

**SPHINGOSINE-1-PHOSPHATE AND THE RGS RHOGEF5 REGULATE
VASCULAR SMOOTH MUSCLE PHENOTYPE**

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Abstract

Sphingosine-1-Phosphate and the RGS RhoGEFs Regulate Vascular Smooth Muscle Phenotype

(Under the direction of Christopher P. Mack, Ph.D.)

The regulation of smooth muscle cell (SMC) differentiation is critical during vascular development, and perturbations in this process contribute to a number of cardiovascular pathologies including atherosclerosis, hypertension, and restenosis. We have shown that activation of RhoA by sphingosine-1 phosphate (S1P) stimulates SMC-specific gene expression by promoting the nuclear localization of the myocardin-related transcription factors (MRTFs).

The aim of this dissertation is to dissect the precise mechanisms by which S1P regulates SMC phenotype. Using a combination of receptor-specific agonists and antagonists we identified S1P2 as the driving S1P receptor sub-type that regulates SMC-specific promoter activity and differentiation marker gene expression in primary SMC cultures. In addition, over expression of $G\alpha_{12}$ or $G\alpha_{13}$ increased SMC specific transcription, a result in excellent agreement with the known G-protein coupling properties of S1P2. Given previous studies on the interaction of $G\alpha_{12/13}$ with the RGS subfamily of RhoGEFs (LARG, PRG, P115), we hypothesized that one

or more of these RhoA activators was important in S1P-mediated SMC differentiation. While expression of each of the RGS RhoGEFs activated SMC specific transcription, LARG exhibited the most robust effect invoking a 10 to 15 fold increase SM22 and SM α -actin promoter activity. LARG expression also resulted in increased stress fiber formation and MRTF-A nuclear localization. Importantly, siRNA-mediated depletion of LARG (by approximately 90%) inhibited activation of RhoA by S1P and also inhibited the effects of S1P on endogenous SMC differentiation marker gene expression and SMC specific promoter activity. Finally, knockdown of LARG promoted SMC migration as measured by scratch wound and transwell assays. These findings indicate that stimulation of RhoA activity by S1P2-dependent activation of LARG plays a critical role in the regulation SMC phenotype. Interestingly P115 RhoGEF appears to regulate SMC migration in opposition to LARG. Despite the importance of RGS RhoGEF signaling little is known about their regulation. This thesis will explore the mechanisms regulating RGS RhoGEFs mediated RhoA activity and how differential RhoA activation may help modulate SMC phenotype. In conclusion these studies have improved our understanding the very complex means by which S1P mediated signaling regulates SMC phenotype and by extension normal and pathological vascular development.

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A certainty, though not such a rare commodity in biology, is an exceedingly rare thing in life. Despite that I am certain of one thing; that I would not have achieved what I have today if not for the love of my life, Crystal Medlin. Crystal is my foundation. She has been a stalwart, enduring, and fearless partner as we have hurtled through life together these past years. Though it is possible that I would have made it through tough times without her, I am certain that I would not have wanted to. All of the wonderful attributes and traits I desire to achieve are exemplified by her and I question every day what I have done to justify having such

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LIST OF ABBREVIATIONS

AA:	Amino Acid
ATCC:	American Type Culture Collection
BCA:	Bicinchoninic Acid
BrdU:	Bromodeoxyuridine
CABG:	Coronary Artery Bypass Graft
CArg:	(CC(A/T)6GG)
cdc42:	Cell division control protein 42
CDR:	Circular Dorsal Ruffle
CRM-1:	Chromosome Region Maintenance 1
CVD:	Cardiovascular Disease
DAD:	Diaphanous Auto-regulatory Domain
DAPI:	4',6-diamidino-2-phenylindole
DbI:	Diffuse B-cell-Lymphoma
DH:	Dbl homology
DMEM:	Dulbecco's modified Eagle's medium
DOCA:	Deoxycorticosterone acetate
DRF:	Diaphanous-related formins
DRhoGEF2:	Drosophila RhoGEF2
EC:	Endothelial Cell
ECT2:	Epithelial Cell Transforming 2

Edg:	Endothelial differentiation gene
eGFP:	Enhanced Green Fluorescent Protein
EV:	Empty Vector
F-actin:	Filamentous Actin
FAK:	Focal Adhesion Kinase
FERM:	4.1 protein Exrin Radixin Moesin
FFSMC:	Floxed FAK Smooth Muscle Cell
FH:	Formin homology
FHOD:	Formin homology 2 domain containing
FITC:	Fluorescein isothiocyanate
FRAP:	Fluorescence Recovery After Photobleaching
FRET:	Fluorescence Resonance Energy Transfer
FRNK:	FAK Related Non-Kinase
G-actin:	Globular-actin
GAP:	GTPase-accelerating protein
GDI:	GDP dissociation inhibitors
GDP:	Guanosine diphosphate
GEF:	Guanine Nucleotide Exchange Factors
GPCR:	G Protein Coupled Receptor
GRK:	GPCR kinases
GTP:	Guanosine triphosphate
HAT:	Histone acetyltransferase

HDAC:	Histone deacetylases
IHC:	Immunohistochemistry
IRB:	Institutional Review Board
KD:	Knock down
KO:	Knock out
LARG:	Leukemia Associated Rho GEF
LDL:	Low Density Lipoprotein
LPA:	Lysophosphatidic Acid
LSM:	Laser Scanning Microscopy
MAPK:	Map-Kinase
MMP:	Matrix Metalloproteinase
mDia:	murine Diaphanous
MRTF:	Myocardin Related Transcription Factor
MyoD:	Myogenic Differentiation
NES:	Nuclear Export Sequence
NET1:	Neuroepithelial Cell Transforming 1
NLS:	Nuclear Localization Sequence
OHRE:	Office of Human Research Ethics
PAS:	Protein A Sepharose
PBS:	Phosphate Buffered Saline
PDGF:	Platelet Derived Growth Factor
PDZ :	PSD-95 Discs-large ZO-1

PH:	Pleckstrin homology
PLC:	Phospholipase C
PRG:	PDZ RhoGEF
Rac1:	Ras-related C3 botulinum toxin substrate 1
Ras:	Rat Sarcoma
RGS:	Regulator of G Protein Signaling
Rho:	Ras Homologous
RIPA:	Radio immunoprecipitation assay
ROCK:	Rho-kinase
RTPCR:	Reverse transcription polymerase chain reaction
S1P:	Sphingosine-1-phosphate
S1PR:	Sphingosine-1-phosphate Receptor
SDS:	Sodium Dodecyl Sulfate
SH2:	Src Homology 2
Shh:	Sonic Hedgehog
siRNA:	Small interfering RNA
SM α-actin:	Smooth Muscle alpha-actin
SMC:	Smooth Muscle Cells
SM-MHC:	Smooth Muscle Myosin Heavy Chain
SphK:	Sphingosine kinases
SRF:	Serum Response Factor
TGF-β:	Transforming Growth Factor-beta

TK: Thymidine kinase

VEGF: Vascular Endothelial Growth Factor

Chapter 1

Background and Significance

Vascular Development and Disease

Proper formation and maintenance of the vasculature is an ongoing and critical process, so important that the vasculature is the earliest organ to develop and requires more reconstruction and maintenance than any other organ system to remain functional. The mature human vasculature is composed of an extensive network of arteries, veins, and capillaries charged with conducting chemical exchange necessary for the maintenance and function of organs and tissues. Disruption of these processes, brought about by perturbations in vascular function, contribute to a substantial portion of human disease.

The genesis and maintenance of vasculature can be defined in three broad categories consisting of, vasculogenesis, angiogenesis, and vascular remodeling. Vasculogenesis begins when hemangioblasts differentiate into angioblasts, which become endothelial cells (ECs), and coalesce into primitive cords and tubes. Once initial vessels are formed the process of angiogenesis or extensive sprouting from preexisting vessels occurs. ECs provide a smooth, non thrombogenic surface to assist in circulation and are critical in the regulation of vasculogenesis, hemostasis, inflammation, and blood pressure. VEGF, Shh, and Notch signaling all play a role in EC-mediated vascular development (see [1] for review). While the role of EC signaling in vascular regulation has been extensively studied the role of SMC signaling in this process is less well understood. Smooth muscle cells are a critical and driving component of the normal and pathological development of mature arterial vasculature (see Figure 1.1). The molecular mechanisms that regulate their phenotypic modulation are of extreme importance in the maintenance of the

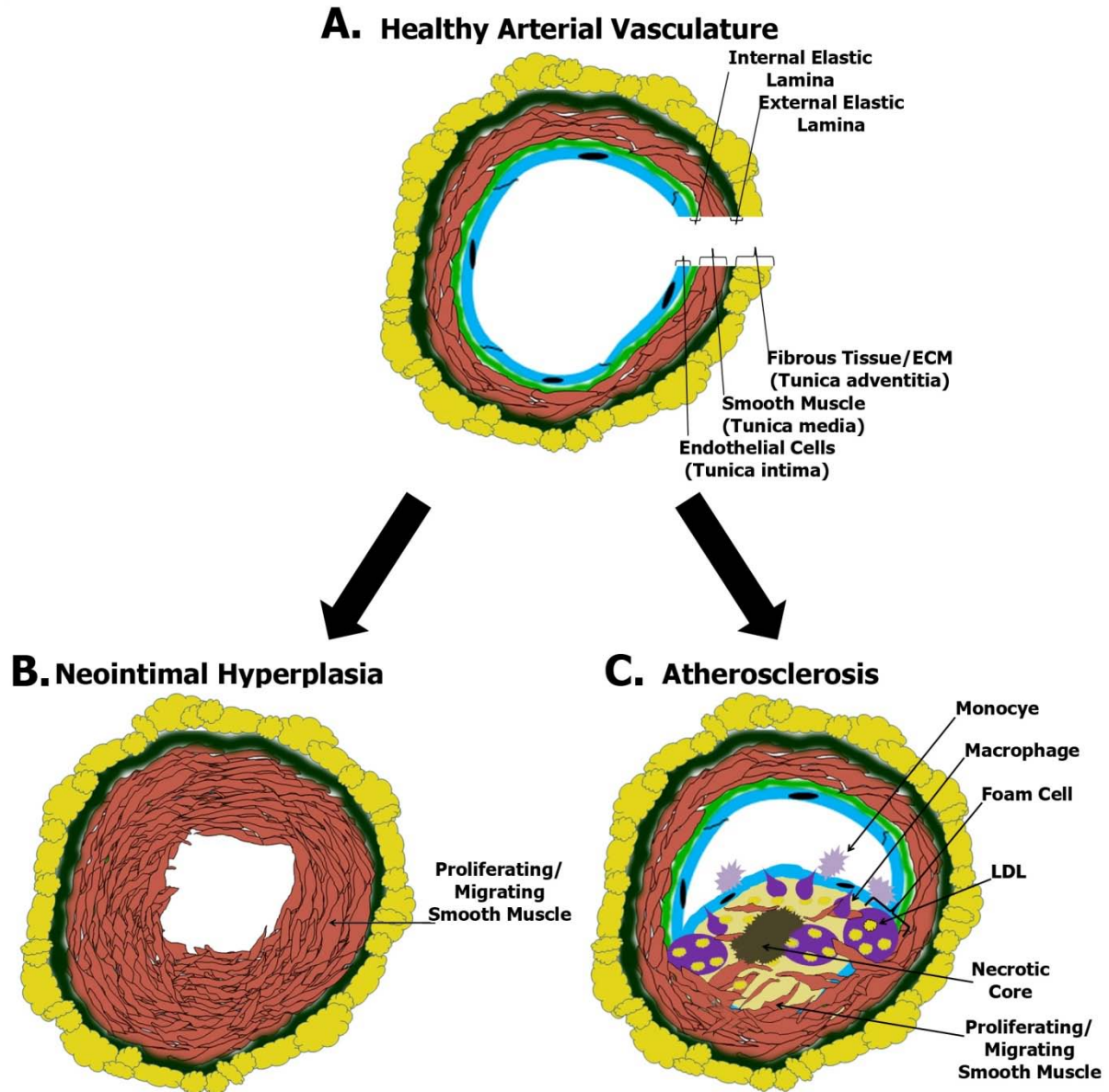


Figure 1.1. Structure of Arterial Vasculature in Healthy and Pathogenic States. **A.** Healthy arterial vasculature consists of 5 distinct layers including an intima composed of endothelial cells, an internal elastic lamina composed mainly of elastin, the tunica media composed of smooth muscle, an external elastic lamina composed mainly of elastin, and the tunica adventitia or externa composed of fibrous tissues and ECM, mainly collagen. **B.** Neointimal hyperplasia is a pathogenic state that occurs after acute arterial injury to the intima. The lesion results from accelerated migration and proliferation of SMC to form a very rigid lesion occluding the intimal space and impeding blood flow. **C.** A complex atherosclerotic lesion composed of monocytes, macrophages and mature foam cells, LDL molecules, SMC, and a necrotic core. SMCs can contribute to plaque instability through migration, proliferation, and even foam cell generation. They also stabilize the fibrous cap, thereby serving a protective role in atherosclerosis.

vasculature and consequently the mortality associated with the disruption of these processes.

Smooth Muscle Cells in Vascular Development and Disease

As embryogenesis proceeds, the demands on the vasculature are greatly increased. Soon the force and pressure associated with providing nutrients to the developing embryo require a more robust vascular network. The dogma surrounding recruitment and investment of SMC in arterial vasculature suggests that increasing demand for vascular integrity promotes signals for recruitment of pericytes and smooth muscle cells. Extensive proliferation, differentiation, and remodeling of vascular SMC is necessary for proper vascular development. The signals that regulate SMC in vascular development, maturation, and homeostasis is complex, varies throughout the vasculature, and is at present poorly understood. The questions about what, where, when, and how these signals are interpreted can only be addressed with a more complete understanding of the multiple origins of SMC during embryogenesis and throughout development, and the extracellular cues that regulate these responses.

The origins that give rise to smooth muscle are extensive and diverse. Fate mapping studies have shown that smooth muscle arises from at least 7 distinct origins in vertebrate embryogenesis. It is impossible to underscore the importance of this revelation in SMC development. One layer of complexity arising from this mosaic tissue type is that smooth muscle arising from differing lineages have differing protein expression patterns and can respond to the same agonist in

completely different ways. The coronary vasculature is thought to arise from the proepicardial mesothelium [2]. SMCs that comprise portions of the ascending aorta arise from the neural crest, SMCs that make up the descending aorta arise from splanchnic mesoderm. Additionally the secondary heart field, pericytes, mesothelium, somites, and mesoangioblasts also contribute to discrete populations of SMC (see [3, 4] for review). It is physiologically relevant that disease progression, under identical circumstances, occurs at different rates depending on the SMC origin [5, 6]. The best example is the propensity for coronary vasculature, a SMC of proepicardial origin, to develop advanced arterial lesions much faster than arteries composed of SMC from other origins [7].

Regardless of the multitude of SMC origins, the tissue as a whole is remarkably similar in both function and phenotype. Smooth muscle differs from cardiac and skeletal muscle in a number of ways. Unlike skeletal and cardiac muscle, smooth muscle is not terminally differentiated. Robust expression of polymerized actin and stress fibers is a hallmark of differentiated SMCs. Structurally these proteins assist in forming the wide, elongated, contractile cells that are critical in supporting arterial vascular and regulating contractility and blood flow. Reversion from a differentiated state includes initiating a decrease in stress fibers and higher order actin filaments, and adhesion complexes, to allow for increased migration, growth, and proliferation. Understanding smooth muscle plasticity is critical in understanding the progression of multiple cardiovascular disease states. Aberrant regulation of smooth muscle differentiation is contributory to both atherosclerosis and neointimal hyperplasia. In these diseases smooth muscle is induced to revert

from a differentiated state leading to an increase in smooth muscle migration, proliferation, and growth. In atherosclerosis smooth muscle cells contribute to fibrous cap formation, foam cell generation, calcification, and other events contributory to disease progression [8-10]. The SMC contribution to neointimal hyperplasia is more overt with the majority of the lesion consisting of SMCs that originally populated the lumen by de-differentiating, migrating, and proliferating prior to re-differentiating to form a rigid occlusion.

SMC Differentiation Signaling

A better understanding of the processes that control SMC growth and differentiation will be very important for our understanding of vascular development as well as the role SMCs play in cardiovascular disease [11-13]. It has become clear that the key to understanding control of SMC differentiation is dependent upon understanding the transcriptional mechanisms that regulate expression of SMC specific marker genes including SM α -actin, SM myosin heavy chain (SM-MHC), SM-22, calponin, etc. Unlike heart and skeletal muscle, which have master regulatory transcription factors (i.e. MyoD, Nkx2.5, Gata-4), SMCs do not have a master transcription factor that completely regulates their differentiation. This lack of a master regulatory factor has hindered our understanding of the control of SMC differentiation by extra-cellular signals. While poorly understood it has been established that SMC growth and differentiation are regulated by a complex array of local environmental cues including growth factors, contractile agonists, integrin-matrix interactions, cell-cell interactions, inflammatory stimuli, and mechanical stresses (see [14, 15] for review). However, few studies have identified the signaling

mechanisms by which these diverse pathways regulate SMC differentiation marker gene expression, and it is completely unknown whether signaling pathways converge to regulate differentiation or whether they exist as independent mechanisms. Recent studies have shown that the processes of growth, migration, and differentiation are not mutually exclusive and that the same extrinsic cues that activate one process can stimulate another simultaneously [16-24]. This revelation both complicates our understanding of the control of SMC phenotype and underscores the importance of determining what intracellular proteins transmit these extrinsic cues.

Despite these complexities, concerted efforts over the last several years have greatly increased our understanding of the complex transcriptional events regulating vascular SMC phenotype. The upstream signals that regulate these newly discovered transcriptional regulation paradigms need further investigation. This dissertation focuses on the upstream factors that regulate these transcriptional events including the cell surface and ligand initiated signaling via G-Protein Coupled Receptors (GPCRs). The benefit of complete understanding of these pathways from ligand binding to transcriptional regulation is the application to the discovery of pharmacologic targets leading to clinically relevant therapies.

The RhoA-MRTF Axis in SMC Transcription

Initial discoveries in the smooth muscle field implicated RhoA and Serum Response Factor (SRF) as a major regulator of SMC specific transcription. SRF regulates SMC-specific transcription by binding to CArG (CC[A/T]₆GG) cis elements

that are found in the promoters of nearly all of the SMC differentiation marker genes [25-31]. Since SRF is ubiquitously expressed and also regulates other CArG-containing genes including the early response genes, c-fos and egr-1, as well as several skeletal- and cardiac-specific genes [32-36], additional mechanisms must be present that regulate SRF activity in a SMC-specific manner.

An important breakthrough came when Wang et al. and others identified the myocardin family of transcription factors that potentiate SMC differentiation specific regulation of SRF driven transcription [37-40]. The MRTFs were shown to be regulated in a RhoA dependent manner by our lab, the Wang lab, and others, thus forming the RhoA-MRTF axis (see Figure 1.2). In brief, conserved RPEL domains are found in the myocardin family members. Actin has been shown to bind these RPEL domains in effect inhibiting myocardin family member nuclear localization and inhibiting SMC specific transcription. RhoA-mediated increases in actin polymerization by its effectors mDia1/2, FHOD1/2, ROCK, and others depletes the cytoplasmic pools of G-actin by increasing actin polymerization causing the release of sequestered cytoplasmic myocardin family members allowing them to localize to the nucleus [41-44].

The specification of SMC differentiation is regulated in part by the myocardin family of transcription factors (myocardin, Myocardin Related Transcription Factors A/B [MRTF-A/B]). The importance of the myocardin family members in governing SMC regulation is exemplified in knockout experiments. The knockout of myocardin in mice resulted in embryonic lethality at E10.5 and was attributed to yolk sac and vascular defects [45]. The MRTF-B knock out embryos die at E13.5. This lethality is

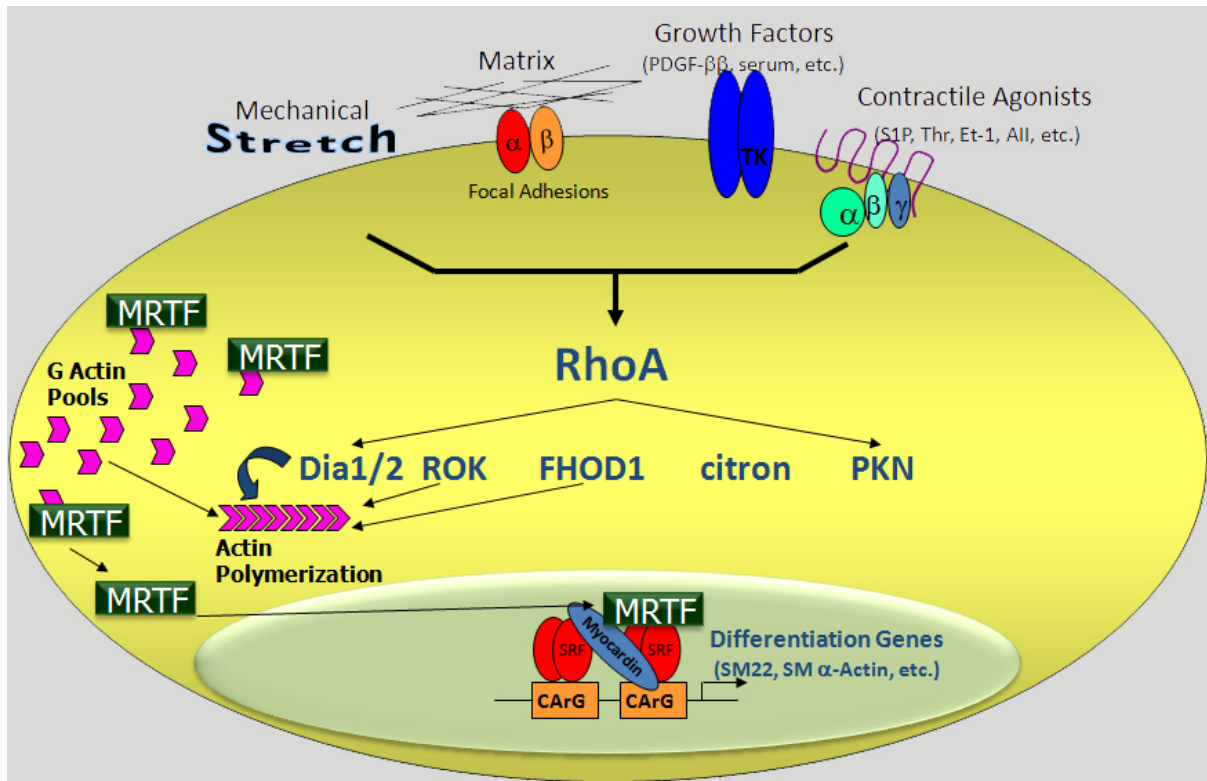


Figure 1.2. The RhoA-MRTF Axis in SMC Transcription. RhoA integrates signals from a number of sources to regulate SMC specific transcription. RhoA activation signals through its effectors to convert globular actin to filamentous actin. Upon actin polymerization RPEL domain mediated binding of the MRTFs to globular actin is interrupted. The MRTFs are then able to translocate to the nucleus and bind the CArG/SRF scaffold and drive SMC specific differentiation.

attributed to vascular defects arising in cells of the neural crest, one of the many originating tissues of differentiated SMC [46, 47]. The MRTF-A knock out is not lethal, possibly due to redundancy with myocardin and MRTF-B, but does exhibit a smooth muscle related phenotype. Female mice that were null for MRTF-A are not able to express milk due to an inability to maintain differentiated mammary myoepithelial cells, a cell type comparable to smooth muscle [48]. Taken together the function of the myocardin family members are crucial in regulating vascular SMC phenotype from a number of origins.

The regulatory mechanism that defines the RhoA-MRTF axis has been further refined in studies that pose a more specific role for nuclear specific regulation of SMC transcription. These studies show that G-actin binding of MRTF-A is required for nuclear export and that the export, and not import, of MRTF-A is the limiting step that governs the MRTF-A mediated transcription of CArG containing SMC genes. Importantly Vartiainen et al. show that globular actin associated with MRTF-A did not prevent transcriptional complex binding, but it did inhibit transcriptional activity [49, 50].

The Rho family of GTPases

The Rho family of GTPases are a major branch of the Ras (Rat Sarcoma) superfamily which comprises over 150 mammalian genes. The Rho family genes are identified by the presence of a Rho specific insert between the G4 and G5 boxes that are involved in the binding of Rho specific regulators and effectors [51]. The small GTPases range from 20-40 kDa with few exceptions [52]. They all have a

nucleotide binding pocket regulated by two highly conserved switch regions that change the overall protein conformation upon nucleotide binding. This conformational shift modulates the signaling capabilities of the protein. Additionally most Rho GTPases are localized to membranes by post-translational farnesylation or geranylgeranylation of a C-terminal cysteine residue. Rho GTPase membrane localization is thought to serve as an additional level of activation control [53, 54].

The Rho GTPases have been linked to the regulation of vesicle trafficking, cytokinesis, migration, cell survival, proliferation, differentiation, gene expression and many other cytoskeletal dependent and independent processes [55-57]. Since the gene for Ras homologous (Rho) was first discovered in the mollusk *Aplysia* in 1985 [58], at least 22 human genes of the Rho family have been described. The majority of investigation has focused on three distinct members RhoA, Rac1, and cdc42. While these GTPases have been shown to play vastly different roles in regulating cellular structure and function classically stress fiber, lamellipodia, and filopodia respectively, the molecular switching that initiates signaling to effector proteins remains remarkably similar in nearly all Rho family GTPases. Rho proteins cycle on and off by toggling between GTP and GDP bound states. When nucleotide free, Rho proteins have a similar affinity for either GDP or GTP nucleotides. However, the ratio of GTP to GDP in the cell results in a higher rate of GTP binding. When GTP bound, Rho proteins are in an active state and signal through their effector proteins responsible for conducting their downstream signaling. Hydrolysis of GTP to GDP molecules, whether occurring slowly through intrinsic hydrolysis or

encouraged by GTPase Activating Proteins (GAPs), inactivates Rho (see Figure 1.3).

