2013

Focal adhesion protein dynamics and the role of endosomes in contractile, fully differentiated, vascular smooth muscle

https://hdl.handle.net/2144/13144

Boston University
BOSTON UNIVERSITY
GRADUATE SCHOOL OF ARTS AND SCIENCES

Dissertation

FOCAL ADHESION PROTEIN DYNAMICS AND THE ROLE OF ENDOSONES
IN CONTRACTILE, FULLY DIFFERENTIATED, VASCULAR SMOOTH MUSCLE

by

RANSOM POYTHRESS

B.S., California Institute of Technology, 2006

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

2013
ACKNOWLEDGEMENTS

There are many people who have made substantial contributions to my doctoral work, without whom this would never have been possible.

First, I’d like to thank my advisor, Dr. Morgan for tolerating my failures, pushing me to think like a scientist, accepting nothing less than the best, and never letting me make excuses for myself. Without your guidance and encouragement, this project would have foundered years ago. Thank you for being honest with me, even about unpleasant things, and for seeing this through to the end with me.

A big thanks to my committee members: Dr. Mahasweta Girgenrath, Dr. Rob Hausman, Dr. Frank Naya, and Dr. Barbara Schreiber. Thank you for your input and suggestions and for always keeping me grounded and realistic. More importantly, thank you for seeing more than just another project and another scientist. Thank you for seeing the person underneath the white lab coat and being interested in me as an individual beyond the four walls of the laboratory.

I cannot imagine completing this project without the help and support of all the lab members, both past and present. Dr. Hak Rim Kim’s input and initial work with VASP really opened the doors for this project in the first place. Dr. Sarah Appel, Dr. Samudra Gangopadhyay, Dr. Rina Yamin, and Dr. Mehtap Yilmaz all deserve thanks for their investments of time and assistance. I also want to particularly thank the undergrads
John Min and Stephy Lin for their input and for reminding me how much fun you can have both in and outside of work. Cindy Gallant deserves special thanks for making everything run smoother. You are the oil in the engine of the lab. You are always there with a cheery word or a smile to get the day back on track (not to mention straightening a few bones and muscles as well). I owe a huge debt of gratitude to Dr. Susanne Vetterkind. You must have the patience of Job. I’ve lost count of the number of times I’ve spun around in my chair to bother you with some silly question, yet you’ve always dealt with me graciously and patiently. I don’t think my experiments would have gone half as well without your tutelage and assistance. Finally, I simply can’t say enough about (soon to be Dr.) Robert Saphirstein. You’ve been here basically since the beginning of grad school and you’ve done it all: friend, ally, gym buddy, groomsman, fellow laborer, confidante, moving helper, and teammate. You have become one of my closest friends and I honestly can’t thank you enough.

I need to especially thank my family. Your love and support has been steadfast and unwavering through thick and thin since the day I was born. I feel incredibly blessed to have you in my life. Justin, I couldn’t have asked for a better brother. Thanks for sharing this crazy thing called life with me.

I’d also like to thank the many friends, old and new, who have helped and supported me during my time here in Boston. I’d particularly like to mention the staff and members of Citylife Church for befriending me and keeping me focused on what really matters. I’d also like to thank Dan Pederson and Tara Martin, some of my oldest friends, for the innumerable things they’ve done for me, but especially in making sure
that I didn’t become a social recluse. Also, a special thanks to Meredith Canode, Diane Wadman, and Danuta Charland. You all work behind the scenes frequently going unnoticed and almost always underappreciated. You are the miracle workers that make all the bureaucratic red tape disappear.

Finally, I’d like to thank my Lord and Savior Jesus Christ: “And I am sure of this, that He who began a good work in you will bring it to completion” (Philippians 1:6). May I always trust in the Lord with all my heart and lean not on my own understanding, but in all my ways acknowledge Him, knowing that He will direct my path (Proverbs 3:5-6).
FOCAL ADHESION PROTEIN DYNAMICS AND THE ROLE OF ENDOSONES IN CONTRACTILE, FULLY DIFFERENTIATED, VASCULAR SMOOTH MUSCLE

(Order No.

RANSOM POYTHRESS

Boston University Graduate School of Arts and Sciences, 2013

Major Professor: Kathleen G. Morgan, Professor of Health Sciences

ABSTRACT

Turnover of focal adhesions (FAs) is known to be critical for cell migration and adhesion of proliferative vascular smooth muscle cells (VSMCs). However, it is often assumed that FAs in non-migratory, differentiated vascular smooth muscle cells (dVSMCs) embedded in the wall of healthy blood vessels are static structures. Recent work from our laboratory has demonstrated agonist-induced actin polymerization and Src-dependent focal adhesion phosphorylation in dVSMCs, suggesting that agonist-induced FA remodeling occurs. However, the mechanisms and extent of FA remodeling are largely unknown in dVSM. Here I show, for the first time, that a distinct subpopulation of dVSM FA proteins, but not the entire FA, remodels in response to the
alpha-agonist phenylephrine. VASP and Zyxin displayed the largest redistributions while beta-integrin and focal adhesion kinase (FAK) showed undetectable redistribution. Vinculin, metavinculin, Rous sarcoma oncogene (Src), p130Crk-associated substrate (CAS), and paxillin displayed intermediate degrees of redistribution. Redistributions into membrane fractions were especially prominent, suggesting endosomal mechanisms. Deconvolution microscopy, quantitative colocalization analysis, and proximity ligation assays revealed that phenylephrine (PE) increases the association of FA proteins with early endosomal markers Rab GTPase 5 (Rab5) and early endosome antigen 1 (EEA1). Endosomal disruption with the small molecule inhibitor primaquine inhibits agonist-induced redistribution of FA proteins, confirming endosomal recycling. FA recycling was also inhibited by cytochalasin D, latrunculin B and colchicine, indicating that the redistribution is actin and microtubule-dependent. Furthermore, inhibition of endosomal recycling causes a significant inhibition of the rate of development of agonist-induced dVSM contractions. Thus, these studies are consistent with the concept that FAs in dVSMCs, embedded in the wall of the aorta, remodel during the action of a vasoconstrictor.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS................................................................................................................................. iv
ABSTRACT ..................................................................................................................................................... vii
TABLE OF CONTENTS ................................................................................................................................. ix
LIST OF TABLES ............................................................................................................................................... xi
LIST OF FIGURES ............................................................................................................................................. xii
LIST OF ABBREVIATIONS .............................................................................................................................. xv
CHAPTER 1: INTRODUCTION .......................................................................................................................... 1
  INTRODUCTION ................................................................................................................................. 1
  CARDIOVASCULAR DISEASES ................................................................................................................. 1
  VASCULAR SMOOTH MUSCLE .................................................................................................................. 4
    Vascular Smooth Muscle Phenotypes .................................................................................................. 8
    Differentiated Vascular Smooth Muscle ........................................................................................... 10
    Ferret as a Model Organism .......................................................................................................... 10
  FOCAL ADHESIONS ............................................................................................................................... 11
    Focal Adhesion Proteins .................................................................................................................. 14
    Focal Adhesion Dynamics in Migrating Cells ................................................................................. 21
    Focal Adhesion Dynamics in Non-Migratory Cells ..................................................................... 23
  ENDOSOMES .............................................................................................................................................. 24
  THESIS RATIONALE/AIMS OF THIS THESIS ....................................................................................... 27
CHAPTER 2: MATERIALS AND METHODS .................................................................................................... 29
  MATERIALS .................................................................................................................................................. 29
    Enzymes and Chemicals .................................................................................................................. 29
    Antibodies ............................................................................................................................................... 30
  METHODS .................................................................................................................................................... 30
    Coomassie Gel Stain for Total Protein .......................................................................................... 30
    Lowry Protein Assay ....................................................................................................................... 31
    Western Blot ........................................................................................................................................... 31
    Contractility Measurements in Serum-Free Organ Culture ....................................................... 33
    Cell Isolation ......................................................................................................................................... 34
    Immunofluorescent Imaging in Mammalian Cells .................................................................... 42
    Differential Centrifugation ............................................................................................................. 44
    Statistical Analysis .......................................................................................................................... 46
CHAPTER 3: FOCAL ADHESION PROTEIN LOCALIZATION ......................................................................... 47
  INTRODUCTION ........................................................................................................................................... 47
  IMMUNOFLUORESCENT IMAGING OF INDIVIDUAL PROTEINS ...................................................... 49
LIST OF TABLES

Table 2.1 Cell isolation enzyme solutions 36
Table 2.2 Cell staining protocol 41
Table 5.1 FA marker protein zyxin shows a quantifiable increase in colocalization with endosome markers in the presence of PE 84
### LIST OF FIGURES

1.1. Role of blood vessels ......................................................... 3  
1.2 Structure of blood vessels .................................................. 5  
1.3 Vascular smooth muscle contraction ....................................... 6  
1.4 Smooth muscle stimuli and signaling pathways ......................... 7  
1.5 Contractile versus migrating smooth muscle .......................... 9  
1.6 Focal adhesion complexity ................................................ 13  
1.7 Focal adhesion structure .................................................. 15  
1.8 Focal adhesion dynamics .................................................. 22  
1.9 Endocytic pathways ....................................................... 26  

3.1 VASP is localized adjacent to vinculin and alpha-actinin at FA-like  
structures .............................................................................. 48  
3.2 Zyxin staining at FA-like structures ....................................... 50  
3.3 Paxillin/pPax co-stain as positive control ............................... 52  
3.4 Paxillin/pLC co-stain as negative control ............................... 54  
3.5 Zyxin and paxillin colocalize ............................................... 56  
3.6 Zyxin and vinculin colocalize ............................................. 58  
3.7 FAK and vinculin colocalize ............................................... 60  

4.1 Immunofluorescent staining reveals no visible movement of zyxin in response  
to PE ...................................................................................... 64
4.2 Phorbol ester DPBA triggers redistribution of some FA proteins

4.3 Differential centrifugation of dVSM tissue distinguishes known cytoskeletal proteins from membrane and cytosolic proteins

4.4 The alpha-agonist PE triggers redistribution of zyxin, VASP, paxillin, filamin, and metavinculin/vinculin to the membrane fraction from the cytosolic fraction.

4.5 CAS and Src redistribute to the membrane fraction from the cytoskeletal fraction in the presence of PE

4.6 FAK, integrin and alpha actinin show no detectable vasoconstrictor-induced redistribution

5.1 FA marker protein zyxin shows increased colocalization with endosome markers in the presence of PE

5.2 Zyxin colocalization with endosome markers increases after PE stimulation

5.3 The amount of zyxin and EEA1 in close proximity increases after PE stimulation

5.4 Primaquine blocks vasoconstrictor-induced membrane redistribution

5.5 Primaquine reduces aortic tissue contractility in serum free organ culture

5.6 Primaquine reduces contractility

5.7 Actin inhibition with cytochalasin D blocks vasoconstrictor-induced redistribution to the membrane fraction

5.8 Actin inhibition with latrunculin B blocks vasoconstrictor-induced redistribution to the membrane fraction
5.9 Microtubule inhibition with colchicine blocks vasoconstrictor-induced redistribution to the membrane fraction 99

6.1 Spatially layered model of focal adhesions 103

6.2 Expected proximity to the plasma membrane suggests degree of redistribution to the membrane fraction 105
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7r5</td>
<td>Cultured rat aortic smooth muscle cells</td>
</tr>
<tr>
<td>AEBSF</td>
<td>4-(2-Aminoethyl) benzenesulfonfyl fluoride</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ARP</td>
<td>Actin-related protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Cytosol</td>
</tr>
<tr>
<td>CAS</td>
<td>p130Crk-associated substrate</td>
</tr>
<tr>
<td>Csk</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Cyt</td>
<td>Cytosol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DPBA</td>
<td>12-deoxyphorbol 13-isobutyrate 20-acetate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>dVSM</td>
<td>Differentiated vascular smooth muscle</td>
</tr>
<tr>
<td>dVSMC</td>
<td>Differentiated vascular smooth muscle cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early endosome antigen 1</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>F-Actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FA</td>
<td>Focal adhesion</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FAT</td>
<td>Focal adhesion targeting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FERM</td>
<td>4.1 protein, ezrin, radixin, moesin</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular actin</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cell line derived from Henrietta Lacks</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>L</td>
<td>Liter(s)</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram(s)</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter(s)</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometer(s)</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>M</td>
<td>Membrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryo fibroblasts</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram(s)</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter(s)</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neural Wiskott-Aldrich Syndrome protein</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer(s)</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PIPES</td>
<td>Piperazine-N,N'-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
</tr>
<tr>
<td>pLC</td>
<td>phospho-myosin light chain</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase-C</td>
</tr>
<tr>
<td>PP2</td>
<td>Protein phosphatase 2</td>
</tr>
<tr>
<td>pPax</td>
<td>Phospho-paxillin</td>
</tr>
<tr>
<td>PQ</td>
<td>Primaquine</td>
</tr>
<tr>
<td>PSS</td>
<td>Phosphate buffered saline solution</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SK</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TX</td>
<td>Triton-X</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>VSM</td>
<td>Vascular smooth muscle</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

INTRODUCTION

Aberrant differentiated vascular smooth muscle contraction (VSM) is an important contributor in the pathogenesis of cardiovascular disease. However, not much is known about the mechanisms and pathways involved in stimulus-induced contraction in this cell phenotype. In non-contractile, migrating cells protein complexes known as focal adhesions (FAs) are responsible for mechanical linkages of the cell to the extracellular matrix (ECM) as well as transmitting outside-in and inside-out signals. This thesis focuses on the role of focal adhesions (FAs) in differentiated vascular smooth muscle (dVSM).

CARDIOVASCULAR DISEASES

Cardiovascular disease is the leading cause of death in the United States (119). As part of the cardiovascular circulatory system, VSM needs to be highly dynamic and versatile to adapt to the changing cellular environment (Figure 1.1); however, it is known that phenotypic abnormalities in VSM make key contributions to atherosclerosis and hypertension, which can then further aggravate or accelerate other, more serious cardiovascular conditions and increase the risk of stroke or heart failure (29, 54, 169). In fact, primary hypertension is present in more than 90% of cardiovascular disease cases (27). Several of the standard treatments involve inhibiting vascular tone and reducing
vasoconstriction in VSM (55). Therefore, it is critical to understand the mechanisms for VSM maintenance of mass, tone, lumen, and contractile capacity (183).
Figure 1.1. **Role of blood vessels.** Breakdown of the dynamic role of blood vessels in response to the changing cellular environment. Schematic of specific blood vessel response and remodeling as a part of normal cellular processes, vascular injury, adaptive response, or contribution to disease pathology. Modified from Humphrey, 2008 (103).
VASCULAR SMOOTH MUSCLE

In a closed circulatory system, large arteries and the smaller associated microvessels play an important role in directing blood flow and regulating blood pressure through alternating constriction and dilation of vessels (10, 137, 210). This effect is achieved primarily by the drastic contraction of vascular smooth muscle cells (VSMCs) which reside in the tunica media, oriented perpendicular to the direction of the overall vessel (210) (Figure 1.2). This contraction occurs via a substantial rearrangement of the cytoskeleton, allowing cells to shorten to 50% of their original length (209) (Figure 1.3). However, much about the signaling pathways and importance of the individual protein constituents involved in cytoskeletal rearrangement remains undiscovered.

