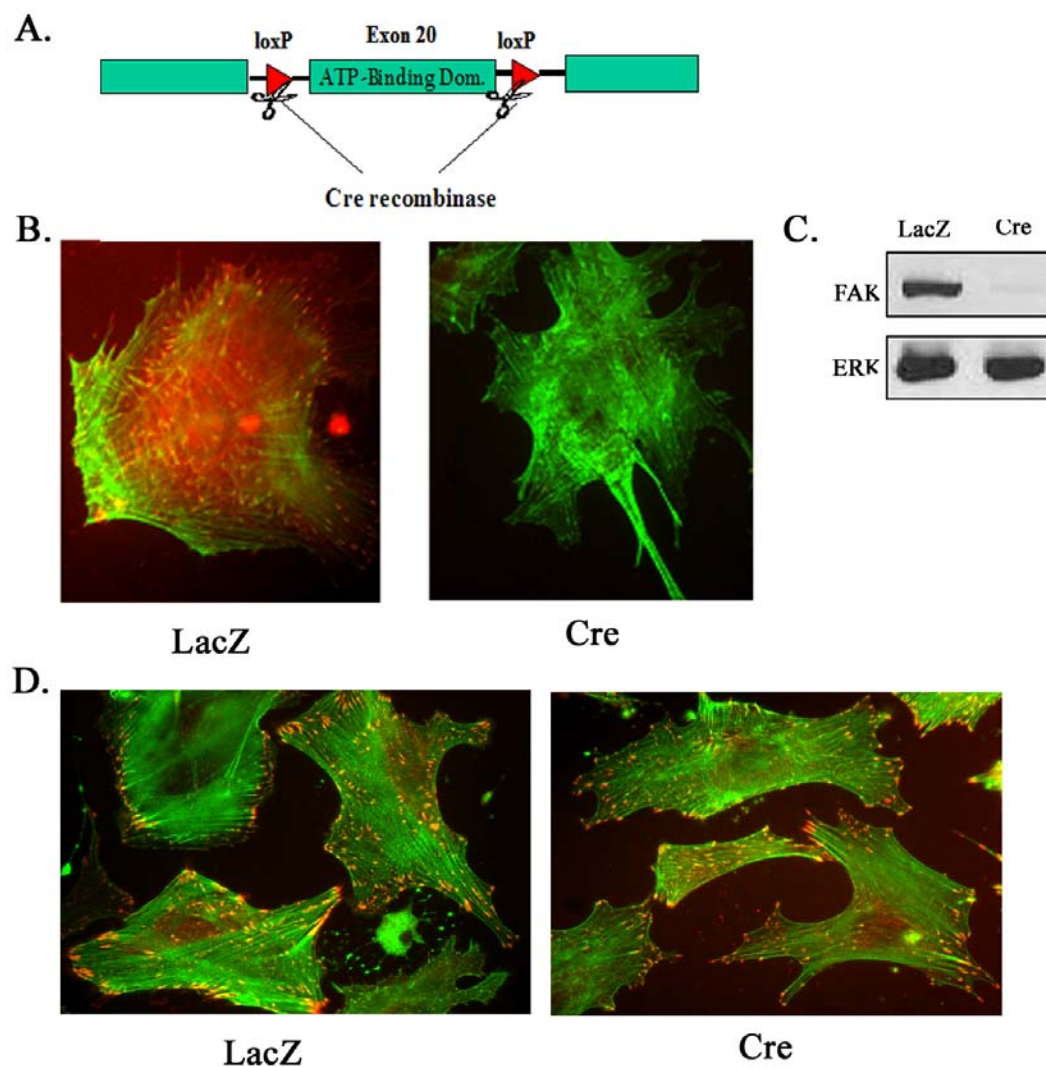
**Plasmids.** All Dia2 constructs were generously provided by Dr. Christopher Mack, and mCherry paxillin was a generous gift of Dr. Klaus Hahn (Dept. of Pharmacology, UNC-Chapel Hill). pEGFP-cortactin was kindly contributed by Dr. James Bear (UNC-Chapel Hill). WAVE2 was purchased from Open Biosystems and subcloned into pEGFP (Promega).

**Statistical Analysis.** For transwell migration assays, the average number of migrated cells per well was calculated from a raw count from four separate fields within the well. Each condition was conducted in duplicate. For dorsal ruffling and polarization quantification, the percentage of cells exhibiting dorsal ruffles or the percentage of polarized cells was calculated from seven fields per slide. For densitometry of glutubulin levels, intensity levels for each band were calculated using NIH ImageJ, then normalized to the corresponding intensity of ERK for each lane. Data shown represents the average of three western blots. For all experiments, p value was calculated using a student's unpaired t-test.

## RESULTS

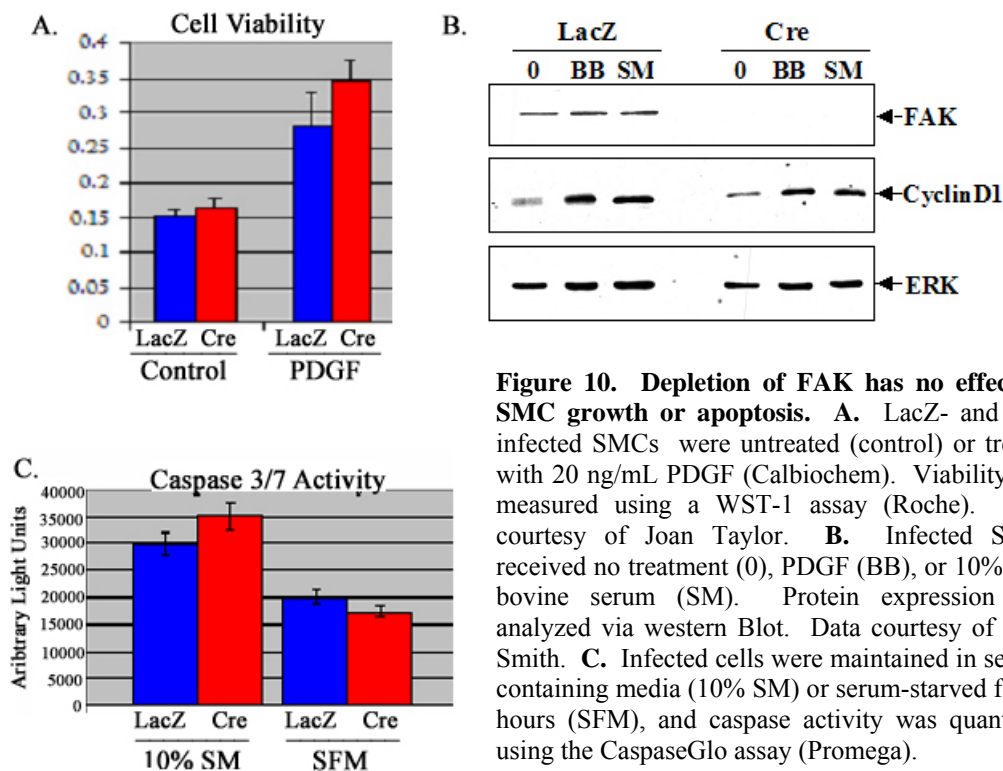
### *Effects of FAK depletion on SMC biology.*

To conditionally ablate FAK in primary SMCs, we have used a published mouse model termed the *fak-flox* mouse<sup>70</sup>, in which loxP sites flank the ATP-binding domain of *fak* exon 20 (Figure 9A). In the presence of Cre recombinase, these loxP sites are cleaved and FAK protein is no longer produced. We isolated aortic SMCs from 8-week FAK<sup>flox/flox</sup> mice and used a replication-deficient adenoviral construct to express Cre recombinase in cultured cells. At 72 hours after viral treatment, FAK expression was effectively ablated in SMCs infected with Cre-expressing virus, but not in those infected with LacZ-expressing virus (Figure 9B-C). Notably, FAK- SMCs exhibit comparable cell spreading and cytoskeletal arrangement to their FAK+ counterparts, contrasting with reports of impaired spreading and aberrant actin structures in endothelial cells, fibroblasts, and keratinocytes following *fak* deletion. Additionally, vinculin staining reveals similar focal adhesion size in FAK-containing and FAK-depleted SMCs (Figure 9D), suggesting that the formation and turnover of these structures is unperturbed in randomly migrating FAK- SMCs. These preliminary observations support the idea that the role of FAK in SMCs is different from its role in other cell types.



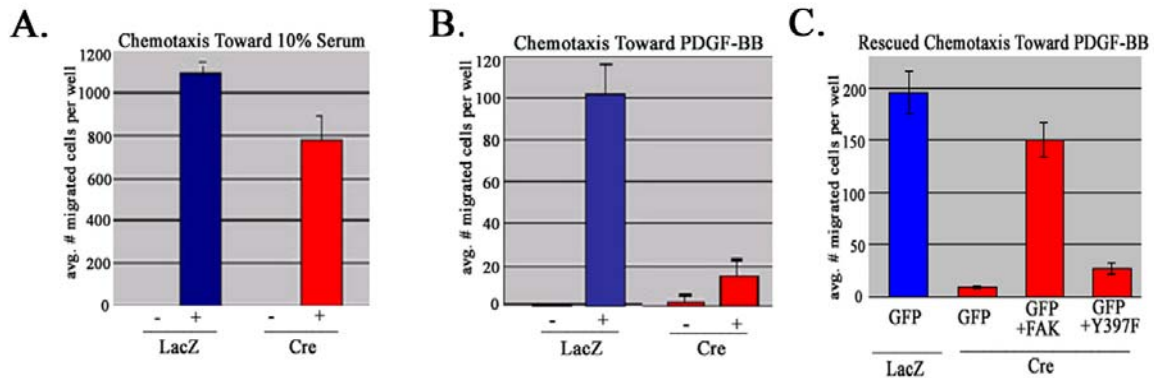
**Figure 9. Conditional depletion of FAK in cultured SMCs.** **A.** Schematic depicting the *fak* gene in the *fak-flox* mouse. **B-D.** Primary aortic SMCs from 8-week FAK<sup>flox/flox</sup> mice were infected with either Cre-expressing or LacZ-expressing (control) adenovirus. Cells were fixed and imaged (**B,D**) or lysed (**C**) 72 hours post-infection. **B.** SMCs were fixed in 4% paraformaldehyde and immunostained with anti-FAK (red) and phalloidin (green). **C.** SMC lysates were separated by SDS-PAGE, and probed for FAK and ERK via western Blot. **D.** SMCs were fixed and immunostained with anti-vinculin (red) and phalloidin (green).

Because FAK has been linked to multiple cellular processes, including proliferation, apoptosis, migration, and differentiation, we investigated the consequences of FAK ablation on these fundamental aspects of SMC biology. A WST-1 assay, which measures cell viability based on mitochondrial function, showed no significant difference in the viability of Cre- and LacZ-infected FFSMCs (Figure 10A). Further, FAK depletion had no effect on mitogen-stimulated cyclinD1 expression (Figure 10B), indicating that FAK is not required for SMC proliferation or most likely cell survival. To assess the impact of FAK depletion on apoptosis, I quantified the activity of caspases 3 and 7, which are early-stage effectors of the apoptotic pathway. At 72 hours post-infection, caspase 3/7 activity was not significantly different in FAK<sup>+</sup> and FAK<sup>-</sup> cells (Figure 10C), suggesting that FAK depletion has no detectable effect on SMC apoptosis.