There are three classes of proteins that regulate Rho GTPase activity. The Rho Guanine nucleotide Exchange Factors (RhoGEFs) activate Rho GTPases by promoting the exchange of GDP for GTP [59]. Conversely GTPase activating proteins or Rho GAPs decrease Rho activity by promoting hydrolysis of GTP to GDP. Lastly another negative regulator of Rho proteins are a protein class known as GDP dissociation inhibitors (Rho GDIs). These proteins sequester Rho in the cytoplasm and prevent nucleotide dissociation, in effect inactivating Rho [60]. In addition to the canonical understanding of GEF, GAP, GDI mediated Rho GTPase regulation there is emerging evidence (much of it specific to RhoA), that subcellular localization of Rho is an important, and oft-employed, method to control Rho signaling. Previously it was assumed that cytosolic Rho was inactive and that activated RhoA was localized exclusively to the plasma membrane. More recent evidence has challenged this view of RhoA localization. For instance some elegant biosensor experiments have shown that there are distinct fractions of RhoA found throughout the cell. These researchers document active RhoA concentrations in a 2 μ m band at the edge of protrusions, in the trailing edge during robust contractile events, and a discrete but inactive perinuclear pool [61, 62]. Despite the power and resolution of these Rho GTPase biosensors their inability to enter the nucleus has left questions concerning RhoA activity in the nucleus. Several GEFs including Net1, LARG, and PRG, and have been shown to localize to the nucleus but

RhoA GTP Cycling

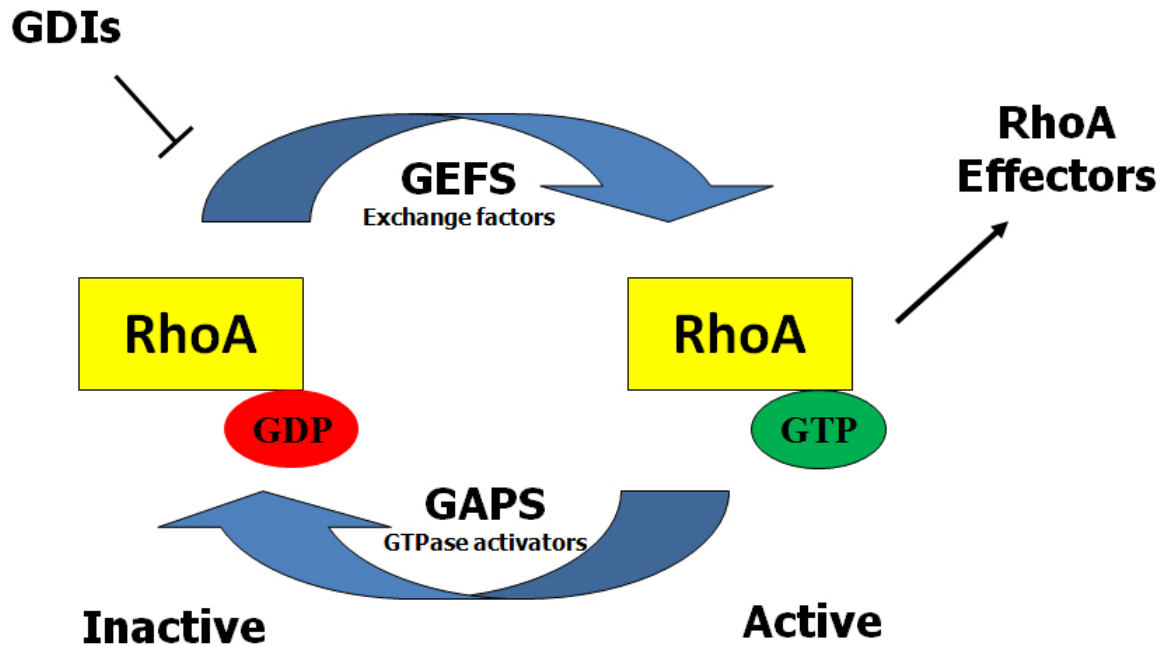


Figure 1.3. RhoA GTPase Regulation. Small RhoA GTPases are considered molecular switches. This switching is regulated by a number of proteins that fit three classifications. GEF activate Rho by increasing RhoGTP binding. GAPs inactivate RhoA by increasing intrinsic GTP hydrolysis to GDP and GDIs bind and sequester RhoA as well as slow the rate of GDP dissociation.

evidence that they are activating RhoA there remains elusive [63, 64]. These data and others highlight the fact that our current understanding of RhoA activation and signaling is incomplete, and the field would benefit by a more thorough understanding of the spatial activation of RhoA in a number of cellular contexts.

GEF Signaling in Mammalian Systems

As previously discussed, the activation of small G proteins is achieved by proteins categorized as Guanine Exchange Factors or GEFs (see [59] for review). The first mammalian RhoGEF was found in Diffuse B-cell-Lymphoma cells, (Dbl) and shown to contribute to neoplastic transformation. Homology to the active site of this gene defines most but not all RhoGEFs [65, 66]. To date there are over 80 identified Human Rho GEFs, curiously around four times more than the Rho GTPases they are designed to activate. The majority of these GEFs share a Dbl homology (DH) domain that confers GEF activity by binding the GTPase and causing the dissociation of GDP and stabilizing the nucleotide free state to allow binding of stoichiometrically dominant GTP. The tandem pleckstrin homology (PH) domains are thought to regulate GEF activity by promoting membrane localization and other, less well described, allosteric mechanisms [67]. The individual specificity of GEFs for specific small GTPases is quite variable and is thought to be determined by variations in the DH domains in combination with variations within the switch regions of the small GTPase where the GEFs have been shown to bind [59, 68-70].

The GEF family is divided into many different sub-families based upon the presence of additional regulatory domains (i.e. SH2, IgG-like, FERM) and very little

is known about how individual GEFs are regulated. What is apparent however is that, given the number of GEFs in the genome, and the number of diseases they are involved in, understanding exactly how they function will be important [63]. The diversity seen in expression and composition of RhoGEFs is also reflected in their GTPase specificity. Some GEFs, have been shown to activate virtually every Rho GTPase they interact with while others are specific to only RhoA, Rac1, cdc42 [59, 71]. The pathways investigated in this dissertation are, in general, RhoA-dependent. One subfamily of RhoGEFs known as the Regulator of G Protein Signaling (RGS) RhoGEFs have repeatedly shown a specificity for RhoA, thus, a major focus of this dissertation will be the study of the signaling properties that govern RGS RhoGEF function.

Structure and Function of the RGS RhoGEFs

There are three RGS RhoGEFs found in humans: P115 RhoGEF (P115), PDZ RhoGEF (PRG) and Leukemia Associated RhoGEF (LARG) (see Figure 1.4). All three RGS RhoGEFs specifically activate RhoA but not the closely-related small GTPases, Rac, and cdc42 [72]. Phylogenetic analysis has revealed that the three RGS RhoGEFs found in humans diverged relatively early in GEF evolution. The specific combination of an RGS like domain coupled with a DH/PH domain is conserved as far back as *C. elegans* in the protein identified as rhgf-1 [73]. The *drosophila* ortholog, which approximates the mammalian RGS RhoGEFs, DRhoGEF2 is essential for the invagination of mesodermal and endodermal primordia during gastrulation [74]. These observations are instructive of the ancient

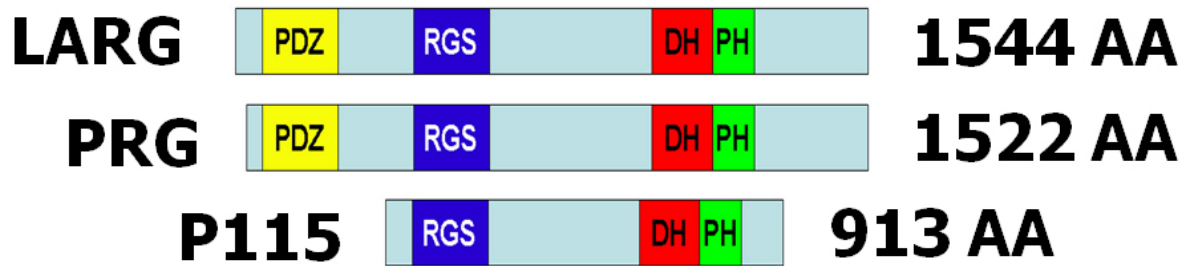


Figure 1.4. Size and Modular structure of the RGS RhoGEFs. **PDZ (PSD-95 Discs-large ZO-1) Domain:** Some PDZs have been shown to bind C-terminal polypeptides; others appear to bind internal (non-C-terminal) polypeptides. **RGS (Regulator of G Protein Signaling) Domain:** RGS family members are GTPase-activating proteins for heterotrimeric G-protein α -subunits that promote GTP hydrolysis by the α subunit of heterotrimeric G proteins, thereby inactivating the G protein and rapidly switching off G protein-coupled receptor signaling pathways. **DH (Dbl Homology) Domain:** About 200 residues shown to encode GEF activity specific for a number of Rho family members. **PH (Pleckstrin Homology) Domain:** About 100 residues that may have multiple functions or different functions in different proteins, including signal transduction, membrane anchoring, and protein-protein interaction. LARG and PRG share 29% amino acid identity and 62% similarity.

importance of this family of RhoGEFs even in comparison to RhoGEFs of other domain structure. The domains they all share in common are a RGS domain and a tandem DH/PH domain. Of the three, PRG and LARG share the closest similarity and both have conserved PDZ domains. They are more similar in size (1544 and 1522 AA, respectively) than the smaller P115 (913 AA). Additionally it should be noted that methods of activating the RGS RhoGEFs, other than $G\alpha_{12}$ and $G\alpha_{13}$, have been observed. For instance the PDZ domain of LARG and PRG is thought to interact with plexins to regulate of growth cone morphology and other RhoA dependent events [75-79].

The addition of an RGS domain to a GEF in order to make one functional protein is intriguing in design. This design bridges signaling between the intracellular signaling of G protein coupled receptors and small Rho GTPases. In brief, upon activation GPCRs allosterically activate $G\alpha$ subunits by exchanging a molecule of GDP for GTP. The RGS domain, which itself is found in over 20 human proteins, acts allosterically to stabilize the transition state of the GTP binding pocket of heterotrimeric $G\alpha$ subunits, in effect increasing the GTPase activity of the proteins, hydrolyzing the GTP nucleotide to GDP and inactivating the G protein. Thus, the addition of an RGS domain to a GEF creates a protein with a duality of purpose, to increase GTP hydrolysis of G proteins in order to activate small G proteins. The RGS domain activity of the RGS RhoGEFs is confined mainly to $G\alpha_{12}$ and $G\alpha_{13}$ subunits [80-82]. The discovery that RGS RhoGEFs are activated by the $G\alpha_{12/13}$ subunits of G protein heterotrimers implicates RGS RhoGEF activity in response to GPCR. The reason for this is that $G\alpha_{12/13}$ are found bound to the

cytoplasmic side of seven trans-membrane proteins. However, there is some controversy about preference of some GEFs for $G\alpha_{12}$ over $G\alpha_{13}$ and vice versa. It should be noted that *drosophila* and mice only carry one ortholog of $G\alpha_{12}$ and $G\alpha_{13}$ (named $G\alpha_{12}$, or *concertina* in *drosophila*). For a more in depth look at $G\alpha_{12/13}$ signaling variations (see [82-85] for review).

While often categorized as ubiquitous, LARG expression in the lung and GI tract of the mouse was specific for the SMC layers in those organs [86, 87]. Regardless we have shown that all three RGS RhoGEFs are expressed in rat aortic SMC (see Figure 1.5). There have been some KO studies conducted on these individual GEFs. The P115 KO mouse showed no lethality but numerous neutrophil defects associated with adhesion, migration, and other actin dependent processes were observed [88]. There are no reports of a global PRG KO studies but PRG KD dHL60 cells were observed to show multiple pseudopods or long tails and to migrate with reduced speed and persistence [77]. The Offermanns lab has generated a LARG KO mouse but it has not been fully characterized. There has been no reported lethality but it has been shown that, unlike wild type mice, the LARG KO mice did not develop hypertension in response to DOCA-salt treatment indicating a protective role in the normal pathological hypertensive response[89].

The individual signaling specificity of the RGS RhoGEFs is still unclear. Though poorly understood, an emerging paradigm with respect to RGS RhoGEF function, is the regulation of migration, adhesion and differentiation, all in a Rho dependent manner in response to GPCR signaling. A family of proteins that link GPCR signaling to events in the vasculature known to regulate the pathogenesis of

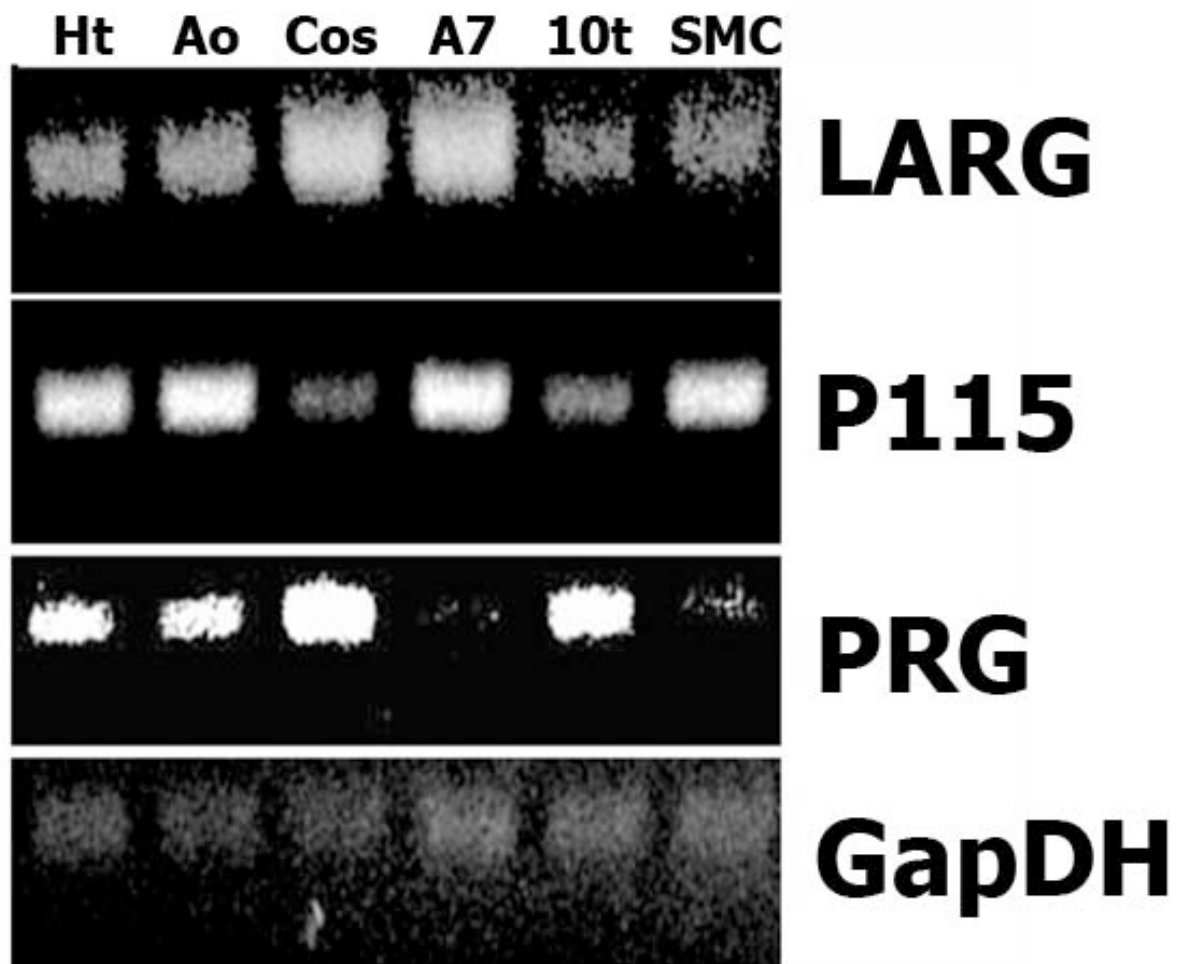


Figure 1.5. Semi-quantitative RT-PCR Expression Profile for LARG, P115 RhoGEF, and PRG. RNA was isolated from mouse tissues and cell lysates in order to construct cDNA transcripts. Left to right the following tissues or cell types were treated: heart, aorta, Cos7, A7R5, 10T1/2, and primary rat aortic SMCs. LARG, P115 RhoGEF, and PRG were all expressed at high levels in the aorta, 10T1/2, and rat aortic smooth muscle cells.

multiple cardiovascular disease states is of immediate therapeutic interest, and a subject of intense study in this dissertation.

S1P Metabolism and Concentration

Sphingosine 1-phosphate (S1P) is a hydrophobic zwitterionic lyso-phospholipid that fits into the class of molecules known as sphingolipids. Sphingolipids were studied and named by J.L.W. Thudichum in 1884 [90]. Etymologically the choice “sphingolipid” was made as an allusion to the mythical Greek Sphinx. Despite the efforts of modern researchers, lipid signaling remains enigmatic in many respects. A review of the literature surrounding S1P signaling often ends with models and theories about system wide S1P signaling with names like “the sphingolipid rheostat” or the “S1P paradox” indicating the difficulty, complexity, and elusiveness of fully explaining how sphingolipid signaling works. This is not to say that progress hasn’t been made but it is fair to say that much of the enigma of S1P signaling remains unsolved [91]. Many of the goals of this dissertation have been to investigate and understand the complexities of S1P signaling in smooth muscle.

S1P is found at very high concentrations in human serum ranging between .5 to 1 $\mu\text{mol/L}$ or more. Despite these concentrations there is a steep gradient between intracellular and extracellular concentrations of S1P [91]. S1P has important biological functions in both intracellular and extracellular compartments [92, 93]. S1P metabolism is well understood. S1P originates from the common membrane component, sphingomyelin which is metabolized to its active form by

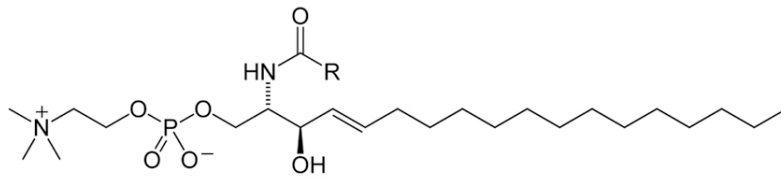
sphingomyelinase to generate two components phosphorylcholine and ceramide. Ceramide is broken down by ceramidase into sphingosine and finally phosphorylated by one of two known sphingosine kinases (SphK1 or 2). S1P can be reversibly cleaved by Sphingosine Phosphatase or irreversibly degraded by S1P lyase into phosphatidylethanolamine and hexadecanal (see Figure 1.6).

S1P Receptor Subtypes

S1P signals through a family of high affinity GPCRs named S1PR1-5. These receptors, formerly known as Edg receptors 1, 5, 3, 6, and 8, respectively, grant S1P the ability to signal in a multitude of pathways. Heterotrimeric G protein binding, receptor expression, affinity, and internalization are all methods utilized by these receptors to govern S1P mediated signaling.

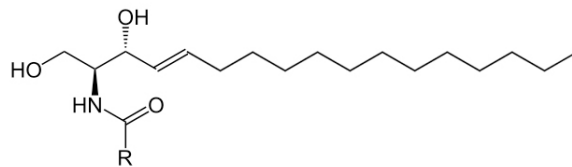
Four of the five S1P receptor knockouts have been made and characterized to varying degrees. The S1PR1 knockout is the only one that alone results in embryonic lethality. This lethality occurs between E12.5 and E14.5 and is attributed to EC mediated vascular maturation defects. The authors show that vasculogenesis and angiogenesis occurred without disruption, but that the deficiency in smooth muscle cell investment of the vasculature causes the observed lethality through systemic hemorrhage [94]. From a pharmacology standpoint this was a seminal finding since it was the first to show that a GPCR was required for vascular maturation and that S1P signaling was essential in development. It is also of note that S1PR1 has been shown to be important in limb development as well as in lymphocyte trafficking [95-98].

Sphingomyelin



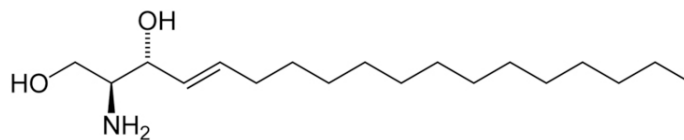
Sphingomyelinase ↓ ↑ **Sphingomyelin synthase**

Ceramide



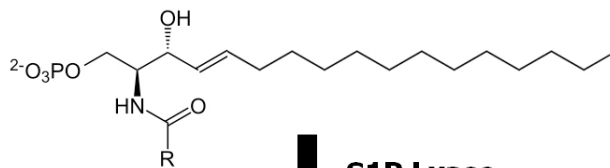
Ceramidase ↓ ↑ **Ceramide synthase**

Sphingosine



Sphingosine kinase ↓ ↑ **S1P phosphatase**

Sphingosine 1-Phosphate



S1P Lyase

Phosphatidyl-ethanolamine

Hexadecanal

Figure 1.6 Sphingosine 1-Phosphate Metabolism. S1P can be generated from the de-novo generation of ceramide or from the ceramide that occurs as the sphingolipid biosynthetic pathway. In the sphingolipid biosynthetic pathway sphingomyelinase cleaved sphingomyelin to generate ceramide which is cleaved by ceramidase to generate sphingosine, which is phosphorylated by one of two sphingosine kinases to generate S1P. S1P can be de-phosphorylated by S1P phosphatase or irreversibly cleaved into phosphatidylethanolamine and hexadecanal by S1P lyase. Complementary synthases can create Ceramide and Sphingomyelin from their metabolite products.

The phenotype of the S1PR2 knock out is much more elusive and less well characterized. Three groups have made S1PR2 $-/-$ mice and observed some similar, but many different effects. The Lee lab were the first to KO S1PR2 and observed no embryonic lethality but estimated seizures in 100% of the mice between weeks 3 and 7, and that approximately 14% of the null mice die due to seizures [99]. The Chun lab independently knocked out the S1PR2 gene and, in their hands, they found little to no observable differences in the S1PR2 KO mice, but did find that the S1PR2/3 double nulls had reduced litter size and marked lethality thorough infancy [100]. The conclusions reached from the Chun lab were that the S1PR2/3 work independently regulating the Rho and PLC/Ca²⁺ pathways respectively but that both of these receptors and pathways are important in development.

The Proia lab, that did the original S1PR1 knockout and characterization, went on to knock out the S1PR2 and 3 receptors as well. In their hands they show that the S1PR1/2 double null and the S1PR1/2/3 triple null showed substantially more severe vascular phenotypes than the S1PR1 KO and that while the S1PR2 or S1PR3 KO mice showed little or no lethality alone the S1PR2/3 double nulls had partial embryonic lethality. This particular line of S1PR2 null mice has been further investigated and a number of vascular pathologies have been observed. Sokura et al. have shown that when S1PR2 $-/-$ neonates were exposed to ischemia-driven retinopathy the normal pathologic neovascularization observed was suppressed [101]. Kono et al. reported a deafness phenotype arising from multiple inner ear pathologies, the earliest of which arises in the stria vascularis, a compartment that harbors the main vasculature of the inner ear [102]. Shimizu et al. also showed that

after injury neointimal hyperplasia is greatly increased in S1PR2 α - mice and that S1P increased RhoA activity in wt but not S1PR2 null mice [103]. This group went on to show that S1PR2 regulates SM α -Actin and SM22 expression in smooth muscle and that S1PR2 regulates SM α -Actin after vascular injury [104]. The S1P receptors 4 and 5 have a much more restrictive expression pattern limited to the lymphoid and lung for the former and brain, skin, and spleen for the latter. S1PR5 has been knocked out but there was no observed phenotype and S1PR4 has been a target of little investigation. While a full dissection of S1P receptor function involves discussion of the 4 and 5 receptors, as they are not expressed in smooth muscle containing tissues, their effects are not germane to this dissertation [105, 106].

S1P has also been shown to regulate a number of cellular processes intracellularly as a second messenger. First, S1P itself can be synthesized through one of the aforementioned methods leading to an increase in intracellular S1P levels. It has been shown that S1P levels can modulate intracellular Ca^{2+} concentrations suggesting that SphK mediated production of intracellular S1P without any ligand-induced signaling can activate intracellular Ca^{2+} stores [107, 108]. S1P also signals intracellularly to suppress apoptosis. This conclusion was reached when intracellular S1P was observed to prevent the appearance of intranucleosomal DNA fragmentation and associated apoptotic morphological changes [109]. Prolonged inhibition of proliferative responses in lymphocytes is an example of intracellular S1P ligand to S1PR1 receptor signaling on the nuclear membrane [92].

Heterotrimeric Subunit Coupling

The most well studied property of the S1P receptors, and possibly all GPCRs, are the intracellular G protein binding properties. The cytoplasmic side of GPCRs are bound to heterotrimeric G proteins consisting of a G α , β , and γ subunits. While G β and γ are known to regulate some signaling little is known about them and it is generally understood that the majority of signaling transduced by GPCR agonist binding is carried out by the G α subunit. There are, at present, 20 known G α subunits separated into four main classifications including G α_i , α_s , α_q , and $\alpha_{12/13}$. While it is not completely understood, and the individual binding affinities lack fidelity, it has been shown that S1P receptor subtypes are bound to specific G α subunits. S1PR1 is thought to bind primarily to G α_i , S1PR2 and 3 have been shown to bind to G α_i , α_q , and $\alpha_{12/13}$ and S1PR4 and 5 have been shown to bind G α_i and G $\alpha_{12/13}$. G α subunit binding is critical as it regulates the majority of intercellular signaling that occurs upon ligand binding. G α_q is thought to signal primarily by activating phospholipase C (PLC). G α_i inhibits the production of cAMP from ATP, G α_s conversely activates the cAMP pathway eventually activating PKA and its associated downstream targets. G $\alpha_{12/13}$ are very important in the regulation of cytoskeletal remodeling most notably through their activation of RhoA. Because of their known association with RhoA activation the G $\alpha_{12/13}$ subunits are closely studied throughout this dissertation. It should be noted that G α_q can also couple to S1PR2 and other S1P receptor subtypes and has been shown to activate RhoA [110-112].