VSM contraction can be achieved through chemical, mechanical, or electrical stimulation. Although all contractile stimuli ultimately affect myosin and/or actin to control cross-bridge cycling, there are several different possible signaling pathways that can be used to arrive at the final target, including both Ca\textsuperscript{2+}-dependent and independent pathways in differentiated vascular smooth muscle (dVSM) (209) (Figure 1.4). Myosin regulation is accomplished almost exclusively through phosphorylation of myosin regulatory light chain (36) whereas actin availability can be controlled by a variety of actin-binding proteins.
Figure 1.2 Structure of blood vessels. Diagrammatic representation of the different cell layers of an artery. The thin, innermost intima layer adjacent to the lumen consists of endothelial cells and a layer or two of fenestrated elastic membrane making up the elastica interna. The middle (media) layer is the thickest layer and is comprised primarily of smooth muscle cells. The outermost adventitial layer is made up of extracellular matrix and fibroblast cells. (reproduced for free from http://commons.wikimedia.org/wiki/File:Anatomy_artery.png).
Figure 1.3 Vascular smooth muscle contraction. Time lapse phaselucent microscope image sequence demonstrating the remarkable contractile capability of VSMCs. Stimulation of an enzymatically isolated dVSMC with 10 μM phenylephrine results in visible cellular contraction over a short period of time to less than 50% of initial cell length. Modified from Vetterkind and Morgan, 2012 (209).
Figure 1.4 Smooth muscle stimuli and signaling pathways. Diagrammatic representation of the various stimuli that can cause a contractile response and the different pathways through which that response can be achieved in dVSM. Mechanical, chemical, or electrical stimuli result in cellular contraction through a Ca\(^{2+}\)-independent increase in actin availability and/or Ca\(^{2+}\)-dependent myosin activation by myosin light chain kinase. Modified from Vetterkind and Morgan, 2012 (209).
Vascular Smooth Muscle Phenotypes

VSMCs can exist in either proliferative, migratory, non-contractile phenotype or a fully differentiated, non-migratory, contractile phenotype (161) (Figure 1.5). Ordinarily, proliferative VSMCs are necessary for embryogenesis and angiogenesis, but uncontrolled division has been implicated in atherosclerosis and hypertension (11, 100). Similarly, the fully differentiated, contractile VSMCs are required for regulation of blood flow and pressure but dysfunction can contribute to hypertension, stroke, and heart failure (214). However, these quiescent, differentiated cells can transform back into a proliferative state in response to inflammatory signals or growth factors (145) and consequently further contribute to the pathogenesis of disease (11, 129). Much of what is known has come through the study of VSMCs in culture, so comparatively little is known about contractile, dVSMCs.
Figure 1.5 Contractile versus migrating smooth muscle. Labeled microscopic images showing some of the differences between synthetic and contractile VSMCs. Contractile VSMCs (right) are elongated, spindle-shaped cells. Upon being cultured, VSMCs de-differentiate into a proliferative, migratory synthetic phenotype (left), losing their elongated cell shape and contractile filaments. The number and size of organelles involved in protein synthesis increases in the de-differentiated, synthetic phenotype. Modified from Rensen et al, 2007 (161).
Differentiated Vascular Smooth Muscle

This study focuses on differentiated as opposed to cultured cells, which necessitates an extra investment of time to dissociate cells with a smaller net yield as well as developing specialized techniques for this system. However, it is well known that dVSMCs undergo a phenotypic change when grown as immortalized cells (19, 80, 123, 161, 205). DVMCs de-differentiate and in the process lose normal contractile responses, assemble a microtubule network, and alter signaling pathways (94). Since it is the fully differentiated, quiescent, contractile cell phenotype that has been implicated in cardiovascular conditions including vasospasm and hypertension, any extra effort is worthwhile to uncover the details in the largely unknown signaling pathways and interactions of dVSMCs. This is understood to be a necessary prerequisite for developing appropriate therapeutics for cardiovascular disorders.

Ferret as a Model Organism

The ferret has been chosen as a model system because, both on the protein level as well as in the overall cardiovascular physiology, ferrets are closely related to humans (7, 30). Because of this close relationship, ferret tissue generally cross-reacts with human-derived reagents and has been used as the model organism for the study of several human diseases (8, 164). Furthermore, this lab has already developed a foundational background in ferrets allowing for potential integration and comparison of these studies to form a more complete picture.
FOCAL ADHESIONS

In order for cells to survive, they must communicate with their environment. Connection or adhesion to the extracellular matrix (ECM) and the subsequent interactions play key roles in cell behavior, regulation, and fate (78, 87). Since the ECM provides the local physical microenvironment, it can serve as a scaffold to guide cell anchoring, migration, and other morphogenic roles (75) as well as to transmit environmental signals to the cell that influence and impact differentiation, growth, and death (15, 76). The sites of ECM adhesion with the cell are loosely grouped together under the title “focal adhesions” (FAs) and were initially discovered by electron microscopy in 1971 (1). FAs are macromolecular protein assemblies thought to mediate stress, stretch, and tension signals into and out of the cell. They are ideally situated as mechanosensors since they are positioned on the inner face of the plasma membrane and connect the ECM to the interior actin cytoskeleton which in turn mediates morphological changes.

FAs are ubiquitous among adherent, migrating cells, but their exact protein composition, structure, size and distribution is widely varied (75). There are more than 100 different proteins that have been associated with FAs (75, 157, 218) (Figure 1.6) and the list continues to grow. The inherent complexity of the system and its regulation is further complicated by the observation that several of the major protein components of FAs have the potential for more than 10 different binding partners (75). Finally, FAs are subject to continual remodeling. An important example of this dynamic character is the
observation that the size of a FA correlates positively with the amount of force acting on it (6), consistent with transducing external stress, stretch, and tension signals.
Figure 1.6 Focal adhesion complexity. Diagram illustrating the complexity of FA protein binding and signaling. FAK and Src (red diamonds) are critical tyrosine kinases involved in FA regulation. Paxillin, zyxin, Cas, talin, and vinculin (purple rectangles) have roles in adaptor protein/signaling function. Beta-integrin (green rectangle) is a trans-membrane adhesion receptor. Actinin, VASP, filamin (purple circles) are actin regulators. Actin (white circle) is the primary cytoskeletal component. Modified from Zaidel-Bar et al, 2007 (218).
Focal Adhesion Proteins

Although there are a vast array of FA proteins, these studies are focused on a few of the more well-studied ones, anticipating that what is already known in migrating, non-contractile cells could inform my studies of what is unknown in non-migrating, contractile tissue (Figure 1.7).
Figure 1.7 Focal adhesion structure. Diagrammatic illustration of a few selected FA proteins of interest, their potential binding partners, and estimated geographical location within the FA. VASP, zyxin, and actinin are thought to be more closely associated with actin regulation. Vinculin, CAS, and Src occupy regions closer to the plasma membrane. Filamin, talin, paxillin, and FAK all potentially compete for binding to transmembrane integrins that are connected to the ECM. Diagram based on compilation of data from several reviews and studies including Kanchanawong et al 2010, Zaidel-Bar et al 2007, and Mitra et al 2005 (107, 138, 218).
**Integrins**

Integrins are transmembrane proteins that connect the ECM to the FAs (105, 155, 182). One subunit consists of a heterodimer of an α and β integrin noncovalently bound to each other (131). The N-termini come together to form a large head that binds to specific amino acid sequences of ECM proteins (collagens, fibronectin, vitronectin, thrombospondin, elastin, tenascin, osteopontin and several laminins) (131). The shorter C-terminal intracellular tail binds to FA proteins and cytoskeletal elements (26). Because they physically connect the cytoskeleton to the ECM, integrins play a critical role in two-way mechanical force transmission as well as signal transduction (78). It has been hypothesized that integrins are critical for regulating blood flow in response to vascular injury (47, 48). There are 24 known integrins, 13 of which have been found in VSMCs although αv and β1 are thought to be the most prevalent subunits (82, 139).

**FAK, Cas, Src**

Focal adhesion kinase (FAK, 125 kDa) is a critical FA tyrosine kinase consisting of a FERM domain (4.1 protein, ezrin, radixin, moesin), a kinase domain, and a focal adhesion targeting (FAT) domain (181). The FAT domains targets FAK to FAs where it is autophosphorylated at Y397 upon integrin binding (138, 197). This activates FAK and exposes a Src (60 kDa) homology SH2 binding site (91, 150, 179, 181). Binding of Src to FAK creates a complex that allows SH3-mediated binding of p130Crk-associated substrate (Cas, 130 kDa) and subsequent phosphorylation (37, 127). Activation and exposure of Cas domains to tyrosine phosphorylation reveals docking sites for other
adaptor proteins and effectors (37, 178). FAK, Cas, and Src can then further activate, inactivate and regulate one another through other phosphorylation sites (101, 111, 140, 168), ultimately controlling FA turnover (138) and downstream activation of the extracellular signal related kinase (ERK) pathway, which activates myosin light chain kinase, which is responsible for contractility (163, 181).

**Paxillin**

Paxillin (68 kDa) is an important adaptor/scaffold protein in FAs (201, 202). It is known to directly bind FAK, vinculin, and Src (193, 203, 213). Once bound, subsequent phosphorylations lead to the recruitment of other adaptor proteins (like Cas), thereby regulating FA dynamics (24, 162), and triggering other signaling cascades (such as mitogen activated protein kinase; MAPK) (118). It has also recently been suggested that paxillin may directly bind integrins (128).

**Filamin**

Filamin (280 kDa) is a scaffolding and actin crosslinking protein that usually functions as a parallel homodimer. Each monomer can bind actin and create a high-angle bend that supports and stabilizes orthogonal actin branching networks at FAs (67). It also competes with talin for direct binding to beta-integrins (112).

**Vinculin/Metavinculin**

Vinculin (117 kDa) is a structural and signaling FA protein head, proline-rich neck, and tail domain (59, 104, 211). In its inactive form, the head and tail region are bound to each other, thus obscuring active binding sites (5, 33, 225, 227). When activated, vinculin opens up, revealing binding sites for actin on the tail and talin on the
head domain (219, 227). Metavinculin (150 kDa) has a 69 amino acid insert in the tail
domain although it is not yet clear what the functional significance of the extra piece is
(25, 81). Although vinculin is ubiquitously expressed, metavinculin has only been found
in muscle and is not as well studied (64, 79, 175).

Zyxin

Zyxin (82 kDa) was first identified as a scaffolding protein in smooth muscle in
1991 (44). Since its discovery however, the majority of characterizing and functional
studies have focused on its role as a mechanosensor in FAs of migrating fibroblasts (217)
and a possible role in costameres (99). Recent work in cultured cells has shown that zyxin
may be regulated by phosphorylation (31), strongly suggesting that, rather than playing a
passive scaffolding role in FAs, it instead is subject to multiple reversible regulatory
modifications. Furthermore, the exertion of mechanical forces facilitates zyxin
recruitment to FAs (97) while decreasing mechanical load increases the unbinding rate
for zyxin at FAs (124). Zyxin knockdown by RNAi in cultured 3T3 and HeLa cells
results in loss of actin stress fibers and reduction of early-stage FAs (86, 93). Finally,
zyxin has been shown to shuttle between FAs and the nucleus (146), and has been
implicated as a mechanosensitive modulator of gene expression (29). Using the growing
body of research on zyxin in migrating cells as a foundational framework to build upon,
these studies address my prediction that the fundamental findings about zyxin in
migrating cells will still be applicable in the functionally different dVSM phenotype.

Vasodilator-stimulated phosphoprotein (VASP)
Vasodilator-stimulated phosphoprotein (VASP, 50 kDa) was first identified in 1989 (89) and characterized in several types of tissues (158) and then suggested as a potential binding partner for zyxin in 1995 (159). Since then the role of this interaction has been well characterized in migrating cells. VASP binds the proline rich region of zyxin (50, 154) through a complicated head-tail interaction (141). Applied mechanical stress induces zyxin recruitment of VASP from FAs to actin stress fibers (215). Zyxin gene deletion results in reduced accumulation of VASP and deficiencies in actin stress fiber remodeling (98). Notably, zyxin colocalizes at FAs and along stress fibers but is not present with VASP at lamellipodia (172). VASP may be recruited to the lamellipodia by interacting with the polyproline sequence in vinculin (77).

In migrating cells, VASP has been implicated as a regulator of actin incorporation at barbed ends of actin filaments (13, 18, 26) at the leading edge tips of lamellipodia, filopodia, and stress fibers (72, 77, 158, 171, 192). VASP has been shown to promote actin polymerization (9, 12) and directly accelerate actin elongation (21, 152). It also works indirectly by recruiting profilin, a known actin polymerization promoter (65, 108). In dVSMCs, VASP is targeted to dense bodies and plaques and is necessary for actin polymerization (115). Even though dense plaque structure and function is somewhat different than in cultured, migrating cells, the known similarities between the role of VASP in differentiated and non-differentiated cells (26, 115) suggest there may be more crossover between cell types. Since VASP’s actin polymerization role is conserved, and the VASP-zyxin interaction in migrating cells is critical for proper actin formation, it seems likely that a VASP-zyxin interaction exists in dVSMCs.
Alpha-actinin

Alpha-actinin (107 kDa) is an actin cross-linking protein that is part of the spectrin family (that includes dystrophin) (49, 60, 185). The two non-muscle isoforms are generally associated with FAs (17, 38, 58) and have been shown to recruit and bind zyxin (45, 126, 160). Less is known about the non-motile muscle isoforms, but it is thought that they may be predominantly associated with the non-contractile actin (63).

Actin

In dVSMCs, actin (42 kDa) makes up about 20% of the overall protein (114) and can be roughly divided into two categories: contractile actin that interacts with myosin to enable cell shortening and non-muscle actin that does not interact with myosin (214). This thesis will focus primarily on contractile actin and its interaction with and regulation by proteins associated with FAs (particularly VASP and zyxin).