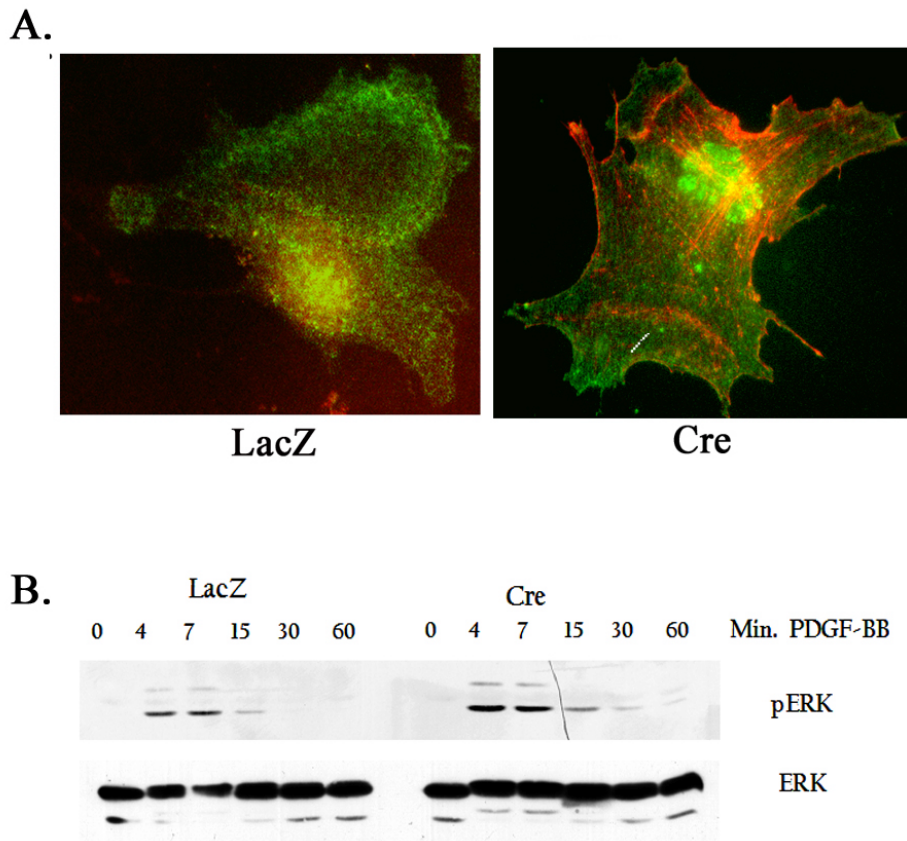
**Figure 10. Depletion of FAK has no effect on SMC growth or apoptosis.** **A.** LacZ- and Cre-infected SMCs were untreated (control) or treated with 20 ng/mL PDGF (Calbiochem). Viability was measured using a WST-1 assay (Roche). Data courtesy of Joan Taylor. **B.** Infected SMCs received no treatment (0), PDGF (BB), or 10% fetal bovine serum (SM). Protein expression was analyzed via western Blot. Data courtesy of Liisa Smith. **C.** Infected cells were maintained in serum-containing media (10% SM) or serum-starved for 16 hours (SFM), and caspase activity was quantified using the CaspaseGlo assay (Promega).

To analyze directed migration of FAK-containing and FAK-depleted SMCs, I used a transwell assay, in which cells crawl through a porous membrane toward a chemoattractant. Interestingly, fibronectin alone was insufficient to stimulate SMC migration, underscoring the unique migratory behavior of SMCs as compared to the more extensively studied fibroblasts. While FAK depletion only slightly impaired SMC migration towards 10% serum (Figure 11A), it dramatically inhibited PDGF-stimulated migration (Figure 11B), suggesting that FAK is critical for this specific agonist signaling pathway. Overexpression of wild-type FAK rescued the ability of FAK- SMCs to migrate towards PDGF (Figure 11C). However, overexpression of FAK Y397F, which cannot be phosphorylated at tyrosine residue 397, failed to rescue the migratory defect, indicating that this major phosphorylation event is critical to PDGF-stimulated SMC migration.



**Figure 11. Impaired 3D migration in FAK-depleted SMCs.** A-C. LacZ- or Cre-infected cells were plated on fibronectin-coated inserts (BioCoat) and allowed to migrate toward 10% FBS (A, courtesy of Liisa Smith), 20 ng/mL PDGF (B,C) for 7 hours. Cells were counted in four fields and averaged. (C) LacZ- or Cre-infected cells were cotransfected with GFP and Flag, GFP and Flag-FAK, or GFP and Flag-FAK Y397F. Twenty-four hours after transfection, chemotaxis was assayed as in (A). The number of migrated GFP-positive cells after 7 hours was normalized to the number of attached GFP-positive cells after 1 hour to account for differences in transfection efficiency. Data shown in (C) provided courtesy of Joan Taylor.

To ensure that FAK was not required for localized activation and/or turnover of the PDGF- $\beta$  receptor, I performed immunofluorescent staining using an antibody specific to the phosphorylated PDGFR. Both LacZ- and Cre-infected cells demonstrated intense staining at peripheral and dorsal membrane ruffles following PDGF treatment (Figure 12A), indicating that PDGFR activation and turnover are FAK-independent processes. Also, PDGF-stimulated ERK activity is not significantly different between FAK+ and FAK-null cells (Figure 12B), supporting the findings that PDGF-stimulated growth is intact in these cells.



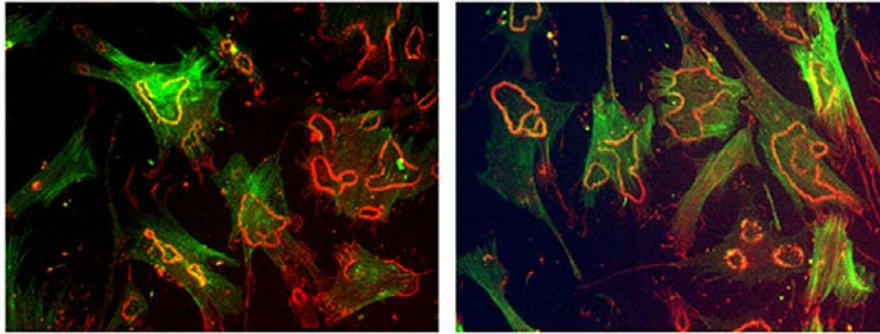
**Figure 12. PDGF receptor activation is FAK-independent.** **A.** SMCs were serum starved for 16 hours, treated with 20 ng/mL PDGF for 5 minutes, fixed in paraformaldehyde, and stained with anti-phospho-PDGF receptor beta (green) and phalloidin (red). **B.** Cells were starved as in (A), and treated at indicated timepoints. Lysates were separated by SDS-PAGE and probed via western Blot for phospho-ERK1/2 (Upstate).



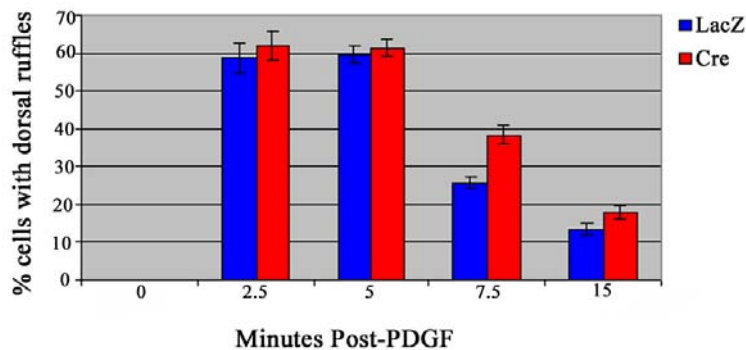
### ***PDGF-induced membrane ruffling and cell polarization.***

Given the dramatic inability of FAK-depleted SMCs to migrate three-dimensionally to PDGF, we used immunofluorescent staining to visualize PDGF-induced actin remodeling in a two-dimensional context. Both LacZ- and Cre-infected SMCs demonstrated dramatic peripheral and dorsal membrane ruffling following PDGF treatment (Fig. 13A). These ruffles colocalized with cortactin, and stress fibers were cleared in the immediately surrounding area. Additionally, the dynamics of dorsal ruffle formation were similar in the presence and absence of FAK, with the response initiating around 2 minutes, peaking around 5 minutes, and subsiding by 30 minutes (Fig. 13B).

**A.**



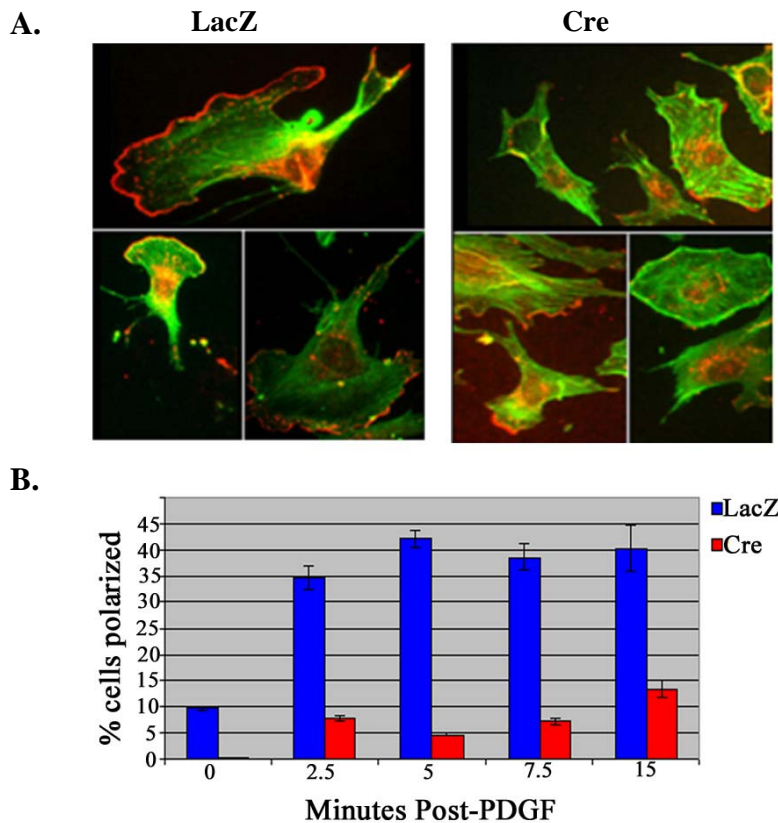
**B.**



**Figure 13. PDGF-induced dorsal and peripheral membrane ruffling is unimpaired by FAK depletion.** LacZ- or Cre-infected SMCs were serum starved for 16 hours, treated with PDGF-BB (20 ng/mL) and fixed in 4% paraformaldehyde at indicated timepoints. **A.** Cells were stained with phalloidin (green) and anti-cortactin (red). Images shown are at 40x magnification. **B.** The percentage of cells exhibiting dorsal ruffles was calculated for seven fields per slide. At 7.5 minutes post PDGF, the difference in ruffling was not significant ( $p = 0.13$ ).