In addition to S1P intracellular signaling, receptor subtype specificity, and G α subunit selectivity, receptor expression can modulate S1P signaling as well. In 2007 Grabski et al showed that S1PR2 regulated neointimal formation in injured mouse

carotid arteries. They went on to show that the S1PR2 regulated the migratory response of the SMC population comprising the lesion but stopped short of a mechanism [104]. Shortly after Wamhoff et al were able to show that arterial injured SMC phenotype was largely controlled by acute changes in S1P receptor subtype expression. They show that S1PR1 is significantly upregulated 24 and 72 hours post injury while conversely, S1PR2 is down regulated during that time. Most interestingly S1PR2 is significantly upregulated 10 days post injury before all of the S1PR expression levels reach equilibrium 14 days after injury. When these expression variations are juxtaposed against a timeline of lesion formation the signals to regulate dedifferentiation, migration, proliferation, and differentiation can largely be explained by the observed changes in S1P mediated signals sent in S1PR specific ways [113].

Receptor internalization after extracellular ligand binding, while previously considered an endpoint in signaling, has recently been suggested to be the beginning of a number of intracellular signaling events. In general GPCR kinases (GRKs) and β -arrestins bind activated receptors and promote their internalization, a process referred to as desensitization [114]. Receptors can then be recycled back to the plasma membrane in a process known as resensitization or transported to lysosomes for degradation. Some GPCRs have even been shown to translocate to the nucleus [115]. There is an intriguing body of evidence that shows S1PR1 and possibly S1PR2 internalize in response to phosphorylation while S1PR3 does internalize but independently of phosphorylation [116, 117]. It is clear that receptor trafficking can differ between receptor subtype. Also that this has functional

implications on S1P dependent signaling that should be considered in any analysis of S1P dependent signaling especially when pharmacologically modified or synthetic ligands are used.

Dissertation Summary

The goal of this dissertation is to improve our understanding of S1P mediated RhoA signaling in SMC. S1P based therapies are just beginning to be utilized in the clinic and represent the next generation of weapons to combat CVD. Deutschman et al have shown that S1P serum levels can be used as an accurate predictor of both the occurrence and severity of coronary stenosis [118]. According to the 2009 AHA update there were 1.3 million angioplasties and 448,000 Coronary Artery Bypass Grafts (CABGs) performed in the U.S. alone. Discoveries made and conclusions that can be drawn from this body of work have direct implications toward the development of pharmacological based therapies to combat the morbidity and mortality associated with CVD. These pharmacologic agents could be used in lieu of, or in conjunction with, the surgical interventions currently employed to treat CVD. Obviously the regulation of S1P-mediated signaling is complex and multifactorial. The hypotheses put forth and the experiments employed through the body of this dissertation provide smooth muscle specific insights to the behavior, and by extension possible manipulations, of SMC in order to build, maintain, and restore healthy functional vasculature.

Chapter 2

S1P Receptor 2 Signals Through the RGS-RhoGEF, LARG, To Promote Smooth Muscle Cell Differentiation

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Abstract

Objective: The goals of this study were to identify the signaling pathway by which S1P activates RhoA in SMC and to evaluate the contribution of this pathway to the regulation of SMC phenotype.

Methods and Results: Using a combination of receptor-specific agonists and antagonists we identified S1PR2 as the major S1P receptor sub-type that regulates SMC differentiation marker gene expression. Based upon the known coupling properties of S1PR2 and our demonstration that over-expression of $G\alpha_{12}$ or $G\alpha_{13}$ increased SMC specific promoter activity, we next tested whether the effects of S1P in SMC were mediated by the RGS-RhoGEFs (LARG, PRG, P115). Although each of the RGS-RhoGEFs enhanced actin polymerization, MRTF-A nuclear localization, and SMC-specific promoter activity when over-expressed in 10T1/2 cells, LARG exhibited the most robust effect and was the only RGS-RhoGEF activated by S1P in SMC. Importantly, siRNA-mediated depletion of LARG significantly inhibited the activation of RhoA and SMC differentiation marker gene expression by S1P. Knockdown of LARG had no effect on SMC proliferation, but promoted SMC migration as measured by scratch wound and transwell assays.

Conclusion: These data indicate that S1PR2-dependent activation of RhoA in SMC is mediated by LARG and that this signaling mechanism promotes the differentiated SMC phenotype.

Introduction

Smooth muscle cell (SMC) differentiation is critical during vascular development, and alterations in SMC phenotype contribute to a number of cardiovascular pathologies including atherosclerosis, hypertension, and restenosis [13]. Although our understanding of SMC differentiation has been complicated by the plasticity of this cell-type and the fact that SMC originate from multiple locations within the embryo [4], the transcription mechanisms involved are starting to become clear. Extensive evidence indicates that Serum Response Factor (SRF) regulates nearly all of the SMC differentiation marker genes by binding to CArG cis elements within their promoters [13]. The discovery of the SRF co-factor, myocardin, was an extremely important advance because this cardiac and smooth muscle selective transcription factor strongly activates SMC-specific transcription in many cell-types and is required for SMC differentiation in the developing aorta [40]. Two Myocardin-Related Transcription Factors, MRTF-A and MRTF-B, have also been identified. Although expressed more widely than myocardin [119], studies have demonstrated that the MRTFs up-regulate SMC-specific transcription and are required for endogenous SMC differentiation marker gene expression in at least some SMC subsets [120, 121]. Importantly, genetic disruption of MRTF-B in the mouse resulted in defective SMC differentiation of the cardiac neural crest cells that populate the brachial arches [46, 47], while loss of MRTF-A inhibited the expression of SM α -actin that occurs in mammary myoepithelial cells during lactation [48, 122].

The identification of the signaling mechanisms that regulate the myocardin factors will be important for our understanding of the control of SMC phenotype. The

Treisman laboratory was the first to demonstrate that MRTF nuclear localization is regulated by the small GTPase, RhoA [123], and we and others have shown that RhoA/MRTF signaling is a critical determinant of SMC-specific transcription [41, 44, 120, 121]. Furthermore, RhoA activity was shown to be required for the induction of SMC differentiation marker gene expression by angiotensin II, TGF- β , intracellular calcium, and mechanical stretch suggesting that this pathway plays an integral role in the regulation of SMC phenotype [124-128].

RhoA activity is tightly regulated by GTPase Activating Proteins (GAPs) that facilitate RhoA's intrinsic GTPase activity (inhibiting RhoA), Guanine Exchange Factors (GEFs) that facilitate exchange of GDP for GTP (activating RhoA), and Rho GDP-dissociation inhibitors (RhoGDIs) that sequester RhoA into an inactive fraction. However, the major regulators of RhoA activity in SMC are not completely clear, and even less is known about the signaling mechanisms by which these proteins are activated. We were the first to demonstrate that the lipid agonist, sphingosine 1-phosphate (S1P), up-regulates SMC-specific gene expression by activating RhoA [120]. The goal of the current study was to identify the signaling pathway that mediates the effects of S1P on RhoA in SMC and to evaluate the contribution of this pathway to the regulation of SMC phenotype.

Materials and Methods

Plasmids and Reagents - S1P, FTY-720, SEW2871, and JTE-013 were purchased from Cayman chemical. LARG, PRG, and P115 cDNAs were kind gifts from Dr. T. Kozasa (University of Tokyo). G α_{12} and G α_{13} cDNAs were kind gifts from Dr. P.

Gierschik (Ulm University, Germany). The $G\alpha_q$ cDNA was a kind gift from Dr. G. Johnson (University of North Carolina, Chapel Hill, NC). All cDNAs were sub-cloned into pcDNA 3.1. Antibodies to LARG and PRG were kind gifts of Dr. Keith Burrige (University of North Carolina, Chapel Hill, NC). The P115RhoGEF Ab was purchased from Santa Cruz.

Transient Transfections and Reporter Gene Assays - The 10T1/2 and SMC cell cultures, transient transfections, and promoter luciferase assays have been previously described [44, 120]. Statistical comparisons between groups were made using the Student's *t* test with statistical significance accepted at $p < 0.05$.

GST G17A Pull-downs - GST-Rho(G17A) was a kind gift of Dr. K. Burrige and pull-downs were performed as previously described [129]. In brief, SMCs were plated and starved for 24 hours and then treated with S1P (10 μ M) or 10% serum for 3.5 or 9 minutes. Protein lysates were incubated with 20 μ g GST-Rho(G17A) beads at 4 °C for 3 hours. Complexes were washed 3X in lysis buffer prior to analysis by Western blot.

Immunofluorescence - 10T1/2 or primary SMCs were plated and transfected in 4-well chamber slides, maintained in 10% serum overnight. Cells transfected with GFP-MRTF-A were serum-starved for 16 h. Cells were fixed in 3.7% paraformaldehyde/PBS for 20 min and permeabilized in 0.5% Triton X-100/PBS for 3-4 min. Cells were then incubated with M2 anti-flag (1:500)(Sigma) in 20% Goat Serum / 3% BSA in PBS for 2 hours. Texas Red, FITC (Jackson ImmunoResearch), conjugated secondary antibodies were used at (1:1000) while

AlexaFluor 546 Phalloidin (Molecular Probes) and DAPI (Molecular Probes) were used at 1:100 and 1nM, respectively

siRNA Knockdowns - siRNA oligos were purchased from Invitrogen and transfections were performed using the Dharmafect transfection reagent as per protocol. RGS-RhoGEF knockdown was confirmed in all experiments by western blotting.

RhoA Activity Assays - To measure RhoA activity in our RGS RhoGEF KD cells we used the luminescence based G-LISA RhoA activation assay kit by Cytoskeleton inc. as per protocol. In brief following 72 hours KD Cells were starved for 24 hours and treated with S1P for 12 minutes, lysed, cleared, equalized and measured for RhoA activity. Concurrent western blots were performed to ensure adequate KD.

Quantitative RTPCR - Briefly primary SMCs were transfected with 19nt siRNA oligos to deplete LARG expression (identical plates were processed for western blots to confirm the level of KD). Trizol was used to achieve RNA extraction and purification and RNAs for control and LARG KD cells were combined with primer and probe sets specific to mouse SM22 and SM α -actin. RNA levels of each SMC marker were normalized to an internal 18S control during analysis.

QRTPCR- Primer sets used were as follows

SM22 Forward- TGCAGTGTGGCCCTGATGT

SM22 Reverse- TGCTCAGAATCACACCATTCT

SM α -Actin Forward- CGCTGTCAGGAACCCTGAGA

SM α -Actin Reverse- CGAAGCCGGCCTTACAGAG

Transwell Assays- In brief SMCs were transfected with control or LARG specific siRNA. After 72 hours of KD cells were split, counted, and equalized. Three thousand cells were plated on the tops of fibronectin coated transwells (8 μ m pore size) in a 24 well format. Cells were allowed to migrate toward DMEM F-12 + 10% Serum in the bottom chamber for 18 hours prior to processing. Chambers were rinsed with PBS, cells were removed from the top chamber by scraping with a cotton swab, and the remaining cells (in the bottom chamber) were fixed in 4% paraformaldehyde for 20 min. Cells that had migrated to the bottom chamber were visualized by crystal violet staining for 4 hours. Data represent the total number of cells in 12 separate fields for each condition through three separate experiments.

Migration Assays - For wound healing assays, confluent cultures of control and LARG knockdown SMC were scraped with a P200 tip and then placed on an inverted microscope equipped with a heated, humidified, and O₂/CO₂ perfused stage. Pictures taken every 3.5 minutes for 9 hours were assembled into movies using Quicktime. At the end of each experiment cell lysates were subjected to western blot to ensure LARG knockdown.

Wound Healing Assay Equipment Specifics- Equipment and instruction was provided by Dr. C. Robert Bagnell, Jr., Ph.D. Professor and Director of the Microscopy Services Laboratory at UNC Chapel Hill.

Olympus IX70 Live Cell Inverted Fluorescence Microscope Description:

The Olympus IX70 is an inverted microscope equipped for bright field, phase contrast, and epi-fluorescence. It is enclosed within an environmental chamber capable of controlling both temperature and relative humidity. It is equipped with a Uniblitz shutter on the transmitted light system and a Ludl stepper motor filter/shutter controller on the epifluorescence system, a BioPrecision motorized stage with linear encoders and motorized focus with servo feedback all controlled by a LEP MAC5000 controller. Image capture is by a Hamatsu ORCA CCD camera. The system is controlled by an Apple IMAC computer using Improvision's Open Lab software. Carbon dioxide supply is provided from a 100% CO₂ tank connected to a flow meter and controlled by a precision needle valve. CO₂ is passed through a water column and delivered through tubing to custom-made tabulated lids that match various cell culture dish configurations.

siRNA KD-All siRNA oligos were designed through, and purchased from, Invitrogen. Transfection of 21NT siRNA oligos was accomplished with Dharmafect transfection reagent as per protocol. A cocktail of two separate siRNA oligos was used for each GEF.

siRNA KD- RGS RhoGEF KD Sequences 5'→3'

LARG1-AAACCAAUGUAUAGAGCUTT

LARG2-CCCACUUGCUGAUUCUGAATT

P1151-AUCUUCUGAGUUCGCCUCCTT

P1152-UUAGAUGUCGACAAUCUGGTT

PRG1-GGGAGAUUCUACACCUGAATT

PRG2-GCAACUGACCGUCAAGCUUTT

BRDU Incorporation -SMCs were plated in 4 well chamber slides and transfected as per protocol with LARG or NTC siRNA. After 48 hours transfection BrdU [10µM] was added for 18hrs. After BrdU incorporation plates were fixed in 3.7% paraformaldehyde/PBS for 20 min, treated with 2M HCL for 1hr, washed with 0.1M borate, permeabilized and incubated with anti-BrdU 1:50 (Abcam cat# ab-9557) in 20% goat serum

Results

S1P signals through S1PR2 to activate SMC differentiation marker gene expression

As shown in figure 2.1, treatment of serum-starved mouse SMC with S1P strongly up-regulated the expression of multiple SMC differentiation marker genes including smooth muscle myosin heavy chain (SM MHC), SM22, SM α -actin and calponin. These results are in excellent agreement with previous studies from our lab and others [104, 113, 120]. S1P signals through a family of G-protein coupled receptors (S1PR1-5) [130]. Because S1P receptor expression levels and coupling properties can vary significantly, it is often difficult to identify the receptor sub-type that mediates a specific S1P-dependent response. RT PCR analysis revealed that our aortic SMC cultures express S1PR1, S1PR2, S1PR3, and low levels of S1PR5 (data not shown). To help determine which of these receptor subtypes was

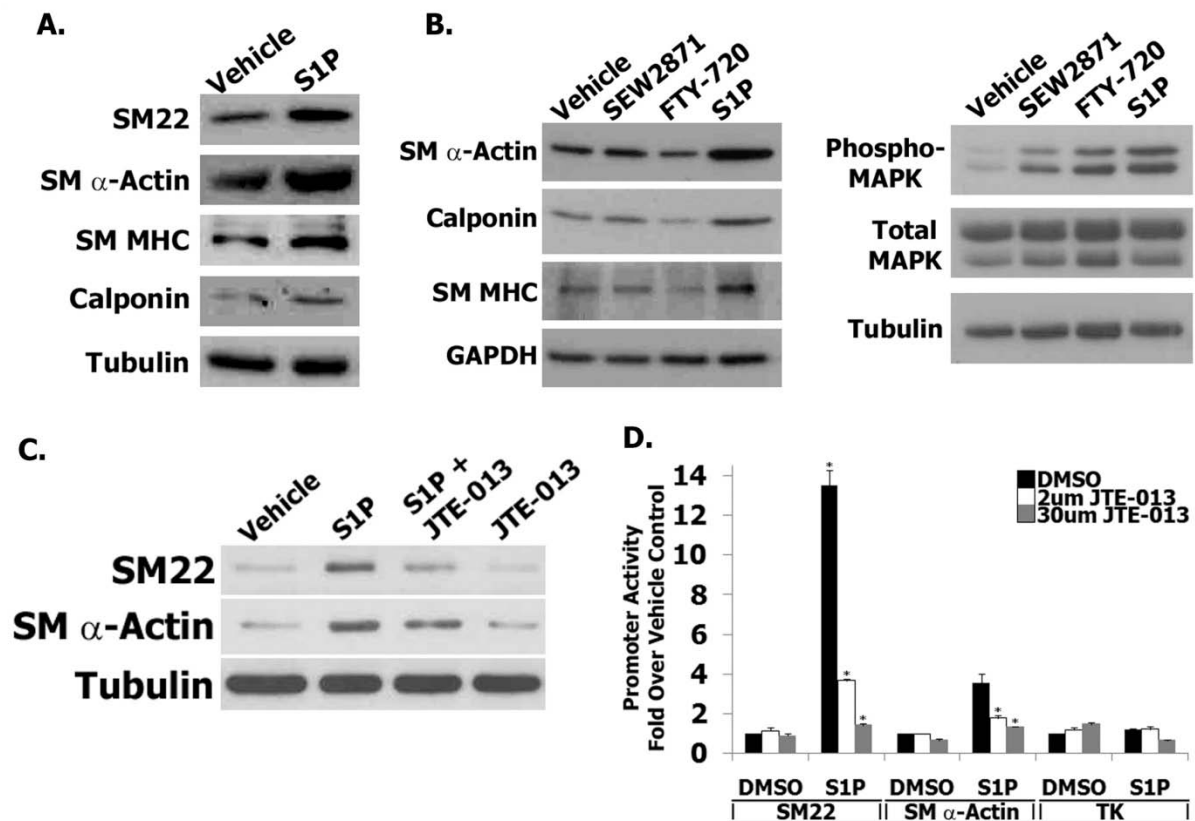


Figure 2.1. S1P stimulated SMC differentiation marker gene expression through S1PR2. **A.** Mouse aortic SMC were serum-starved for 24h and then treated with S1P (10 μ M) for 24 hours. Cell lysates were separated on an SDS-page gel, transferred, to nitrocellulose and probed with antibodies to the indicated SMC differentiation marker genes. **B.** Western Blots were performed on SMCs that were serum-starved and then treated for with S1P [10 μ M], SEW2871 [5 μ M] (stimulates only S1PR1), or FTY-720 [10 μ M] (stimulates all S1P receptors except S1PR2). SMC marker expression represents 24 hours agonist treatment. MAPK activation control represent 12.5 minute agonist treatment **C.** Western blots were performed on SMC treated with S1P for 24 hours +/- pre-treatment with JTE-013. **D.** 10T1/2 cells transfected with the indicated promoter luciferase construct were treated with S1P +/- pretreatment with the S1PR2 antagonist, JTE-013. Luciferase activity was measured 24 after treatment and is expressed relative to untreated cells. * $p < 0.05$ vs S1P-treated in the absence of JTE-013.

responsible for activating SMC-specific transcription, we utilized several receptor sub-type specific agonists and antagonists. SMC differentiation marker gene expression in SMC was not enhanced by SEW2871, an S1PR1-specific agonist, or FTY720, an S1P agonist that activates all S1P receptors but S1PR2 (Figure 2.1b). However, as expected by the known coupling patterns of the S1P receptors (see below), these agonists did activate MAPK. To examine the role of S1PR2 more directly, we pre-treated SMC cells with the S1PR2-specific antagonist, JTE-013. Importantly, JTE-013 inhibited the effects of S1P on SM α -actin and SM22 protein expression in SMC (Figure 2.1c). To determine whether these effects were mediated transcriptionally we also tested JTE-013 on S1P-dependent SMC-specific promoter activity in multi-potential 10T1/2 cells. This SMC precursor line is frequently used to study SMC-specific gene activation [131-134] and our RT PCR analyses indicated that 10T1/2 cells express all 5 S1P receptors (data not shown). As shown in figure 2.1d JTE-013 dose-dependently inhibited SM22 and SM α -actin promoter activity. JTE-013 did not affect the expression of housekeeping genes or the activity of a minimal thymidine kinase promoter in these studies.

G $\alpha_{12/13}$ and the RGS-RhoGEFs stimulate SMC-specific transcription

The G-protein-dependent signaling pathways activated by S1PR1-3 have been fairly well-characterized in a number of heterologous cell culture systems (see [130] for review). S1PR1 couples almost exclusively to G α_i and regulates MAPK activity and cell growth while S1PR2 and S1PR3 have been shown to couple somewhat promiscuously to G $\alpha_{12/13}$ and G α_q . Since both G $\alpha_{12/13}$ and G α_q have been associated with RhoA activation (see [135] for review), we tested whether over

expression of these G-proteins was sufficient to stimulate SMC-specific transcription. As shown in figure 2.2, over-expression of $G\alpha_{12}$ (Q231L) or $G\alpha_{13}$ (Q226L) in SMC activated SM22 and SM α -actin promoter activity approximately 2-3 fold while expression of $G\alpha_q$ (Q209L) had no effect. In contrast, over-expression of $G\alpha_{12}$ (Q231L) or $G\alpha_{13}$ (Q226L) did not activate the c-fos promoter while $G\alpha_q$ (Q209L) did suggesting at least some specificity between the transcriptional responses to these G-protein sub-types.

Previous studies have shown that RhoA activation by $G\alpha_{12/13}$ -coupled receptors is mediated by the Regulator of G-protein Signaling (RGS) sub-family of RhoGEFs that includes P115RhoGEF (P115), PDZ-RhoGEF (PRG), and Leukemia Associated Rho GEF (LARG) [135]. The RGS-RhoGEFs bind specifically to $G\alpha_{12/13}$ (through the RGS domain) and function as GAPs for these G-proteins. Importantly, this interaction also stimulates the GEF activity of RGS RhoGEFs providing a direct mechanistic link between $G\alpha_{12/13}$ -coupled receptors and RhoA activation. All three RGS-RhoGEFs are expressed in rat aortic SMC [86, 87] and interestingly, Becknell et. al. demonstrated that LARG expression in the lung and GI tract of the mouse was specific for the SMC layers in those organs [87]. Of additional importance to our studies, the RGS-RhoGEFs, unlike most other GEFs, specifically activate RhoA but not the closely-related small GTPases, Rac and Cdc42. Based on these observations and our data implicating S1PR2 and $G\alpha_{12/13}$ in the activation of SMC-specific gene expression, we hypothesized that one or more of the RGS-RhoGEFs mediated the effects of S1P on RhoA in SMC and that this family of RhoA activators were important regulators of SMC phenotype.

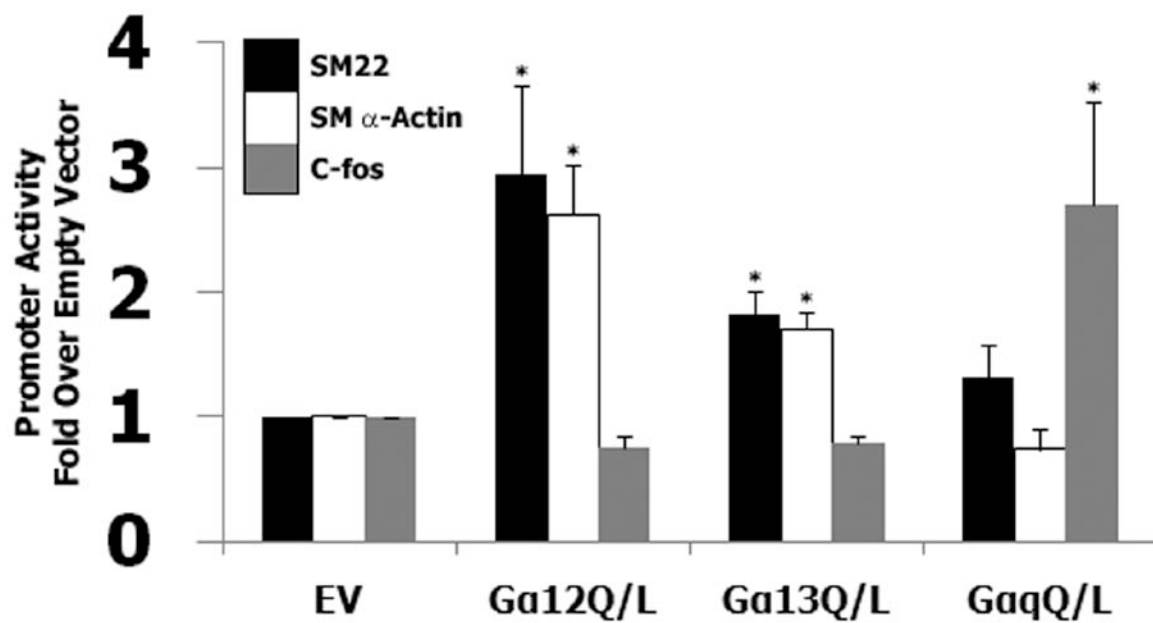


Figure 2.2. $G\alpha_{12}$ and $G\alpha_{13}$ increased SMC transcription. SMCs were co-transfected with the indicated promoter-luciferase construct along with $G\alpha_{12}$ (Q231L), $G\alpha_{13}$ (Q226L), $G\alpha_q$ (Q209L), or empty expression vector (EV). Luciferase activity was measured 24h after transfection. * $p < 0.05$ vs EV.

To begin to examine the role of the RGS-RhoGEFs in SMC, we tested whether ectopic expression of these proteins in 10T1/2 cells could up-regulate SMC-specific promoter activity. As shown in figure 2.3 over expression of LARG strongly increased (8-16 fold) the activities of the SM MHC, SM α -actin, and SM22 promoters. The effects of PRG were somewhat less robust (~4-7 fold), but this difference may reflect slightly lower PRG expression levels in our experiments (see inset). Somewhat surprisingly, expression of P115 had only minor effects on SM MHC and SM22 promoter activity and no significant effect on SM α -actin promoter activity. Co-expression of C3 toxin, completely inhibited the transcriptional effects of all three RGS-RhoGEFs strongly supporting the involvement of RhoA in this response (data not shown). Since the effects of RhoA signaling on SMC-specific transcription are thought to be due to alterations in actin polymerization that regulate MRTF nuclear localization, we also tested the effects of the RGS-RhoGEFs on these parameters. As shown in figure 2.3, over-expression of either LARG, PRG, or P115 in 10T1/2 cells enhanced actin polymerization (Figure. 2.3b) and localization of GFP-MRTF-A to the nucleus (Figure. 2.3c).