Actin cytoskeleton remodeling is important for a wide range of cellular functions including migration, axon guidance, motility, contraction, and lamellipodia formation (35, 114, 117, 156, 199). Actin polymerization is regulated by multiple pathways including decreased depolymerization, increased new filament branching, and increased or accelerated elongation of existing filaments (121, 156, 224). Recent studies have shown that, while not as fast or extensive as in migrating cells, actin cytoskeleton remodeling occurs in dVSMCs (114) and regulates diameter, compliance, and contractile force (68, 69, 134). This can occur at any site in the cell with barbed actin filaments, which, in smooth muscle, includes membrane-localized FAs.
**Focal Adhesion Dynamics in Migrating Cells**

Actin polymerization at the leading edge of a migrating cell typically produces protrusions in the direction of movement. Integrins in the cell bind to ECM proteins (like collagen and elastin), clustering together and recruiting early FA proteins like talin and vinculin (216). This early stage FA, sometimes referred to as a “focal complex” can either mature into a fully formed FA or disappear. Maturation involves recruitment of cytoskeletal and cytoplasmic proteins for both signaling and structural integrity. FA growth is tension dependent and its composition and size are constantly regulated by tyrosine phosphorylations of constituent signaling proteins (73, 217). As the cell moves forward, these mature FAs help the cell adhere to the ECM. Eventually, FAs detach and the protein components are broken down (14, 120). This process is shown in Figure 1.8.

It should be noted that most studies of FA mechanics and behavior have been performed on two-dimensional substrates. FA behavior, although similar in general, changes markedly when cells are placed in a three-dimensional environment (46). This again highlights how important it is to study FA dynamics in dVSMCs. Although I expect similarities, FA mechanics in an intact three-dimensional (3-D) tissue environment in non-migratory cells are almost certain to be significantly different from what is observed in cultured, migratory cells.
Figure 1.8 Focal adhesion dynamics. Illustration of the life cycle of FAs in migrating cells. New focal adhesions form at the leading edge of a migrating cell. They grow and mature into fibrillar adhesions by recruiting more proteins through kinase signaling. As the cell moves over the FA, the FA travels to trailing edge of the cell where it is disassembled and the proteins broken down for eventual reuse at new FAs at the leading edge. Modified from Broussard et al, 2008 (22).
Focal Adhesion Dynamics in Non-Migratory Cells

Dynamic remodeling of FAs and the associated actin cytoskeleton is known to be critical for cell function in proliferative and migratory cells. FA formation at the leading edge, gradual maturation, and eventual turnover have been extensively studied (16, 22, 212). However, the degree to which FAs in non-migratory, fully differentiated cellular phenotypes are dynamic, and the functions of those FAs embedded in tissue, have yet to be fully clarified.

Recent studies in our lab and elsewhere have demonstrated that the actin cytoskeleton in non-migrating, contractile VSM is not a totally static structure (For reviews, see (88, 113, 209, 214)). In fully dVSMCs, actin polymerization increases in response to the vasoconstrictor phenylephrine (PE) (114). Furthermore, it has been shown that the actin elongation factor, VASP (vasodilator-stimulated phosphoprotein), is necessary for alpha-agonist induced actin polymerization in VSM (115). Interestingly, in airway smooth muscle, Neural Wiskott-Aldrich Syndrome protein (N-WASP) activation of the Actin-related protein (Arp) 2/3 complex has been shown to be necessary for actin polymerization and acetylcholine-induced contractility (224). However, the functional specifics of how cytoskeletal remodeling is involved in the VSM contractile response remain unclear.

Likewise, evidence exists that FAs in VSM may not be purely structural, fixed elements. In airway smooth muscle, agonist-induced vinculin (102), paxillin (195), FAK, talin (148), and alpha-actinin (223) redistribution occurs and are necessary for active
tension development with cholinergic agonists. In dVSM, VASP shifts from the soluble fraction to the insoluble fraction in response to a phorbol ester (115), and Src and CAS redistribute between insoluble and soluble fractions in response to an alpha-agonist (136). Finally, noradrenaline or endothelin-induced stimulation of small mesenteric arteries causes redistribution of paxillin from a soluble fraction to an insoluble fraction (147). Beyond this work, it is unclear whether other FA proteins in dVSM remodel in response to contractile stimuli. Furthermore, the degree of movement, the mechanism, and the precise targets are largely unknown (167).

ENDOSOMES

Endocytosis, a process by which cells use the plasma membrane to engulf extracellular fluids or particles, is used to acquire and transport nutrients (189). This involves three steps: invagination and pinching off of the plasma membrane, transportation of the vesicles, targeting and tethering to final destination (132). Endocytosis can be divided into phagocytosis (uptake of particles) and pinocytosis (uptake of fluids). Pinocytosis can be further divided into macropinocytosis [membrane patches > 1 μm (116)], clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis (41, 144).

After an internalization (which is assisted by barbed end actin growth to physically pinch off the vesicle (40, 106, 188), endosomes travel along existing actin (short-range) or microtubule (long-range) tracks. Movement is facilitated by either myosin, dynein, or kinesin motors (3, 130). Regulation and targeting is accomplished by
various adaptor complexes and a range of Rab GTPases (monomeric G-proteins that regulate membrane traffic through active or inactive GTP or GDP-bound forms respectively) \((74, 142, 170, 198, 221)\). Generally speaking, vesicles are first sent to the early endosome/sorting endosome where cargo fate is decided. Proteins can either return to the plasma membrane via recycling endosomes or go on to the late endosome. From there, they go to either the trans-Golgi network or lysosome for degradation \((85, 189)\) (Figure 1.9).
Figure 1.9 Endocytic pathways. Simplified diagram of the various endosome pathways, the molecular motors involved, and a few of the accessory proteins of interest. Dynamin constriction and actin filament elongation actively facilitate vesicle budding off of the plasma membrane. Vesicles travel along microtubule or actin networks to fuse with early/sorting endosomes. From there, proteins can be directed to the late endosome and eventually the lysosome or trans-golgi network, or be recycled back to the plasma membrane through the recycling endosome. All of these processes are regulated and modulated by various adaptor and motor proteins, particularly specific Rab GTPases. Based on reviews by Soldati and Schliwa 2006 and Grant and Donaldson 2009 (85, 189).
It is known that endocytic pathways mediate FA protein recycling in migrating, proliferating cells. This has been studied most thoroughly in the case of the FA regulator c-Src (109, 110, 176, 200). Endocytic recycling of transmembrane integrins and its affect on FA regulation is also well studied (4, 28, 32, 61). Some recent studies have implicated endocytic recycling in shuttling of membrane-associated proteins in cultured myocytes (96, 133). However, endocytic recycling of FA proteins in the dVSMC phenotype has not been examined, to the best of my knowledge. At first glance, endosomal traffic is expected to be minimal in non-synthetic cell types such as dVSMCs, but no alternate mechanism has been suggested for FA protein translocation in these cells.

**THESIS RATIONALE/AIMS OF THIS THESIS**

Although dVSMCs are known to be critical in the progression and pathogenesis of cardiovascular disease, much of the mechanisms and regulation of contractile activity remains unclear. In cultured, migrating fibroblasts, FAs play an essential role in cellular development, migration, and proliferation. Although, FAs exist in dVSMCs, since this cell phenotype is non-migratory, I wanted to know in what way focal adhesions in dVSM are similar to and different from FAs in migrating cells. Understanding the role of FAs in dVSM could better inform our understanding of cardiovascular disease and present therapeutic targets for the treatment of disease.

This thesis tests the hypothesis that FA proteins in dVSMCs dynamically redistribute in response to an alpha-agonist and that this redistribution is dependent on
microtubule and actin-reliant endocytic recycling. In chapter 3 the data show that, like their migrating counterparts, FA proteins exist in dVSMCs and are concentrated in regularly distributed punctate points. In chapter 4 experiments are performed that quantify the stimulus-induced distribution and re-distribution of several major FA proteins in dVSM. I show that some, but not all, FA proteins are mobile. This finding is in contrast to the behavior of FA proteins in migrating fibroblasts which undergo turnover and complete breakdown as they move from the leading edge to the trailing edge of the moving cell.

Chapter 5 presents evidence that inhibiting endocytic recycling prevents agonist-induced redistribution. This is accomplished either by inhibiting budding, or ablation of microtubule or actin networks necessary for endosome movement. Additional experiments show increased proximity of FA proteins to endocytic markers after stimulation. Finally, I demonstrate that endocytic components are important contributors in determining the strength and speed of VSM tissue contraction.

These novel findings open up new possibilities for research and targeted drug therapies. Showing how important FA proteins and endocytic recycling are for contractile response will hopefully encourage future research into these areas and shed light on potential targets to alleviate developing cardiovascular disease.
CHAPTER 2: MATERIALS AND METHODS

MATERIALS

Enzymes and Chemicals

Isoflurane purchased from VEDCO (St. Joseph, MO). Adenosine 5’-triphosphate disodium salt (ATP), colchicine, Cytocholasin D, 12-deoxyphorbol 13-isobutyrate 20-acetate (DPBA), N-ethylmaleimide (NEM), primaquine bisphosphate, Sigmacote, Triton X-100, and Trypsin Inhibitor purchased from Sigma-Aldrich (St. Louis, MO). Bovine Serum Albumin (BSA) Fraction V was purchased from gibco by Life Technologies (Grand Island, NY). Collagenase Type 2 was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Elastase Grade II lyophilizate purchased from Roche Diagnostics (Indianapolis, IN). Coomassie Brilliant Blue G-250 purchased from Thermo Scientific (Rockford, IL). Donkey and goat blocking serum purchased from Vector Laboratories (Burlingame, CA). Tween-20 and tetramethylethylenediamine (TEMED) purchased from Biorad (Hercules, CA). FluorSave and Insolution protein phosphatase 2 (PP2) purchased from Calbiochem (San Diego, CA). FM4-64FX membrane stain and all Alexa-Fluor secondary antibodies purchased from Invitrogen (Eugene, OR). Latrunculin B purchased from A. G. Scientific Inc. (San Diego, CA).

Unless otherwise stated, lab chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).
Antibodies

Antibodies directed against GAPDH (rabbit, 1:2000) and vinculin (mouse, 1:4000) were from Sigma-Aldrich (St. Louis, MO); Rab5 (mouse, 1:500), paxillin (mouse, 1:500), CAS (mouse, 1:500), and EEA1 (mouse, 1:500) were from BD Transduction Laboratories (San Jose, CA); zyxin (goat, 1:1000), Src (rabbit, 1:500), and FAK (rabbit, 1:100) were from Santa Cruz Biotechnology (Santa Cruz, CA); alpha-actinin (rabbit, 1:1000) was from Abcam (Cambridge, MA); pan-actin (rabbit, 1:2000) was from Cytoskeleton Inc. (Denver, CO); VASP (rabbit, 1:1000), phospho-paxillin (1:500), and phospho-myosin light chain (1:1000) were from Cell Signaling Technology (Danvers, MA); and Filamin (mouse, 1:500) and beta-integrin (rabbit, 1:500) were from Millipore (Billerica, MA).

METHODS

Coomassie Gel Stain for Total Protein

Gel was placed in a Coomassie blue staining solution (25% isopropanol, 10% acetic acid, 0.1% Coomassie) for one hour while covered and rocking at slow speed. The staining solution was then replaced with a destaining solution consisting of 10% isopropanol and 10% acetic acid and set on a rocker for 20 minutes. The destaining solution was replaced with fresh destaining solution every 20 minutes for 5 cycles. The gel was then left in destaining solution overnight and washed with distilled water 2 times before being scanned and analyzed in the same manner as a western blot (see below).
Lowry Protein Assay

Lowry kit was purchased from Biorad (Hercules, CA). All Lowry measurements were done in duplicate. A calibration curve was created with 0, 3, 7.5, 15, 22.5, and 30 µg of BSA per 1.5 mL microcentrifuge tubes added to 3 µL of the buffer the samples were stored in. Enough ddH$_2$O was then added to each tube to bring the final volume to 30 µL. Three µL of each test sample was added to tubes with 27 µL ddH$_2$O. Solution B (1.2 mLS per well) was prepared in a 24 well plate. One hundred fifty µL of a Solution A and Solution S mixture (50:1 ratio, respectively) were added to each sample, mixed and then, the now 180 µL, was transferred to the wells with Solution B. The mixtures in the wells were immediately mixed and incubated for 15 minutes at room temperature (the A/S mixture gets cloudy if allowed to sit too long). UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan) readings were taking at 750 nm. Sample protein concentration estimates were made based on the BSA calibration curve.

Western Blot

SDS PAGE gel electrophoresis: the night before the experiment, a 10% separating/resolving gel was poured into a Biorad apparatus (Hercules, CA) (2.89 mL ddH$_2$O, 187.5 µL glycerol, 1.875 mL lower Tris buffer (36.33 g Tris, 0.4% SDS, ddH$_2$O to 200 mL, pH=8.8), 2.5 mL of a 30% acrylamide/0.8% bis-acrylamide weight/volume solution (National Diagnostics, Atlanta, GA), 75 µL of a 10% ammonium persulfate (APS) solution, 7.5 µL N,N,N',N'-tetramethylethylenediamine (TEMED)). APS and TEMED were added and mixed just before pouring. Gels were covered with a thin layer
of ddH$_2$O to even out the gel and prevent drying. The following day, the ddH$_2$O was removed and the stacking gel with combs inserted was poured (4.0 mL ddH$_2$O, 1.565 mL upper Tris buffer (12.11 g Tris, 0.4% SDS, ddH$_2$O to 200 mL, pH 6.8), 625 µL of a 30% acrylamide/1.6% bis-acrylamide solution, 625 µL APS, 6.25 µL TEMED) and allowed to polymerize. During this time, frozen tissue homogenates were slowly thawed on ice. Small amounts of each sample were combined with 1x sample buffer (8% SDS, 40% glycerol, 0.25 M Tris (pH=6.8), 10% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 6 minutes, and 5 µL of 10 mM N-Ethylmaleimide (NEM) was added to each sample. Combs were removed from gels and gels were placed into running apparatus filled and surrounded by 1X running buffer (25mM Tris, 0.192 M glycine, 0.1% SDS). Samples were loaded into the gel with a marker lane using Precision Plus Protein Dual Color Standards (Biorad, Hercules, CA). The gel was then run at constant current (10-15 mA) on a GE Healthcare Biosciences Electrophoresis Power Supply (Pittsburgh, PA) until the samples entered the separating gel. The current was then increased to between 20 and 25 mA until the samples reached the bottom of the gel.