Taken together, these observations indicate that cortactin-mediated branched actin polymerization and subsequent membrane protrusion are uninhibited in the absence of FAK.

Along with membrane protrusion, a critical step in migration is polarization of the cell body<sup>29</sup>, as it dictates the directionality of cell movement. Importantly, it has been shown that a distinct chemotactic gradient, such as that established in a transwell assay, is not required for cell polarization<sup>38</sup>. Consistent with this, we observed dramatic SMC polarization following PDGF treatment in a two-dimensional system, regardless of the homogenous distribution of agonist. Interestingly, FAK<sup>-</sup> cells appeared markedly less polarized following PDGF stimulation (Figure 14A).

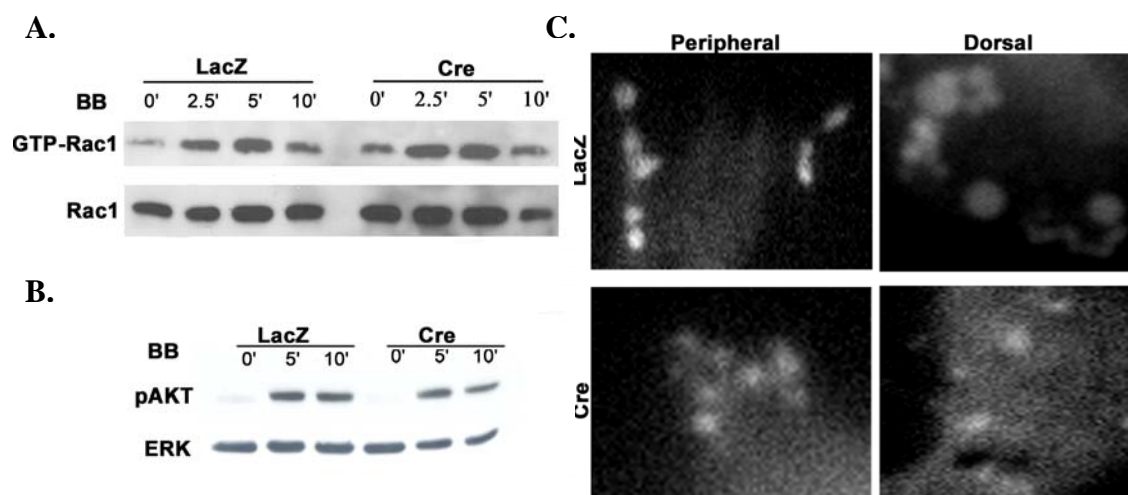


**Figure 14. PDGF-induced cell polarization is reduced five-fold in FAK-SMCs.** LacZ (blue)- or Cre (red)-infected SMCs were serum starved for 16 hours, treated with PDGF-BB (20 ng/mL) and fixed in 4% paraformaldehyde at indicated timepoints. **A.** Cells were stained with phalloidin (green) and anti-cortactin (red). Images shown are at 40x magnification. **B.** Polarized cells were counted in seven fields per slide, then averaged. For all timepoints shown,  $p \leq 0.01$ .

Polarized FAK<sup>+</sup> cells were characterized by a broad, cortactin-rimmed leading edge and a stress fiber-rich, tapered trailing edge, while FAK<sup>-</sup> cells formed some cortactin-containing lamellapodia, but failed to form the clear leading and trailing edges indicative of directional movement. Quantitatively, polarization following PDGF treatment was reduced approximately five-fold in the absence of FAK (Figure 14B), indicating that FAK is critical for this aspect of PDGF-stimulated cytoskeletal remodeling.

***PDGF-induced Rac1 signaling in FAK-depleted SMCs.***

A considerable body of evidence indicates that FAK can facilitate cell motility by regulating components of the Rac signaling pathway, such as Cas, PI3 kinase, and DOCK180<sup>35,71,72</sup>. To explore this pathway in FAK-depleted SMCs, we first analyzed global Rac1 activation in response to PDGF by using a GST-PBD pulldown assay. Consistent with previous reports<sup>73,74</sup>, PDGF rapidly and robustly activated Rac1 within 2.5 minutes, and activation began to subside at 10 minutes (Figure 16A). Further, this activation was comparable in the presence and absence of FAK, indicating that FAK is dispensable for global activation of Rac1 in PDGF-treated SMCs.



**Figure 15. Rac1/PI3 Kinase/AKT signaling is not perturbed by FAK depletion.** **A.** Serum-starved SMCs were treated with PDGF for the timepoints indicated, then lysed and rotated with 30 ug of GST-PBD-conjugated sepharose beads for 30 minutes. Rac1 immunoblot was performed for bead-bound Rac (upper) and 3% input lysates (lower). **B.** Serum-starved SMCs were treated for the timepoints indicated, then scraped into sample buffer and electrophoresed. Immunoblot was performed for pAKTSer473 and ERK1/2 (loading control). **C.** Infected SMCs were transfected with GFP-WAVE2, starved, then treated with PDGF and imaged in real time. Images shown represent 5 minutes post-PDGF.

Despite this similarity in global Rac1 activation, we analyzed two downstream targets of Rac1 to explore possible specific signaling defects that might not be detectable by GST pulldown assay. PI3 kinase, which interacts directly with FAK, plays a critical role in cell polarization and directional chemotaxis in multiple cell types via its downstream target, AKT<sup>75-77</sup>. To determine if this signaling pathway might be perturbed in the absence of FAK, we analyzed AKT phosphorylation in PDGF-treated SMC lysates. In LacZ- and Cre-infected SMCs, AKT was similarly phosphorylated following PDGF treatment (Figure 16B), suggesting that FAK depletion has no significant effect on PI3 kinase-AKT signaling.

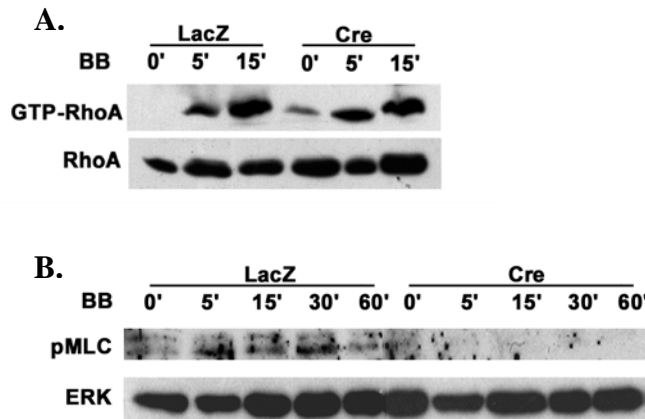
As shown in Figure 13, we found that FAK was dispensable for dorsal ruffling, but required for cell polarization. WAVE2, a member of the Wiskott - Aldrich Syndrome

Protein family, has been shown to be required for PDGF-induced polarization, but not dorsal ruffling<sup>78</sup>. Further, this activity was shown to depend on PI3 kinase. To determine if FAK might be required for WAVE2 activation, we used a GFP-tagged variant of WAVE2 to visualize localization of this protein in live SMCs. In both LacZ- and Cre-infected SMCs, WAVE2 was rapidly and robustly recruited to both dorsal and peripheral ruffling (Figure 16C), suggesting that FAK is not required for PDGF-induced WAVE2 activation. This finding is also consistent with the unperturbed PI3 kinase signaling shown in Figure 16B.

***Global activation of RhoA and myosin light chain.***

It has been suggested that FAK may regulate focal adhesion turnover by suppressing Rho activity<sup>33</sup>. To assess RhoA activity in FAK-depleted SMCs, we used an approach similar to that described in Figure 16. Interestingly, a GST pulldown assay indicated that RhoA was substantially activated by PDGF in SMC, in contrast to reports conducted in other cell types<sup>79-81</sup>. Like Rac1, RhoA was similarly activated in the presence and absence of FAK (Figure 17A). Notably, basal RhoA activity prior to PDGF treatment was slightly elevated in the absence of FAK, consistent with observations of FAK-null fibroblasts<sup>14</sup>. These data suggest not only that FAK is dispensable for global activation of RhoA in response to PDGF, but also that RhoA may play a unique role in PDGF signaling in vascular SMCs.

Contraction of the cell body is a critical component of polarization, and is in part mediated by the conventional myosin, myosin II<sup>82</sup>. Myosin II, which consists of a light chain and a heavy chain, is activated by serine phosphorylation of the myosin light chain (MLC). RhoA can promote MLC phosphorylation through its effector, Rho kinase (ROCK) by two principle mechanisms<sup>83</sup>. First, ROCK can directly phosphorylate myosin light chain. Second, it can inhibit the activity of myosin light chain phosphatase, thereby promoting the phosphorylated state of myosin light chain. We assessed MLC phosphorylation in PDGF-treated SMCs to determine if impaired contractility might underlie the defective polarization of FAK-depleted cells. PDGF induced modest phosphorylation of MLC, peaking at 30 minutes and subsiding by 60 minutes (Figure 17B). Interestingly, this phosphorylation was dramatically attenuated in FAK-depleted SMCs, despite comparable basal levels prior to treatment. Thus, while RhoA activation