The effects of S1P were mediated by LARG

To identify the RGS-RhoGEFs that were activated by S1P in SMC, we used an assay described by the Burrige lab that employs a nucleotide free variant of RhoA (G17A) to precipitate activated RhoGEFs from cell lysates [129]. As shown in figure 2.4a, treatment of SMC with S1P resulted in a dramatic increase in the amount of LARG present in GST-RhoA(G17A) precipitates suggesting that S1P

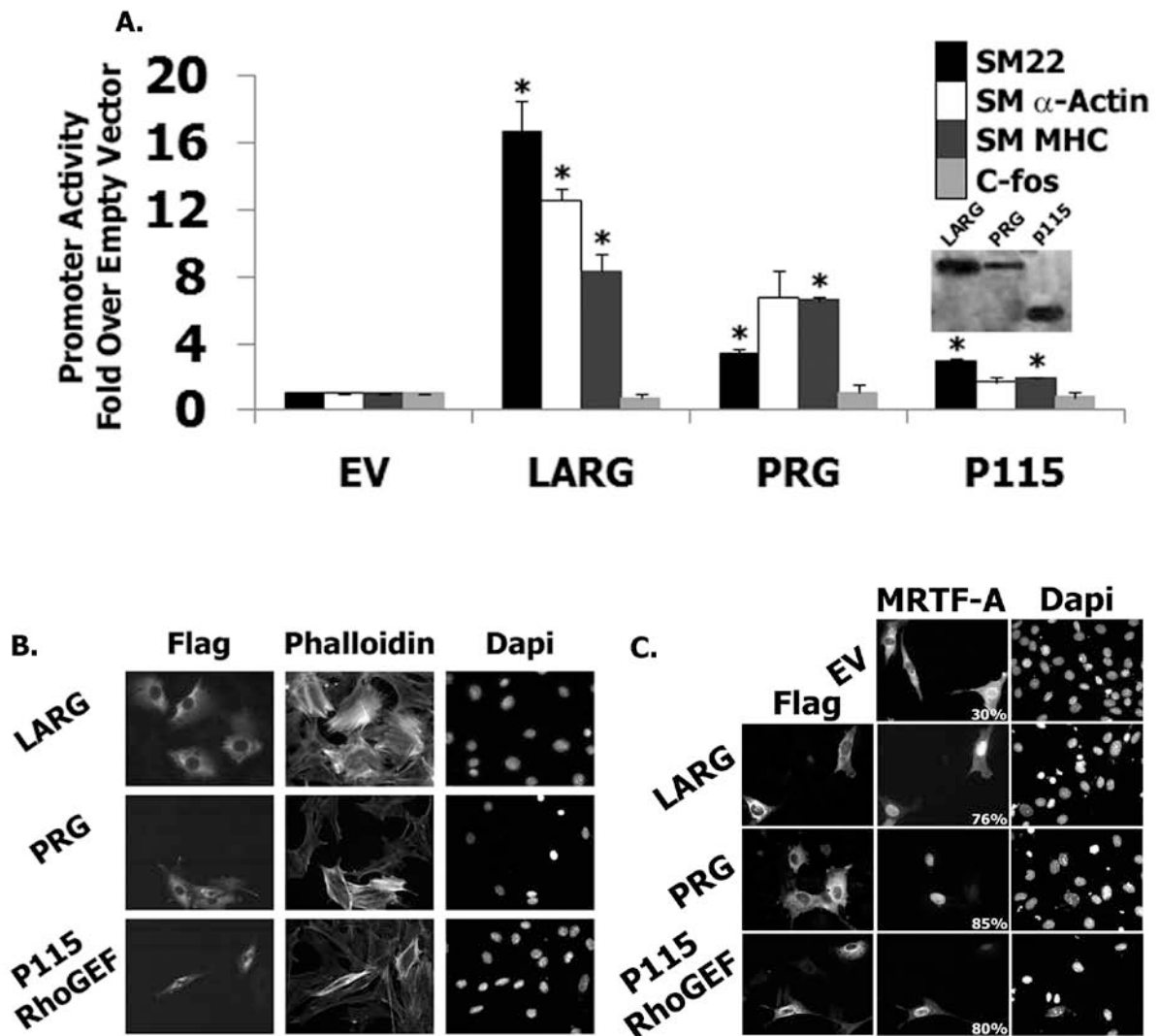


Figure 2.3. RGS-RhoGEF over-expression increased SMC transcription, stress fiber formation, and MRTF-A localization. **A.** 10T1/2 cells were co-transfected with the indicated promoter-luciferase construct along with LARG, PRG, P115, or empty expression vector (EV). Luciferase activity was measured 24h after transfection. * $p < 0.05$ vs EV. **B.** 10T1/2 cells expressing the indicated flag-tagged RGS-RhoGEF were stained with phalloidin to visualize actin polymerization. **C.** 10T1/2 cells were co-transfected with the indicated RGS-RhoGEF and GFP-MRTFA. Cell counts (>100 cells per condition) demonstrated that RGS-RhoGEF expression increased the percentage of cells that exhibited mainly nuclear MRTF-A localization (see lower right hand corner of micrographs in the middle column).

activates LARG in this model. LARG was also activated by 10% serum to a somewhat lesser extent. We observed little to no activation of PRG or P115 by S1P or serum.

We next used a siRNA approach to test whether LARG was required for S1P-mediated activation of RhoA in SMC. In parallel experiments we also knocked-down all three RGS-RhoGEFs in combination to examine potential compensatory effects within this family. We consistently achieved greater than 90% knock-down of LARG when compared to SMC transfected with a control siRNA that targets GFP, and we observed no up-regulation of either PRG or P115 in LARG knock-down cells (Figure 2.4b). As measured by the Rhotekin-based assay (Cytoskeleton), knockdown of LARG in SMC inhibited S1P-dependent RhoA activation by approximately 65% (Figure 2.4c). In addition, depletion of LARG was as effective as the triple knock-down providing further evidence that LARG is the major GEF within this family that mediates the activation of RhoA by S1P. LARG knockdown had no effect on S1P- or PDGF-bb mediated activation of MAPK (Figure 2.4d).

Importantly, knockdown of LARG in SMC significantly inhibited S1P-dependent activation of the SM22 and SM α -actin promoters (Figure 2.5a) and S1P-dependent activation of endogenous SMC differentiation marker gene expression as measured by quantitative RT-PCR (Figure 2.5b). Western blotting also demonstrated that LARG knockdown significantly inhibited S1P mediated increases in SM22, MHC, and SM α -actin protein levels by 45%, 52%, and 27%, respectively (see Figure 2.5c). In good agreement with the results from the RGS RhoGEF and RhoA

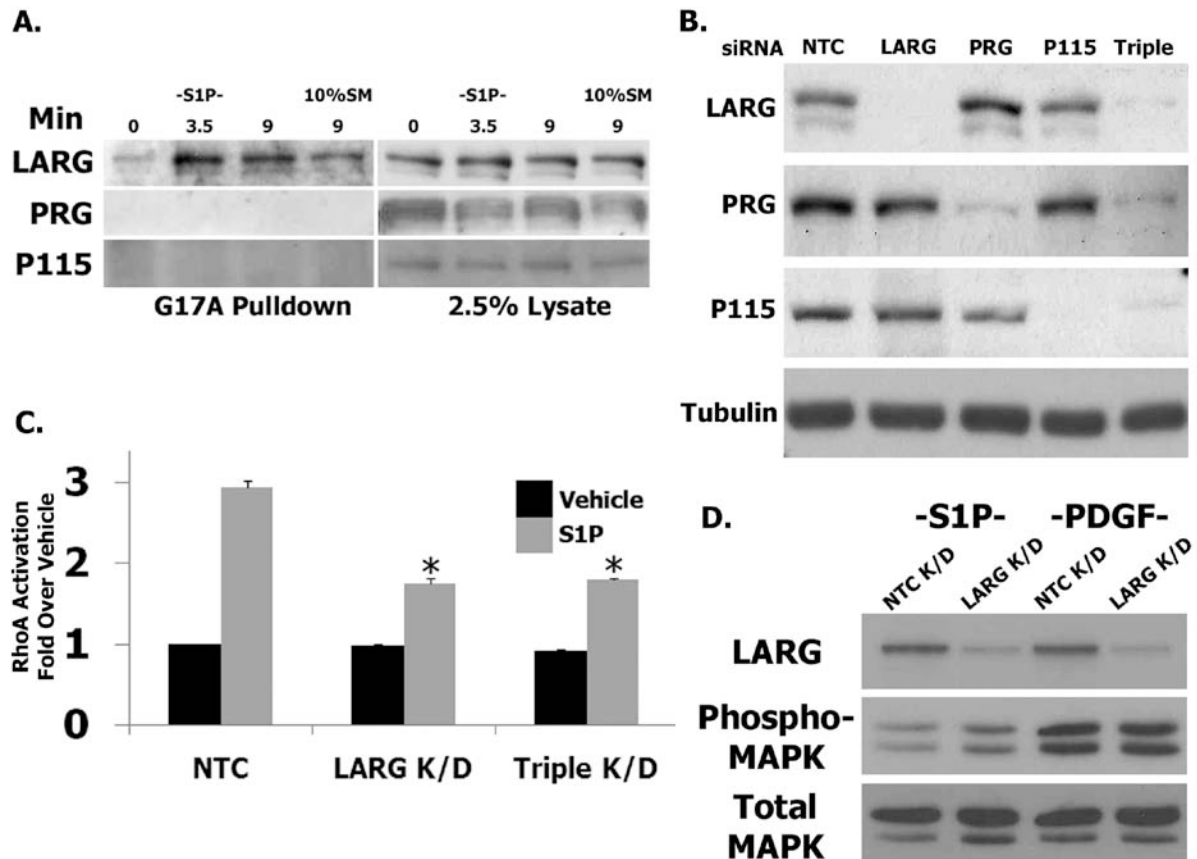


Figure 2.4. S1P induced RhoA activation in SMC was mediated by LARG. **A.** SMCs were starved for 24 hours and then treated with S1P or 10% serum for 0, 3.5, or 9 min. Cell lysates were incubated with GST-Rho(G17A) coated beads and precipitates were analyzed for the presence of the indicated RGS-RhoGEF by Western Blot. The amount of RGS-RhoGEF protein present in cell lysates is presented at right. **B.** Western Blot demonstrating significant knockdown of all three RGS-RhoGEFs using our siRNA transfection protocol. (NTC = Non-Targeted Control siRNA) **C.** RhoA activation was measured in control, LARG, and triple knockdown cells using the G-LISA™ assay (Cytoskeleton) and is expressed relative to vehicle-treated cells. At least 90% RGS-RhoGEF knockdown was ensured by Western Blot in all experiments (not shown). * p < 0.05 vs S1P-treated NTC. **D.** Western blot of Control and LARG knockdown SMCs serum starved for 24 hours, treated for 12.5 min, and probed for phosphorylated and total MAPK.

activity assays, single knockdowns of PRG or P115 had no effect on S1P-dependent SMC differentiation marker gene expression (data not shown). Taken together these results strongly indicate that S1P activates RhoA in SMC by activating LARG and that this signaling mechanism promotes SMC differentiation marker gene expression.

LARG inhibited SMC migration

Phenotypically modulated SMC exhibit increased migration, and it is well known that RhoA plays an important, yet complicated role in the regulation of this process. RhoA stimulates the formation of the stress fibers and focal adhesion complexes that promote firm cell adhesion. However, RhoA also regulates actin-myosin based contractility that is required for trailing edge retraction, and RhoA activity has been detected at the leading edge where it likely contributes to membrane protrusion by stimulating linear actin polymerization (see [136] for review). The mechanisms that regulate this shifting balance between cellular adhesion and cell movement are incompletely understood, and studies have shown that RhoA can inhibit or promote cell migration depending upon cell context [137-139]. Given our demonstration that LARG promoted the differentiated SMC phenotype, we hypothesized that it may also inhibit SMC migration. To test this directly, we measured serum-induced migration in control and LARG knock-down SMC using a transwell assay. As shown in figure 2.6, migration was significantly increased in LARG knock-down SMC cells suggesting that LARG is a limiting factor in this assay. We also assessed the effects of LARG on SMC migration in a scratch

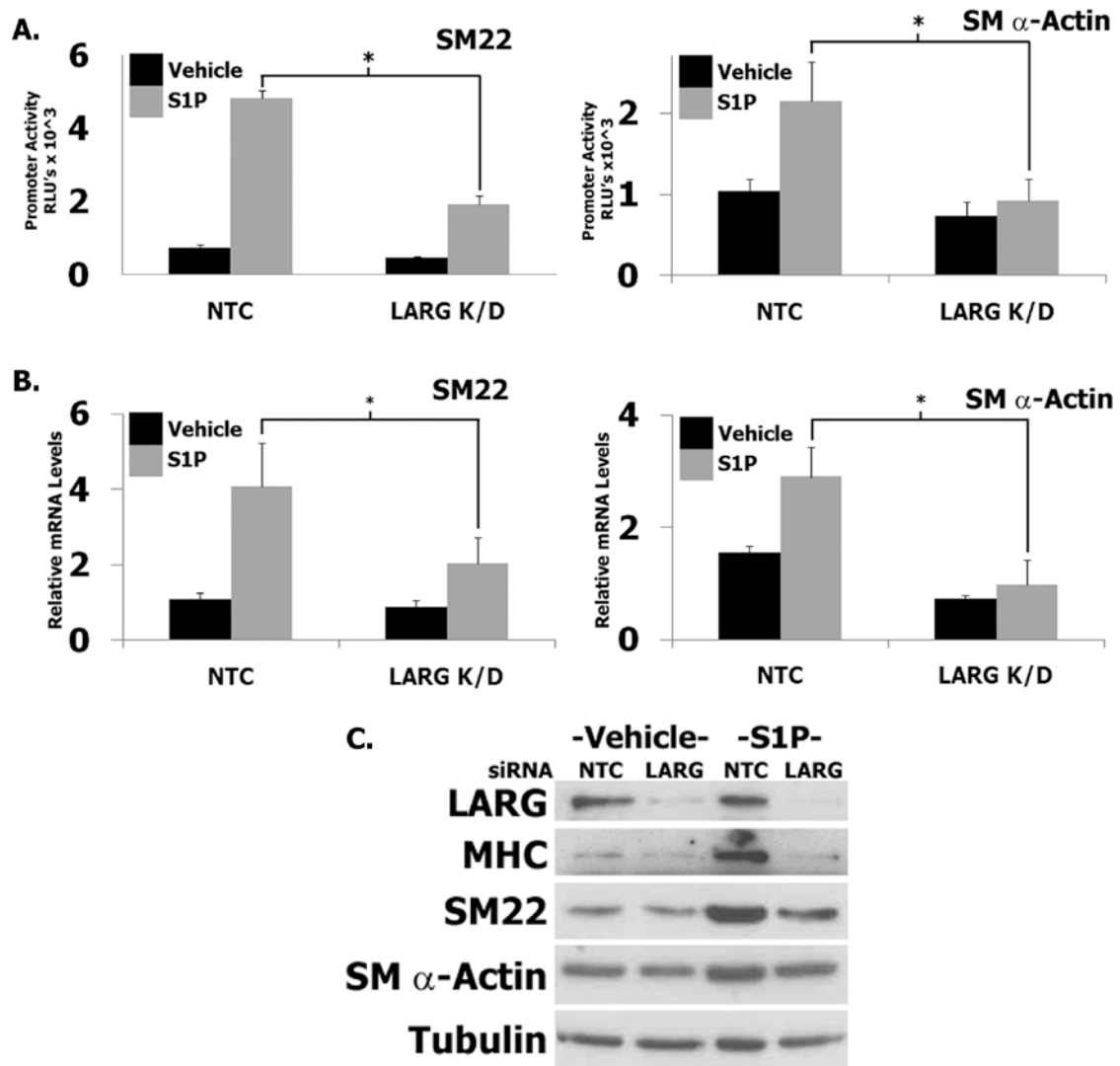


Figure 2.5. Knockdown of LARG inhibited S1P-induced up-regulation of SMC differentiation marker gene expression. **A.** Control and LARG knockdown SMC were transfected with SM22 or SM α -actin specific promoter luciferase constructs and then serum-starved for 24 hours. Luciferase activity was measured after 8h of S1P treatment. LARG knockdown was confirmed by Western Blot in all experiments (not shown). * p<0.05 vs. S1P-treated NTC. **B and C.** Control and LARG knockdown SMC were serum-starved for 24 hours and then treated with S1P for an additional 24 h. Expression of the indicated SMC differentiation marker genes was measured by quantitative RT PCR (B) and Western Blotting (C).

wound assay using live cell imaging. As shown in figure 2.6c, wound closure by SMC transfected with control siRNA was much slower than that in LARG knock-down SMC which was virtually complete by 9h. Since wound closure can also be affected by changes in cell proliferation we used BrdU incorporation assays to determine cell proliferation indices in control and LARG knock-down SMC (see Figure 2.7). As shown in figure 6b knock-down of LARG did not affect SMC proliferation strongly suggesting that the effects of LARG observed in the scratch wound model were due to decreased SMC migration.

Discussion

A growing body of evidence indicates that S1P regulates vascular function by controlling the growth, migration, contraction, and cell-cell interactions of endothelial cells and SMC (see [140] for review). The identification of the S1P-dependent signaling mechanisms involved has been complicated by the expression of multiple S1P receptors in the vessel wall and the promiscuous coupling of those receptors to G-proteins that have dramatically different effects on cell function. We were the first to demonstrate that S1P increased SMC differentiation in a RhoA dependent manner [120], and the current study supports previous studies implicating S1PR2 in this response [104, 113]. More importantly, we show that S1PR2-dependent activation of RhoA in SMC is mediated by LARG and that this RGS RhoGEF is a key component in the control of SMC phenotype. The identification of this signaling mechanism provides significant insight into the control of SMC function and into the vascular/SMC phenotypes observed in a variety of knockout models

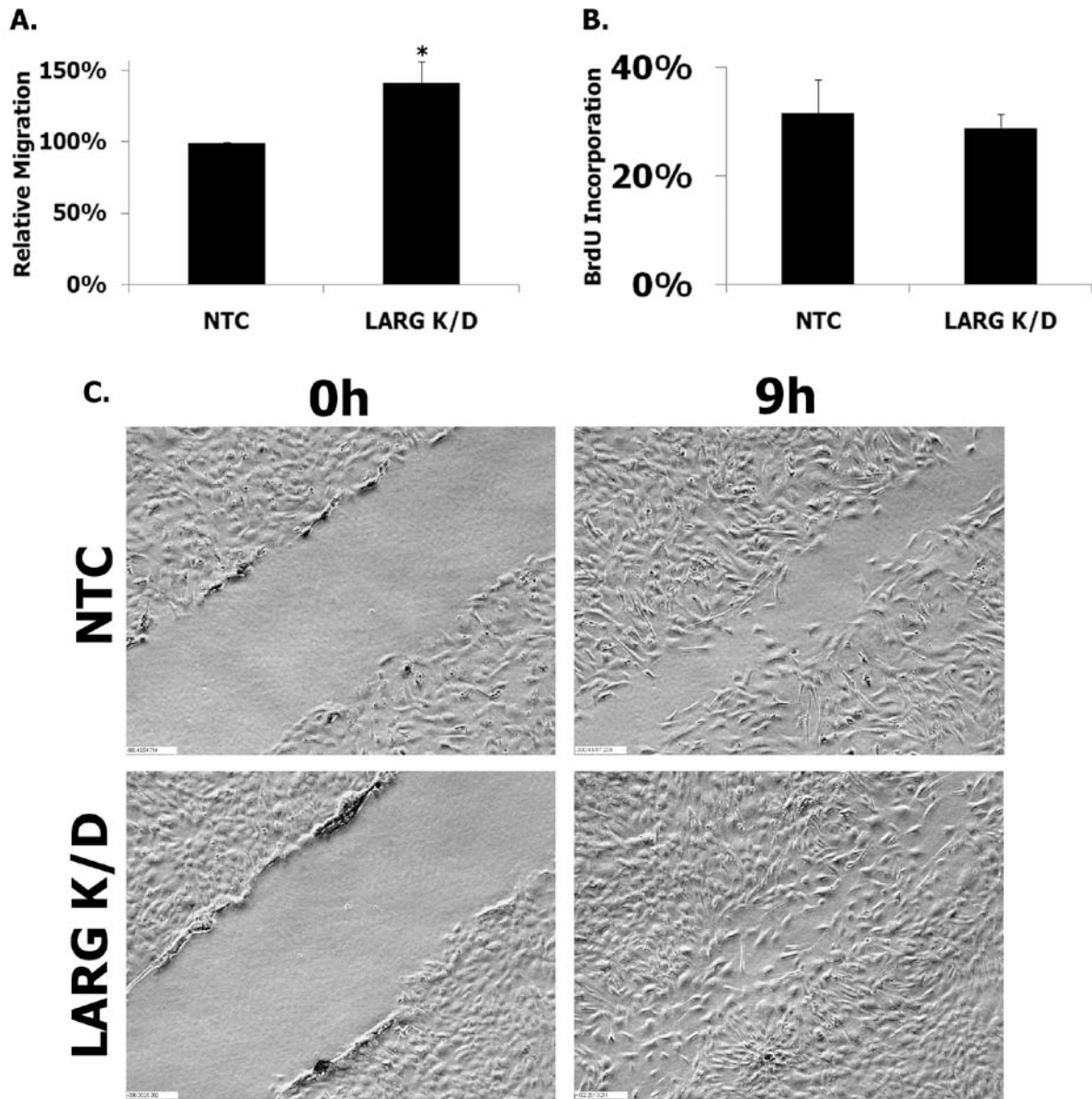


Figure 2.6. Knockdown of LARG increased SMC migration. **A.** Equal numbers of Control and LARG knockdown cells were plated on fibronectin coated transwell inserts and were allowed to migrate toward 10% serum-containing media for 18 h. Following fixation the number of cells that had migrated to the bottom chamber were visualized by crystal violet staining. SMC migration was evaluated in 3 separate experiments and is expressed relative to migration of control cells set to 1. * $p < 0.05$ versus control. **B.** Control and LARG knockdown SMC were treated with BrdU for 18 h prior to fixation. The percentage of BrdU-containing nuclei in each group was determined by immunohistochemistry. **C.** Confluent cultures of Control and LARG knockdown SMC were scraped with a P200 tip and then placed on an inverted microscope equipped with a heated, humidified, and O_2/CO_2 perfused stage. Pictures taken every 3.5 minutes for 9 h were assembled into movies using Quicktime (0h and 9h frames shown).

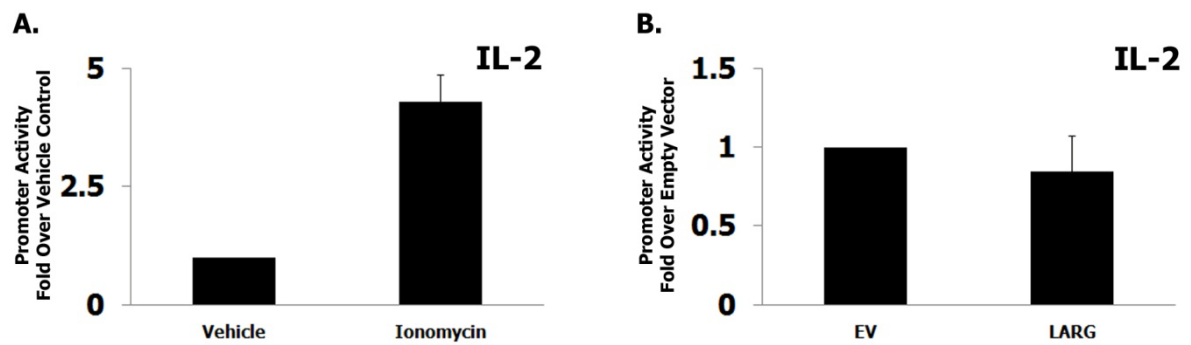


Figure 2.7. LARG over-expression has no effect on IL-2 promoter activity. **A.** SMCs were transfected with control and IL-2 luciferase fused promoter construct. After 24 hours cells were starved for 8 hours and treated with vehicle or Ionomycin [5 μ m]. Luciferase activity was measured 8 hours after treatment (N=3). **B.** SMCs were co-transfected with control and IL-2 luciferase fused promoter construct and empty expression vector or LARG. Luciferase activity was measured 24h after transfection (N=3).

The analysis of S1P receptor sub-type-specific knockout mouse models has yielded important information on the role of S1P receptor signaling in the vasculature. S1PR1-deficient mice die around E13.5 and have a defect in SMC investment of the dorsal aorta [94]. However, because a similar phenotype was observed in EC-specific S1PR1 knockouts [141], this effect was likely secondary to defects in EC tube maturation and not to defects in SMC differentiation per se. Vascular abnormalities were not observed in S1PR2 deficient mice [100], but the earlier lethality and increased hemorrhage observed in S1PR1/S1PR2 and S1PR2/S1PR3 double knockouts [142] suggests that S1PR2 may have an independent role in the establishment and maintenance of a mature vasculature. Although it has been difficult to determine whether SMC differentiation during development was affected by the loss of S1PR2, Shimizu et. al. have directly implicated S1PR2 in the regulation of SMC phenotype in adult animals [103]. Using a carotid artery ligation model these authors demonstrated that S1PR2 deficient mice had larger neointimas [103] and reduced SM α -actin expression following vessel injury [104].

Our results also implicate $G_{12/13}$ in the regulation of SMC differentiation. When coupled with the branchial arch SMC defect observed in MRTF-B deficient mice [46, 47], it is intriguing to postulate that $G_{12/13}$ signaling to RhoA plays a critical role in the differentiation of cardiac neural crest cells into SMC. Interestingly, neural crest cell-specific deletion of $G_{12/13}$ signaling resulted in proximal outflow tract defects and the development of an aneurysm-like structure in the septal branch of the left coronary artery [143]. Because cell tracing analyses demonstrated that

neural crest cell migration to these structures was not impaired, these phenotypes may have resulted from defects in the differentiation/maturation of these cells into SMC. In addition, deletion of endothelin receptor A, another $G\alpha_{12/13}$ -coupled receptor that activates SMC contraction and SMC differentiation marker gene expression [144, 145], also resulted in defective outflow tract development [146].

Our demonstration that S1PR2 signals through LARG may have important implications on the control of vascular tone. Extensive evidence indicates that RhoA regulates SMC contraction by inhibiting myosin phosphatase (see [147] for review), and several in vitro studies have shown that S1P constricts vessels by a RhoA dependent mechanism [148, 149]. Adult S1PR2 deficient mice have relatively normal systemic blood pressure, but flow measurements showed decreased resistance in mesenteric and renal vascular beds especially in the presence of adrenergic stimulation [150]. It has also been postulated that the deafness observed in S1PR2 deficient mice may be due to dilation of the spiral modiolar artery that supplies blood to the inner ear [102]. LARG knock-out mice also have relatively normal blood pressure, but were shown to be less susceptible to salt-sensitive hypertension [89]. Thus, we feel that S1PR2-dependent activation of LARG could serve a critical role in blood pressure regulation and that targeting this pathway could be beneficial in the treatment of hypertension. S1P also affects vascular tone by increasing in intracellular calcium, an effect most likely mediated by S1PR3 [100]. Interestingly, recent studies have demonstrated that calcium may also be important for SMC differentiation marker gene expression and that cross-talk between calcium and RhoA may be involved [113, 126]. In our model over-expression of LARG in

SMC did not activate the calcium/calcineurin-dependent IL-2 promoter suggesting that LARG does not significantly activate calcium signaling (see Figure 2.7). However, it is possible that these two pathways act in parallel to regulate SMC-specific transcription or that they intersect further downstream.