The gel was then carefully removed and added to a “sandwich” for transfer. From the bottom up, there were two sheets of blotting paper (VWR International, West Chester, PA) pre-soaked in Anode I buffer (0.3 M Tris, 10% methanol), one sheet of blotting paper soaked in Anode II buffer (25 mM Tris, 10% methanol), activated membrane (Millipore Immobilon-FL transfer membrane (Billerica, MA) soaked in methanol and rinsed with ddH$_2$O), gel, and two sheets of blotting paper soaked in Cathode buffer (25 mM Tris base, 40mM glycine, 10% methanol). The whole sandwich was placed on the
Hoefer SemiPhor semi-dry transfer unit (Amersham Pharmacia Biotech AB, San Francisco, CA) and run at 120 mA (per gel) constant current for 90 minutes.

At the end of this time, the membrane was carefully removed, trimmed and placed in Odyssey Infrared Imaging System Blocking Buffer (LI-COR Biosciences, Lincoln, NE) while rocking for one hour. The membrane was then incubated with primary antibodies in Tris buffer saline with Tween (TBST) (18g NaCl, 2.424 g Tris, ddH$_2$O to 1L, pH 7.4, 1 mL Tween-20, pH=7.4) overnight at 4°C. The following day, the membrane was rinsed 4x for 5 minutes each in TBST, and incubated while rocking and protected from light in LI-COR secondary antibodies diluted 1:000 in TBST. Following 6 rinses, 5 minutes each in TBST, immunostained membranes were imaged with an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln NE), allowing simultaneous imaging of two separate color channels. Quantitative densitometric analysis was performed with Odyssey software (V. 3.0.16) by comparing band intensity summations after consistent background subtraction.

**Contractility Measurements in Serum-Free Organ Culture**

All procedures in this study were performed in accordance with and after approval from the Boston University Institutional Care and Use Committee. Male sable ferrets (*Mustela putorius furo*) from Marshall Farms, North Rose, NY, were euthanized with an inhalant overdose of 15 mL of isoflurane. The thoracic segment of the aorta was quickly excised and placed in an ice-cold oxygenated physiological Krebs salt solution (PSS) consisting of 120 mmol/L NaCl, 5.9 mmol/L KCl, 1.2 mmol/L NaH$_2$PO$_4$, 25 mmol/L NaHCO$_3$, 11.5 mmol/L dextrose, 1 mmol/L CaCl$_2$, and 1.4 mmol/L MgCl$_2$. The tissue
was then dissected in the oxygenated (95% O₂, 5% CO₂) PSS solution at room temperature to remove fat, connective tissue, and endothelium. Aortic rings 4 mm in length were attached to force-displacement transducers (Grass Instrument Co FT03, Quincy, MA) in a 37°C PSS Myobath organ bath (World Precision Instruments, Sarasota, FL) and allowed to equilibrate for one hour after being stretched to 2g passive resistance. The rings were then stimulated with a 51 mmol/L KCl PSS in which 51mmol/L NaCl was replaced with 51mmol/L KCl to test for viability. After 10 minutes, the tissue was washed three times with PSS and allowed to relax for one hour before experimentation began. Force read outs and data were analyzed using LabChart 7 v7.3.3 software (ADInstruments, Colorado Springs, CO). At the end of the experiment, tissue rings were quick-frozen by immersion in an acetone/dry ice slurry containing 10 mmol/L dithiothreitol (DTT) and placed in -80°C degree freezer in preparation for further processing.

**Cell Isolation**

Single dVSMCs were enzymatically dissociated from ferret aorta tissue according to a previously published method (114, 115). One liter of a Hanks physiological salt solution was prepared fresh the morning of experimentation consisting of 137 mmol/L NaCl, 5.4 mmol/L KCl, 0.42 mmol/L NaH₂PO₄, 0.44 mmol/L KH₂PO₄, 4.17 mmol/L NaHCO₃, 10 mmol/L Hepes, 5.55 mmol/L glucose, pH=7.4. An appropriate amount was combined with BSA and sucrose for a total volume of 100 mL of a 0.2% BSA and 300 mM sucrose solution. This solution was then combined with collagenase (12 mg of 360 U/mg activity with 4.14 mL of Hanks/BSA/sucrose), elastase (11.5 mg of 4.10 U/mg
activity with 4.00 mL of Hanks/BSA/sucrose), and trypsin inhibitor (5 mg/mL) into three different aliquots labeled T1, T2, and T3 (Table 2.1).
<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase</td>
<td>1.38mL</td>
<td>1.38mL</td>
<td>1.38mL</td>
</tr>
<tr>
<td>Elastase</td>
<td>1.33mL</td>
<td>0.67mL</td>
<td>0.67mL</td>
</tr>
<tr>
<td>Trypsin Inhibitor</td>
<td>50 μL</td>
<td>50 μL</td>
<td>50 μL</td>
</tr>
<tr>
<td>Hanks/BSA/Suc</td>
<td>4.79mL</td>
<td>5.45mL</td>
<td>5.45mL</td>
</tr>
<tr>
<td>Tot Volume</td>
<td>7.5 mL</td>
<td>7.5 mL</td>
<td>7.5 mL</td>
</tr>
</tbody>
</table>

**Table 2.1. Cell isolation enzyme solutions.** Quantities and contents of three different enzymatic time point solutions for single cell isolation. Equal final volumes with differing amounts of elastase are used for the three different enzymatic incubations.
Male sable ferrets (*Mustela putorius furo*) from Marshall Farms, North Rose, NY, were euthanized with an inhalant overdose of 15 mL of isoflurane. The abdominal segment of the ferret aorta was quickly excised and placed in an ice-cold oxygenated physiological Krebs salt solution (PSS) consisting of 120 mmol/L NaCl, 5.9 mmol/L KCl, 1.2 mmol/L NaH$_2$PO$_4$, 25 mmol/L NaHCO$_3$, 11.5 mmol/L dextrose, 1 mmol/L CaCl$_2$, and 1.4 mmol/L MgCl$_2$. The tissue was then dissected in the oxygenated (95% O$_2$, 5% CO$_2$) PSS solution at room temperature to remove fat, connective tissue, and endothelium. The strip was cut down to a length of 3.5 cm (approximately 150 mg). One longitudinal cut was made down the length of the strip and the tissue was unfolded, flattened, and pinned. Using small, circular motions, the endothelium was lightly brushed off with a rubber policeman. The strip was then cut horizontally into 11-12 pieces. Each of those pieces was cut into thirds in the opposite direction for a total of about 35 pieces. All pieces were then gently transferred to an oxygenated, pre-siliconized 125 mL flask that included T1. The flask was then stoppered with oxygen line still inside and placed in 34°C Magni Whirl constant temperature water bath (Blue M Electric a unit of General Signal, Watertown, WI) and set to shake back and forth 60 times per minute.

Flasks were siliconized by pouring 25 mL of Sigmacote back and forth between 2 flasks while slowly rotating the flasks for 20 minutes. The flasks were then dried overnight, baked for 1 hour at 110 F, soaked in ddH$_2$O overnight, and then left to air dry for several days.
After 80 minutes of shaking, the T1 solution with the tissue strips was carefully poured through a mesh square (mesh opening 500 µm, % open area 39, thickness 610 µm) (Spectrum Laboratories, Inc., Rancho Dominguez, CA) into another siliconized 125 mL flask while rinsing with 10 mL Hanks/BSA/Suc. The mesh square was then flipped over on top of original flask and T2 solution was emptied onto it, to wash the tissue back into flask. This was then put back in shaking water bath after plugging with an oxygen line fed inside. The T1 solution with cells was evenly distributed amongst the wells of a 6 well flat bottom plate with low evaporation lid (Becton Dickinson Labware, Franklin Lakes, NJ) with 22 x 22 mm glass coverslips (VWR International, West Chester, PA) lying on the bottom of the wells and placed on ice in a closed container while oxygenated. Coverslips were pre-etched by shaking in a 2N NaOH solution for 2 hours before rinsing several times with ddH₂O. This was repeated for T2 and T3 after incubations of 30 min and 20 min respectively. The plates with the cell solution were then allowed to sit on ice for one hour to give the cells time to adhere to the coverslips.

Optional Pre-Permeabilization

At this point, there was an optional pre-permeabilization step before fixation. Cells were incubated for one minute at room temperature in a cytoskeletal stabilization buffer (50 mM NaCl, 3 mM MgCl₂, 10 mM PIPES, 2 mM EGTA, 30 mM Sucrose, pH 6.8) with 0.1% Triton-X.

After examining one coverslip from each of the different time points under bright field 4x magnification on a Nikon Diaphot microscope, the batch with the highest quality (length and shape) and quantity of cells was selected for further processing. The
remaining coverslips from the selected batch were transferred to new 6 well plates at room temperature and covered with a 4% paraformaldehyde solution for 10 minutes.

Two grams of paraformaldehyde were mixed with 5 mL ddH$_2$O and heated to 60°C while stirring. Fourteen drops of 1N NaOH solution were added until the entire solution cleared. This was then added to 45 mL of a 0.1M sodium phosphate buffer (10.374 g Na$_2$HPO$_4$, 1.559 g NaH$_2$PO$_4$, 500 mL ddH$_2$O). An additional 0.1 g of NaCl was added and the pH was adjusted to 7.4 if necessary.

After 10 minutes, the paraformaldehyde was aspirated and replaced with 2 mL of a 0.1mM glycine in 1% BSA Hanks PSS solution per well. After 5 minutes, the glycine solution was aspirated out and replaced with fresh glycine. After another 5 minutes this solution was replaced with 0.1% Triton X-100 in 1% BSA Hanks PSS and the cells were placed on a slow rocking platform for 10 minutes (VWR International, West Chester, PA).

Cells were then washed 3 times for 5 minutes each in 0.05% Triton X-100 in 1% BSA Hanks PSS before being placed in a 0.05% Triton X-100, 1% BSA Hanks PSS with 10% blocking serum and gently rocked for one hour. Coverslips were then incubated overnight at 4°C with primary antibodies and 2% blocking serum, 0.05% Triton X-100, and 1% BSA Hanks PSS.

The following day, all coverslips were washed 3-6 times for 10 minutes each time in 0.05% Triton X-100 1% BSA Hanks PSS. They were then incubated in the dark for 30 minutes with secondary antibodies in Hanks PSS (1:500). All secondaries were Alexa-Fluor secondaries from Invitrogen (Eugene, OR). After three more 5 minute washes in
Hanks PSS, the coverslips were dipped in ddH$_2$O and carefully affixed to slides with FluorSave Reagent (Calbiochem, Darmstadt, Germany). This whole process is summarized in Table 2.2.
<table>
<thead>
<tr>
<th>Length (minutes)</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>4% paraformaldehyde</td>
</tr>
<tr>
<td>5</td>
<td>0.1 mM glycine in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>5</td>
<td>0.1 mM glycine in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>10</td>
<td>0.1% Triton X-100 in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>5</td>
<td>0.05% Triton X-100 in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>5</td>
<td>0.05% Triton X-100 in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>5</td>
<td>0.05% Triton X-100 in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>60</td>
<td>10% Block Serum + 0.05% Triton X-100 in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>Overnight</td>
<td>1' antibody + 2 % goat serum +0.05% Triton X-100 in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>10</td>
<td>0.05% Triton X-100 in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>10</td>
<td>0.05% Triton X-100 in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>10</td>
<td>0.05% Triton X-100 in 1%Bsa Hanks PSS</td>
</tr>
<tr>
<td>30</td>
<td>Secondary fluorescent conjugated antibody in Hanks PSS no Bsa</td>
</tr>
<tr>
<td>5</td>
<td>Hanks PSS</td>
</tr>
<tr>
<td>5</td>
<td>Hanks PSS</td>
</tr>
<tr>
<td>5</td>
<td>Hanks PSS</td>
</tr>
</tbody>
</table>

**Table 2.2 Cell staining protocol.** Summary of the steps and time lengths involved in fixing and staining single cells in preparation for microscopic analysis. Paraformaldehyde fixation is followed by glycine quenching, detergent permeabilization, blocking, primary antibody incubation, fluorescent secondary antibody incubation, and finally coverslip mounting.
Immunofluorescent Imaging in Mammalian Cells

Deconvolution Microscopy

For deconvolution microscopy, 3-D image stacks were acquired with a Nikon Eclipse TE 2000-E inverted microscope equipped with a Nikon Plan Apochromat 60XA (NA 1.4) oil immersion objective. Images were recorded by a high-resolution fluorescence CCD camera (CoolSNAPTM HQ2, Photometrics®) with NIS-Elements Advanced Research (Nikon) software. Out-of-focus fluorescent blur was removed by deconvolution of Z-stacks (Richardson-Lucy algorithm, constrained iterative-maximum likelihood estimation algorithm) as previously described (115). For dual labeling experiments, no detectable cross talk was confirmed by exchanging excitation/emission filters on single labeled coverslips.

Quantitative Colocalization

Cells that were >50 μm long and > 4 μm wide (enough to resolve individual FA structures) were selected for analysis. Thresholding was set to optimize the resolution of the labeled structures on visual display. Using NIS-Elements Advanced Research (Nikon) software, quantification of overlapping pixels by a logical ‘AND’ calculation was applied to a center section, excluding the nucleus and the ends of the cell. The percentage of colocalized pixels versus the total number of pixels labeled was calculated and reported as percent colocalization. At least three different animals were used for each condition.

Proximity Ligation Assay
Steps for PLA were identical to aortic cell isolation through overnight incubation with primary antibodies. Cells were then washed 3 times for 5 minutes each time in Hanks PSS + 1% BSA + 0.05% TX-100 in six well plates. The coverslips were then incubated for 2 hours in PLUS and MINUS Duolink PLA probes (e.g. mouse PLUS and goat MINUS) (Olink Biosciences, Uppsala, Sweden) (186), diluted 1:5 in antibody dilution buffer (Hanks + 1% BSA + 0.05% TX-100) (30 μL buffer, 10 μL PLUS probe, 10 μL MINUS probe per cover slip).

To conserve PLA probes, 50 μL of solution was used per square coverslip. The solution was first placed on a Parafilm sheet that was stretched on the inside of the lid of a six well plate and then each coverslip was carefully lowered onto the droplet with cell side down, facing the solution (this small volume and Parafilm method was also used for the ligation and detection steps that follow). To protect from evaporative drying, another six well cover was placed on top of the cover with the Parafilm to function as a lid and the two together were rocked slowly at room temperature.

After 2 hours, the coverslips were rinsed 3 times for 5 minutes each in Hanks PSS + 1% BSA + 0.05% TX-100 in a six well plate. The ligation buffer was warmed to room temperature ahead of time to dissolve precipitates, then diluted 1:5 in water. Ligase was added (1:40) right before use. Coverslips were incubated on the ligation droplets for 30 min at 37°C. They were then washed at room temperature 2x 2 minutes in Hanks-Tween (0.05%) in six well plate.