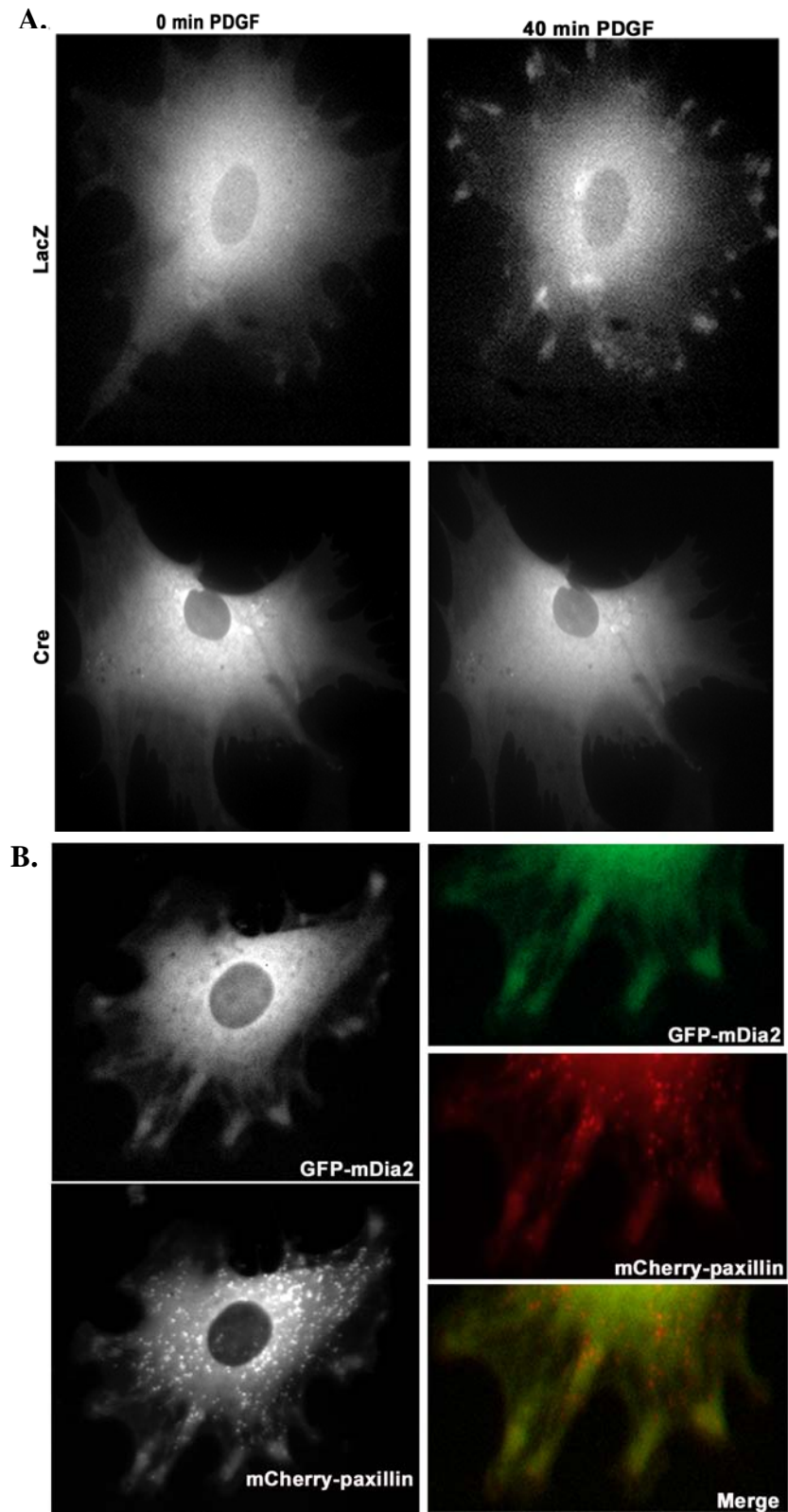


**Figure 16. RhoA/myosin light chain signaling is perturbed by FAK depletion.** **A.** Serum-starved SMCs were treated with PDGF for the indicated timepoints, then lysed and rotated with 40 ug of GST-RBD beads for 30 minutes. Immunoblot was performed for bead-bound RhoA (upper) and 5% input (lower). Blot provided courtesy of Joan Taylor. **B.** Serum-starved SMCs were treated with PDGF for the indicated timepoints, then lysed and immunoblotted for pMLCSer19 and ERK1/2 (loading control).

appears FAK-independent at the global level, this finding indicates perturbed signaling to a specific RhoA target.

### ***PDGF-induced localization of Dia2.***

The formin Dia2 has been well characterized *in vitro* as a promoter of unbranched actin. However, the *in vivo* function of the DRFs is still poorly understood. Further, the role of the DRFs in PDGF signaling has not been investigated. We hypothesized that Dia2 might be important in SMC migration to PDGF, and assessed this by imaging live cells expressing YFP-tagged Dia2. Surprisingly, PDGF treatment redistributed Dia2 dramatically (Figure 18A, upper). At baseline, Dia2 was diffusely distributed. Following PDGF treatment, however, it was driven to prominent streaks at the periphery of the cell, the localization and appearance of which were consistent with those of mature focal adhesions. Moreover, this localization pattern was absent in FAK-depleted SMCs (Figure 18A, lower). To confirm that these structures were focal adhesions, we cotransfected SMCs with YFP-Dia2 and mCherry-paxillin, a known marker of focal adhesions. Indeed, following PDGF treatment, the Dia2-containing streaks colocalized with mCherry paxillin, while immature focal contacts were highlighted by paxillin alone (Figure 18B). Together, these observations indicate that FAK is required for localization of Dia2 to mature focal adhesions in response to PDGF.

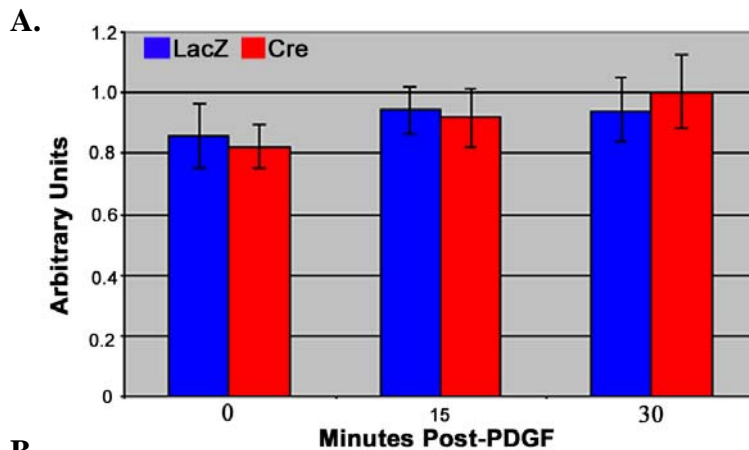


**Figure 17. PDGF drives Dia2 to focal adhesions only in the presence of FAK.** **A, B.** SMCs were transfected with YFP-Dia2 (A) or YFP-Dia2 and mCherry-paxillin (B), then starved, treated with PDGF, and imaged in real time. Images shown represent 35 minutes post-PDGF.



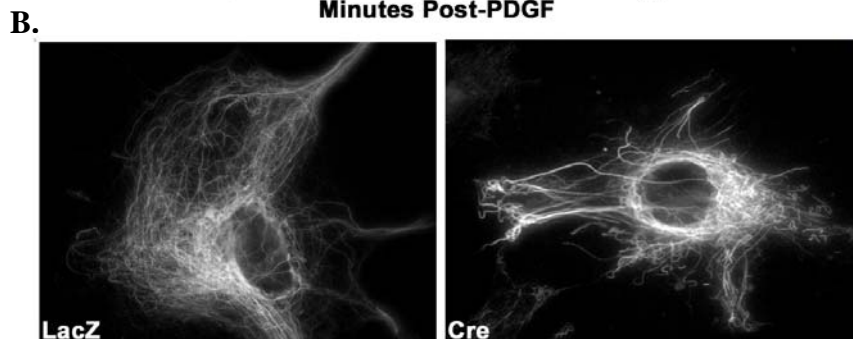
### *Stabilized microtubules in FAK-depleted SMCs.*

Since the above data show that Dia2 is highly responsive to PDGF, we next wondered what the specific biological role of Dia2 in SMC migration might be. It was previously reported that overexpression of FRNK ablated stabilized microtubules in migrating fibroblasts, and that this defect could be rescued by overexpression of constitutively active Dia1<sup>34</sup>. To determine whether FAK-Dia signaling might play a similar role in SMCs, we performed Western blot analysis and immunohistochemical staining of detyrosinated tubulin (glu-tubulin), a marker of stabilized microtubules. By western blot, PDGF treatment had no significant effect on glu-tubulin levels in either LacZ- or Cre-infected cells (Figure 19A), consistent with previous reports in fibroblasts<sup>84</sup>. Furthermore, glu-tubulin was abundant in both LacZ- and Cre-infected cells at baseline (Figure 19B), suggesting that the FAK-Dia signaling axis is not critical for microtubule stabilization in vascular SMCs.



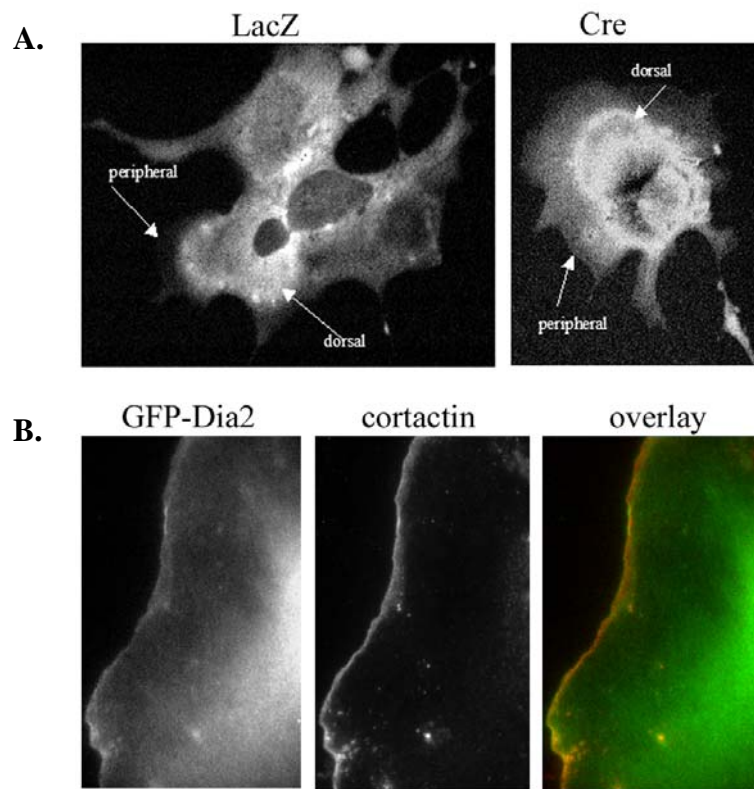
**Figure 18. FAK-Dia signaling is dispensable for microtubule stabilization.**

**A.** Densitometry of western blot for glu-tubulin at various timepoints post-PDGF was calculated using NIH ImageJ. Data shown represents the average of three blots. **B.** Infected SMCs were serum-starved, fixed in methanol, then stained for glu-tubulin and imaged at 40x mag.



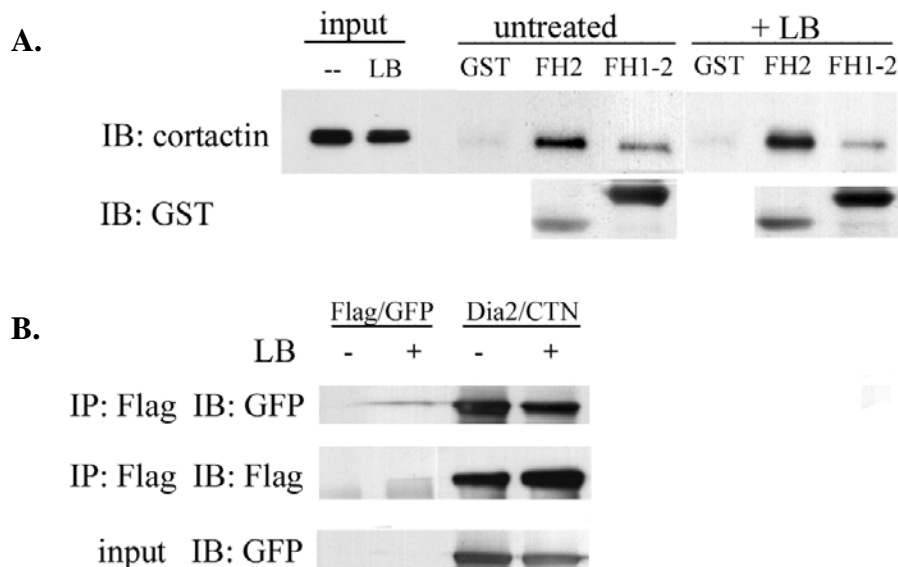
### *A novel interaction between Dia2 and cortactin.*

In addition to focal adhesion localization, we noted a second response of YFP-Dia2 to PDGF treatment in live SMCs. Namely, like cortactin and WAVE2, Dia2 localized to areas of membrane ruffling in a robust manner (Figure 20A). Further, GFP-Dia2 and endogenous cortactin are both enriched at membrane ruffles in serum-maintained SMCs, as demonstrated by immunocytochemistry (Figure 20B). Interestingly, unlike focal adhesion targeting, this localization pattern was FAK-independent. Given this observation, and a previous report implicating cortactin as a formin binding protein, we wondered whether Dia2 and cortactin might interact in SMCs.