The residual S1P-dependent RhoA activity observed in LARG deficient SMC could be due to incomplete knockdown of LARG expression but could also reflect the contributions of S1PR3 coupling to $G\alpha_q$. $G\alpha_q$ has been shown to activate RhoA and SRF-dependent transcription through a separate family of RhoGEFs that includes Trio, Duet, and p63RhoGEF [151]. In support of this idea, Ishii et al. detected residual S1P-dependent RhoA activity in S1PR2 deficient fibroblasts but no S1P-dependent RhoA activity in fibroblasts isolated from S1PR2/S1PR3 double knockouts [100]. The increased lethality and hemorrhage observed in the S1PR2/S1PR3 double knockout mice also supports this concept [142]. Surprisingly, G_q did not stimulate SMC-specific transcription in SMCs, but it is possible that these RhoGEFs are not highly expressed in these cells. Compensatory RhoA activation may also explain the lack of a significant SMC phenotype in S1PR2 and LARG knockout mice during development and why SMC phenotypes have been revealed only under conditions of vascular stress (i.e. artery ligation or salt-induced hypertension).

Although all three RGS-RhoGEFs are expressed in SMC and are sufficient to increase actin polymerization and EGFP-MRTF-A localization when over-expressed in 10T1/2 cells, we detected little to no RGS-RhoGEF activity in serum-starved SMC, and only LARG was activated by S1P. The latter result supports previous studies

demonstrating that specific $G\alpha_{12/13}$ -coupled agonists activate specific RGS-RhoGEFs. For example, activation of RhoA by thrombin in PC-3 prostate cancer cells was primarily mediated by LARG [152] while activation of RhoA by lysophosphatidic acid in HEK293 cells was mediated by PRG [153]. Although the molecular interactions within the agonist-receptor- $G\alpha_{12/13}$ complex that mediate this specificity are currently unknown, agonist-specific RGS-RhoGEF activation could provide an additional level of control over RhoA activity and could help integrate multiple RhoA-dependent signals. Further supporting differential activation of the RGS-RhoGEFs, Guilluy et. al. recently demonstrated in SMC (using the G17ARhoA pull down assay) that angiotensin II treatment specifically activated P115 [154]. These authors also described a novel mechanism of P115 activation that was mediated by JAK2-dependent tyrosine phosphorylation. In our experiments P115 did not activate SMC-specific promoter activity as strongly as LARG or PRG but did result in increased actin polymerization and MRTF-A nuclear localization. These data suggest that additional signals might be required to fully activate MRTF-A dependent transcription or that the RGS-RhoGEFs might act on separate pools of RhoA that differentially affect MRTF-A activity. Interestingly, qualitative assessment of P115-expressing cells revealed a more cortical pattern of actin polymerization. P115 lacks the PDZ domain present in LARG and PRG, but whether this difference significantly affects P115 activation, function, and/or localization has not been directly tested in our model.

Our demonstration that LARG decreases SMC migration provides mechanistic insight into the control of cell migration by RhoA and helps explain the

anti-migratory effects of S1PR2-dependent signaling observed in several of other models [155, 156]. Importantly, our results are in excellent agreement with the previous demonstration that S1PR2 deficient SMC exhibit increased migration [103], and strongly suggest that this effect is due, at least in part, to decreased LARG activity. Ong et al. have recently shown that expression of LARG in breast and colorectal cancer cells markedly inhibited migration [157] providing further evidence that LARG inhibits this process. The precise mechanisms for the anti-migratory effects of LARG are not completely understood but may involve increased cell adhesion. For example, Dubash et.al. have shown that siRNA depletion of LARG decreases stress fiber and focal adhesion formation [158]. Clearly, additional studies will be necessary to identify the spatial and temporal patterns of RhoA activation that are necessary for cell migration and to determine whether LARG (and other RhoGEFs) activate RhoA within specific cellular compartments.

Chapter 3

The RGS RhoGEFs LARG and P115 Regulate Dorsal Ruffle Formation in Vascular Smooth Muscle Cells

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Abstract

Actin based cytoskeletal rearrangements regulate a multitude of cellular physiological responses and signaling events. Dorsal ruffles establish polarity and initiate migration transitioning cells from a static to motile state. Despite the importance of these events the molecular regulation of these structures has remained elusive. While studying the RhoA mediated transcriptional response of SMCs we serendipitously discovered the presence of two RGS RhoGEFs, LARG, and P115, in dorsal ruffles. We used immunofluorescence and Laser scanning confocal microscopy to confirm co-localization of multiple dorsal ruffle markers with LARG and P115 in PDGF-bb-induced SMC dorsal ruffles. Endogenous depletion by siRNA mediated knockdown revealed a differential effect on the rate of dorsal ruffle formation. In comparison to control knockdown cells LARG depletion inhibited dorsal ruffle formation while P115 depletion increased the occurrence of dorsal ruffles.

Since dorsal ruffles are known to regulate motility we studied the contribution of LARG and P115 to the regulation of directional migration as well as wound healing. Again we observed differential effects compared to control cells with LARG depletion increasing migration and P115 depletion inhibiting migration possibly due to polarity defects. We hypothesize that the difference in signaling is a result of preferential interaction of downstream signaling proteins with one of these GEFS. The mDia formin is a well known actin regulator and RhoA effector. We show that LARG interacts with mDia in SMC and that both mDia 1 and 2 localize to dorsal ruffles. Understanding the molecular regulation of dorsal ruffles will provide insight

to the establishment of polarity and initiation of migration in SMC and by extension the processes that govern vascular development and the pathogenesis of CVD.

Introduction

Actin based structures regulate cell shape, adhesion, contractility, proliferation, and motility. Understanding the physiologic role, structure, and molecular composition of these structures is the key to understanding the mechanisms that drive their functional specificity. Traditionally our understanding of these actin based structures has been that stress fibers regulate cell contractility, filopodia direct growth and initiate adhesion, and lamellipodia form the leading edge. Furthermore it has been understood that RhoA, cdc42, and Rac1 regulate these processes respectively. Dorsal ruffles represent a fourth, unique, cytoskeletal structure whose impact on cell behavior is equally profound, but in comparison to other actin based structures the regulation of dorsal ruffles is poorly understood. What is now becoming evident is that the small GTPases of the Rho family often act in concert, with a balance of additive and opposing effects on one another, and that regulation of cytoskeletal events ultimately depends upon the spatiotemporal juxtaposition of Rho family GTPase activity and a host of interacting and scaffolding proteins.

Stress fibers, lamellipodia, filopodia, and dorsal ruffles are all actin based structures that carry out different roles in cellular physiology. The Rho family of small GTPases are well known regulators of these structures. Stress fibers are regulated by the small GTPase RhoA. The physiological role of stress fibers is to

anchor within cells and regulate cell adhesion by allowing myosin motors to generate force during contraction. This force can be used to power migration or to conduct mechanotransduction [159]. Stress fibers are higher order structures composed of polymerized and bundled actin filaments, crosslinking proteins i.e. filamin, α -actinin, and myosin II motors.

Lamellipodia formation is regulated by Rac1 signaling. However, in these structures the actin takes the form of multi nucleated webs or networks constantly turning over driving a wide meshwork outward in a thin membrane bound sheet of cytoplasm. Globular actin is quickly converted into thinner polymerized actin filaments that are often nucleated to create multiple branch points generating an expanding network. As quickly as nucleation drives branched actin polymerization, other proteins regulate the disassembly at the rear of these actin structures increasing actin turnover providing additional g-actin to drive meshwork expansion. The Arp2/3 complex and associated scaffolding proteins like WAVE and WASP drive leading edge protrusion while capping proteins as well as gelsolin or ADF/cofilin are also important in the posterior disassembly of actin filaments [160, 161].

Filopodia formation is driven by the small GTPase cdc42. These structures function as sensory organelles or as a precursor of adhesion sites and stress fiber formation [162-165]. Filopodia, also known as microspikes, play an important role in neurite outgrowth, wound healing, and cell migration. Structurally, filopodia take the shape of actin filled membrane projections of around 100-300nm in diameter. Filopodia are composed of polymerized actin strands and crosslinking proteins like fimbrin. Other proteins found in filopodia promote un-branched elongation.

ENA/VASPs for instance have anti capping activity while formins like Dia1/2 increase processive barbed-end nucleation and elongation [166-168].

A major goal of this work was to characterize sub cellular activation of RhoA in SMC. As a first step we used immunofluorescence to determine the effects of agonist stimulation on Leukemia Associated Rho GEF (LARG) localization. Serum treatment at the shortest time points revealed localization of LARG at circular structures forming on the cells. Realizing that the structures resembled dorsal ruffles, time courses were shortened to the 5-20 minute range, PDGF-bb was used as an agonist, and a number of other proteins known to localize to dorsal ruffles were probed for, concurrent with LARG and P115.

The function of dorsal ruffles is not completely clear. The term dorsal ruffle was first used in 1983 to describe the dynamic actin based structures that occurred in glial cells as a response to PDGF-bb treatment [169]. Since then these structures have been observed in a number of cell types including fibroblasts, endothelial cells, hippocampal neurons, lymphocytes, tumor cells, and vascular smooth muscle cells, indicating structural relevance in a number of physiological contexts [169-175].

Visually the protrusion of a dorsal ruffle is reminiscent of the linear, unbranched, bundled formations that compose filopodia or even stress fibers. Dorsal ruffles form on the dorsal surface of a cell extending upward and are likely to function in the degradation of stress fibers both establishing polarity and initiating conversion of cells from a static to motile state. The upward push and eventual internalization of dorsal ruffles results in detachment of adhesions to the extracellular matrix.

Moreover stress fiber disassembly provides a surplus of unbundled cortical actin

useful in lamellipod extension [172, 176]. When this surplus of G-actin is localized to one side of the cell, polarity is established and lamellipodia formation is directed at the dorsal ruffle forming edge of the cell [177, 178]. The evidence of dorsal ruffle induced polarity was best described by McNiven et al. who show with time lapse photography that as dorsal ruffles constrict to a reduced diameter there is a concomitant protrusion at the cell periphery where the ruffle disappeared that becomes smooth and polarized forming a single large lamellipodia [172].

Additionally receptor internalization, plasma membrane recycling, and macropinocytosis have been proposed as secondary functions to occur during dorsal ruffle resolution[171, 179-182]. Despite their appearance the composition of dorsal ruffles is similar to that of lamellipodia as both of these structures include Arp2/3, Wave/WASP, and gelsolin. The components are not identical however as Rab5 as well as Rac1 has been shown to regulate dorsal ruffle formation [183]. Two interesting proteins unique to dorsal ruffles are the large G proteins $G\alpha_{12}$ and $G\alpha_{13}$. It has been shown that $G\alpha_{12}$ and $G\alpha_{13}$ both co-localize to dorsal ruffles. These authors show that fibroblast cells that are deficient in $G\alpha_{12}$ and/or $G\alpha_{13}$ show similar ability to form dorsal ruffles upon PDGF treatment but that the dorsal ruffles in the $G\alpha_{12/13}$ null cells took much longer to disassemble. The authors conclude that $G\alpha_{12/13}$ accelerate dorsal ruffle turnover. The proposed mechanism the authors put forth, based on Rac1 FRET analysis in these $G\alpha_{12/13}$ null cells, is that the G proteins control dorsal ruffle disassembly by shortening the duration of Rac1 signaling, though they stop short of explaining how [184].

Dorsal ruffles differ from other actin based cytoskeletal structures in a few ways. Unlike lamellipodia or membrane waves which are very transient and reform quickly, a dorsal ruffle will form only once. Unlike podosomes or invadosomes (other circular actin based cytoskeletal rearrangements) which are MMP rich and function to anchor or invade a matrix, dorsal ruffles form on the dorsal surface of a cell extending upward and do not assist in invasion or anchorage. Dorsal ruffles are the earliest cytoskeletal rearrangements to occur in a cell and assemble within minutes upon agonist stimulation with dorsal ruffles lasting for 5 to 20 minutes [185]. Dorsal ruffles form in response to a number of receptor-tyrosine-kinase growth factors, most notably in response to platelet-derived growth factor (PDGF) but also form in response to TPA and neomycin [170, 186].

Though dorsal ruffles are purportedly mediated in a Rac1 dependent manner, multiple Rho family GTPases have been observed in dorsal ruffles including Rab5, Rac, Ras, and Arf6 [183, 187]. In fact, Wang et al. showed that although dominant negative Rac expression blocked PDGF-induced dorsal ruffling, constitutively active Rac1 over expression was incapable of inducing dorsal ruffles. This indicates that though Rac is required for dorsal ruffles it, alone, is not sufficient. Multiple Rho GTPase regulatory proteins have also been observed in dorsal ruffles. ACAP1/2 as well as ASAP1, each GAPs for ARf6 have been observed in circular dorsal ruffles (CDR) [187, 188]. Two Rac specific GEFs Swap70 and β -PIX have been observed in dorsal ruffles and are believed to positively regulate their formation [189, 190].

Our demonstration of LARG and P115 in dorsal ruffles suggests that the spatiotemporal regulation of RhoA can further explain the complicated role that the

GTPase plays in the regulation of cell migration. RhoA was originally shown to be responsible for the formation of the stress fibers and focal adhesion complexes that regulate cell shape and adhesion [191]. However, RhoA and the RhoA effectors such as the Diaphanous proteins can mediate actin polymerization that drives cell extension at the leading edge. RhoA activity is also required for retraction of the trailing edge of cells during directional movement because it regulates actin-myosin based contractility [192]. The mechanisms that regulate this shifting balance between cellular adhesion and cell movement are not well known understood but probably involve localized activation of each of the small GTPases. Interestingly, some studies have shown that expression of a dominant negative RhoA attenuated membrane ruffling and lamellipodia formation, focal complexes/adhesion turn-over, and cell migration [139], while others have shown that RhoA activity correlates with the strength of cell adhesion to the ECM and that decreased Rho activity is permissive for increased migration [138]. Clearly it will be critical to identify the mechanisms that regulate RhoA's many different functions during the process of migration.

In addition to the spatiotemporal regulation of RhoA there is increasing evidence that significant cross talk exists between many of the Rho family GTPases and that Rac1 and RhoA specifically have extensive regulatory effects on one another [193-198]. Indeed in other Rac mediated cytoskeletal rearrangements RhoA has been shown to play an important role, Cascone et al. have shown that Tie-2 (a tyrosine kinase receptor)-mediated shape change requires cooperative activation of both RhoA and Rac1[199]. Also Sepp et al. have shown that both RhoA and Rac1

have distinct but interdependent roles in glial cell migration and nerve ensheathment [200]. These examples of RhoA/Rac1 cross talk have been proven relevant in the regulation of actin based cytoskeletal structures but the mechanisms that govern their antagonism have not been investigated.

The goal of this study is to understand the role that the RGS RhoGEFs play in the regulation of dorsal ruffle formation as well as migration and to determine the mechanism or proteins involved in differentially regulating LARG and P115 associated dorsal ruffles. We hypothesize that spatiotemporal regulation of RhoA activation, possibly through a Rac1 antagonism mechanism elicits the increase in dorsal ruffle formation observed in P115 KD cells and that LARG mediated actin polymerization localized by interaction with mDia1/2 explains the decrease in dorsal ruffle formation observed in LARG KD cells.

Materials and Methods

Plasmids and Reagents- LARG, PRG, and P115 cDNAs were kind gifts from Dr. T. Kozasa (University of Tokyo). All cDNAs were sub-cloned into pcDNA 3.1.

Antibodies to LARG and PRG were kind gifts of Dr. Keith Burridge (University of North Carolina, Chapel Hill, NC). Primary antibodies for endogenous detection include P115RhoGEF (SantaCruz sc-20804), vinculin (Sigma V4505), cortactin (Millipore 4F11), mDia1(Santa Cruz sc-10886), and mDia2 (Santa Cruz sc-10894). Alexa Fluor 555 or 488 (Molecular Probes) secondary antibodies and DAPI (Molecular Probes) were used at 1:2000 and 1nM, respectively.

Cell Culture, Transfections, and Reporter Assays- The 10T1/2 and SMC cell cultures, transient transfections, and promoter luciferase assays have been previously described [44, 120].

Immunofluorescence - SMCs were plated and transfected in 2 or 4-well chamber slides, maintained in 10% serum overnight. Cells were serum-starved for at least 16 hours and treated with PDGF-bb for 7 or 15 min, fixed in 3.7% paraformaldehyde/PBS for 20 min and permeabilized in 0.5% Triton X-100/PBS for 3-4 min. Transfected cells were incubated with M2 anti-flag (1:500)(Sigma) or LARG or P115 antibodies in 20% Goat Serum / 3% BSA in PBS for 2 hours

Immunoprecipitation- Cultured SMC lysates were purified and pre-cleared with PAS beads. 1.2mg of lysates were then incubated with 10ul of anti-LARG for 2 hours. PAS beads were added to mix for 2 hours prior to washes and westerns were performed.

siRNA knockdowns - All siRNA oligos were designed using Invitrogen algorithms, and purchased from, Invitrogen. Transfection of 21NT siRNA oligos was accomplished with Dharmafect transfection reagent as per protocol. A cocktail of two separate siRNA oligos was used for each GEF.

siRNA KD- RGS RhoGEF KD Sequences 5'→3'

LARG1-AAACCAAUGUAUAGAGCUTT

LARG2-CCCACUUGCUGAUUCUGAATT

P1151-AUCUUCUGAGUUCGCCUCCTT

P1152-UUAGAUGUCGACAAUCUGGTT

Transwell Assays- In brief SMCs were transfected with control LARG pr P115 specific siRNA. After 72 hours of KD cells were split, counted, and equalized. Three thousand cells were plated on the tops of fibronectin coated transwells (8µm pore size) in a 24 well format. Cells were allowed to migrate toward DMEM F-12 + 10% serum in the bottom chamber for 18 hours prior to processing. Chambers were rinsed with PBS, cells were removed from the top chamber by scraping with a cotton swab, and the remaining cells (in the bottom chamber) were fixed in 4% paraformaldehyde for 20 min. Cells that had migrated to the bottom chamber were visualized by crystal violet staining for 4 hours. Data represent the total number of cells in 12 separate fields for each condition through three separate experiments.

Migration Assays - For wound healing assays, confluent cultures of control and LARG knockdown SMC were scraped with a P200 tip and then placed on an inverted microscope equipped with a heated, humidified, and O₂/CO₂ perfused stage. Pictures taken every 3.5 minutes for 15 hours were assembled into movies using Quicktime. Individual frames were isolated from quicktime movies and analyzed by image J software to calculate the wound area at time=0 and time=15hours. The area at 15 hours was divided by the initial wound area. That percentage was subtracted from 1 to determine the percent wound closure. At the end of each experiment cell lysates were subjected to western blot to ensure LARG knockdown.

Wound Healing Assay Equipment Specifics- Equipment and instruction was provided by Dr. C. Robert Bagnell, Jr., Ph.D. Professor and Director of the Microscopy Services Laboratory at UNC Chapel Hill.

Olympus IX70 Live Cell Inverted Fluorescence Microscope Description: The Olympus IX70 is an inverted microscope equipped for bright field, phase contrast, and epifluorescence. It is enclosed within an environmental chamber capable of controlling both temperature and relative humidity. It is equipped with a Uniblitz shutter on the transmitted light system and a Ludl stepper motor filter/shutter controller on the epifluorescence system, a BioPrecision motorized stage with linear encoders and motorized focus with servo feedback all controlled by a LEP MAC5000 controller. Image capture is by a Hamatsu ORCA CCD camera. The system is controlled by an Apple IMAC computer using Improvision's Open Lab software. Carbon dioxide supply is provided from a 100% CO₂ tank connected to a flow meter and controlled by a precision needle valve. CO₂ is passed through a water column and delivered through tubing to custom-made tabulated lids that match various cell culture dish configurations.

Confocal Laser Scanning Microscopy- Cell preparation and Immunofluorescence procedures were identical to those previously outlined. A Carl Zeiss Pascal Laser scanning confocal scope was used to obtain images resolved to 0.46 μ m in the z-axis. Scanning was delayed between 488 (FITC) and 555(Rhodamine) channels to ensure no residual signal between scans. Zeiss LSM Image browser version 4.2.0.121 was used to process images generated and conduct overlay analysis.

Results

Development of IF Methods for endogenous RGS RhoGEF Localization

RGS RhoGEF localization is likely important in regulating RhoA mediated cytoskeletal structures. To comprehensively examine the endogenous localization of these GEFS we needed to optimize our immunofluorescence procedures for compatibility with endogenous GEF antibodies. The LARG and P115 antibodies used in these studies have not been thoroughly tested for use in immunofluorescence based applications. We first tested the specificity of these antibodies in our immunofluorescence protocols. In figure 3.1a, we transfected 10T½ cells with flag-tagged plasmids for LARG and P115, and then probed with an anti-flag antibody and antibodies specific to LARG and P115. As you can see cells that express a flag tag have a highly elevated expression level of either LARG or P115 indicating specificity for the endogenous antibodies.

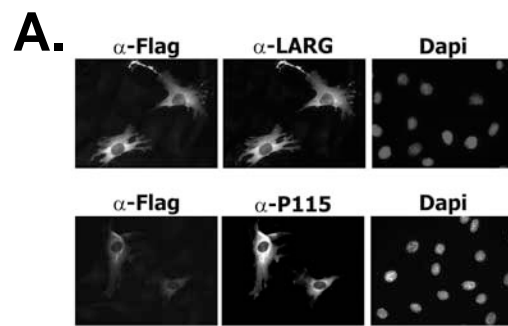
LARG and P115 localize to dorsal ruffles

In our initial examination we observed occasional RGS RhoGEF localization to circular structures after 15-20 minutes of serum stimulation. To test whether these structures were dorsal ruffles we grew primary smooth muscle cells to approximately 70% confluence, and treated the cells with PDGF-bb, a known inducer of dorsal ruffles. Cells were fixed and stained or probed for known components of dorsal ruffles including polymerized actin (via phalloidin), cortactin, and vinculin along with LARG or P115 [170, 172, 187, 201]. Using confocal laser scanning microscopy we show that both LARG and P115 co-localize phalloidin,

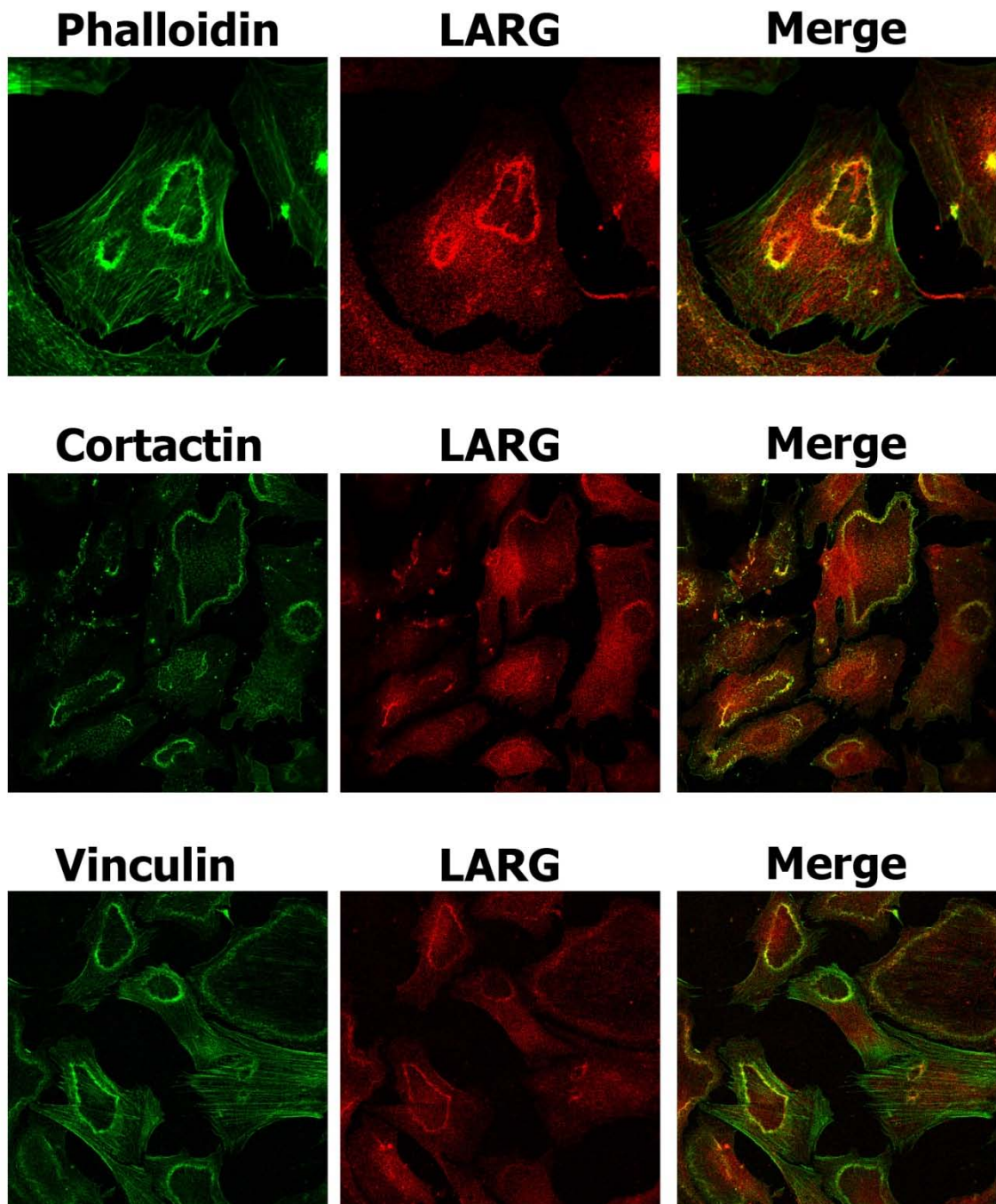
cortactin, and vinculin upon PDGF-bb induced dorsal ruffle formation (Figure 3.1b and c).