Detection buffer was thawed then diluted 1:5 in water. Polymerase was added right before use (1:80). Coverslips were incubated on 50 μL detection droplets for 90-120
minutes at 37°C. During this time coverslips were rocked gently by hand every 20 minutes. To protect against evaporation, the six well plate cover and lid were wrapped in Parafilm to seal the edges. Coverslips were protected from light from now through mounting. After the detection incubation, coverslips were washed 2 times for 10 minutes each in Hanks PSS and once in ddH₂O before mounting on slides with FluorSave.

The resulting red fluorescent PLA dots were counted using NIS Elements AR 2.30 software (Nikon, Melville, NY), and images were processed with Photoshop CS3 software (Adobe Systems, Mountain View, CA) as previously described (208).

**Differential Centrifugation**

*Ferret Tissue*

The method used here is similar to that of Kim et al 2010 (115) but has been modified to result in 3 rather than 2 fractions. One sample of quick-frozen ferret tissue was pulverized with pre-cooled mortar and pestle in liquid nitrogen. The ground tissue pieces were transferred, by careful scraping, to a glass homogenization tube on ice containing 0.22 mL buffer I (Tris-HCl 20 mmol/L pH 7.5, NaCl 50 mmol/L, sucrose 250 mmol/L, DTT 10 mmol/L, EGTA 3 mmol/L, MgCl₂ 5 mmol/L, ATP 1 mmol/L, and 15 μL protease inhibitor cocktail). Protease inhibitor cocktail consisted of 0.11mM leupeptin, 0.11mM pepstatin A, 40mM Na₃VO₄, 20mM NaF, 0.4mM AEBSF, and 0.4 mM aprotinin. This was immediately homogenized by hand for 30 seconds, avoiding up and down motions. The homogenization tube was then put into a 600 mL glass beaker packed with a slurry of ice and water and mechanically homogenized using three bursts of 18 seconds set at the lowest possible constant speed on a Glas-Col Variable speed
homogenizer (Terre Haute, IN). There was a 2 minute cool down period between bursts. The ground up tissue solution was transferred to a 1.5 mL ultracentrifugation polyallomer tube on ice (Beckman, Palo Alto, CA). This process was repeated for any other remaining tissue samples.

Tubes were then evenly balanced in a pre-chilled rotor and placed in a Beckman Coulter Optima TLX Ultracentrifuge (Fullerton, CA). Samples were spun at 4°C at 100,000xg for 1 hour. The supernatant was collected as the cytosolic (C) fraction. The remaining pellet was resuspended in 0.22 mL buffer II (Tris-HCl 20 mmol/L pH 7.5, sucrose 250 mmol/L, Triton X-100 0.5%, DTT 10 mmol/L, EGTA 3 mmol/L, MgCl₂ 5 mmol/L, ATP 1 mmol/L, and protease inhibitors), extracted at 4°C for 1 hour, and centrifuged at 100,000xg for 1 hour. This supernatant was collected as the Triton X-soluble or membrane (M) fraction. The pellet was resuspended in 0.22 mL buffer III (Tris-HCl 20 mmol/L pH 7.5, sucrose 250 mmol/L, Triton X-100 0.5%, sodium dodecyl sulfate (SDS) 1.2%, DTT 10 mmol/L, EGTA 3 mmol/L, MgCl₂ 5 mmol/L, ATP 1 mmol/L, and protease inhibitors), extracted at 4°C for 1 hour, and spun down on a tabletop microfuge (6 minutes at 13,000 RPM) to remove nuclei and unbroken cells. This final supernatant was collected as the Triton X-insoluble or cytoskeletal (SK) fraction. It should be noted that this method differs from the method previously used by our lab to monitor actin polymerization (114), most notably in the temperature used, since actin polymerization is optimal at 37°C but, here, as is generally done, the centrifugations were performed at 4°C to minimize any potential degradation or post-translational modifications of the proteins during processing.
Statistical Analysis

Data are expressed as mean +/- standard error (SE) in all graphs. Significance of difference between two individual sets of data was evaluated by a two-tailed, paired Student’s t-test (significant differences reported at p < 0.05), unless indicated otherwise. At least 3 separate ferrets were used for all experiments.
CHAPTER 3: FOCAL ADHESION PROTEIN LOCALIZATION

INTRODUCTION

FAs and the regulatory and functional role of the individual protein constituents has recently become an intense area of research (76, 107, 153). Although the literature is not quite as extensive, Dr. Susan Gunst and colleagues have made strides in understanding the role of FA proteins in airway smooth muscle (102, 148, 194, 223). Our lab has recently had success in demonstrating the dynamic nature of the cytoskeleton in VSM (114, 115). Furthermore, it was shown that the actin elongation factor, VASP, is localized adjacent to FA markers alpha-actinin and vinculin (Figure 3.1). To further pursue the possibility that FAs in dVSMCs may remodel in response to stimuli, I attempted to visualize FA protein localization in dVSMCs.
Figure 3.1 VASP is localized adjacent to vinculin and alpha-actinin at FA-like structures. Enzymatically dissociated dVSMCs were costained for alpha-actinin and VASP or vinculin and VASP. Deconvolved, merged, center sections of cells were used for analysis. (A) alpha-actinin (green top). (D) vinculin (green bottom). (B,E) VASP (red) (C,F) merged. White arrows indicate spots where vinculin and VASP colocalize. Scale bar 10 μm. Modified from Kim et al, 2010 (115).
IMMUNOFLUORESCENT IMAGING OF INDIVIDUAL PROTEINS

Zyxin

I then extended these experiments to zyxin, a known binding partner of VASP in migrating cells (50, 159). Because of zyxin’s binding to and targeting of VASP (98), immunofluorescent imaging was expected to show staining patterns similar to that of VASP (115). Both portal vein and aortic cells were enzymatically dissociated, isolated, fixed and stained for zyxin (Figure 3.2). Immunofluorescent images showed low levels of cytosolic background signal interspersed with a regular distribution of bright punctate spots near the membrane at one micron intervals (Figure 3.2). These findings are in line with FA protein imaging previously published in our lab (115).
Figure 3.2 Zyxin staining at FA-like structures. Portal vein tissue was enzymatically dissociated into individual cells, fixed, and stained for zyxin. Deconvolved cell center sections were examined by fluorescent microscopy. Images showed zyxin staining in portal vein cells at regularly spaced one micron intervals near the membrane at FA-like structures indicated by white arrows. Scale bar 10 μm.
COLOCALIZATION

Proteins that are known to localize to FAs in migrating cells were examined to confirm that these proteins associate with FAs in dVSMCs. Furthermore, colocalization studies were undertaken to demonstrate that these FA proteins localized together, at the same FAs.

Paxillin/pPax

To begin colocalization assessment, positive and negative controls were established for comparison. As a positive control, freshly dissociated aorta cells were fixed and co-stained with antibodies against paxillin and phospho-paxillin (Figure 3.3). Paxillin phosphorylation at Y118 was selected since it is a known target of Src during FA activation in response to stimulation (147, 173). Immunofluorescent imaging after stimulation showed paxillin localization in a filamentous pattern with regularly spaced, punctate spots of bright fluorescence near the membrane indicative of FA-like staining. The mostly punctate phospho-paxillin staining clearly made up a subset of the total paxillin staining.
Figure 3.3 Paxillin/pPax co-stain as positive control. Enzymatically dissociated dVSMCs were co-stained for paxillin and phospho-paxillin after 10 min 10 μM PE stimulation. Deconvolved cell bottom sections were imaged for colocalization staining. Phospho-paxillin (green) stains a subset of overall paxillin (red) at regularly distributed FA-like punctae. Blue staining is nuclear DAPI signal. Scale bar 5 μm.
**Paxillin/pLC**

As a negative control, paxillin, which localizes to FAs at the end of actin stress fibers (203), was co-stained in freshly isolated aorta cells with phospho-myosin light chain (pLC) (Figure 3.4). Myosin light chain phosphorylation at serine 19 was selected since myosin chains are expected to be localized with the contractile filaments in the interior of the cell and are not thought to interact with FA proteins. In stimulated cells, the punctate staining of paxillin was clearly separable from the more filamentous pLC staining. Paxillin staining showed distinct localization to the membrane with the predicted FA-like, regularly dispersed, bright punctate dots. Phospho-myosin light chain staining, on the other hand, appeared more cytosolic and filamentous with brighter punctate dots at intervals along the filamentous stretches. There appeared to be no significant overlap between the two protein populations, as expected.
Figure 3.4 Paxillin/pLC co-stain as negative control. Enzymatically dissociated dVSMCs were co-stained for paxillin and phospho-myosin light chain after 10 min 10 µM PE stimulation. Deconvolved cell center sections were imaged for colocalization staining. Phospho-myosin light chain (green) punctae localized along intracellular filamentous structures do not overlap with overall paxillin (red), localized at bright punctae near the membrane. Blue staining is nuclear DAPI signal. Scale bar 5 µm.
Zyxin/Paxillin

As previously discussed, zyxin and paxillin are both known to localize to FAs in migrating cells. Immunofluorescent imaging in dVSMCs appeared to show localization of both zyxin and paxillin at FA-like structures (Figures 3.2 and 3.4 respectively). If they localize at FAs in dVSMCs, I would expect them to localize at some of the same FAs and therefore demonstrate at least partial colocalization in imaging experiments. To test this hypothesis, freshly dissociated dVSMCs were co-labeled with antibodies against zyxin and paxillin and viewed under fluorescence microscopy (Figure 3.5). Both zyxin and paxillin again, individually showed the typical FA-like, punctate staining pattern near the membrane. A deconvolved, merged center section showed zyxin and paxillin dots at approximately one micron intervals near the cell membrane. These dots either colocalized (at the 140 nm resolution of deconvolution microscopy (149)) or were closely adjacent to each other.
Figure 3.5 Zyxin and paxillin colocalize. Enzymatically dissociated dVSMCs were co-stained for zyxin and paxillin. Deconvolved cell center sections were imaged for colocalization staining. Zyxin (red) and paxillin (green) show partial overlap at FA-like structures near the membrane (white arrows). Where they do not overlap, paxillin and zyxin punctae localize adjacent to each other next to the membrane. Blue staining is nuclear DAPI signal. Scale bar 10 μm.
Zyxin/Vinculin

Previous studies in migrating cells have shown that zyxin and vinculin colocalize at FAs (104, 135). Therefore, I performed colocalization studies for these two proteins in dVSM. Immunofluorescent co-staining of freshly dissociated dVSMCs with antibodies against zyxin and vinculin revealed partial colocalization similar to that seen with zyxin and paxillin (Figure 3.6). Vinculin staining appeared brightest at regular punctate regions bordering the cell membrane. Likewise, zyxin staining was strongest at FA-like punctae at the cell membrane. A deconvolved, merged image section clearly showed partial overlap of some of the distinct vinculin and zyxin punctae.
Figure 3.6 **Zyxin and vinculin colocalize.** Enzymatically dissociated dVSMCs were co-stained for zyxin and vinculin. Deconvolved cell center sections were imaged for colocalization staining. Zyxin (red) and vinculin (green) show partial overlap at FA-like structures near the membrane (white arrows). Where they do not overlap, vinculin and zyxin punctae localize adjacent to each other next to the membrane. Blue staining is nuclear DAPI signal. Scale bar 10 μm.
FAK/Vinculin

Finally, vinculin colocalization with FAK was examined. FAK has been shown to be a major component of FAs in migrating cells (138). For this reason, FAK and vinculin were chosen as another colocalization pair to compare the structure of FAs in dVSMCs to those in cultured cells. Freshly isolated dVSMCs were co-stained with antibodies against FAK and vinculin (Figure 3.7). FAK displayed easily identifiable bright punctate spots at regular intervals, particularly near the cell membrane. The brightest vinculin staining, although not as well localized in this set of images, was also punctate near the cell membrane. A deconvolved, merged center section shows partial overlap of zyxin and FAK punctae, indicative of colocalization (Figure 3.7A). This is especially evident in a merged cell image section taken near the top of the cell (Figure 3.7B). This section shows a “slice” off the top, essentially only showing protein localization near the membrane (and minimizing the cytosol or intracellularly localized fluorescent signal).
Figure 3.7 FAK and vinculin colocalize. Enzymatically dissociated dVSMCs were co-stained for zyxin and vinculin and imaged by immunofluorescent microscopy. (A) Deconvolved cell center sections were imaged for colocalization staining. FAK (green) overlaps with a subset of vinculin (red) at FA-like structures near the membrane (white arrows). (B) Deconvolved cell top section showing FAK colocalization with a subset of vinculin staining (white arrows) at FA-like structures associated with a membranous top cellular slice. Scale bar 10 μm.
SUMMARY

Here I have shown that some of the same proteins that are known to associate with FAs in cultured, migrating cells also localize to FA-like structures in dVSMCs as shown by in immunofluorescent imaging of several FA proteins. Furthermore, although each protein has its own unique staining pattern, at least a subset of each protein seemed to colocalize with the other FA proteins at distinct FA-like structures. Taken together, this suggests that the protein make up and structure of FAs in dVSMCs is similar to the FA architecture in proliferating, migrating cell types. However, this raises an important question. How far do the similarities extend? If FAs in dVSM are structurally related to FAs in other cell types, are they functionally analogous as well? Or, alternatively, do FAs in dVSM use a common FA structural basis to accomplish functional tasks unique to their cell type?
CHAPTER 4: FOCAL ADHESION PROTEIN DYNAMICS

INTRODUCTION

FAs are known to be highly dynamic structures in migrating fibroblasts (216, 220). Recent studies have suggested that they may be similarly dynamic even in fully differentiated cell phenotypes, just that their function may be different. Research from the laboratory of Dr. Susan Gunst has shown a variety of FA proteins seemingly translocating in response to cholinergic agonists (148). As a means of examining the role of FA proteins in VSM contraction, our lab has shown that Src inhibition by the small molecular inhibitor PP2 (10 µM) (90, 125) or morpholino knockdown during tissue contraction (10 µM PE) in serum free organ culture slows contraction and decreases steady-state contractile force amplitude (136). Recently, published work from our lab has also shown agonist-induced redistribution of VASP (115). I suspected that these were not isolated examples and wanted to investigate whether other FA proteins, particularly those that are thought to be associated with VASP, might be exhibiting dynamic redistribution and whether this movement has any effect on contractile function.