**Figure 19. GFP-Dia2 and cortactin are enriched at membrane ruffles.** **A.** Infected SMCs were transfected with GFP-Dia2, serum starved, and treated with 20 ng/mL PDGF. Live cell images shown represent 10 minutes after PDGF at 40x magnification. **B.** SMCs were transfected with GFP-Dia2 (green), then fixed and stained for endogenous cortactin (red). Images shown are at 100x mag.

Initially, we hypothesized that the SH3 domain of cortactin would bind to the proline-rich region of the FH1 domain in Dia2, as suggested by a previous report<sup>85</sup>. However, using a GST pulldown assay, we found that the FH2 domain alone was capable of pulling down cortactin, indicating that the proline-rich FH1 domain was dispensable for this interaction (Figure 21A). To ensure that Dia2 and cortactin were not simply interacting by virtue of their common association with F-actin, we repeated the pulldown in SMCs treated with latrunculin B, an inhibitor of actin polymerization. Surprisingly, the interaction persisted in latrunculin-treated lysates, indicating that F-actin does not mediate the Dia2-cortactin interaction. Moreover, Flag-Dia2 and GFP-cortactin associated strongly in COS lysates before and after latrunculin B treatment, as shown by coimmunoprecipitation (Figure 21B). Taken together, these data suggest that the FH2 domain of Dia2 binds to cortactin independently of F-actin.



**Figure 20. Dia2 and cortactin associate independently of F-actin.** **A.** SMCs received no treatment or 0.5 uM latrunculin B for 1 hour. For each reaction, 800 ug of lysate was incubated with 10 ug of GST alone, GST-FH2, or GST-FH1FH2. **B.** COS-7 cells were cotransfected with Flag and GFP empty vectors, or with Flag-Dia2 and GFP-cortactin. Cells received no treatment or latrunculin B for 1 hour. Triton. For each reaction, 1 mg lysate was rotated with 35 uL of Flag-M2 agarose beads. Input lanes represent 20 ug for both A and B.

## DISCUSSION

### *Understanding the biological role of FAK: the importance of cell type.*

Based on previous evidence, it is clear that FAK is critical for cell migration in various cellular and organismal systems. However, it is also clear that the specific manner in which FAK mediates migration varies between cell types, as summarized in Table 2. Additionally, several lines of evidence suggest that FAK might have a unique biological role in SMCs, as discussed previously. Given this conceptual framework, we sought to perform the first conditional FAK knockout in differentiated SMCs. While some overlap exists between the phenotype described in this work and those described in Table 2, our study highlights several aspects in which the biological role of FAK in SMCs differs from that described in other cell types.

Cell Type	Phenotypic Consequence of FAK Inhibition
<b>Fibroblasts</b>	Rounded morphology; more focal adhesions <sup>86</sup> ; reduced focal adhesion turnover <sup>33</sup> ; violent trailing edge release <sup>87</sup> ; multiple leading edges <sup>30</sup> ; reduced microtubule stability <sup>32</sup>
<b>Endothelial cells</b>	No membrane ruffles or lamellipodia; spiky peripheral actin protrusions; nonpolarized cell movement <sup>19</sup>
<b>Epithelial cells</b>	Inhibited actin/microtubule reorganization at leading edge; fewer focal adhesions <sup>88</sup>
<b>Keratinocytes</b>	Disorganized actin cytoskeleton; increased stability of focal adhesions and microtubules <sup>20</sup>
<b>Macrophages</b>	Elevated protrusive activity; reduced lamellipodial persistence; altered adhesion dynamics <sup>89</sup>

**Table 2. Effects of FAK inhibition on migration of different cell types.** FAK inhibition can alter focal adhesion turnover, microtubule stability, actin organization, and lamellipodia formation, depending on cell type.

First, FAK depletion had no obvious effects on SMC growth. This contrasts with studies conducted in melanoma cells<sup>90</sup>, fibroblasts<sup>91</sup>, and endothelial cells<sup>92</sup>, in which suppression of FAK signaling correlates with impaired cell proliferation. Second, FAK depletion did not significantly impact apoptosis, in contrast to studies in epithelial cells<sup>93,94</sup>, fibroblasts<sup>95</sup>, leukemic cells<sup>96</sup>, and glioblastoma cells<sup>97</sup>, which suggest both positive and negative regulatory roles for FAK in apoptosis. Thirdly, I saw no obvious change in the size or number of focal adhesions in FAK-depleted SMCs, while studies in fibroblasts have shown that FAK depletion results in increased focal adhesion formation and decreased focal adhesion turnover<sup>33,86</sup>. Additionally, FAK-depleted SMCs displayed no impairments in membrane ruffling, cell spreading, microtubule stability, or organization of the actin cytoskeleton, all of which have been described in fibroblasts and keratinocytes<sup>20</sup>. Taken together, our findings underscore the importance of cell type as a determinant of FAK function, and support the concept of a SMC-specific role for FAK.

Above, I identified Dia2 and myosin light chain, both of which are activated by RhoA, as critical mediators of FAK-dependent SMC chemotaxis. Additionally, I found that PDGF globally activates RhoA in SMCs, while it does not in other cell types. These observations combine to implicate RhoA as a key mediator of FAK-dependent migration in differentiated SMCs. This implication is of particular interest given evidence that Rho signaling is critical to the process of SMC differentiation<sup>98-101</sup>. Moreover, it has recently been published that the RhoA-Dia2 signaling axis is of particular importance in activating transcription of SMC-specific genes<sup>44</sup>. Given the special role of RhoA in determining SMC fate, one could hypothesize that it might also facilitate a unique FAK signaling pathway in migrating SMCs.

### ***Understanding FAK-dependent migration: variability in experimental systems.***

Along with cell type, the mechanism by which FAK signaling is suppressed is important to consider when comparing studies of FAK-dependent migration. In this study, we used the *fak*<sup>fllox/fllox</sup> model to completely abrogate production of the active FAK protein. This technique differs from pharmacological inhibition of FAK by agents such as PP2, overexpression of point mutations blocking FAK phosphorylation or protein-protein interactions, or microinjection of interfering peptides or antibodies. Importantly, it also differs from overexpression of FRNK, a commonly used method of FAK inhibition. While FRNK undoubtedly acts as a dominant negative for FAK, it may also sequester various C-terminal binding partners for FAK, which likely impacts SMC biology differently than ablation of endogenous FAK. The use of FRNK as a tool for FAK inhibition is further complicated by our poor understanding of the regulation and function of endogenous FRNK.

Different methods of FAK inhibition could help explain discrepancies within the SMC literature. For instance, we and others have shown that overexpression of FRNK suppresses not only migration to PDGF, but also PDGF-stimulated proliferation<sup>18</sup>. Further, it has been proposed that FRNK overexpression attenuates SMC migration to PDGF by downregulating ERK signaling<sup>15</sup>. In contrast to this, I have found no changes in PDGF-stimulated growth or ERK activation following FAK ablation. Our lab also reported that FRNK overexpression attenuates Rac activation in PDGF-treated SMCs<sup>16</sup>, while I have found Rac activation to be FAK-independent in the floxed-*fak* system.

Additionally, while most studies of FAK-dependent migration have focused on adhesion-driven haptotaxis, this study has focused solely on PDGF-stimulated chemotaxis. Although these two processes share several basic mechanical processes (i.e., polarization, focal adhesion turnover, lamellipodial formation, etc), the molecular mechanisms by which these events are carried out can differ between extracellular stimuli. Finally, adhesion-driven studies usually monitor cells maintained in serum-containing media, which contains a host of soluble factors that regulate diverse signaling pathways. As suggested in Figure 11, some of these factors can stimulate SMC chemotaxis independently of FAK. Thus, inclusion of FBS can obfuscate the role of a specific molecular component in cell motility. In this study, I used serum starvation to dampen FBS-induced signaling before applying PDGF to stimulate chemotaxis.

***FAK as a regulator of Rho-mediated cell contractility.***

Above, I present evidence that a FAK-RhoA-ROCK signaling axis facilitates contraction of the cell body during SMC migration. Consistent with previous reports<sup>102,103</sup> I found that pharmacological inhibition of ROCK with compound Y-27632 blocked PDGF-stimulated migration in a transwell assay (data not shown). Interestingly, inhibition of MEK, p38, or PI3 kinase, all of which have been implicated as critical to cell migration in various systems<sup>104</sup>, resulted in little to no impairment. This suggests that ROCK is indeed a critical regulator of PDGF-driven SMC migration, and supports our claim that abrogated MLC activation could underlie impaired migration in FAK-depleted cells.

The current literature presents varying concepts of the FAK-ROCK relationship, depicting FAK as both upstream and downstream of ROCK, and as both a positive and negative regulator of ROCK. It was recently shown that impaired trailing edge retraction induced by FAK inhibition is rescued by overexpression of ROCK-activating constructs<sup>87</sup>. It has also been reported that inhibition of ROCK attenuates FAK phosphorylation in migrating HeLa cells<sup>105</sup>, and that inhibition of FAK elevates ROCK activity in endothelial cells<sup>106</sup>. ROCK inhibition has been shown to abrogate phosphorylation of FAK in neutrophils<sup>107</sup> and cardiomyocytes exposed to cyclic stretch<sup>108</sup>. Finally, Src and FAK were recently demonstrated to be necessary for MLC phosphorylation in ephrinA1-treated carcinoma cells<sup>109</sup>.

While myosin activation is the best understood mechanism for generating contractile force, a growing body of evidence suggests that actin polymerization may play a distinct role in this biological process, specifically in SMCs<sup>110</sup>. Thus, one can imagine that the Rho GTPases, which facilitate various modes of actin polymerization, might regulate SMC contractility in this second manner. Indeed, it has been reported that contractile agonists stimulate RhoA-mediated actin polymerization in airway and cerebral artery SMCs<sup>111,112</sup>. It has also been shown that pharmacological inhibition of actin polymerization results in a profound reduction in contractility, despite having no effect on myosin phosphorylation<sup>113</sup>. While FAK-depleted SMCs display no global abnormalities in stress fiber organization or membrane protrusion, it is possible that a more subtle defect in actin remodeling could contribute to impaired contractility in these cells. Such a scenario could involve spatiotemporally distinct activity of actin polymerizing factors, such as the diaphanous-related formins. It is well documented that



contractile stimuli induce the recruitment of multiple factors, such as alpha-actinin, vinculin, and paxillin, to adhesion complexes at the SMC cortex<sup>110</sup>. Here, it is thought, these proteins help reorganize actin-matrix connections, thereby creating a stable network for the transmission of force.