LARG and P115 depletion differentially effect dorsal ruffle formation

After discovering the presence of these GEFs in dorsal ruffles we wanted to test whether these GEFs play a role in the regulation of their formation. $G\alpha_{12}$ and $G\alpha_{13}$ have been shown to interact with these GEFs and to localize to dorsal ruffles. Functional analysis of dorsal ruffle duration in $G\alpha_{12}$ and $G\alpha_{13}$ null fibroblasts revealed that the duration of dorsal ruffles was increased indicating $G\alpha_{12}$ and $G\alpha_{13}$, when active, increase dorsal ruffle disassembly. The RGS domains of LARG and P115 are known to increase GTP hydrolysis of $G\alpha_{12/13}$ subunits inhibiting their signals to downstream effectors. Based on these data we hypothesized that depletion of the GEFs LARG and P115 would increase the ratios of GTP or activated $G\alpha_{12}$ $G\alpha_{13}$ increasing dorsal ruffle turnover. To investigate this possibility we depleted LARG and P115 in smooth muscle cells by transient transfection of siRNAs. After siRNA mediated depletion we starved primary smooth muscle cells, treated with PDGF-bb, and scored the number of dorsal ruffles in non-target control, LARG KD, and P115 KD SMCs. Our initial hypothesis was supported by the observation that the number of dorsal ruffles was reduced in the LARG KD cells but we were surprised to observe that P115 depletion increased the incidence of dorsal ruffle formation (Figure 3.2).



B.



C.

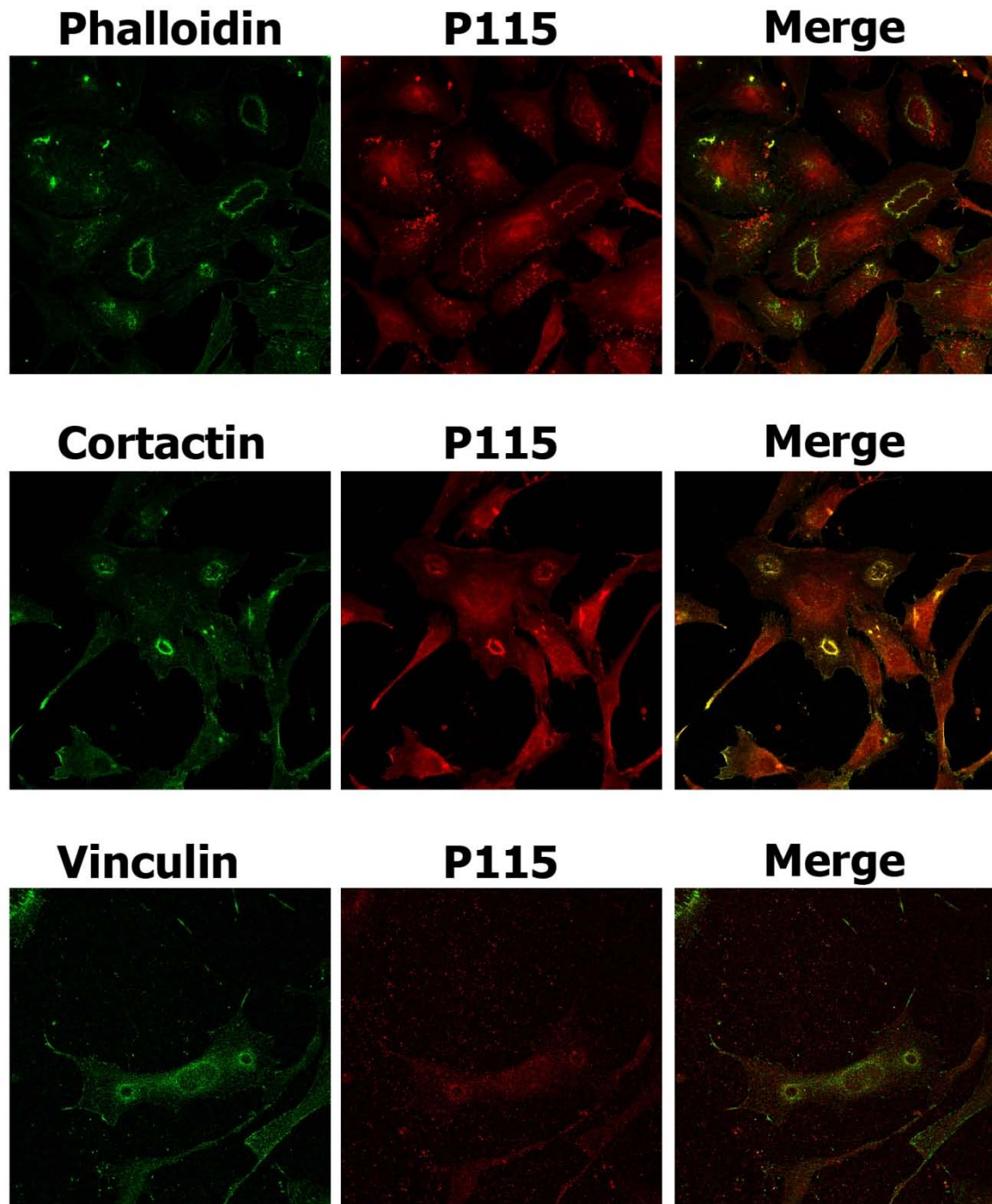


Figure 3.1. LARG and P115 localize to dorsal ruffles. **A.** SMCs were transfected with flag tagged versions of LARG and P115. Cells were probed with flag and LARG or P115 antibodies and visualized with Alexa Fluor conjugated secondary antibodies. **B.** Smooth muscle cells were grown to near confluence, starved over night, stimulated with PDGF-bb for 7 minutes and probed for LARG and indicated dorsal ruffle associated proteins. Confocal images are depicted. **C.** Cells were processed identically to those in 3.1b but probed for P115 instead.

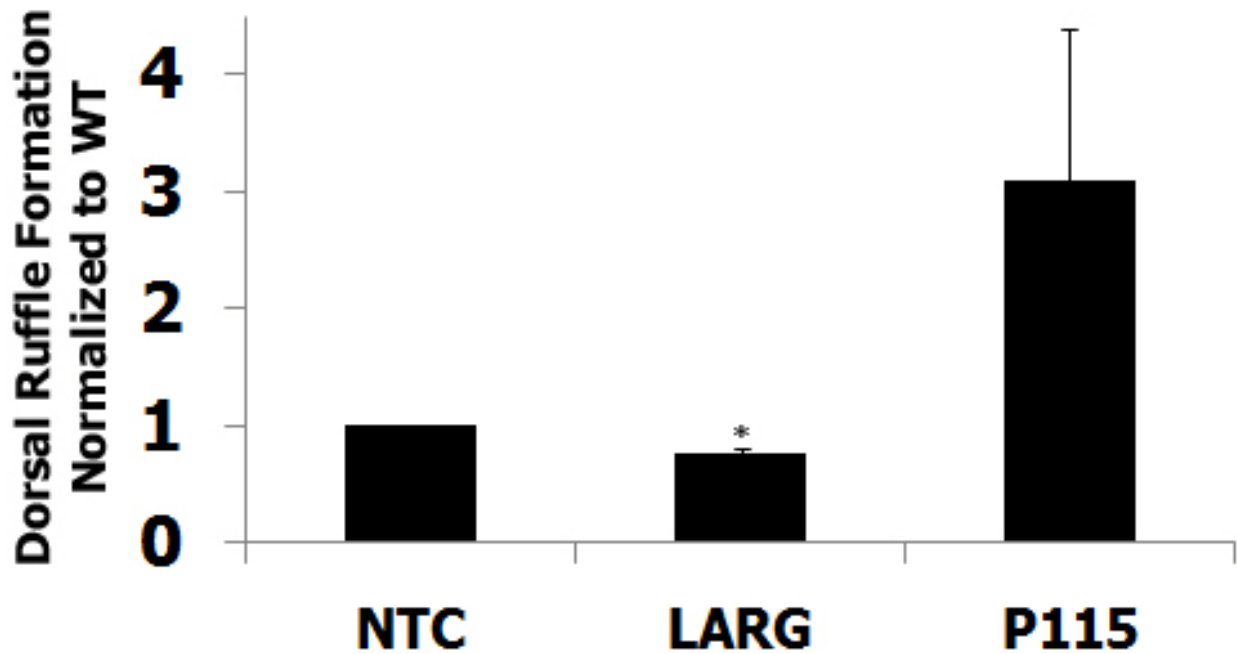


Figure 3.2. LARG and P115 knock down differentially effect dorsal ruffle formation. A. LARG, P115, and control siRNA oligos were transfected in SMCs plated on chamber slides. After 3 days cells were starved and treated with PDGF-bb for 7 minutes. Cells were fixed and stained for phalloidin. Dorsal ruffle formation was normalized to control cells. Fields were chosen by dapi stain and counts were done independently among different experiments to avoid bias. * $P < 0.05$

LARG and P115 differentially regulate SMC migration

Two of the physiological functions of dorsal ruffles are the establishment of polarity and the initiation of migration. We have previously shown that LARG depletion increases SMC migration indicating that LARG negatively regulates this process [202]. Conversely, P115 KO studies in neutrophils concluded that P115 is required for normal polarization and directional migration. To test whether depletion of LARG and P115 would differentially effect SMC migration we transfected siRNA oligos into primary SMC and measured directional migration and wound healing.

Directional migration measured by transwell assay, as shown in figure 3.3a, reveals that depletion of LARG causes a nearly 150% increase in migration whereas depletion of P115 results in an equally dramatic decrease in migration. These changes are mirrored in wound healing assays as well. Scratch wounds are induced in monolayers of control or GEF depleted SMCs and live cell imaging was done to construct time lapse movies of migration. Figure 3.3b shows frames taken at time 0 and after 15 hours of migration. Cell transfected with a non-target control show an intermediate capacity for wound closure compared to LARG KD which healed more quickly and P115 KD cells which healed comparatively slowly. Quantification of the initial wound area compared to the remaining wound area after 15 hours is shown in figure 3.3b.

LARG and mDia 1 and 2 bind and co-localize to dorsal ruffles

The RhoA effectors mDia 1 and 2 are members of the subfamily of diaphanous-related formins (DRFs) which are identified by conserved formin

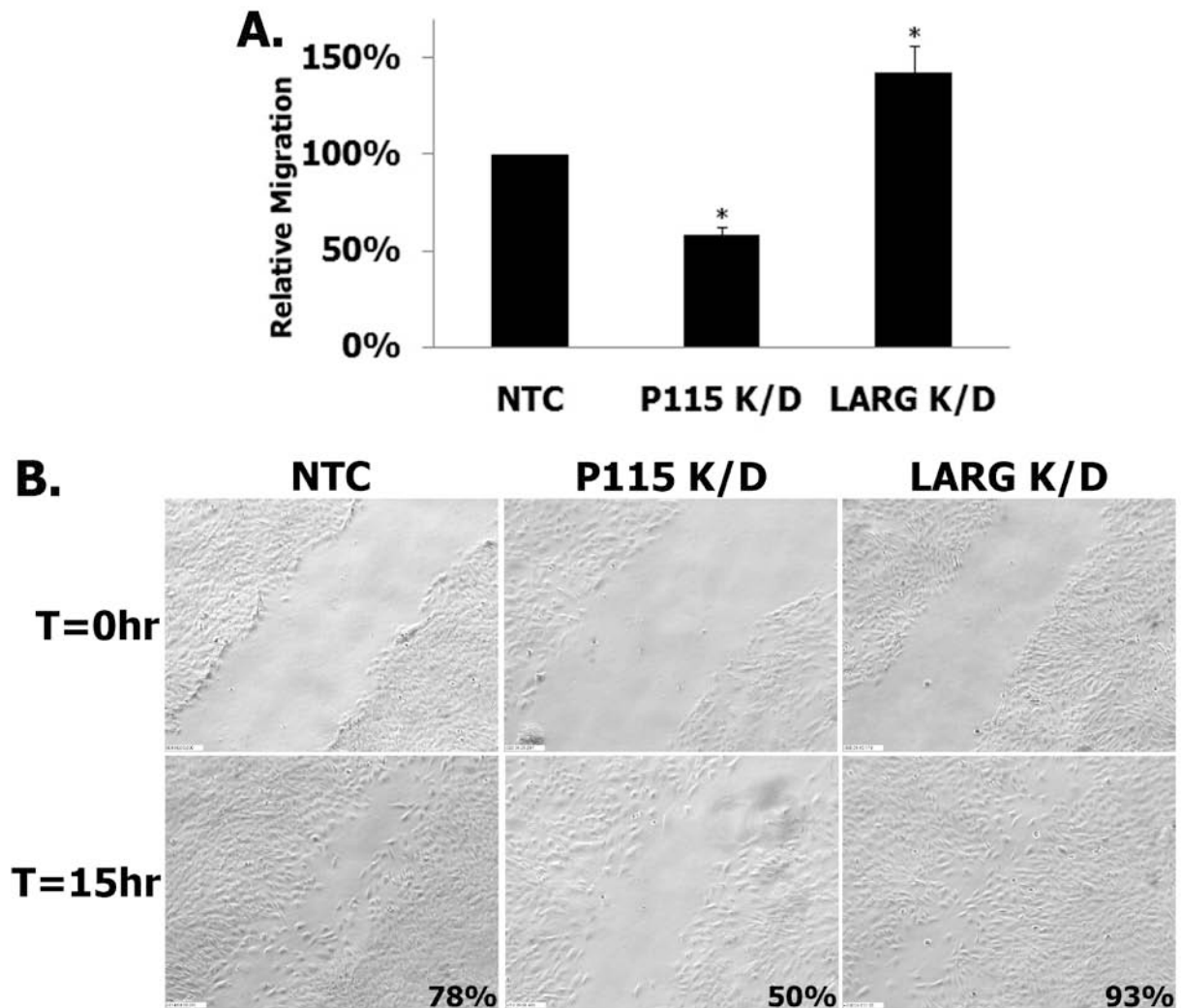


Figure 3.3. LARG and P115 Differentially Regulate SMC migration. **A.** Equal numbers of control, LARG, and P115 knockdown cells were plated on fibronectin-coated transwell inserts and were allowed to migrate toward 10% serum-containing media for 18 hours. Following fixation, the number of cells that had migrated to the bottom of the chamber were visualized by crystal violet staining. SMC migration was evaluated in three separate experiments is expressed relative to migration of control cells set to 1. * $P < 0.05$ **B.** Confluent cultures of control, P115, and LARG knockdown SMC were scraped with a P200 tip and then placed on an inverted microscope equipped with a heated, humidified, and O₂/CO₂ perfused stage. Pictures taken every 3.5 minutes for 15 hours were assembled into quicktime movies. (0 and 15 hour frames shown) Image j was used to quantify wound area in control, P115, and LARG knockdown SMCs before and after 15 hours of migration. Final area was divided by initial area and that ratio was subtracted from 1 to determine percent wound closure as indicated in the lower right corner of 15hr panels.

homology (FH) domains, a conserved GTPase binding domain (GBD) that interacts with Rho family GTPases, and a Diaphanous Auto-regulatory Domain (DAD). It has been well defined that binding of the GBD by RhoA activates Dia in SMC promoting actin polymerization [203]. mDia mediated actin polymerization is an important step in cytoskeletal regulation. For instance, mDia has been shown to increase processive barbed-end nucleation and elongation in filopodia formation.

There are four published papers that suggest a LARG/Dia interaction. One study that highlights Dia LARG interaction shows that the *Drosophila* LARG homolog dRhoGEF2 co-localizes with Dia at the furrow canal during cellularization [204]. A second study suggests a role for LARG and Dia upstream of RhoA. These authors use LPA, an agonist that stimulates $G\alpha_{12/13}$ coupled receptors to show that Dia can stimulate LARG mediated activation of RhoA creating a positive feedback loop [203]. These authors show that Dia1 binds LARG and determine the general region in which these proteins bind but stop short of determining the exact residues. Another study conducted in neutrophils showed that LARG and mDia1 co-localize at the leading edge during migration and that Dia facilitates the recruitment. They also show that this interaction is important upstream of RhoA by showing that in Dia $-/-$ cells LARG is less efficient at activating RhoA [205]. In the fourth study, Gourlimari et al suggest LARG and mDia1 link $G\alpha_{12/13}$ to cell polarity and microtubule dynamics [206]. All of these studies link Dia and LARG to either RhoA activation or actin dynamics or both but they are all limited to mDia1 and none of the studies were conducted in smooth muscle.

Previously we have shown that SMC specific transcription and RhoA activity is preferentially activated by LARG in SMC when compared to P115 but the mechanisms governing this difference were not explored. It is possible that the difference in dorsal ruffle regulation observed by LARG and P115 depletion is the result of a divergence in their individual binding partners and downstream signaling pathways. Given the possibility that these differences are achieved by RhoA effectors that regulate actin dynamic we chose to look at the mDia formins and their interaction with LARG.

To determine co-localization of mDia1 and 2 with LARG in dorsal ruffles we serum starved primary aortic SMCs, treated with PDGF-bb, and probed with LARG and mDia1 and 2 specific antibodies. In figure 3.4a we show that both mDia1 and mDia2 co-localize with LARG to dorsal ruffles. The association of LARG and mDia1 is well documented but to confirm the interaction of LARG and mDia2 we performed co-immunoprecipitations with anti-LARG and probed for mDia2. In figure 3.4b we show by IP with anti-LARG that LARG associates with mDia2.

Discussion

We have shown that the RhoA-specific GEFs LARG and P115, localize to dorsal ruffles upon PDGF stimulation. Depletion studies of LARG and P115 both had an effect on dorsal ruffle formation. The loss of LARG in SMC showed impaired ability to form dorsal ruffles whereas depletion of P115 resulted in an increased rate of dorsal ruffle formation. Transwell and wound healing assays of LARG and P115 KD SMCs also revealed differential effects. When compared to control cells LARG

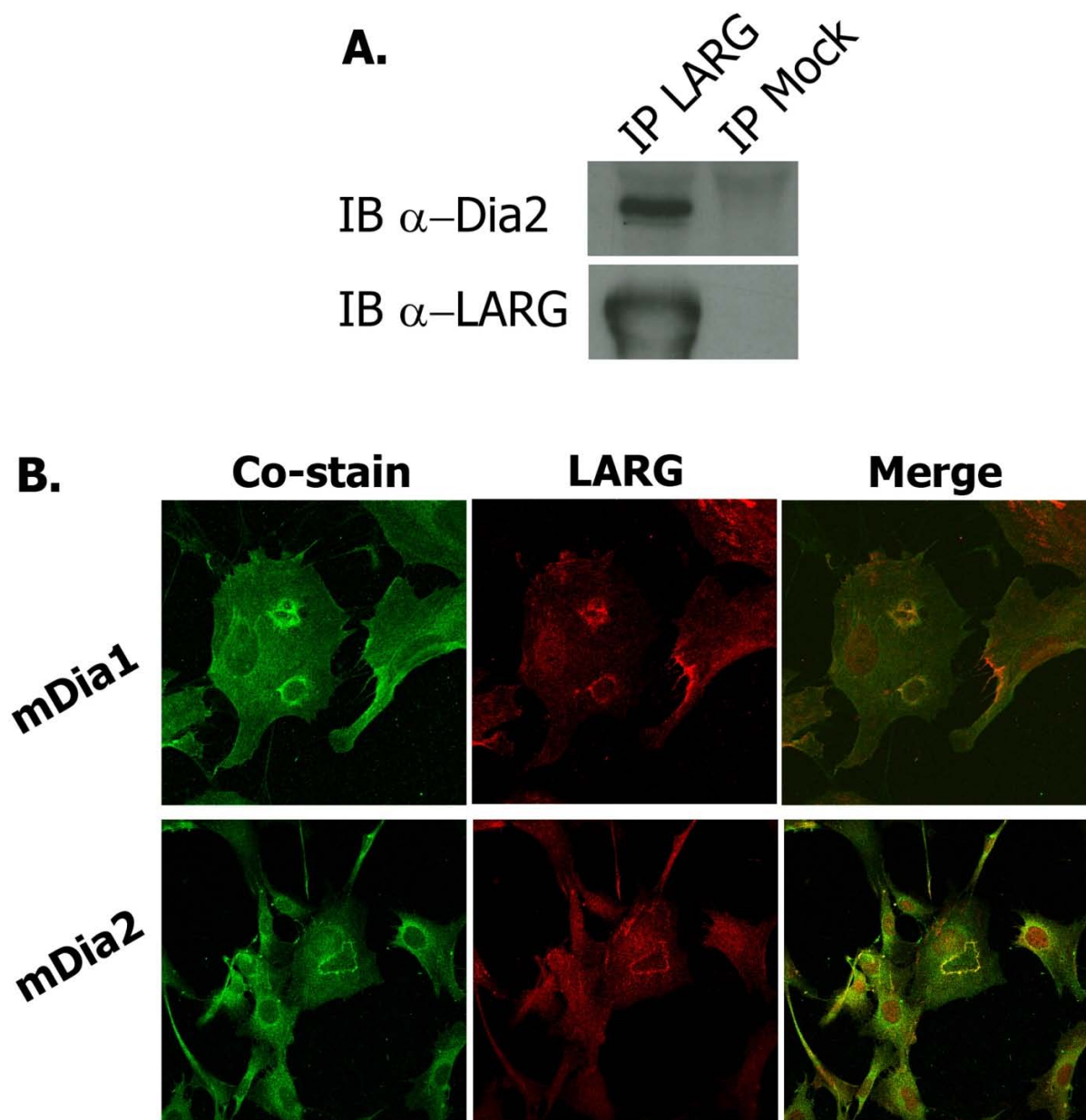


Figure 3.4. LARG and mDia 1 and 2 bind and co-localize to dorsal ruffles. A. FFSMC lysates were purified and incubated with LARG antibody and anti-rabbit conjugated PAS beads or just anti-rabbit conjugated PAS beads. Westerns were run and probed for Dia2 and LARG. **B.** Smooth muscle cells were grown to near confluence, starved over night, stimulated with PDGF-bb for 7 minutes and probed for LARG and mDia1 or 2. Confocal images are depicted.

depleted cells showed increased directional migration and wound closure, whereas P115 depleted cells showed reduced directional migration and wound healing. Furthermore we show that the actin polymerizing formin Dia binds to LARG and co-localizes to dorsal ruffles.

While identified and coined in 1983 dorsal ruffles have enjoyed relatively little scrutiny in comparison to their structural cousins stress fibers, lamellipodia, and filopodia. Despite their understudied status, dorsal ruffles, with their ability to establish polarity and initiate migration, are structures of significant physiological relevance [172, 176-178]. In this study we discovered two RhoA regulating proteins of the RGS RhoGEF family in circular dorsal ruffles. Interestingly LARG and P115 had differential effects on dorsal ruffle formation. To our knowledge this is the first report to implicate RhoA in the regulation of these structures.

Somewhat surprisingly we discovered that these highly similar proteins differentially regulate dorsal ruffle formation, a finding that mirrored LARG and P115's ability to inhibit and promote migration respectively. The most intriguing finding of this study raises the obvious question, how can two similar proteins elicit differential effects on dorsal ruffle formation? The literature reveals two proteins, known to regulate dorsal formation, which can theoretically justify positive or negative regulation of dorsal ruffles by RGS RhoGEFs. $G\alpha_{12}$ and $G\alpha_{13}$ have been shown to regulate dorsal ruffle disassembly [184]. The RGS domain found in LARG and P115 increase $G\alpha_{12/13}$ GTP hydrolysis inactivating the protein. One logical hypothesis would be that RGS RhoGEF mediated $G\alpha_{12/13}$ inactivation would increase dorsal ruffle duration/stability. Our LARG depletion data support this

hypothesis. The P115 data however is contrary to this understanding. This dorsal ruffle response in P115 KD cells can be reconciled if an imbalance exists between the $G\alpha_{12/13}$ signaling and P115 activation. For instance $G\alpha_{12}$ has been shown to preferentially bind LARG compared to $G\alpha_{13}$ [207]. If $G\alpha_{12}$ is preferentially regulating dorsal ruffle turnover in SMCs then one might expect that depletion of LARG would have a greater impact the number of dorsal ruffles observed than depletion of P115. Further studies will require investigation of differential $G\alpha_{12}$ and $G\alpha_{13}$ signaling in SMC dorsal ruffle formation.

Conversely Rac1 is a well documented as a positive regulator of dorsal ruffle formation [190, 208, 209]. There are a number of studies that suggest increased RhoA activity inhibits Rac1 signaling. Since both LARG and P115 have been shown to increase RhoA activity it would make sense that endogenous depletion of the GEF would lead to decreased RhoA activity and increase Rac1 mediated dorsal ruffle formation. Indeed it is the case that in P115 knockdown dorsal ruffle formation is increased, which would be the expected outcome if RhoA Rac1 antagonism regulated dorsal ruffle formation, however this is not the case with LARG knockdown. Again if P115 mediated RhoA activation could somehow bias increase Rac1 inactivation we could explain the differential P115 effect. A spatiotemporal explanation for the differential effect of P115 will be investigated with future studies. RhoA biosensor studies, pioneered by Klaus Hahn, allow for exquisite visualization of real time RhoA activity in migrating cells. Work from the Danuser lab asserts that in lamellipodia RhoA activation occurs in a band $2\mu\text{m}$ from the edge and that cdc42 and Rac1 act $1.8\mu\text{m}$ from the edge. Furthermore temporally RhoA activation occurs

initially and after a 40 second delay (relative to protrusion) Rac1 and cdc42 reach their peak activation [62]. It is conceivable that discrete differences in localization of LARG and P115 to established actin regulating microdomains within dorsal ruffles could account for substantial differences in RhoA activation, Rac1 antagonism, and dorsal ruffle dynamics. For instance if the localization of P115 differs from the localization of LARG in dorsal ruffles by a fraction of a micrometer it is conceivable that RhoA mediated Rac1 inactivation could be biased by the individual GEF binding.