IMAGING IN UNTREATED VS. PE STIMULATED CELLS

I tested the hypothesis of FA protein translocation using those techniques that have already proven successful in this field. I began by looking for visible signs of
stimulus-induced redistribution through immunofluorescent imaging. Freshly enzymatically dissociated cells were left untreated or stimulated with 10 μM PE for 10 minutes, stained for zyxin, and then inspected for changes in location, intensity, or other staining patterns. Unfortunately, these experiments did not show any consistent difference between untreated and PE-treated cells (Figure 4.1). This could be because high background staining masks slight changes, that this type of immunofluorescent analysis is simply not sensitive enough to observe change, that the pool of dynamic proteins is too small to be detected this way, or that the proper landmarks were not used to detect movement.
Figure 4.1 Immunofluorescent staining reveals no visible movement of zyxin in response to PE. Freshly dissociated ferret aorta cells stained with antibodies for zyxin, with (bottom panel) or without (top panel) 10 minutes of 10 μM PE, treatment reveal no obvious visual evidence of movement. Deconvolved center sections were used for analysis. Blue staining in the top panel is nuclear DAPI signal. Empty, dark area of the cell in the bottom panel is probably occupied by the nucleus, but this cell was not stained with DAPI to confirm. Scale bar 10 μm.
DIFFERENTIAL CENTRIFUGATION

Assays up to this point had failed to reveal any significant agonist-induced changes in VSM contraction. So, I chose to expand on previously successful procedures in our lab which took advantage of multiple tissue fractions. Kim and colleagues in our laboratory have used two-fraction differential centrifugation to demonstrate phorbol ester agonist induced redistribution of VASP (115). I sought to confirm and expand these findings with a two and then three-fraction analysis of several FA proteins.

DPBA

Differential centrifugation of aortic tissue homogenates was used to separate proteins into soluble (cytosolic, Cyt) and Triton X- insoluble (cytoskeletal, Csk) fractions. FA-associated proteins in the tissue fractions were identified by immunoblot and the results analyzed for changes in the distribution between fractions in response to the phorbol ester, DPBA. Results confirmed the previously seen shift of VASP from cytosol to cytoskeleton. Furthermore, my data show, for the first time, a significant shift from cytosol to cytoskeleton of zyxin and metavinculin, and a similar trend for vinculin and CAS but no change for alpha-actinin and actin (Figure 4.2). There is a certain degree of variability in the method that may have prevented vinculin and CAS from reaching statistical significance.
Figure 4.2 Phorbol ester DPBA triggers redistribution of some FA proteins. Densitometric analysis of western blots of FA proteins in cytosolic (Cyt) and cytoskeletal (Csk) fractions normalized as a percentage of the total protein amounts from unstimulated (Rest) or DPBA (10 μmol/L) stimulated (Stim) tissues (n=5). *p<.05 as determined by a two tailed, paired t-test compared to resting values.
After successfully demonstrating translocation with DPBA, I wanted to expand on my findings by establishing a differential centrifugation protocol that would result in three fractions (cytosolic, membrane, and cytoskeletal) using the more physiologically relevant vasoconstrictor, phenylephrine (PE).

**Total gel stains**

To confirm the validity of this procedure and proper segregation of the fractions, a Coomassie blue-stained protein gel of the three fractions at four different treatment time points (unstimulated (unstim), 10 μmol/L PE for 2 min, 5 min, and 10 min) was run and indicates, as expected, that a large proportion of total cellular protein was located in the cytoskeletal (SK) fraction at all time points (Figure 4.3A). Average densitometry from such gels is shown in Figure 4.3B, where it can be seen that more than half of the overall protein was in the cytoskeletal fraction.

As positive controls, three proteins with known intracellular distributions were examined by western blot (Figure 4.3C). As expected, about 80% of GAPDH was in the cytosolic (C) fraction (Figure 4.3D), in agreement with previous findings (143). Almost 40% percent of Rab5, a marker protein for early endosome recycling (23, 83, 190), was in the membrane (M) fraction (Figure 4.3E). About 60% of total cellular actin is in the cytoskeletal (SK) fraction (Figure 4.3F) as has been found by others (147) and it was largely unchanged under these conditions.
Figure 4.3 Differential centrifugation of dVSM tissue distinguishes known cytoskeletal proteins from membrane and cytosolic proteins. (A) Typical Coomassie stained blot of proteins in fractions (kD, kilodaltons). (B) Graph of quantitative densitometry from panel A. n=3. cytosolic (C), membrane (M), and cytoskeleton (SK) (C). Western blots of unstimulated or PE-(10 μmol/L) stimulated tissues of three different proteins typically associated with the three different subcellular compartments of interest. (D,E,F). Quantitative densitometric analysis of western blots of total GAPDH, Rab5, and Actin (n=3-11), proteins characteristically associated with cytosol, membrane, and cytoskeleton respectively. Brightness has been uniformly altered for visual display. *p<.05 as determined by a two tailed, paired t-test compared to resting values.
In addition, after differential centrifugation of 6 different aortic rings from 2 different animals, the final pellet was further solubilized in 1N NaOH. The recovered protein from the pellet made up 5.9% +/- 0.97% of the total protein based on densitometric analysis of Coomassie gel stains. The remaining protein in the pellet was likely residual unbroken nuclei and chromatin as well as highly insoluble ECM proteins like elastin. To confirm this was the case, immunoblot analysis of pellet fractions (solubilized in 1N NaOH) after differential centrifugation of both resting and PE-treated tissue was performed and the data show that the pellet contributed an average of 0.43% +/- 0.17 overall zyxin and an average of 0.57% +/- 0.25 overall VASP indicating negligible loss during differential centrifugation.

Remodeling proteins

- Some FA proteins move out of the cytosolic fraction

Differential centrifugation of aortic tissue homogenates was used to separate proteins into soluble (cytosolic), Triton X-soluble (membrane), and SDS-soluble (cytoskeletal) fractions. FA-associated proteins in the tissue fractions were identified by immunoblot (Figure 4.4A) and the results analyzed for changes in the distribution between fractions in response to the vasoconstrictor, PE. Zyxin, a FA protein (44) that facilitates actin filament elongation in response to mechanical forces in cell culture (97), resides to a large extent in the cytosolic fraction in dVSM (Figure 4.4B, left panel). Between 2-10 minutes after addition of PE, zyxin redistributed, leaving the cytosolic fraction (83% to 56%) and increasing in both the membrane and cytoskeletal fractions. At
10 minutes, zyxin levels increased fourfold in the membrane fraction and doubled in the cytoskeleton (Figure 4.4B, right panel). No statistical significance was calculated for graphs normalized to resting protein since such a procedure eliminates experimental variability in unstimulated protein levels.

VASP, a protein that binds to and is targeted by zyxin (50), behaved similarly to zyxin (Figure 4.4C, left panel). In the absence of a stimulus, about half of the total VASP localized in the cytosol, but after PE exposure, VASP gradually left the cytosolic fraction and redistributed to the membrane fraction. After 10 min of PE treatment, 12% of total VASP has shifted to the membrane fraction. This represents a 2.5 fold increase in the amount of protein in the membrane fraction (Figure 4.4C, right panel). Note that although Figure 4.4C suggests that the significant increase of VASP in the membrane fraction (left panel) at 2 and 5 minutes may come from a decrease in the cytosolic fraction, the variability in the method prevented us from making that conclusion since no statistically significant changes were observed in the VASP content of the cytosolic fraction at early time points.

Metavinculin, a multifunctional FA signaling/crosslinking protein (Figure 4.4D) (135, 227), and paxillin, which interacts with FAK and promotes downstream signaling pathways (24, 202) (Figure 4.4E), similarly redistributed from the cytosolic to the membrane fraction upon addition of PE. Finally, vinculin and filamin showed small, but significant redistribution to the membrane as well (Figure 4.4 F,G).
Figure 4.4 The alpha-agonist PE triggers redistribution of zyxin, VASP, paxillin, filamin, and metavinculin/vinculin to the membrane fraction from the cytosolic fraction. (A) Typical blots illustrating the time course of PE stimulus-induced changes in protein subcellular distribution. Blank space is a spliced out empty lane. (B-G) Densitometric analysis of western blots of FA proteins in cytosolic, membrane, and cytoskeletal fractions normalized as a percentage of the total protein (left panels) or as a percentage of unstimulated protein (right panels) from unstimulated or PE-(10 μmol/L) stimulated tissues (n=4-17). Data for each experiment are from a single gel. Brightness has been uniformly altered for visual display. *p<.05 as determined by a two tailed, paired t-test compared to resting values.
Some FA proteins move out of cytoskeletal fraction

Not all FA-associated proteins exhibit the same redistribution pattern. It has previously been reported that CAS and Src, which are both signaling molecules associated with FAs, redistribute to the membrane from the cytoskeletal fraction (136). I have confirmed those data and showed increasing levels of CAS and Src in the membrane fraction over a time course of PE treatment (Figures 4.5A,B,C). The change in CAS was a small percentage of the total (11%) (Figure 4.5B, left panel), but this represented a 67% increase in the membrane fraction after 10 minutes of PE exposure (Figure 4.5B, right panel). It is also interesting to note that even though Src and CAS may be closely associated in the FA and exhibit similar relocalization patterns, their general intracellular distributions as measured by this method were quite different in that CAS in unstimulated samples was 46% cytosolic compared to just 14% for Src.
**Figure 4.5 CAS and Src redistribute to the membrane fraction from the cytoskeletal fraction in the presence of PE.** Time course of stimulus-induced changes in protein subcellular distribution. (A) Typical blots. Densitometric analysis of CAS (B) and Src (C) in cytosolic, membrane, and cytoskeletal fractions normalized as a percentage of the total protein amounts (left panels) or as a percentage of unstimulated amounts (right panels) from unstimulated or PE-(10 μmol/L) stimulated tissues (n=6-18). Data for each experiment are from a single gel. Blank space is a spliced out empty lane. Brightness has been uniformly altered for visual display. *p<.05 as determined by a two tailed, paired t-test compared to resting values.
Static Proteins

Notably, the FA protein FAK showed no significant PE-induced relocalization (Figure 4.6A,B). Furthermore, almost no FAK was detectable in the membrane fraction despite its known interaction with beta-integrins (180), transmembrane proteins that reside to a large extent in the membrane fraction in this assay (Figure 4.6C). These results suggest that FAK is more strongly bound to the actin cytoskeleton than to the integrin-associated membrane (138). It is particularly striking that beta-integrin showed no noticeable agonist-induced redistribution with this assay (Figure 4.6A,C). This is in contrast to the well documented recycling of integrins in migrating cells (4, 165). This is suggestive of a qualitative functional difference of FAs between migrating and non-migrating cells.

The lack of detectable movement of alpha-actinin is also interesting (Figure 4.6A,D). Since alpha-actinin is known to associate with zyxin (45), one might expect it to behave similarly.
Figure 4.6 FAK, integrin and alpha actinin show no detectable vasoconstrictor-induced redistribution. (A) Typical blots. Densitometric analysis of FAK (B), beta-integrin (C), and alpha-actinin (D) in cytosolic, membrane, and cytoskeletal fractions normalized as a percentage of the total protein amounts from unstimulated or PE-(10 μmol/L) stimulated tissues (10 min) (n=4-12). Data for each experiment are from a single gel. Blank space is a spliced out empty lane. Brightness has been uniformly altered for visual display. *p<.05 as determined by a two tailed, paired t-test compared to resting values.
SUMMARY

Here I have demonstrated the dynamic redistribution of several FA proteins in response to stimuli from two different agonists (VASP, zyxin). Expanding my study to more FA proteins revealed a pattern of redistribution in response to PE. However, there were several proteins (FAK, beta-integrin, alpha-actinin) that deviated from this pattern, and from what is known about their behavior in migrating cell phenotypes, by showing no redistribution. This is indicative of a functional difference between the FA proteins in dVSM and other cell and tissue types. Interestingly, the FA proteins that did redistribute in this assay all showed some degree of movement into the membrane fraction.
CHAPTER 5: FOCAL ADHESION PROTEINS AND ENDOSONES

INTRODUCTION

The findings of agonist-induced redistribution of FA proteins were exciting and perplexing and raised questions. If these proteins are moving, where are they moving and why? Why do some move and not others? Of particular interest was the realization that there is a consistent pattern of redistribution to the membrane, which was unexpected. Review of the literature showed that endosomal trafficking of some FA proteins was known to occur in non-muscle cells. I hypothesized that redistribution to a membrane fraction might reflect FA protein recycling through association with an endocytic process.

IMAGING

Immunofluorescent Colocalization

In order to see if the PE-induced redistribution of dVSM FA proteins to the membrane fraction observed here is linked with endocytic processes, I performed co-localization studies for zyxin and endosome markers by deconvolution immunofluorescence microscopy of freshly enzymatically isolated aorta dVSMCs. Since zyxin is largely cytosolic, all images were uniformly thresholded to increase the resolution of zyxin at FA punctae and visually remove the cytosolic signal. As seen in Figure 5.1A, zyxin showed an increased degree of colocalization with Rab5, an early
endosome marker (23, 83), after PE stimulation. Zyxin staining at the cell edge that did not colocalize (arrowheads) likely represents FA localization. Zyxin colocalized with Rab5 in the cell interior (white arrows), consistent with endocytosis. Not all Rab5-labeled endosomes colocalized with zyxin. Similarly, not all zyxin is colocalized with Rab5, suggesting that only a fraction of total zyxin is in transition (which corroborates the results from differential centrifugation).