Currently, the physiological role of the DRFs in SMCs is poorly understood, and their involvement in SMC contractility remains wholly unexplored. Dia2 was recently shown to localize to focal adhesions at the leading edge of migrating epithelial cells, where it appears to regulate focal adhesion turnover by maintaining a stable pool of cortical actin<sup>114</sup>. Others have proposed that Dia1 facilitates turnover by targeting microtubules to focal adhesions<sup>49</sup>. However, in FAK-depleted cells we find that mislocalization of Dia2 is not associated with defective protrusion or disorganized microtubules, but rather a contractile impairment that likely contributes to a nonpolarized phenotype. Given the emerging paradigm suggesting that actin polymerization is particularly relevant to SMC contractility, it is reasonable to hypothesize that localization of Dia2 to focal adhesions could mediate this process in SMCs. Further experiments will be required to determine (a) if Dia2 does indeed mediate contractility by localizing to adhesion complexes; (b) the molecular mechanism by which it does so; and (c) how this pathway is regulated by FAK.

Finally, it is worth noting that I detected aberrations in both ROCK and Dia2 signaling, which are canonically understood as separate pathways, but found no change in global RhoA activity. These findings may suggest that the ROCK and Dia2 pathways are not in fact separate, but influence each other. Christopher Mack and colleagues have recently gathered evidence that ROCK directly phosphorylates Dia2, suggesting the

existence of molecular crosstalk between these two RhoA effectors (personal communication). Similarly, while myosin activation and actin polymerization are currently understood as separate modes of contractile force generation, the possibility that ROCK-Dia2 crosstalk could unite these two physiological mechanisms merits further study.

### ***New roles for Dia2 in cytoskeletal remodeling.***

Copious biochemical evidence has characterized the DRFs as promoters of unbranched actin polymerization. However, the physiological activity of these proteins is not well understood. In this work, I show that Dia2 localizes to focal adhesions dependently of FAK, and to membrane ruffles independently of FAK, and provide evidence of an interaction between Dia2 and cortactin. To date, two other studies have shown interactions between Dia2 and Arp2/3 activators<sup>115,116</sup>. Most recently, Dia2 was shown to bind directly to WAVE2 in complex with Arp2/3<sup>115</sup>. Interestingly, a previous report conducted in mouse melanoma cells detected no interaction between Dia2 and WAVE<sup>116</sup>. Instead, Dia2 bound to Abi1, another component of the WAVE/Abi1/Nap1/PIR121/HSP300 (WANP) complex, which activates Arp2/3. The authors proposed that Abi1 targets Dia2 to the leading edge, where it participates in lamellipodial extension. Notably, neither WAVE2 nor Abi1 interacted with the formin homology domains of Dia2, while my data suggest that cortactin interacts with the FH2 domain. Thus, while WAVE2, Abi1, and cortactin are all Arp2/3 activators, they appear to interact with Dia2 in different ways. Further experiments are required to determine the specific region of cortactin that interacts with Dia2.

This emerging body of data begs the question: if the only function of Dia2 is to polymerized unbranched actin filaments, why does it interact with proteins that promote branched actin polymerization? Beli et al. propose that WAVE2 prevents filopodia formation by sequestering Dia2, and show that overexpression of WAVE2 mutants unable to bind Dia2 increases filopodia formation following EGF treatment. Conversely, Yang et al. demonstrated a requirement for Dia2 in lamellipodia formation, and showed that Dia2 knockdown results in a disorganized dendritic actin network at the cell periphery, indicating that Dia2 is actively involved in the process of branched actin formation. The authors propose that Dia2 may facilitate protrusion by creating stable “mother filaments” that form a platform for Arp2/3, and/or by protecting elongating filaments from capping. They also present evidence that Dia2 participates in an alternative form of filipodial formation known as convergent extension. In this process, Dia2 forms filipodia by bundling existing lamellipodial actin, rather than generating unbranched filaments *de novo*. Clearly, the biochemical dogma of Dia2 function is being challenged by current *in vivo* studies.

At present, we have yet to explore the biological function of the Dia2-cortactin interaction. I hypothesize that the two types of PDGF-induced Dia2 localization (i.e., membrane ruffles and focal adhesions) are mutually exclusive, and therefore it is possible that the Dia2-cortactin interaction must be disrupted for Dia2 to translocate to focal adhesions. If this is true, then Dia2-cortactin binding might be enhanced in FAK-depleted cells. Further investigation is needed to determine the extracellular cues and signaling events that regulate Dia2-cortactin binding. Additionally, electron microscopy could reveal if and how these two proteins polymerize actin differently when associated

with each other, and whether blockade of their interaction induces formation of aberrant actin structures. Undoubtedly, the interaction I have identified in this work represents an exciting opportunity to further our limited understanding of Dia2 function in cell migration.

## **APPENDIX: KNOCKDOWN OF LEUPAXIN IN VASCULAR SMCs**

## **ABSTRACT**

Members of the paxillin family of LIM domain-containing proteins are focal adhesion-targeted factors that play important roles in cytoskeletal remodeling and cell motility. Leupaxin, a recently identified member of this family, has also been shown to regulate motility in hematopoetically derived cells. Our lab previously reported that leupaxin is enriched in vascular SMCs, binds FAK, and may promote SMC differentiation by interacting with serum response factor (SRF) in the nucleus. Here, we present siRNA-mediated knockdown of endogenous leupaxin in human aortic SMCs, and the effects of leupaxin depletion on SMC motility. Transfection with an siRNA specific to leupaxin significantly diminished leupaxin levels after 72 hours, but had no effect on the expression or localization of paxillin or Hic-5. Leupaxin-depleted cells were unable to migrate to serum-containing media in a three-dimensional transwell assay, and exhibited reduced cell speed and directional persistence in a two-dimensional context. Further, cortactin staining revealed ragged, spiky membrane ruffles at the periphery of leupaxin-depleted SMCs. These preliminary findings point to an important role for leupaxin in SMC motility, and invite further investigation into the function of this protein during development.

## INTRODUCTION

Leupaxin, a tyrosine kinase substrate recently identified in leukocytes, is a member of the paxillin family of proteins<sup>117</sup>. The C-terminus of leupaxin, which shares 70% identity with paxillin, contains four 50-60 amino acid regions known as LIM domains. LIM domains contain two zinc finger motifs, which are composed of conserved arrangements of cysteine, histidine, and aspartate residues. LIM domains facilitate focal adhesion targeting of paxillin<sup>118</sup>, and can interact with other LIM domains and tyrosine-containing motifs<sup>119</sup>. The leupaxin C-terminus also possesses three LD motifs, which are named for their characteristic N-terminal leucine/aspartate pair. In paxillin, LD motifs have been identified as binding sites for FAK and vinculin<sup>118,120</sup>, and FAK-LD binding has been linked with focal adhesion targeting of FAK<sup>118</sup>. While the functions of leupaxin's LIM domains remain unknown, the LD2 and LD3 motifs have been shown to interact with c-Src<sup>121</sup> and Lyn<sup>122</sup>, respectively, and nuclear export sequences were recently identified in LD3 and LD4<sup>123</sup>.

To date, the leupaxin knowledgebase is extremely limited. Table 3 summarizes the proposed binding partners and biological roles of leupaxin identified in the current literature. While the majority of leupaxin studies have been conducted cells of hematopoietic origin, recent reports have detected leupaxin expression in metastatic prostate cancer cells<sup>121,123</sup> and vascular SMCs<sup>124</sup>. Like paxillin and Hic-5<sup>125,126</sup>, leupaxin

localizes to focal adhesions and appears to regulate cytoskeletal remodeling and cell motility. In bone-derived prostate cancer cells, overexpression of leupaxin results in increased Rho GTPase activity and increased cell migration<sup>121</sup>, while siRNA-mediated knockdown inhibits cell invasion and motility<sup>123</sup>. In osteoclasts, leupaxin overexpression enhances cell adhesion, induces a motile phenotype, and enhances resorption<sup>127</sup>, while knockdown inhibits resorption<sup>128</sup>, a process dependent on adhesion and assembly of actin-rich podosomes. Leupaxin associates with several known regulators of the actin cytoskeleton, including the Arf GAP, p95 PKL<sup>128</sup>, further supporting a role for leupaxin in cell motility.

<b>Binding Partner</b>	<b>Cell Type</b>	<b>Putative Biological Function</b>
<b>PYK2</b>	Macrophages <sup>117</sup> , osteoclasts <sup>128</sup> , prostate cancer cells <sup>121</sup>	Focal adhesion adapter protein
<b>PTP-PEST</b>	Spleen <sup>129</sup> , osteoclasts <sup>127</sup> , prostate cancer cells <sup>121</sup>	Regulation of antigen receptor signaling; podosomal remodeling
<b>c-Src</b>	Osteoclasts <sup>127</sup> , prostate cancer cells <sup>121</sup>	Podosomal complex signaling, osteoclast activation
<b>p95 PKL</b>	Osteoclasts <sup>128</sup>	Podosomal remodeling
<b>FAK</b>	Vascular SMCs <sup>124</sup> , osteoclasts <sup>128</sup>	Podosomal complex signaling
<b>Lyn</b>	B-cell lymphoma <sup>122</sup>	Regulation of B-cell receptor signaling
<b>SRF</b>	Vascular SMCs <sup>124</sup>	Promotion of SMC differentiation

**Table A1. Identified binding partners of leupaxin, and putative biological functions of each interaction.** Mounting evidence implicates leupaxin as an important mediator of adhesion, migration, and invasion.

Our lab recently reported that leupaxin interacts with FAK in vascular SMCs, and may promote SMC differentiation<sup>124</sup>. Overexpression of leupaxin enhanced transcription of SMC-specific genes, and this activity was attributed to a leupaxin-SRF interaction within the nucleus. This study provides evidence of an important role for leupaxin in



SMC biology, and lends further credence to the proposed connection between cytoskeletal remodeling and SMC differentiation.

## EXPERIMENTAL PROCEDURES

**Cell Culture.** Primary human aortic SMCs, culture media, and supplements were all purchased from Clonetics. Cells were cultured in SmBM smooth muscle basal medium, supplemented with the SmGM-2 bullet kit (amphotericin/gentamicin, recombinant human insulin, recombinant human epidermal growth factor, human fibroblast growth factor-B, and 5% fetal bovine serum). At the time of purchase, cells had been passaged six times, and were not used beyond 7 additional passes following purchase.

**siRNA Design and Transfection.** BLOCK-iT standard siRNA oligos were designed and purchased through Invitrogen. Oligos were targeted to the 3' untranslated region of *Homo sapiens* leupaxin mRNA (accession number NM\_004811), at nucleotide position 1732 (GCAUCUCCUUCAGUGUAU). Non-targeted control sequence was the Dharmacon siControl Non-targeting siRNA #1 (UGGUUUACAUGUCGACUAA). Oligos were transfected into SMC using Dharmafect-1 reagent (Dharmacon) according to manufacturer's instructions. Experiments were performed 72 hours following siRNA transfection.