Obviously, investigation to conclusively locate or discount the presence of RhoA in dorsal ruffles will be of critical importance to future studies. Pertz et al. based on biosensor experiments, claim that RhoA is not present in dorsal ruffles [61]. One caveat of this study is that these observations are based on over expressed RhoA in these cells. RhoA over expression has been shown to complicate physiological relevance [210]. Additionally these studies were conducted in MEFs not smooth muscle cells. Since these GEFs are selectively expressed in SMC this can only increase the possibility that SMC dorsal ruffles are unique in their composition and may contain RhoA while other cell types do not. One future experiment to determine if RhoA is contributory toward dorsal ruffle formation in SMCs would be to treat with the RhoA inhibiting compound Y-27632 prior to PDGF- β treatment. If RhoA signaling is indeed decreasing Rac1 activity and dorsal ruffle formation we would expect to see increased numbers of dorsal ruffles with Y-27632 treatment.

It is also possible that signaling proteins that interact exclusively with LARG or P115 could cause differential regulation of CDRs. Four separate publications show that LARG and the RhoA effector and actin polymerization protein mDia interact. Further bolstering the role for a small GTPase mediated employment of mDia to drive actin polymerization in cytoskeletal structures is the link between cdc42 and mDia2 in filopodia formation [211]. It is possible that selective activation of actin polymerization by LARG mediated recruitment of mDia1 and 2 could stabilize or increase the rate of dorsal ruffle formation, and that selective recruitment of mDia by LARG and not P115 could explain why a decrease in dorsal ruffle formation is observed upon endogenous depletion of LARG and not P115.

Our demonstration that the RGS RhoGEFs, LARG and P115, are present in dorsal ruffles, provide mechanistic insight into the establishment of polarity and the regulation of cell migration in SMC. Clearly, additional studies will be necessary to identify the mechanism that allow for differential regulation of dorsal ruffle formation by two highly similar GEFs. Disparate signaling protein interactions and discrete microdomain localization of LARG and P115 are both mechanisms capable of eliciting the observed response and will be the focus of further investigation.

Chapter 4

FAK Does Not Phosphorylate LARG in SMC

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Introduction

It is known that LARG, PRG, and P115 are activated by association with $G\alpha_{12/13}$ subunits through their respective RGS domains. However, the precise mechanisms that regulate LARG or other RGS RhoGEFs in any cell type (including SMC) are poorly understood. All RGS RhoGEFs contains a Dbl homology (DH) domain and a neighboring Pleckstrin homology (PH) domain. The DH domain is found in nearly all RhoGEFs and is thought to enhance the GTP exchange reaction through allosteric mechanisms [67]. Although less understood, the PH domain is thought to serve a modulatory function and has been shown to bind to membrane lipids, the β/γ subunits of heterotrimeric G-proteins, and phosphorylated Ser/Thr residues. Additionally, LARG and PRG but not P115 have a PSD-95/Dlg/ZO-1 (PDZ) domain that is thought to mediate its interaction with additional proteins including the IGF1 receptor and plexins [212-214] as well as a C-terminal region that allows for both homo and hetero-oligomerization [72]. LARG has been shown to be phosphorylated by the tyrosine kinases, Focal Adhesion Kinase (FAK), and TEC, and these phosphorylation events have been associated with LARG activation [215]. While the precise site has not been mapped, evidence suggests that FAK may phosphorylate the LARG DH domain.

Our interest, based on the expression and effects of PRG in SMC, are focused on P115 and LARG signaling in SMC. A series of experiments by Suzuki et al. showed that LARG was indeed phosphorylated and that a mutant lacking the N-terminus was incapable of being phosphorylated [83]. Despite convincing evidence of phosphorylation these studies were conducted in COS cells and TEC was the

kinase shown to phosphorylate LARG not FAK. TEC has not been observed in smooth muscle however, limiting the importance of TEC mediated LARG phosphorylation studies in our SMC based tissue culture systems.

Previous studies in our lab demonstrated that FAK signaling regulates SMC phenotype [216, 217]. Based on the available evidence of LARG phosphorylation the goal of these studies was to test whether FAK-dependent tyrosine phosphorylation of LARG is an important mechanism that regulates RhoA activity in SMC. Our lab has a number of FAK related reagents useful in testing this hypothesis including dominant negative and constitutively active forms of FAK. The most powerful tool used to dissect endogenous function of FAK is a FAK deficient primary mouse SMC culture. These cultures are isolated from floxed FAK mice using standard techniques, and FAK is then deleted in culture by adenoviral-mediated expression of Cre recombinase (see Figure 4.1). This method avoids selective pressures that are common in cells isolated from conventional knock-out mice.

Materials and Methods

Transient Transfections and Reporter Gene Assays- 10T1/2 were obtained from ATCC. Floxed FAK primary aortic smooth muscle cells (FFSMC) were isolated as previously described [218]. For transfections, cells were cultured in 24 or 48 well plates, maintained in 10% serum, and infected with Cre or control virus for 24-48 hours. After infection cells were transfected 24 h after plating at 50-60% confluence using the transfection reagent, TransIT-LT1 (Mirus, Madison, WI), as per protocol.

The SM22, SM α -actin, and c-fos promoter luciferase reporter constructs have been previously described [120]. FAK, SuperFAK, and FRNK constructs were have been previously described [217].

Immunoprecipitation- Cultured FFSMC lysates were purified and pre-cleared with PAS beads. 1.2mg of lysates were then incubated with 10ul of anti-LARG for 2 hours. PAS beads were added to mix for 2 hours prior to washes and westerns were performed.

Western Blots- Cells were lysed in radio immunoprecipitation assay (RIPA) buffer plus protease and phosphatase inhibitors. Protein concentrations were determined using the BCA assay (Pierce). Protein lysates were run on an 7 or 10% SDS polyacrylamide gel, transferred to nitrocellulose, and probed with indicated antibodies Anti-LARG (A kind gift of K. Burrige, UNC-CH) or P-Tyr (Millipore 05-321). To achieve separation of 240-260kd proteins 7% mini gels were run until 150kd proteins were at the bottom of the gel.

Results

FAK over expression had no effect on SMC transcription

To begin to test our hypothesis that FAK was important in regulating LARG mediated RhoA activation and SMC specific differentiation we over expressed FAK,

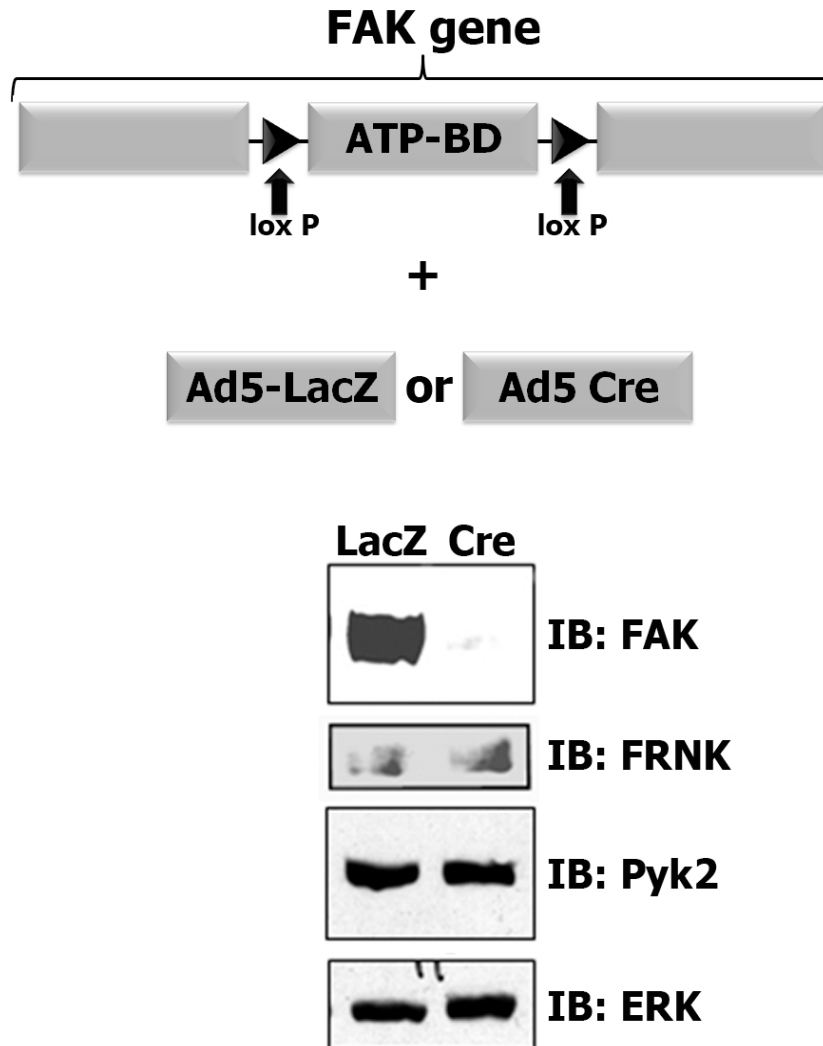


Figure 4.1. Generation of FAK-null Cells. Multiple aortas are extracted from mice that contain the floxed FAK transgene. Adventitial layers are removed and aortas are digested in elastase and collagenase. After plating cells are infected with Cre adenovirus or control (LacZ) virus to ablate expression of endogenous FAK. Western blots contributed by Liisa Smith Ph.D.

SuperFAK (a construct with increased activation potential), and FRNK (dominant negative) concomitantly with LARG in our tissue culture system. The effects generated by co-transfecting LARG and FAK were unremarkable and revealed little effect on SMC specific transcription. (see Figure 4.2)

Depletion of FAK has no effect on RGS RhoGEF mediated SMC transcription

After seeing no effect with FAK over expression we hypothesized that the endogenous levels of FAK were at or above a threshold for maximal FAK mediated activation and that FRNK over expression was not adequate at inhibiting FAK mediated activation of LARG. To determine if endogenous FAK was required for RGS RhoGEF mediated transcriptional activation we cultured primary aortic SMC isolated from mice that possess a floxed FAK transgene. After Cre mediated FAK depletion we co-transfected RGS RhoGEF constructs and smooth muscle specific promoters fused to luciferase reporters. Both wild type and FAK depleted SMCs showed similar increases in smooth muscle specific transcription in response to RGS RhoGEF depletion. (see Figure 4.3)

FAK does not phosphorylate LARG in SMC

To determine if LARG was significantly phosphorylated on tyrosine residues in SMC we conducted immunoprecipitation experiments with LARG and phosphotyrosine antibodies. Figure 4.4 shows that a substantial amount of LARG was immunoprecipitated from SMC lysates as evidenced by the amount of LARG compared to the input lane. A phosphotyrosine band was undetectable in that IP lane. We tried the reverse IP using phosphotyrosine antibodies which again

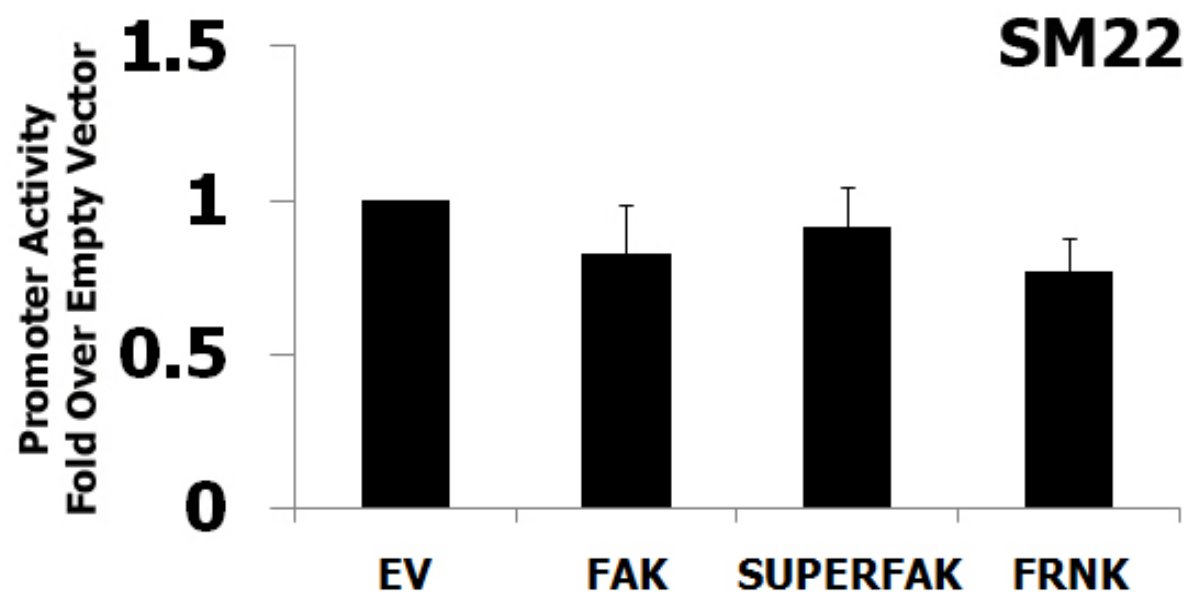


Figure 4.2. FAK over expression has no effect on SMC specific transcription. FAK, potentiated FAK (SuperFAK), and dominant negative FAK (FRNK) were co-transfected with a luciferase fused SM22 promoter into 10T1/2 SMC precursors. Luciferase activity was measured and expressed as fold increase over empty vector and promoter (N=3).

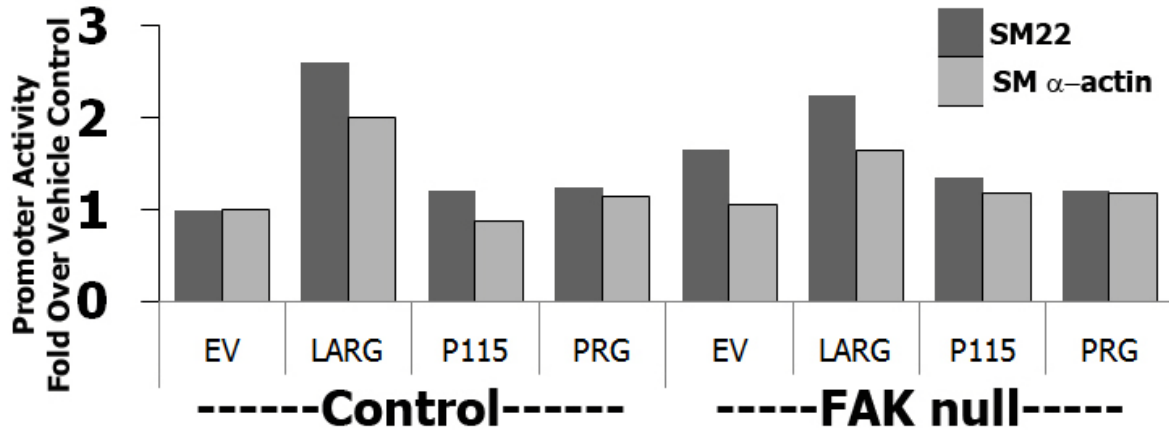


Figure 4.3. Effects of endogenous FAK depletion on RGS RhoGEF mediated SMC transcription. Floxed FAK SMCs were infected with control or Cre virus to deplete FAK expression. Cells were then co-transfected with the indicated RhoGEF and luciferase fused SMC promoters SM22 or SM α -actin. Luciferase activity was measured and expressed as fold increase over control virus transfected with empty vector and promoter (N=3).

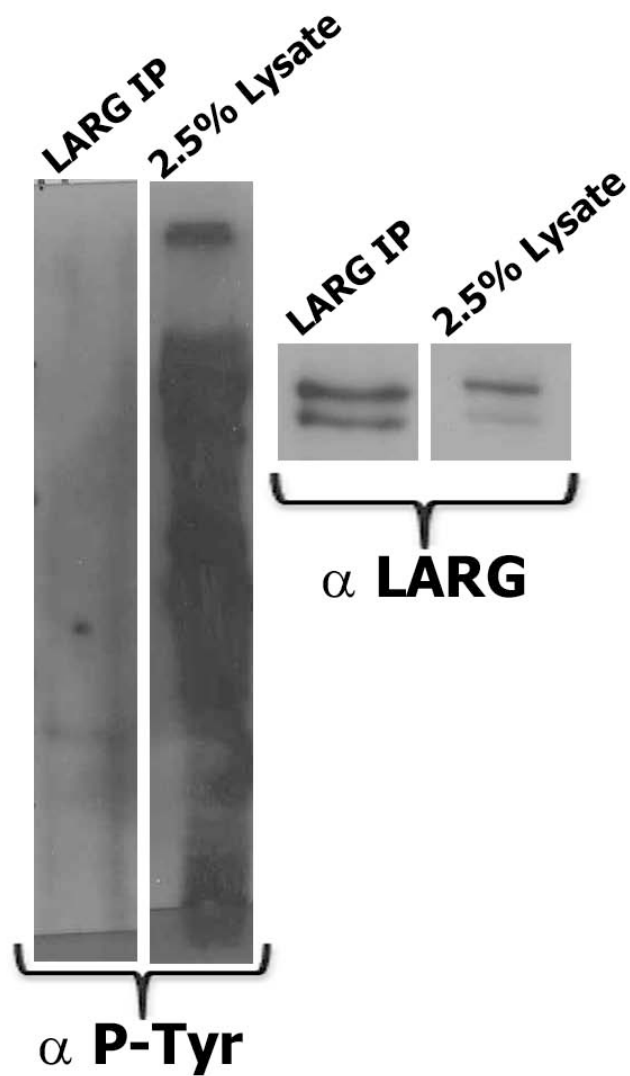


Figure 4.4. LARG is not tyrosine phosphorylated in SMC. 1.2mg of SMC lysate were pre-cleared with PAS beads and incubated with anti-LARG antibody for 1 hour prior to IP. No P-Tyr signal was detected in LARG IP lane.

showed that there was no phosphorylated LARG in SMC lysates. When a western blot is performed on SMC lysates with a phosphotyrosine antibody there is a substantial phosphor-tyrosine band that emerges at around 250 KD. Under close investigation these bands reveal that there are actually two substantial phosphor-tyrosine bands at approximately 260 and 240 KD and that there is no phosphor-tyrosine band that correlates with LARG in SMC lysates (Figure 4.5).

Discussion

Our original hypothesis was that FAK-dependent tyrosine phosphorylation of LARG was an important mechanism that regulates RhoA activity in SMC. However, we did not observe detectable levels of LARG tyrosine phosphorylation in our studies, a result in disagreement with previous reports. Iwanicki et al show, in fibroblasts, that PRG is tyrosine phosphorylated by FAK in response to LPA treatment[219]. Chikimi et al. claim that both LARG and PRG are phosphorylated by FAK but focus mainly on PRG and only show one instance of LARG phosphorylation in an IP with an input of ~50mg. Additionally their studies were conducted in HEK 293 cells not SMC. Suzuki et al. do show that TEC can phosphorylate LARG but these studies were done in COS lysates and TEC is not expressed in smooth muscle [83]. Our negative data can be reconciled by two possibilities. The first is that tyrosine phosphorylation of LARG does occur in cell types other than SMC. The second possibility is that other groups who have observed tyrosine phosphorylation of LARG were mistaken.

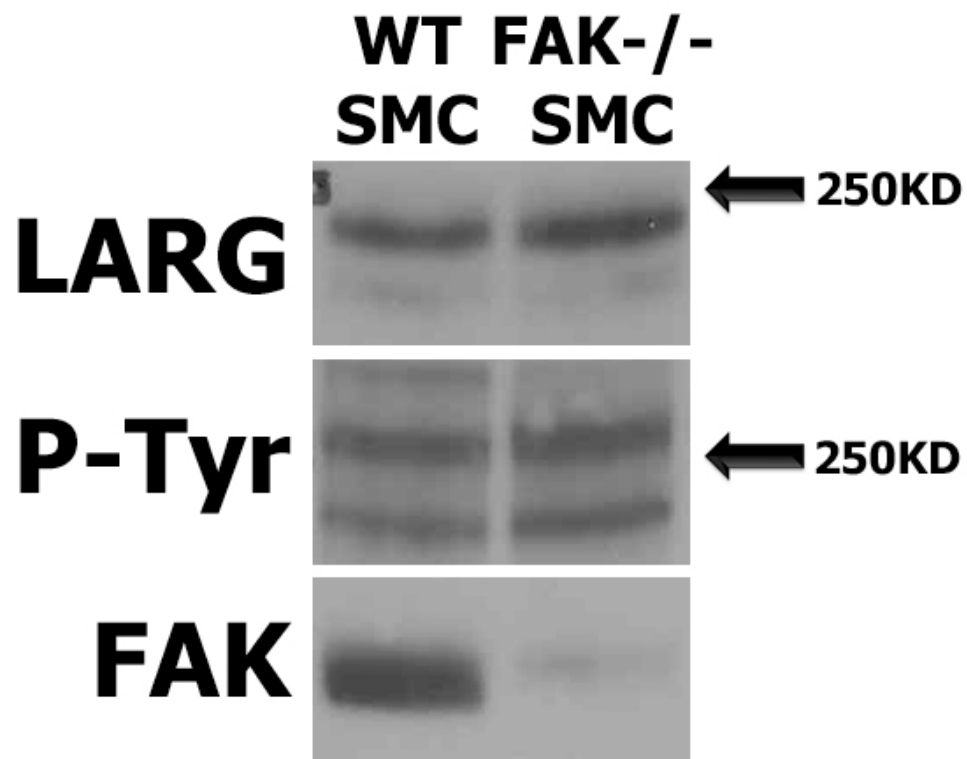


Figure 4.5. FAK depletion has no effect on observed P-tyr bands near 250 KD. FFSMC were treated with control or Cre virus to deplete endogenous FAK expression. 8% SDS Page gels were extensively run to reveal that the observed P-tyr bands ~ 260 KD and 240 KD bracket LARG at 245 KD.

LARG and PRG are immense proteins of 1544 and 1522 AA respectively. Logistically running and separating these ~250 kiloDalton proteins can be difficult. Further confounding the ability to study these two GEFs on an individual basis, LARG and PRG have been shown to heterodimerize [72]. Because of their similarity in size and heterodimerization it is possible that if large amounts of LARG were immunoprecipitated then residual tyrosine phosphorylated PRG could have co-immunoprecipitated and contaminated the samples. It should be noted that in later fractionation experiments using super resolved gels we observed that the P-tyr band at 240 KD correlates with the nuclear specific PRG (data not shown).

Given the similarity with LARG there is a likelihood that PRG can function redundantly or in lieu of LARG. Indeed in previous RhoA activity assays there was a small but consistent contribution of PRG in S1P mediated RhoA activation. One intriguing prospect is that tyrosine phosphorylation, possibly mediated by FAK, could preferentially activate or inhibit PRG over LARG. Given size and domain structure one would expect these proteins to function and localize identically but this is not the case. In fact in fractionation experiments LARG and PRG have both been shown in the nuclear and cytoplasmic fractions but a robust P-tyr band was seen in the nuclear fraction that corresponded exactly with one of the PRG doublet bands and not LARG. While it would be premature to draw the conclusion that the PRG band seen in the nuclear fraction is a preferentially tyrosine phosphorylated version of the protein it is an interesting theory and could illuminate a mechanism to target two otherwise very similar proteins to different compartments of the cell. Regardless PRG expression levels are fairly low in SMC and over expression generated

underwhelming effects on SMC transcription. Because of this PRG has been of limited interest in these studies and the project was abandoned.

Chapter 5

Subcellular Localization Regulates LARG Activity

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Introduction

An increasing number of studies outline the importance of LARG signaling in a number of physiological conditions including growth, migration, and differentiation [75, 76, 83, 87, 89, 124, 157]. While the importance of LARG signaling has been underscored we have a poor understanding of how LARG localization regulates its activity. The evolutionary processes that have driven the divergence from one common RGS RhoGEF like the DRhoGEF in *drosophila* or rhgf1 in *C. elegans* to the three RGS RhoGEFs found in higher order vertebrates had a reason to do so. The significance of RGS RhoGEF divergence and the signaling specific to LARG is not a topic that has been thoroughly studied, and understanding the domains that regulate LARG function is imperative in understanding protein function as a whole. LARG contains two N-terminal NLSs, PDZ, RGS, and DHPH domains as well as an extreme C-terminal coil coil region. Some PDZs have been shown to bind C-terminal polypeptides; others appear to bind internal (non-C-terminal) polypeptides. The RGS domain is found in GTPase-activating proteins for heterotrimeric G-protein α -subunits that promote GTP hydrolysis by the α subunit of heterotrimeric G-proteins, thereby inactivating the G protein and rapidly switching off G protein-coupled receptor signaling pathways. The DH domain contains about 200 residues shown to encode GEF activity specific for a number of Rho family members and the PH domain contains about 100 residues that may have multiple functions or different functions in different proteins, including signal transduction, membrane anchoring, and protein-protein interaction [220]. This combination of domains results in a protein with a number of signaling properties. Complete understanding of LARG

signaling will require studying subtle changes in domain structure and composition, post translational modifications, and subcellular localization.

Grabocka et al. have shown that there is a functional and unique NLS in the N-terminus of LARG and that oligomerization, regulated by a C-terminal coil coil region, prevents nuclear localization of LARG. They went on to show that leptomycin treatment results in accumulation of LARG in the nucleus suggesting that LARG cycles between the cytoplasm and nucleus in a CRM-1 dependent manner [64]. The authors, however, stop short of mapping the NES and conduct their studies primarily in COS cells. Additionally these authors stop short of posing a mechanism for the observed variations in localization. Specific functions for P115 RhoGEF are evident by the differences in domain structure and expression pattern, lymphocyte and neuronal and apoptotic specific effects for instance [221-224]. The differences between LARG and PRG function are much less well known.

Nuclear localization has previously been considered an important property for transcription factors, chromatin modification proteins like HDACs and HATs, and other DNA binding/modifying proteins. There is mounting evidence however that the nucleus can function as a microdomain for specific cellular events not restricted to transcriptional regulation. There is evidence to suggest that RhoA is present and may be active in the nucleus. Balboa et al. showed in cell fractionations that RhoA is present in the nuclear fraction [225]. More recently Li et al. showed in several cell types that RhoA is localized in the nucleus with high concentrations in the nucleolus and that the concentration of nuclear RhoA increases when cells undergo tumorigenesis [226]. Most importantly other GEFs known to activate RhoA including

ECT2 and NET1 have been detected in the nucleus [63, 227]. Work from our own lab has shown that the actin polymerizing RhoA effector Dia is also present in the nucleus. Together these data represent an intriguing picture by placing together the necessary components to orchestrate RhoA mediated actin dynamics in the nucleus. We have shown that LARG localization is tightly regulated by an N-terminal NLS and a C-terminus that regulates nuclear localization by an unknown mechanism. This goal of this study was to determine how nuclear localization of LARG is regulated and determine how this localization governs its ability to regulate smooth muscle specific gene transcription. Based on the emerging evidence of nuclear microdomain signaling we hypothesize that high nuclear concentrations of LARG will increase SMC specific transcription in a RhoA dependent manner.