I also tested whether zyxin colocalized with EEA1 (Figure 5.1B), another protein marker of early endosomes (39, 143, 184). EEA1 exhibited vesicular, punctate staining typical of endocytic vesicles and similar to that seen for Rab5. It also showed increased colocalization with zyxin after treatment with PE (white arrows). Finally, using the vital cell membrane tracking dye FM4-64FX (92), I again saw increased colocalization of zyxin with the membrane dye after PE stimulation (Figure 5.1C).
Figure 5.1 FA marker protein zyxin shows increased colocalization with endosome markers in the presence of PE. (A) Immunofluorescent colocalization of Rab5 and zyxin before (left panel) and after (right panel) 10 min 10 μmol/L PE stimulation. Arrows indicate colocalization. Arrowheads show lack of colocalization and zyxin localization at FA-like structures. (B) Colocalization of zyxin and EEA1 before (left panel) and after (right panel) 10 min 10 μmol/L PE stimulation. Arrows indicate colocalized points. (C) Colocalization of zyxin and membrane tracking dye FM4-64FX before (left panel) and after (right panel) 10 min 10 μmol/L PE stimulation. Arrows indicate colocalized points. Blue staining is nuclear DAPI signal. Scale bar, 5 μm.
Quantitative Colocalization

Quantitative colocalization analysis was performed (Figure 5.2). Phalloidin and α-actin colocalization was studied as a positive control, and α-actin and DAPI colocalization was studied as a negative control. The percent of zyxin that colocalized with both Rab5 and EEA1, respectively, increased in a statistically significant manner after PE treatment, consistent with endocytic redistribution. In both cases, at least a doubling in the amount of colocalization was seen. As expected, the colocalization did not reach values seen with the positive controls, which would be indicative of complete colocalization (Table 5.1).
**Figure 5.2 Zyxin colocalization with endosome markers increases after PE stimulation.** Graphical depiction of zyxin colocalization with two different endosome markers (Rab5, EEA1) shows significant increases after PE stimulation. Quantitative colocalization under both unstimulated and 10 min 10 μM PE stimulated treatment conditions is at least 20% less than the positive control (α-actin/phalloidin) (not complete colocalization) but at least 10% more than the negative control (α-actin/DAPI). *p<.05 as determined by a two tailed, paired t-test.
Table 5.1 FA marker protein zyxin shows a quantifiable increase in colocalization with endosome markers in the presence of PE. Data are the percentage of the first protein that colocalizes with the second protein +/- SE. 10-14 cells were analyzed in each case. See methods for further details. *p<.05 as determined by a two tailed, paired t-test compared to resting values.

<table>
<thead>
<tr>
<th></th>
<th>Unstim</th>
<th>Stim</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-actin with Phalloidin</td>
<td>53.8±2.2</td>
<td></td>
</tr>
<tr>
<td>Phalloidin with α-actin</td>
<td>57.0±2.0</td>
<td></td>
</tr>
<tr>
<td>α-actin with DAPI</td>
<td>8.3±1.4</td>
<td></td>
</tr>
<tr>
<td>DAPI with α-actin</td>
<td>9.3±1.4</td>
<td></td>
</tr>
<tr>
<td>Rab5 with Zyxin</td>
<td>17.5±2.6</td>
<td>39.1*±3.2</td>
</tr>
<tr>
<td>Zyxin with Rab5</td>
<td>18.1±2.8</td>
<td>38.4*±2.8</td>
</tr>
<tr>
<td>EEA1 with Zyxin</td>
<td>16.8±1.7</td>
<td>38.1*±3.0</td>
</tr>
<tr>
<td>Zyxin with EEA1</td>
<td>16.9±1.7</td>
<td>38.4*±3.1</td>
</tr>
</tbody>
</table>
**Proximity Ligation Assay (PLA)**

Duolink proximity ligation assays (PLA) were performed on freshly isolated dVSMCs to determine the proximity (as an indicator of the potential association) of zyxin and EEA1 (70, 186, 187, 226). PLA results in easily detectable and quantifiable fluorescent dots when proteins are within 40 nm of each other (Figure 5.3A). There is some background nuclear autofluorescent signal in each cell also seen in previous studies (208). A negative control of incubating with the zyxin antibody alone, displayed a negligible number of fluorescent dots per \( \mu \text{m}^2 \) (n=17) (Figure 5.3B). In unstimulated cells, there was a statistically significant increase in the number of fluorescent dots compared to the negative control (n=31). This indicates that a certain subpopulation of zyxin and EEA1 resides close enough to each other to interact. After 10 minutes of PE stimulation, the amount of zyxin and EEA1 in close proximity increased 2.5 times compared to resting (n=41). The magnitude of this increase is similar to the PE-induced increase in fluorescent colocalization seen in Table 5.1. Furthermore, zyxin and EEA1 proximity dots appeared to be located in the cell interior (Figure 5.3A, bottom panel), which is suggestive of endocytic association.
Figure 5.3 The amount of zyxin and EEA1 in close proximity increases after PE stimulation. Confirmation by proximity ligation assay of close association that increases after 10 min PE stimulation. (A) Freshly dissociated dVSMCs were fixed and incubated with zyxin and EEA1 antibodies or zyxin alone as a negative control. Representative images of each condition are shown. Scale bar, 10 μm. Panels have been inverted to gray scale for better visualization. Cell outline based on visible light capture. See methods for further details. (B) The number of signal dots, as a measure for proximity of an antigen pair, was analyzed based on 17-41 cells per experimental condition in 3-5 independent experiments. *p<.05, **p<.01, ***p<.001 as determined by a two tailed t-test compared to resting values unless otherwise indicated.
SMALL MOLECULE INHIBITORS

Primaquine

*Differential Centrifugation*

To further test the hypothesis that dVSMC FA proteins recycle through an endocytic mechanism, I used primaquine (PQ), a small molecule inhibitor of endosomal budding (95, 206). PQ-treated samples showed, in all cases examined, a marked inhibition of the previously demonstrated PE-induced protein redistribution to the membrane fraction. In the presence of PQ and PE, the amount of zyxin and VASP in the membrane fraction returned to the levels seen in the absence of PE (Figure 5.4A,B). Src and CAS distributions were altered by PQ but to different degrees. Src levels in the membrane fraction were reduced with PE and PQ, but not to untreated amounts (Figure 5.4C), perhaps reflecting association with nonvesicular membranes such as the plasmalemma. With CAS, levels in the membrane fraction returned to pre-PE levels in the presence of PE and PQ in a dose-dependent manner (Figure 5.4D). FA proteins with smaller overall redistribution to the membrane (paxillin, filamin, vinculin, metavinculin) all showed similar trends in response to PQ but did not all reach significance, probably due to the natural variability inherent within the methodology (Figure 5.4E,F,G,H). PQ had no significant effect on membrane distribution of “static” proteins FAK, beta-integrin, actin, or alpha-actinin (Figure 5.4I,J,K,L).
Figure 5.4 Primaquine blocks vasoconstrictor-induced membrane redistribution. (A-H) Pretreatment with two different concentrations of PQ reduce PE-induced protein redistribution to the membrane in response to 10 min 10 μM PE stimulation. (A-D,G) Zyxin, VASP, Src, CAS, and vinculin all show significant inhibition of redistribution to the membrane after PQ treatment. (E,F,H) Paxillin, filamin, and metavinculin trend towards inhibition of redistribution. (I-L) FAK, beta-integrin, actin, and alpha-actinin show no significant changes in subcellular localization in unstimulated, PE stimulated, or PQ pretreated tissues. N=4-18, +p<.05 compared to PE; *p<.05 compared to untreated.
Contractility

To test the hypothesis that FA protein recycling may significantly modulate tissue contractility, dVSM tissue was treated with PQ while recording contractile force. One hour of 150 μmol/L PQ pretreatment before PE stimulation noticeably altered tissue contractility (Figure 5.5).
Figure 5.5 Primaquine reduces aortic tissue contractility in serum free organ culture. Force readout showing the marked reduction in speed and strength of tissue contractility in response to PE after pretreatment with PQ. Blue colored trace (top) shows force exerted over time by a single 4mm aortic ring treated with 10 μM PE. Pink colored trace (bottom) shows force exerted over time by a 4mm aortic ring pretreated with 150 μM PQ and then stimulated with 10 μM PE.
A statistically significant slowing of the rate of force development apparent as almost a doubling of the time it takes for tissue to reach 50% of maximal contraction was seen (Figure 5.6A). Additionally, a statistically significant inhibition of steady state contractile force was seen with PQ pretreatment (Figure 5.6B). Since all force generated by the contractile filaments in dVSM is channeled through the FAs to be transmitted to the vessel wall, even a partial disruption of FA structure would be expected to impair contractile function.
Figure 5.6 Primaquine reduces contractility. (A), Effect of PQ on the time to 50% steady state contraction amplitude. N=6, *p<.05. (B), PQ significantly reduces maximal steady state contractile force in response to PE stimulation. N=6, *p<.05.
Actin Polymerization Inhibitors

Differential Centrifugation with Cytochalasin D

It is known that actin polymerization and associated cytoskeletal remodeling in dVSMCs occurs during vasoconstrictor-induced regulation of tissue contractile function (115), and in cultured cells, it is known that actin dynamics play a role in some endocytic pathways, particularly early endosomal events (40, 53, 84). To test the hypothesis that actin dynamics might be involved in facilitating endocytic recycling of FA proteins in dVSM, tissue was treated with two inhibitors of actin polymerization. Concentrations of 500 nmol/L and below of cytochalasin D are known to specifically inhibit actin filament elongation by capping the barbed ends while leaving the existing filaments intact (42). Treatment with cytochalasin D for one hour prior to PE stimulation had no effect on protein distribution for unstimulated tissue, but significantly inhibited PE-induced membrane redistribution of zyxin, VASP, paxillin and CAS (Figures 5.7A,B,C,D). Cytochalasin D had no effect on actin distribution (Figure 5.7E).
Figure 5.7 Actin inhibition with cytochalasin D blocks vasoconstrictor-induced redistribution to the membrane fraction. (A-D) Membrane fraction analysis after differential centrifugation of tissue samples that are either untreated, PE-stimulated, or Cytochalasin D pre-treated and PE-stimulated. (E) Actin shows no significant subcellular redistribution with or without Cytochalasin D pretreatment. N=3-17, +p<.05 compared to PE; *p<.05 compared to untreated.
**Differential Centrifugation with Latrunculin B**

Pretreatment with 0.1 μmol/L latrunculin B, which inhibits actin branching by sequestering G-actin (43), had no effect on protein distribution for unstimulated tissue. However, in a manner similar to cytochalasin D, latrunculin pretreatment of PE-stimulated tissue inhibited protein redistribution to the membrane fraction, with amounts in the membrane fraction returning to untreated levels (Figure 5.8A,B).

**Contractility**

Cytochalasin D has already been shown to decrease PE-induced contractility in dVSM tissue (115), and a similar effect was seen with latrunculin B (Figure 5.8C). However, it is unclear based on these experiments, to what extent the effect on contractility can be attributed to endocytic processes as compared to other actin-dependent processes. Altogether, however, these data point to a role for actin polymerization in the regulation of FA protein recycling.
Figure 5.8 Actin inhibition with latrunculin B blocks vasoconstrictor-induced redistribution to the membrane fraction. (A,B) Densitometric analysis of western blots stained for VASP and zyxin after differential centrifugation of latrunculin B treated tissue shows significant inhibition of PE-induced redistribution into the membrane fraction. N=3-19, +p<.05 compared to PE; *p<.05 compared to untreated. (C) 0.1 μmol/L Latrunculin B pretreatment for 1 hour significantly inhibits maximal tissue contraction strength. N=3, *p<.05.
Microtubule Network Inhibitor

*Colchicine*

It is known in proliferative cells that endocytic recycling relies on microtubule networks and their associated motors to transport vesicles throughout the cell (3, 130, 189). To test the hypothesis that stimulus-induced FA reorganization also relies on a microtubule dependent endocytic recycling pathway in dVSMCs, I treated tissue with colchicine at two different concentrations to disrupt microtubule networks (66, 222). Treatment with colchicine for one hour prior to PE stimulation and differential centrifugation significantly inhibited previously observed PE-induced membrane redistribution in zyxin and VASP (Figure 5.9A,B).

These experiments confirm previous studies that indicate that colchicine does not significantly inhibit vasostimulator-induced contractile force [Figure 5.9C and (66, 222)]. However, increasing concentrations of colchicine had a significant slowing effect on the speed of force development (Figure 5.9D). At higher concentrations, it took twice as long to generate the same force. It is unclear, based on these experiments, to what extent the effect on speed can be attributed to endocytic processes as compared to other microtubule-dependent processes. Altogether, however, these data point to a role for microtubule networks in the regulation of both FA protein recycling and tissue contractility.
Figure 5.9 Microtubule inhibition with colchicine blocks vasoconstrictor-induced redistribution to the membrane fraction. (A,B) Densitometric membrane fraction analysis after differential centrifugation of tissue samples that are either untreated, PE-stimulated, or 15 μmol/L colchicine treated and PE-stimulated. N=3-17, +p<.05 compared to PE; *p<.05 compared to untreated. (C) Colchicine pretreatment at either 15 μM or 100 μM concentrations for 1 hour did not affect maximal tissue contraction to PE; (D) Colchicine pretreatment did significantly increase the time to 50% maximal contraction in response to PE in a dose dependent manner. N=3, *p<.05.
SUMMARY

Here I have shown a significant increase in the colocalization of zyxin with two different early endosome markers after treatment with PE. This was accomplished both through quantitative immunofluorescent colocalization and PLA. In addition, using a range of small molecular inhibitors to target and disrupt various stages of endocytic recycling resulted in a significant inhibition of the previously observed FA protein redistribution to the membrane. PQ, a specific inhibitor of endosome budding, also severely disrupted normal tissue contractile response. The actin and microtubule inhibitors I used (cytocholasin D, latrunculin B, Colchicine) induced similar effects, but to a lesser degree. This may be due to pathway redundancies and off-target effects (actin and microtubule networks are involved in more than just endocytic pathways). Taken as a collected whole, I believe these experiments present a compelling case for endocytic recycling as a means to redistribute FA proteins in dVSMCs as part of a contractile response.
A SUBSET OF FA PROTEINS IN DVSM REMODEL IN RESPONSE TO AN ALPHA-AGONIST

The major finding of the present study is that a distinct subset of dVSM FA proteins redistribute in response to the vasoconstrictor, PE. In migrating cells the development and function of FAs has been extensively studied. FAs in proliferative cells provide structural and adhesive support as well as initiate signaling cascades. Typically, focal contacts coalesce at the leading lamellipodial edge, mature into anchoring FAs as the cell moves forward, and then completely break down as the leading edge migrates forward (151). In contrast, non-migratory dVSMCs have no need for complete FA turnover. However, it is known that dVSM cells display remarkable cytoskeletal plasticity, with the ability, when unrestrained and activated by an agonist, to shorten to 50% of their initial length (204). Recycling/relocation of a subset of dVSM FA proteins may allow maintenance of plasticity of the connection to the contractile filaments while maintaining a stable FA protein core and a linkage to the matrix in the vessel wall. Such plasticity will also allow retention of the ability to quickly shift proteins to sites of high stress to strengthen and/or increase signaling responses without requiring an energetically costly and time consuming process of completely breaking down and rebuilding FAs in these non-synthetic, non-proliferative cells. It is well known that dVSMCs have low rates of protein synthesis compared to proliferative cells (115). Thus, it is more energetically
efficient for these cells to recycle proteins to sites where they are needed than to proteolyze proteins and then synthesize and target new proteins.