**Antibodies.** The monoclonal antibody to human leupaxin was a generous gift from Dr. Don Staunton of the ICOS Corporation, Bothell, Washington State. The antibodies to paxillin and Hic-5 were purchased from Transduction Laboratories. All

three antibodies were used at a dilution of 1:1000 for western blot and 1:500 for immunohistochemistry. Anti-cortactin was purchased from Upstate and used at a dilution of 1:500 for immunohistochemistry. The antibody to total C-terminal ERK 1/2 was purchased from Upstate and used at 1:1000 for western blot.

***Immunofluorescent Staining.*** Human aortic SMCs were plated onto LabTek II chamber slides (Nunc) and transfected with siRNA the following day. 72 hours after transfection, chamber slides were fixed in 4% paraformaldehyde for 20 minutes at room temperature. Slides were blocked in 20% goat serum and 3% BSA in 1X PBS for 30 minutes at RT, then incubated in primary antibody for 1 hour at RT and washed thrice in 1X PBS for 4 minutes each. Slides were incubated with FITC-phalloidin (Molecular Probes) at a dilution of 1:500 and Texas Red-anti-mouse IgG (Jackson ImmunoResearch) at 1:1000 in 1X PBS for 1 hour at RT, then washed thrice with PBS for 4 minutes each. Following a final aqueous rinse, slides were mounted and coverslipped.

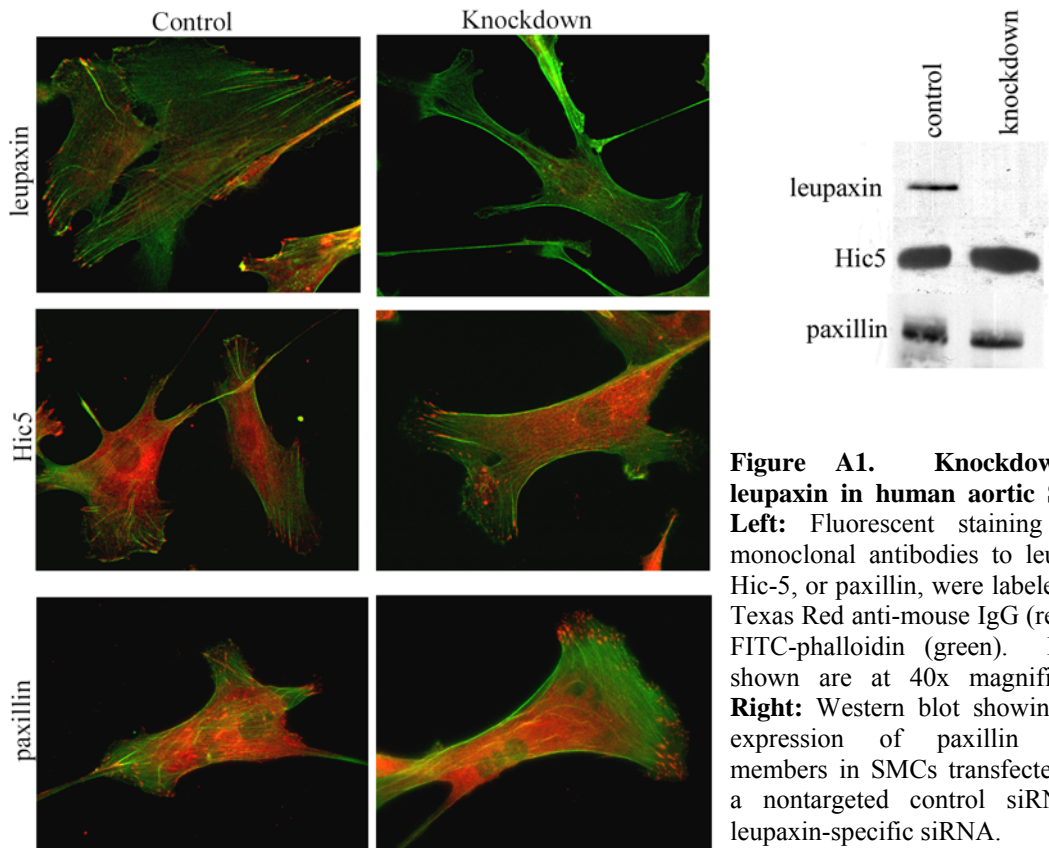
***Transwell Assays.*** Human aortic SMCs were transfected with nontargeted control or siRNA or anti-leupaxin siRNA. 72 hours after transfection, cells were plated onto BD Biocoat transwell inserts (8 micron pore size) coated with 20 ug/mL fibronectin (Sigma) at a density of 15K/insert. Fetal bovine serum was added to the bottom well, and cells were allowed to chemotax overnight (approximately 16 hours). Transwells were fixed in 4% paraformaldehyde for 20 minutes at RT, rinsed thrice, then stained for 1 hour in 1% crystal violet at RT. Insert tops were scraped with a cotton swab and rinsed with 1X PBS three times, and migrated cells were counted at 10x magnification over 4 fields in 3 separate wells.

***Microscopy and Live Cell Imaging.*** Immunofluorescence images were acquired using an Olympus BX51 upright microscope equipped with a 40x oil immersion lens and a Affymetrix CoolSnap CCD camera. Images were processed using Adobe Photoshop. For random motility assays, cells were imaged using an Olympus IX70 inverted microscope encased in a Plexiglas housing and the internal environment was kept at a temperature of 34.5° C and a relative humidity of 60%. Dishes were perfused with 100% CO<sub>2</sub> at a flow rate sufficient to maintain optimal pH. Tracking images were acquired at 4x magnification every 5 minutes by an Optronix DEI 750 CCD camera using OpenLab software (Improvision). Tracking measurements and motion path images were generated using the MultiTracker plugin for NIH ImageJ.

## PRELIMINARY RESULTS

### *Knockdown of endogenous leupaxin in human aortic SMCs.*

Given our recent finding that leupaxin is enriched in vascular SMCs<sup>124</sup>, we transfected human aortic SMC with an siRNA oligo targeted to the human leupaxin gene to observe the effects of leupaxin silencing on SMC biology. Transfection with a leupaxin-targeted siRNA abrogated leupaxin expression, but had no effect on expression or localization of paxillin or Hic-5 (Figure A1).



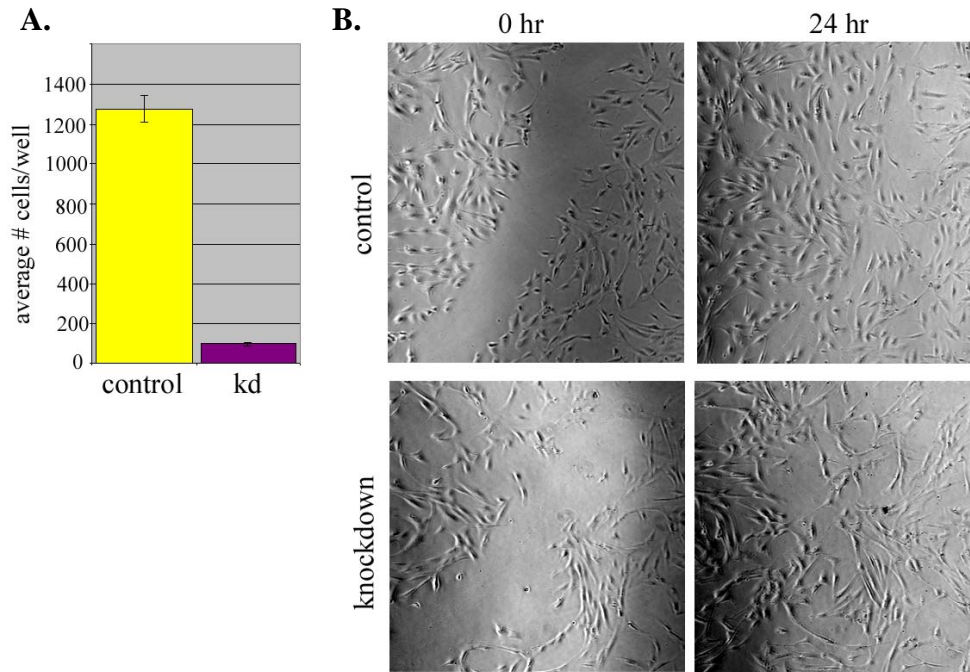
**Figure A1. Knockdown of leupaxin in human aortic SMCs.** **Left:** Fluorescent staining using monoclonal antibodies to leupaxin, Hic-5, or paxillin, were labeled with Texas Red anti-mouse IgG (red) and FITC-phalloidin (green). Images shown are at 40x magnification. **Right:** Western blot showing total expression of paxillin family members in SMCs transfected with a nontargeted control siRNA or leupaxin-specific siRNA.

By eye, leupaxin knockdown cells demonstrated no rounding, apoptosis, or loss of adhesion as described in other reports, but were noticeably sparser and consistently yielded less total protein than control-transfected SMCs. While we have not performed quantitative assays to confirm this effect, this preliminary observation could suggest that leupaxin knockdown induces growth arrest in SMCs. Because we recently showed that leupaxin overexpression upregulates SMC gene expression, we expected that SM marker genes might be downregulated in leupaxin-silenced cells. However, western blotting for SM22 and smooth muscle alpha-actin yielded inconclusive results, possibly due to passage-specific effects on SMC biology. Thus, it remains unclear whether leupaxin regulates SMC differentiation in its endogenous state.

#### ***Impaired motility in leupaxin-depleted SMCs.***

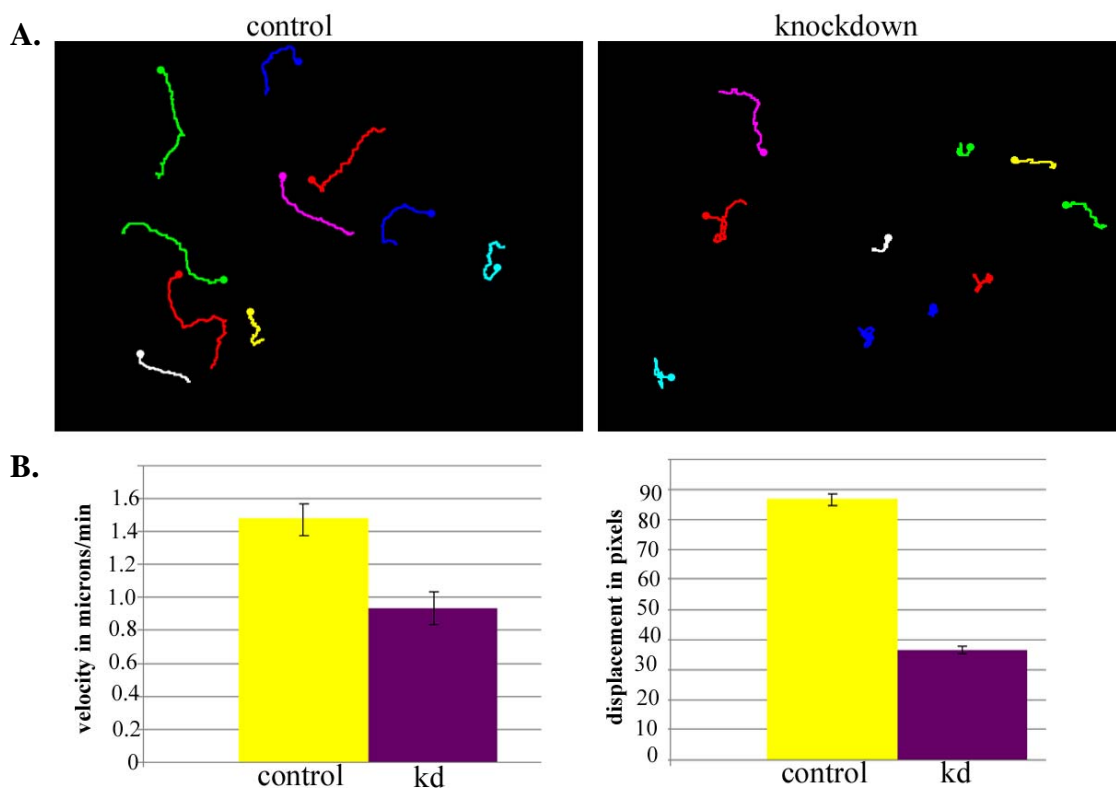
The paxillin family members are known regulators of cytoskeletal dynamics, due to their status as focal adhesion adapter proteins<sup>130</sup>. Enhanced or diminished leupaxin expression has been shown to correlate with increased or decreased cell motility, respectively. We used a transwell assay to assess the impact of leupaxin silencing on SMC motility. Remarkably, leupaxin-depleted cells were unable to migrate to serum-containing media, indicating a gross defect in three-dimensional migration that is not specific to a certain agonist (Figure A2.A).