MODEL FOR DVSM REMODELING DEPENDENCE ON FA ARCHITECTURE

With super-resolution light microscopy (iPALM, a combination of photoactivated localization microscopy and simultaneous multi-phase interferometry of photons) the Waterman group has recently measured out a spatially layered architecture for FAs of mouse embryo fibroblasts (MEF) cells. They quantitated “integrin signaling”, “force transduction”, and “actin regulatory” layers of proteins at increasing distance from the plasma membrane (107) (Figure 6.1).
Figure 6.1 Spatially layered model of focal adhesions. Interferometric photoactivated localization microscopy was used to map focal adhesion protein spatial distribution on a nanoscale level. Based on the literature, FA proteins were separated into three functional groups: integrin signaling proteins, force transduction proteins, or actin regulatory proteins. Based on average quantifiable z-distance from the membrane and known binding interactions in the literature, this model was generated. Modified from Kanchanawong et al, 2010 (107).
The FA of the dVSM cell appears to display some features in common with this model but also exhibits some differences. If I use the PE-induced redistribution to the membrane fraction (Figure 6.2A) is used as an indicator of FA remodeling, then it appears that the proteins identified by Kanchanawong et al. to be associated with the integrin signaling layer (Figure 6.2B) undergo the least amount of remodeling (Figure 6.2C). In dVSM, this is functionally appropriate, since shifting of critical FA proteins (especially beta-integrin and FAK) would constitute a major disruption of the integrity of the connection of the cell to the matrix of the vessel wall. This finding is also of interest since FAK has been reported to undergo an agonist-induced redistribution in airway cells from the cytosol to a cell surface distribution. The difference in these findings may point to functional and structural differences between the tissues (148).
Figure 6.2 Expected proximity to the plasma membrane suggests degree of redistribution to the membrane fraction. (A) Diagramatic representation of hypothesized role of endosomes in FA protein redistribution.Disconnected FA proteins are joined with budding vesicles which then fuse with early endosomes and are recycled to different FAs at the plasma membrane. (B) Potential spatially-layered FA architecture based on Kanchanawong et al. model. The integrin signaling proteins, force transduction
proteins, and actin regulatory proteins are organized at increasing distance from the plasma membrane. (C) Percent increase in the membrane fraction after 10 min 10 μM PE stimulation compared to resting amounts. Proteins are arranged by expected distance from the plasma membrane based on Kanchanawong et al. model (107).

Alpha-actinin also displayed a lack of detectable movement in these studies. Interestingly, alpha-actinin has been reported to translocate in imaging experiments in response to acetylcholine stimulation of tracheal smooth muscle (223). Therefore, the stationary nature of alpha-actinin in dVSM appears to demonstrate a difference between vascular and airway smooth muscle function. In fact, the general protein distribution pattern of alpha-actinin more closely resembles total actin (20% cytosolic, 20% membrane, 60% cytoskeleton) than VASP or zyxin (50% and 80% cytosolic, respectively) with which it is known to interact. However, most of the dVSM cellular actin is in the contractile filaments, and alpha-actinin is also a marker for the dense bodies into which the contractile filaments insert (63). Thus, in dVSMCs, alpha-actinin may be playing a larger role in the relatively less dynamic contractile filament domain than in the cortical non-muscle cytoskeleton/FA domain.

On the other hand, VASP and zyxin, both of which have been identified by Kanchanawong et al. as components of the actin regulatory layer, exhibit the highest degree of remodeling. The large difference in mobility between the actin regulatory proteins and other FA proteins is suggestive of a disconnect, breaking-off point, or a point of release of the actin cytoskeleton from the FA, in response to PE. This is supported by the model suggested by Kanchanawong et al., indicating that zyxin and VASP lie on the outskirts of the FA, where they might more easily disengage. As
mentioned previously, alpha-actinin in dVSM may be playing a larger role in the dense bodies which insert into the less dynamic contractile filaments and, therefore, may not be as mobile as proteins associated with the cortical cytoskeleton. In keeping with the idea of a point of disconnect, there appears to be a second such point between the integrin signaling layer proteins and the force transduction layer proteins (Figure 6.2C). Proteins categorized as force transduction proteins, such as vinculin, display intermediate levels of mobility in our system as measured by membrane redistribution. They do not seem to disengage as easily as zyxin or VASP but also are not bound as immobile as the more integral FA proteins. Again, in airway smooth muscle apparently greater redistribution of vinculin has been reported and the direction of movement was from the cell center to the cell surface, as determined by immunofluorescent imaging of freshly dissociated cells. This may reflect differences in tissue function or in methodology. Of note, Eddinger et al. also imaged vinculin in airway muscle and saw little or no agonist-induced redistribution in cryosections of frozen tissue as opposed to when freshly dissociated airway cells were studied (56, 57).

FA PROTEIN REMODELING IS DEPENDENT ON ENDOCYTIC PROCESSES

So, if cells are capable of both disengaging the actin cytoskeleton from FA proteins and FA proteins from each other and then reallocating them to sites of active tension and stress, the question arises as to how the proteins are transported. Another major finding of the present study is that endocytic recycling is necessary for PE-induced FA protein recycling in dVSM. Active endocytic pathways are known to be characteristic
of synthetic, migratory, proliferative cells, but here, for the first time, I show an essential role for endocytic processes in a non-proliferative, non-migratory, fully differentiated, contractile cell phenotype. Several studies have pointed to a connection between FA proteins and the endocytic pathway in cultured cells (176, 177, 200), which led me to hypothesize that dVSMCs might use endocytic pathways to disengage proteins from the FAs and shuttle/recycle them to regions with increased cellular tension. In support of this concept, immunofluorescence microscopy colocalization and PLA experiments showed that zyxin increased its colocalization with Rab5 and EEA1, early endosome recycling markers after stimulation with PE. Disruption of endocytic recycling with 150 mmol/L PQ caused a marked reduction in the speed of contraction as well as a decrease in contraction strength. It also eliminated or diminished the PE-induced redistribution of FA proteins, consistent with the concept of a connection between FA proteins, endosomal recycling, and the regulation of dVSM contractility. Fifty mmol/L PQ indistinguishably decreased the redistribution of zyxin, VASP, and Src, but caused a smaller inhibition of CAS redistribution and perhaps that of other FA proteins, which may explain why this lower concentration of the recycling inhibitor had a diminished effect on contraction speed and strength.

Another surprising aspect of this study is that paxillin, often considered a FA marker and a known cytoskeletal protein that associates with FAs (203) in at least some non-muscle cells and in Schwann cells (34), was here found to be largely excluded from the cytoskeletal fraction. In the absence of a stimulus, 76% of total paxillin was in the cytosolic fraction and only 6% was seen in the cytoskeletal fraction. Similar results were
seen with vinculin. This agrees with earlier findings in small arteries but points to unique properties of the dVSM cells of the vascular wall.

FORCE GENERATION AND MAINTENANCE IN DVSM IS ACTIN AND MICROTUBULE DEPENDENT

I also report here that endosomal redistribution in dVSM is actin and microtubule dependent. Others in our group previously showed that actin filament elongation is necessary for normal vascular contractility and that a cortical, non-muscle actin population is involved (115). Recent studies have shown that increased membrane tension in MDCK cells increases actin dependency of clathrin-mediated endocytosis. By inhibiting actin polymerization with cytochalasin D or latrunculin B, endosomal trafficking in dVSM was impaired. The mechanism could involve an inhibition of the construction of actin tracks along which the endosomes might move, or it could involve inhibition of actin filament-mediated pinching off of new endosomes either by pushing the vesicle away from the plasma membrane or constricting the neck in an Arp 2/3 complex-mediated mechanism (408, 480). The actin dependency may help identify the specific endocytic pathways involved, since not all endocytic pathways are dependent on actin polymerization (2, 20, 71, 84, 174). Similarly, endocytic dependency on microtubule networks has been well documented in cultured cells (3, 189), but here, for the first time, I report similar findings in dVSMC.

Thus, in the present study we report that FAs in dVSMCs embedded in the wall of the aorta remodel during the action of a vasoconstrictor and that the remodeling involves
an actin and microtubule-dependent endocytic pathway. These results may have significant implications for understanding the mechanisms underlying dVSM contractile function and vascular compliance and may provide new targets for drug discovery studies aimed at creating new therapeutics for the treatment of cardiovascular disease.

FUTURE PERSPECTIVES/RESEARCH

Although the work done here extends our understanding of the mechanisms and processes involved in smooth muscle contraction and focal adhesion dynamics, it needs to be confirmed, focused and extended.

Confirming FA Dynamics

My findings would be bolstered by additional experiments confirming FA protein association with endosomes after stimulation. This could be accomplished by demonstrating increased tethering of endosome markers with FA proteins after stimulation via immunoprecipitation. Duplicating my findings with lower reversible concentrations of stimulants and demonstrating not only FA protein redistribution to the membrane, but also demonstrating reversal back to original protein distributions after relaxation, would strengthen my results further. Live-cell imaging visibly showing FA protein redistribution, or immuno-gold EM imaging of FA proteins in membranous vesicles would also help support my hypothesis.
Focusing on FA Dynamics and Endocytic Recycling.

Despite my findings on FAs and endosomes, there is still much that remains to be clarified and elucidated. Future work on FA dynamics could include comparing protein redistribution in normal vs. diseased models. It would also be useful to expand the list of FA proteins studied to get a better sense of the overall picture and potentially clarify and delineate the specific “points of release.” Once these have been more fully defined, specifically targeting binding sites that connect these points could reveal how important these connections are in contributing to FA remodeling and contractile response.

Endosome recycling is a complex process and it would be enlightening to determine the specific pathways involved in FA protein recycling (189). By combining specific inhibitors of different steps of the various endocytic processes, I could develop a more precise picture of the pathways involved. For example, inhibition with methyl-β-cyclodextrin could help distinguish between clathrin and caveolae-dependent or independent endocytosis (51, 166, 191, 196). Inhibition of specific molecular motors (kinesins, myosins, dynein, dynamins) could tell me which endocytic steps are involved (62, 130, 188). Furthermore, could I increase the amount of FA recycling in response to stimulation by increasing lipid content or stabilizing microtubule networks (taxol)? Inducing endocytic trafficking at 20°C prevents vesicle transfer from early to late endosome and would provide more specific data on which pathways are implicated (52, 122). Finally, tracking protein uptake (avidin or biotin) or subcellular distribution of fluorescent endocytic probes (transferrin, lactosylceramide) could provide information about the pathways involved (207).
Potential Clinical Applications.

By developing a better understanding of the importance of FA dynamics and endocytic recycling in regulating dVSM contraction, drug companies may be able to find new candidate targets for therapies (55). Other work in our lab has already been successful developing specific, dominant-negative, cell-permeable peptide inhibitors (115). A clearer understanding of the specifics of the endocytic pathways involved and which FA proteins are most important for contractile response in dVSM would give me a better idea of which proteins for which to design peptide inhibitors. These findings could potentially provide new options for targeted drug therapies for alleviation of cardiovascular disease.

In addition, the FA protein complex in dVSM has similarities to the beta-dystroglycan complex and costameres in skeletal muscle. If some of my general findings are consistent across cell phenotypes, it could open avenues for new research into alleviation of various dystrophies.
LIST OF JOURNAL ABBREVIATIONS

Adv Exp Med Biol – Advances in Experimental Medicine and Biology
Am Heart J – American Heart Journal
Am J Pathol – American Journal of Pathology
Am J Physiol – American Journal of Physiology
Am J Physiol Cell Physiol – American Journal of Physiology: Cell Physiology
Am J Physiol Gastrointest Liver Physiol – American Journal of Physiology: Gastrointestinal and Liver Physiology
Am J Physiol Heart Circ Physiol – American Journal of Physiology: Heart and Circulatory Physiology
Annu Rev Cell Dev Biol – Annual Review of Cell and Developmental Biology
Antiviral Res – Antiviral Research
Arch Intern Med – Archives of Internal Medicine
Arterioscler Thromb Vasc Biol – Arteriosclerosis, Thrombosis, and Vascular Biology
Auton Autacoid Pharmacol – Autonomic and Autacoid Pharmacology
Biochem Biophys Res Commun – Biochemical and Biophysical Research Communications
Biochem J – Biochemical Journal
Biochem Soc Trans – Biochemical Society Transactions
Biochim Biophys Acta – Biochimica et Biophysica Acta
Cardiovasc Clin – Cardiovascular Clinics
Cardiovasc Res – Cardiovascular Research
Cell Biochem Biophys – Cell Biochemistry and Biophysics
Cell Death Differ – Cell Death and Differentiation
Cell Mol Life Sci – Cellular and Molecular Life Sciences
Cell Motil Cytoskeleton – Cell Motility and the Cytoskeleton
Cell Struct Funct – Cell Structure and Function
Circ Res – Circulation Research
Curr Atheroscler Rep – Current Atherosclerosis Reports
Curr Biol – Current Biology
Curr Opin Cell Biol – Current Opinion in Cell Biology
Dev Biol – Developmental Biology
Dev Cell – Developmental Cell
EMBO J – EMBO Journal
Eur J Biochem – European Journal of Biochemistry
Eur J Cell Biol – European Journal of Cell Biology
Exp Cell Res – Experimental Cell Research
FEBS Lett – FEBS Letters
Front Biosci – Frontiers in Bioscience
ILAR – The ILAR Journal
J Appl Physiol – Journal of Applied Physiology
J Biol Chem – Journal of Biological Chemistry
J Cell Biochem – Journal of Cellular Biochemistry
J Cell Biol – Journal of Cell Biology
J Cell Mol Med – Journal of Cellular and Molecular Medicine
J Cell Physiol – Journal of Cell Physiology
J Cell Sci – Journal of Cell Science
J Histochem Cytochem – Journal of Histochemistry and Cytochemistry
J Mol Biol – Journal of Molecular Biology
J Neurosci – Journal of Neuroscience
J Physiol – Journal of Physiology
J Struct Biol – Journal of Structural Biology
Mamm Genome – Mammalian Genome
Mol Biol Cell – Molecular Biology of the Cell
Mol Cell – Molecular Cell
Mol Cell Biol – Molecular and Cellular Biology
Mol Ther – Molecular Therapy
Nat Biotechnol – Nature Biotechnology
Nat Cell Biol – Nature Cell Biology
Nat Methods – Nature Methods
Neth Heart J – Netherlands Heart Journal
Physiol Rev – Physiological Reviews
Proc Natl Acad Sci U S A – Proceedings of the National Academy of Sciences, USA
Respir Res – Respiratory Research
Tissue Eng Part B Rev – Tissue Engineering Part B: Reviews
Trends Cardiovasc Med – Trends in Cardiovascular Medicine
Trends Cell Biol – Trends in Cell Biology


7. **Ball RS.** Issues to consider for preparing ferrets as research subjects in the laboratory. *ILAR J* 47: 348-357, 2006.