To determine whether this impairment was evident in a two-dimensional context, we used live cell imaging to visualize SMC motility in the presence and absence of leupaxin. In a wound healing assay, both control and leupaxin knockdown SMCs closed a scratch wound completely by 16 hours (Figure A2.B), indicating that some directed



**Figure A2. Three-dimensional migration, but not wound healing, is abrogated by leupaxin knockdown.** **A:** Human aortic SMCs were transfected with nontargeted control or leupaxin-specific siRNA, then plated onto fibronectin-coated transwell inserts and allowed to migrate to 10% FBS overnight. Migrated cells were fixed, stained, and counted in triplicate. **B:** Cells were transfected as above, then plated on uncoated tissue culture dishes in serum-containing media and wounded with a pipet tip.

two-dimensional movement is possible in the absence of leupaxin. However, when cells were sparsely plated, cell tracking revealed that the paths of leupaxin-depleted cells were visibly circuitous in nature and were less persistent than those of control cells (Figure A3.A). Quantitatively, knockdown cells exhibited slower cell speed by approximately 35%, and net displacement from their point of origin was reduced by about 55% (Figure A3). Thus, in sparsely plated cells, leupaxin silencing appears to result in disoriented two-dimensional SMC motility.



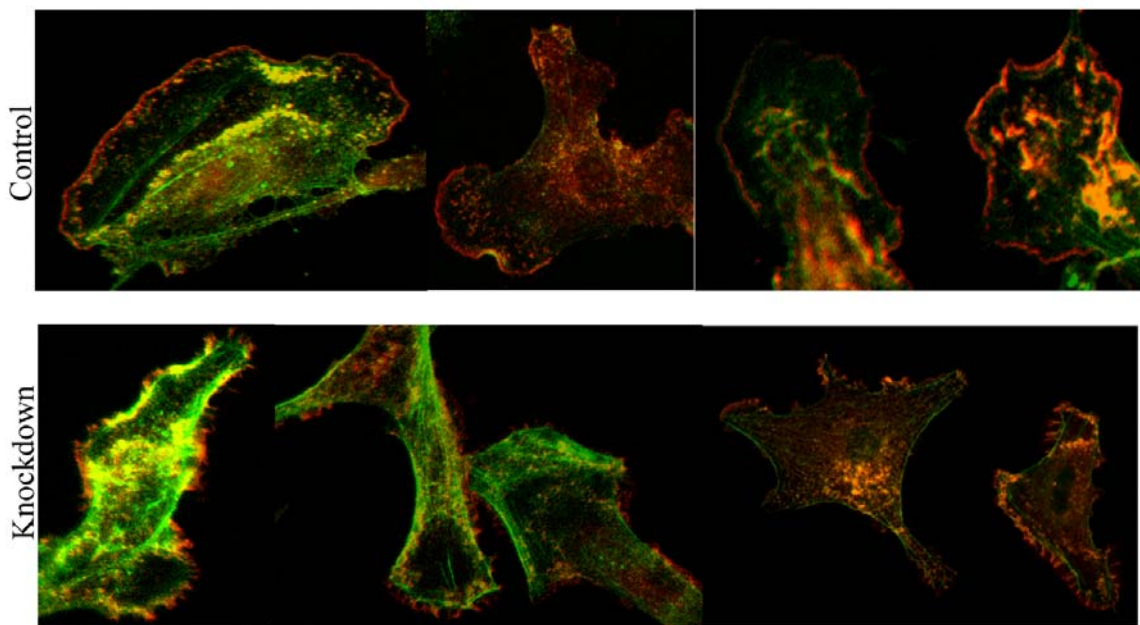
**Figure A3. Disoriented two-dimensional motility in sparsely plated leupaxin knockdown cells.** A,B. SMCs were transfected with control or leupaxin-specific oligos for 72 hours, then trypsinized and replated at equal densities on uncoated tissue culture dishes in serum-containing media. Cells were imaged every 5 minutes over a span of 8 hours. Tracking measurements and cell paths were generated using the ImageJ MultiTracker plugin. A. Each colored line represents the path of a single cell over 8 hours.

### ***Malformed membrane ruffles in leupaxin-depleted SMCs.***

In light of this motility defect, we used immunofluorescent staining to visualize cytoskeletal organization and remodeling in leupaxin knockdown cells. Basally, no aberrations were evident in stress fiber organization, and there were no obvious differences in cell shape or size. However, cortactin staining, which highlights membrane ruffles, revealed malformed membrane ruffling in leupaxin knockdown cells stimulated with PDGF (Figure A4). In contrast to the smooth, continuous cortactin staining shown by control cells, leupaxin-depleted SMCs exhibited ragged, disconnected



staining at the cell periphery, suggesting that leupaxin is required for proper lamellipodial formation. Interestingly, knockdown cells were still capable of forming dorsal ruffles, indicating that the machinery responsible for driving branched actin polymerization is activated to some degree in the absence of leupaxin. Preliminary kymographic analysis showed that protrusions of leupaxin-depleted cells extended at a slower rate and for a shorter duration than control cells. These findings suggest that depletion of leupaxin results in malformed, unstable lamellipodia, which likely contribute to the dramatic motility defect displayed by these cells.



**Figure A4. Malformed membrane ruffles in PDGF-treated leupaxin knockdown cells.** Human aortic SMCs were transfected with nontargeted control or leupaxin-specific siRNA oligos for 72 hours, then serum starved for 16 hours and treated with 20 ng/mL PDGF for 5 minutes. Cells were fixed and stained for cortactin (red) and phalloidin (green), then imaged at 40x magnification.

## CONCLUSIONS AND FUTURE DIRECTIONS

The study of leupaxin is still in its infancy, yet the existing body of literature provides convincing evidence that this protein plays an important role in cell motility. Here, we have conducted the first SMC-specific knockdown of leupaxin, and collected preliminary data that implicates it as critical for migration in this cell type. It is interesting to note that the migrational phenotype of leupaxin-depleted SMCs is even more severe than that of FAK-depleted SMCs, described in the main body of this work. While FAK appears necessary for PDGF-specific SMC migration, leupaxin is critical for migration to serum-containing media, which contains a vast array of chemotactic signals. Also in contrast to FAK-depleted cells, leupaxin knockdown cells display a clear defect in lamellipodial formation. Thus, it is reasonable to hypothesize that leupaxin may play a broader role than FAK, and regulate some of the basic cytoskeletal machinery required for general SMC motility. Additionally, preliminary observations strongly suggest that leupaxin depletion arrests SMC growth. Taken together, the experiments we have conducted thus far suggest that leupaxin plays an important role in several aspects of SMC biology and clearly merits further study in this cell type.

The motility experiments conducted here highlight an important consideration in the field of cell migration: mechanistic differences between three-dimensional and two-dimensional systems. Scratch wound assays indicate that leupaxin is not required for

SMCs to migrate into a wound, yet transwell assays reveal a dramatic inability of knockdown cells to chemotax towards serum-containing media. This could suggest a special function for leupaxin in three-dimensional invasion, a concept that is supported by the critical role of this protein in osteoclastal podosome formation and resorption. It is well documented that distinct molecular and biomechanical events, such as matrix metalloprotease secretion and invadopodia formation, are involved in 3D migration<sup>131</sup>. Furthermore, it has been reported that focal adhesions are constructed and organized differently on 3D matrices than 2D substratum<sup>132</sup>. This could be especially relevant to the function of leupaxin, given its localization to focal adhesions.

Our lab recently published that overexpressed leupaxin undergoes nuclear/cytoplasmic shuttling in SMCs, and promotes SMC marker gene expression via direct interaction with SRF. Concurrently, others reported a similar phenomenon in prostate cancer cells, where overexpressed leupaxin activates transcription of the androgen receptor, purportedly regulating cancer cell apoptosis and invasion<sup>123</sup>. Thus far, we have been unable to obtain consistent readouts of SMC marker gene expression following endogenous leupaxin knockdown (data not shown). In human aortic SMCs, which are derived from the neural crest, leupaxin knockdown cells have shown unchanged or increased SMC marker gene expression. In human coronary SMCs, which are derived from the proepicardial organ, knockdown cells have shown unchanged or decreased marker gene expression. Interestingly, we have also seen changes in basal leupaxin expression levels from passage to passage. These findings suggest that the role of leupaxin in SMC differentiation may depend on cell passage as well as SMC lineage, and invite *in vivo* assessment of leupaxin expression during embryonic development.

Using an animal model, one could determine if leupaxin is differentially expressed in distinct cell lineages, and/or at specific stages of SMC differentiation. Obviously, it is also important to consider differences between endogenous protein manipulation and protein overexpression as contributors to our conflicting results. Lastly, quantitative real time RT-PCR may provide a more sensitive and reliable tool for SMC marker gene analysis in leupaxin knockdown cells.

Given the clinical relevance of SMC proliferation and invasion to atherosclerosis and restenosis, it is exciting to consider leupaxin, which appears to regulate both of these biological processes *in vitro*, as a potential therapeutic target. In addition to revealing the role of leupaxin during vasculogenesis, *in vivo* studies could elucidate the significance of this protein in the initiation and progression of vascular disease. In my opinion, it will be important to continue human SMC experimentation in tandem with small mammal studies. While a human SMC system is more technically limiting than a murine system, it also helps provide evidence for the relevance of leupaxin in the human vasculature. We have previously shown that leupaxin is expressed in human vascular SMCs *in vivo* as well as *in vitro*. Others have recently reported that leupaxin is overexpressed in invasive human prostatic cancers. Future analysis of human tissue samples could determine if leupaxin is similarly upregulated in vascular disease states.

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