Materials and Methods

Plasmids and Reagents- Full Length LARG and PRG were generous gifts of T. Kozasa (University of Tokyo, Tokyo, Japan) LARG and PRG were then subcloned into pcDNA 3.1 and eGFP expression vectors. Antibodies to LARG and PRG were kind gifts of K. Burrige (UNC Chapel Hill). FL LARG spans AA 1-1544, Δ NLS LARG AA 41-1544, Δ N LARG AA 360-1544, Δ C LARG AA 1-1161, Δ NLS Δ C LARG AA 41-1161, Δ N Δ C LARG AA 1079-1161. All LARG constructs were engineered with 5' KPN and 3' APA sites and shuttled between pcDNA 3.1 and eGFP expression vectors.

Cell Culture, Transfections, and Reporter Assays- The 10T1/2 and SMC cell cultures, transient transfections, and promoter luciferase assays have been previously described [44, 120].

Localization by Immunofluorescence- 10T1/2 cells were transfected with GFP tagged versions of indicated LARG truncations. At least 100 cells were scored from three separate experiments under three criteria nuclear, cytoplasmic, or diffuse defined as mostly nuclear, a defined nuclear shadow, and an overall even distribution, respectively.

Results

LARG localization is tightly regulated between nuclear and cytoplasmic fractions in SMC

As discussed in chapter 2 of this dissertation, LARG was shown to play a dominant role in S1P mediated SMC specific transcription. For these reasons emphasis was placed on LARG above PRG and P115 in the study of subcellular localization and possible mechanisms for spatial restriction of LARG protein. LARG consists of several different domains and is modular in structure. Analysis for conserved domains suggests that, from N to C terminus there are two nuclear localization sequences, a PDZ domain, and RGS domain, a DH/PH or GEF domain a coil coil domain on the extreme C terminus. Five truncations were made and subcloned into expression vectors using standard subcloning techniques (see Figure 5.1). These truncations were subcloned into a GFP expression vector and over-expressed in

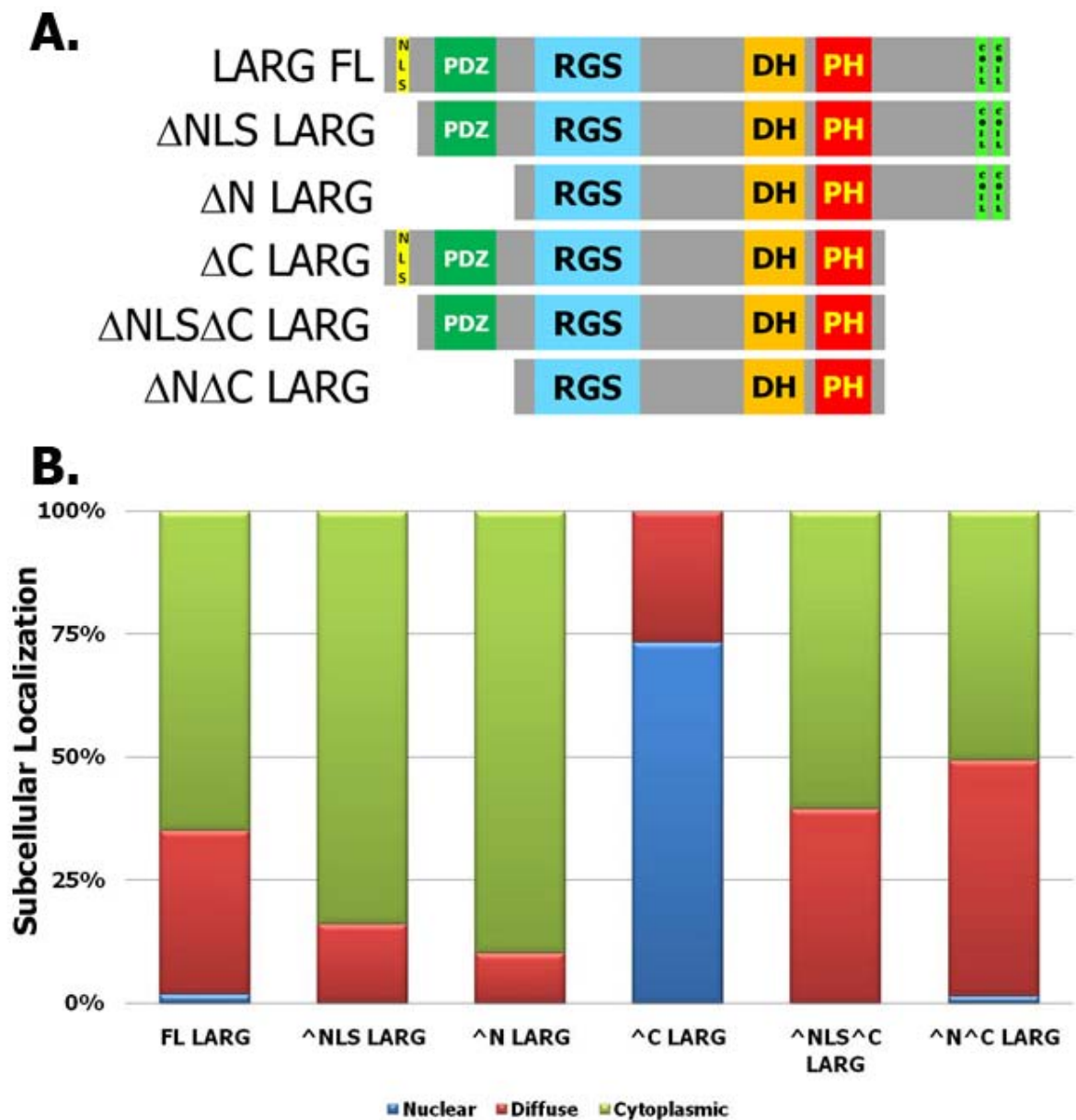


Figure 5.1. Sub-cellular localization of LARG is regulated by N and C-terminal domains. **A.** Schematic representing six LARG variants made to study localization and transcriptional activation. **B.** Quantification of LARG variant localization scored for nuclear, cytoplasmic, or diffuse patterns.

10T1/2 SMC precursors. At least 100 cells were counted and scored under three categories, cytoplasmic, diffuse, and nuclear, with diffuse defined as an equal distribution amongst the entirety of the cell and either cytoplasmic or nuclear defined as an observable gradient existing between the compartments. Full length LARG was mostly cytoplasmic or diffuse with only a small fraction nuclear, around 2%. Truncation of the most extreme N-terminal NLS achieves the expected result inducing increased cytoplasmic localization. Additional truncation of the N-terminus slightly increases the cytoplasmic localization. This result is not surprising based on a predicted weak NLS in this region. Additionally the Δ N truncation removes the PDZ domain. This suggests that, upon over expression, the PDZ domain itself does little to regulate nuclear localization. The most robust change in localization occurs when the extreme C terminus of the LARG protein is cleaved which results in a shift from 2% nuclear to 73% nuclear with the remainder of cells exhibiting a diffuse expression pattern. Cleaving the C-terminus removes a coil coil region which regulates homodimerization and possibly heterodimerization with PRG.

Over expression of nuclear LARG increases SMC specific transcription

To determine what effect LARG localization had on SMC specific transcription A luciferase based system was used to assess the effect over expression had on promoter firing. Three promoters were tested including SM22 and SM α -actin both indicators of smooth muscle specific transcription and C-fos as an indicator of growth specific transcription. Results were normalized against a TK minimal response promoter. We did not observe significant changes with a c-fos luciferase reporter with co-transfection of a LARG and LARG truncation constructs. The full

length LARG as well as the Δ NLS LARG and the Δ N LARG all had similar effects generating a 3-5 fold increase in smooth muscle specific transcription. As seen with SM22 and SM α -Actin the most robust effect was seen with the Δ C LARG construct. This mainly nuclear construct increased SM α -actin and SM22 transcription by approximately 6 and 8 fold respectively or roughly twice the effect of full length LARG (see Figure 5.2).

Discussion

We have shown that nuclear targeted LARG is twice as effective at increasing SMC specific transcription as the more cytoplasmic full length LARG. The initial observations of this study support the work done in COS cells by Grabocka et al. that characterized the localization of LARG [64]. The most interesting and novel finding of these studies was that the nuclear targeted version of LARG (Δ C LARG) was by far the most effective at increasing RhoA mediated increases in SMC differentiation gene expression.

Additional studies related to nuclear localization of LARG in SMC have focused on the endogenous localization states. Conclusive data answering these important questions has remained elusive. Cellular fractionation and subsequent western blotting suggest that there are nearly equal proportions of endogenous nuclear and cytoplasmic LARG throughout the cell (data not shown). This is in sharp contrast to over expression studies, by our lab and others, that suggest LARG is predominantly found in the cytoplasm unless a C-terminal truncations is induced.

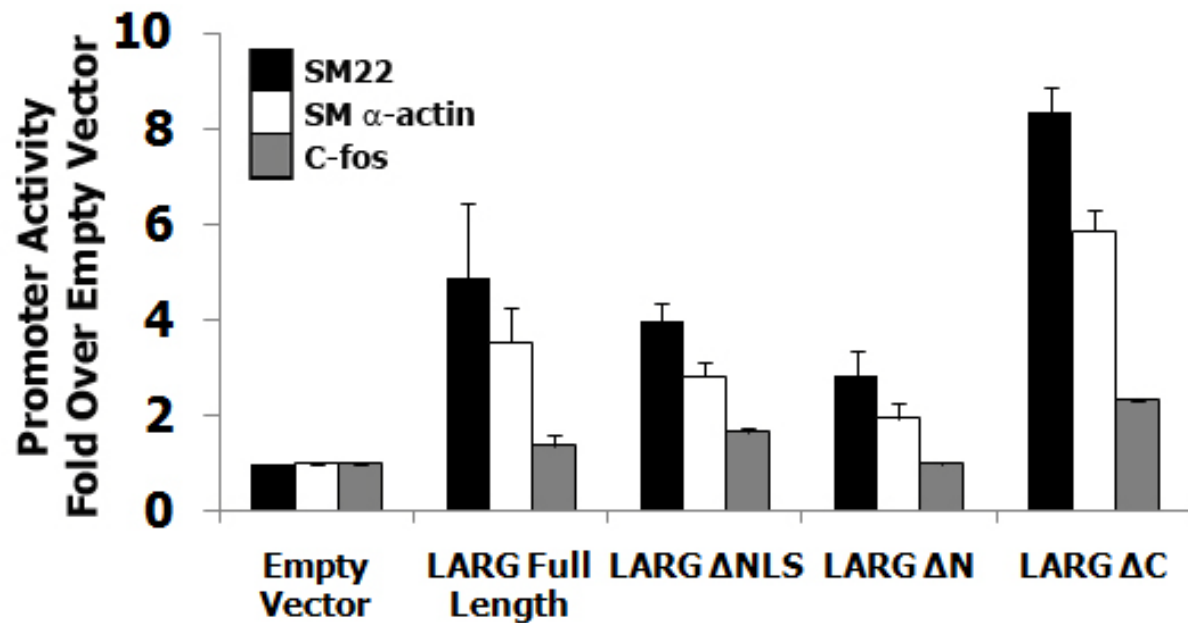


Figure 5.2. C-terminal truncation of LARG increases SMC specific transcription. LARG truncations were co-transfected with luciferase fused SMC specific promoters into 10T1/2 SMC precursors. Data is expressed as fold increase over empty vector co-transfection (N=3).

This discrepancy in observed localization could be attributed to artifacts of over expression. Grabocka et al suggest that the C-terminal is required for nuclear export of LARG but are not able to conclusively show the mechanism that regulates this export. It is evident that LARG localization is an active process but whether the C-terminal deletion causes decreased nuclear export or increased nuclear import remains in question. These questions could be addressed by tracking photo activatable pools of LARG and LARG truncations or inversely through FRAP experiments. We also examined whether LARG localization was regulated by various agonists. So far none of the reagents studied have been able to significantly increase either cytoplasmic or nuclear endogenous LARG localization in SMC. Likewise, to address if cytoplasmic or nuclear pools are differentially activating RhoA nucleotide free G17A Rho pulldowns could be performed on fractionated lysates previously stimulated with various agonists.

The robust localization variation in LARG and the multiple domains and motifs within the protein that allow for regulation of localization make it an attractive GEF to conduct spatially regulated RhoA activation studies. Using the RhoA biosensor and FRET based approach has been attempted but obtaining reliable results has been difficult. The rigors of siRNA transfection in a cell type already crippled by robust RhoA over expression limits the reliability of biosensor based data.

One worthwhile series of experiments to arise from these studies came when testing if serum stimulation increased LARG localization on short time scales. While there were no significant changes in nuclear versus cytoplasmic LARG we did observe for the first time LARG in circular dorsal ruffles. The function of LARG in

these structures is the subject of intense investigation previously discussed in this dissertation.

Chapter 6

Translational Perspectives of S1P Signaling in Arterial Vasculature

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Introduction

The largest single class of pharmacological targets are G-Protein Coupled Receptors (GPCRs) with estimates ranging from 27% to 60%; a class to which the S1P receptors belong [228, 229]. According to the CDC National Vital Statistics Report of 2006 the leading cause of death in the U.S. is cardiovascular disease. The body of this dissertation has explored and explained signaling pathways that regulate SMC phenotype. One of the most important revelations in SMC biology, studied in depth in this dissertation, is that S1P regulates SMC differentiation and by extension the pathogenesis of many cardiovascular disease states. This has direct implications toward the development of pharmaceuticals targeted at regulating vascular homeostasis.

Despite an intense focus on finding new categories and classes of targets for pharmacological intervention, relatively incremental progress has been made in the last several years. The utilization of huge screening protocols as opposed to methodical investigation of the components and kinetics of signal transduction is one factor that has complicated the refinement of drug discovery in recent years. There are many drugs, now in use, whose mode of action or specific pharmacology is poorly understood. This lack of understanding has hampered our ability to predict the off target effects of many drugs currently on the market, and has only increased the incidence of clinically relevant polypharmacology associated with newly identified compounds [228]. Consequently the push to further investigate and develop compounds with a high degree of specificity to functional pharmacological targets with well defined pathways is ever increasing. The discoveries surrounding S1PR2

mediated regulation of SMC differentiation exemplifies this confluence of pharmacological relevance and biological understanding.

James Black, winner of the 1988 Nobel Prize in Physiology or Medicine, said “the most fruitful basis for the discovery of a new drug is to start with an old drug” [230]. If this is indeed true than the discovery of S1PR specific drugs should prove exceptionally productive. While still in their infancy S1PR targeted drugs are of intense interest and are just beginning to be developed and fully investigated. Their use in traditional Chinese medicines dates back centuries. Extracts from the fungus *Isaria sinclairii* have revealed a sphingosine mimetic compound named FTY720 or fingolimoid (see [231, 232] for review). While initially tested as an immunosuppressant for kidney transplants more recent testing has shown a particular efficacy for immunosuppressive treatment of multiple sclerosis. The compound was so effective that in June 2010 the FDA unanimously recommended FTY720 be approved as the first oral medication for MS.

FTY720 has shown a high affinity for S1PR 1, 3, 4, and 5 with relatively little affinity for S1PR2. The specifics of the FTY720 mode of action beyond S1PR binding are a matter of some debate in the field. The emerging consensus shows an initial wave of high specificity binding and activation [233]. Additional evidence suggests that while S1P promotes a reversible internalization, the internalization induced by FTY720 binding is irreversible, triggering ubiquitination and proteasome mediated degradation, rendering endogenous S1P unresponsive [234, 235]. Beyond the binding affinities and receptor dynamic specifics the ability for FTY720 to inhibit lymphocyte egress from lymphatics is unmistakable.

While the role of FTY720 and other S1P analogs has been defined in lymphocyte trafficking, the role these compounds play in the regulation of vascular homeostasis and disease is not well understood. Mouse model evidence suggests that the S1P receptors are viable targets for modulating vascular patterning. Keul et al. were able to show, in an atherosclerosis prone Apo-E mouse, that FTY720 treatment can reduce lesion volume by 63% [236]. Also dosage studies suggest that stent-delivered high concentrations of FTY720 at the sites of acute vascular injury may inhibit restenosis and hyperplasia [237]. Other vaso-protective functions have been observed with FTY720 including anti-hypertensive effects and protection from ischemia/reperfusion injury [238, 239].

S1P levels have proven to be a strong indicator of cardiovascular disease in general [118, 240]. But given the high basal level of S1P signaling we hypothesize that general negative regulatory methods are a major factor in directing pathway specification. Furthermore varying the expression levels of S1P receptors is a means that has already been shown to preferentially increase S1P mediated signaling [113]. While extensive in-vitro and mouse model studies have been done there is little or no published data concerning S1P receptor expression in human atherosclerotic plaques. The goal of this study was to determine if S1PR2 expression varies between healthy and diseased arterial vasculature in humans.

Materials and Method

Human Aortic Protein Isolation- Office of Human Research Ethics (OHRE) and Institutional Review Board (IRB) approval was obtained to procure aortas, from

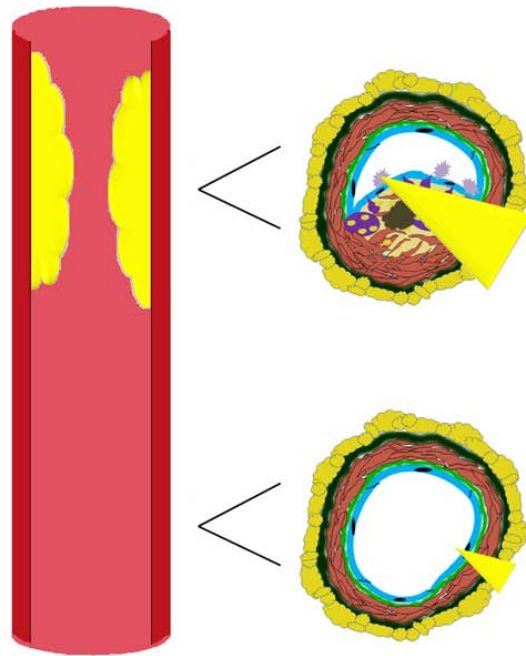
human cadavers, which presented with advanced atherosclerotic plaques. The fat was removed from the aorta and all remaining layers were kept intact. Rings approximately 5mm wide were sliced from the aortas in “healthy” areas with no observed plaque formation as well as in areas with advanced lesion formation. Transmural wedges were cut, snap frozen, and homogenized in RIPA buffer. The tissue samples were lysed in RIPA and purified by centrifugation and equalized by BCA assay as per protocol. Equal amounts of protein were loaded on an SDS PAGE gel and western blots were performed.

Result

S1PR2 expression is up regulated in atherosclerotic vasculature

In a resected section of aorta from a human cadaver we show that atherosclerotic vasculature has increased S1PR2 expression and decreased SM α -Actin expression compared to neighboring non-diseased aorta (see Figure 6.1) SMC with no adventitia, plaque, fibrous cap, adventitia only, and fat only layers were also separated and individually probed for S1PR2 and SMC markers. Plaque tissues (which included SMC layer) from the diseased aorta showed the most robust expression of S1PR2.

A.



B.



Figure 6.1. S1PR2 and SM α -Actin levels in healthy and atherosclerotic human aortas. A) Schematic of disc and wedge resection method for procuring protein from tissue samples. **B)** S1PR2 and SM α -Actin expression in healthy and diseased aorta.

Discussion

Given the abundance of S1P in circulation we have hypothesized that negative regulation of receptor expression is a viable means of directing S1P specific signaling. Despite the interest in S1P mediated regulation of CVD progression, very little is known concerning receptor expression variation in human models of disease. Previously our lab and others have shown that the S1PR2 specifically drives the S1P mediated differentiation of SMCs and that this differentiation is important in development as well as disease. Given these findings we were curious what changes might occur in S1PR2 expression in an advanced atherosclerotic lesion. The data we have generated to address this question reveals a very large delta with respect to S1PR2 in healthy and diseased aortic tissue. This degree of expression variation begs a host of questions and additional experiments.

One caveat of the human aorta western blot experiment relates to the composition of a plaque. An atherosclerotic plaque, by nature, is a very complex lesion consisting of a number of cell types. It is possible that the variation in expression is due to cell type specific expression of S1PR2. During dissection of the aortas, an attempt was made on separate areas of healthy and diseased aortas, to separate the individual layers and assess protein expression in a layer specific manner. It did appear that protein purified from just the medial SMC and intimal EC lacking the fat and adventitial layers of healthy and atherosclerotic tissue mirrored the gross protein purifications. Additionally purified protein isolated from just the fibrous cap of the lesion showed very little expression of S1PR2 (data not shown). If the changes in S1PR2 are attributable to cells other than those of smooth muscle

origin they would need to be those that compose the inner layers of the plaque excluding the tissue that composes the fibrous cap. The most direct way to answer these questions would be to conduct IHC analysis of S1PR2 expression in situ, with other markers that would allow identification of SMCs, lymphocytes, fibroblasts, macrophages, and other cell types. Additionally these data bring into question what, if any, changes are made in S1P receptor expression in other vascular disease states including hypertension and restenosis, and if these expression changes occur preferentially in SMC of specific origins.

Based on previous studies surrounding S1P receptor expression and signaling in cell culture and animal models we have a few interesting hypothesis about how S1P treatments could be employed to combat CVD. Given the already high molar concentration of S1P in serum, combined with the very dynamic changes in receptor expression, systemic delivery of S1P would not be the preferable method of delivery. Given the unknowns surrounding receptor expression and origin-specific smooth muscle signaling the possibility off target effects resulting from systemic delivery would be too great. In short there are just too many variables to contend with. Systemic prophylaxis with a S1PR2 specific agonist aimed at inhibiting migration and proliferation of SMC may show some merit. Based on results from our lab and others, increasing S1PR2 specific activation in SMC should serve to stabilize SMC populations by increasing the differentiated state and inhibiting proliferation and migration. While this would not be preferable during development as the aforementioned processes are critical in proper vascular patterning,

administration of an S1PR2 specific agonist after the initial formation of a fatty streak could conceivably inhibit progression to clinical atherosclerosis.

In instances such as balloon injury, coronary artery bypass grafts (CABG), and other acute arterial injury, localized delivery of an S1PR specific drug by a drug eluting stent is also worthy of clinical consideration. If a coating could be engineered to elute agonist specific to S1PR1 and 3 for a short window preceding elution of an S1PR2 specific agonist SMCs could be directed to first populate and proliferate at the area of injury then pushed toward a differentiated state to quickly and efficiently construct matured robust arterial vasculature capable of handling elevated arterial pressure. Another area ripe for the inclusion of S1P assisted vascular maturation is in the biopolymer based tissue scaffolding arena. A number of pathologies require arterial replacement including instances of CABGs and patients with kidney failure who require peripheral grafts for dialysis access. There are many instances when patients need engineered tissues because of a lack of conduit. S1PR specific direction of xenograft development prior to implantation could be very effective in the production of synthetic grafts. Taken together the efficacy of S1P analogs as pharmacologic agents is unmistakable and the potential for attenuation of CVD progression at the level of S1P receptors is high. These facts beg further investigation of S1P signaling as a means to treat CVD.

Dissertation Overview and Conclusions

The entirety of this dissertation was borne out of the question, how does S1P regulate SMC phenotype? Attempts to answer that question revealed three relevant

areas of study explored in this dissertation. The second, fourth, and fifth chapter of this dissertation are intensely focused, and definitively explain, how S1P regulates smooth muscle differentiation. The third chapter of this dissertation explores the importance of the S1PR2 downstream effectors, LARG and P115, in regulating the transition of SMC from a static to motile state and eventual de-differentiation. The last chapter of this dissertation examines the clinical relevance of these findings.

Prior to beginning this thesis work little was known about the upstream regulators of RhoA in the context of smooth muscle specific transcription. An intense focus on the signaling events downstream of RhoA activation of SMC revealed the RhoA-MRTF axis as the regulatory mechanism for SMC specific differentiation. These findings, along with the realization that S1P activated RhoA in a variety of cell types, revealed a gap in our understanding of the signaling that regulates SMC differentiation. Chapters 2, 4, and 5 of this dissertation fill this gap in the SMC field. Chapter Two is a reiteration of a paper published in ATVB and is titled S1PR2 Signals Through LARG to Promote SMC Differentiation. This paper parses the signaling that occurs after S1P binding to surface receptors at the plasma membrane until SMC differentiation specific genes are transcribed. In brief the results conclusively show that SMC differentiation is regulated by S1P through S1PR2 and that $G\alpha_{12/13}$ and the RGS RhoGEFs, upon overexpression, can stimulate SMC-specific transcription. Finally we go on to show that LARG alone is necessary in generating significant S1P mediated increases in RhoA activity as well as SMC specific transcription, message, and protein expression.

Navigating the intricacies of a thesis project is not, nor should it be, a strictly linear progression. During the course of the studies important questions were asked and answered that didn't necessarily fit into a larger publication. Chapters 4 and 5 represent these types of findings. Other cell types have shown a role for FAK in regulating LARG activity. We chose to test if FAK phosphorylated LARG in SMC. We show that FAK over expression or depletion had no significant effects on LARG mediated SMC transcription and that, in our hands, FAK does not phosphorylate LARG in SMC. This negative data did not fit well into our planned publications nor was it worth the effort needed to develop into a standalone publication. Despite these caveats the inability of FAK to phosphorylate LARG in SMC is an important observation and is worthy of inclusion with this dissertation. Chapter 5 focused on the subcellular localization of LARG and the effects this had on the protein's ability to activate RhoA. We show that LARG localization is tightly regulated between nuclear and cytoplasmic fractions and that the subcellular localization of LARG has substantial effects on SMC specific transcription. While these findings alone are short of publication readiness, this is interesting data worthy of further investigation. The emerging paradigm of subcellular localization, nuclear localization in particular, of small GTPases as a regulatory mechanism would make LARG an attractive GEF to study in this respect. Time considerations and other studies forced us to shelve this project, though the questions surrounding this project would likely yield interesting and possibly compelling results.

Chapter 3 of this dissertation approaches the regulation of SMC phenotype with a focus on the processes that regulate the de-differentiation of SMC. The

clinical relevance of these studies are more tangible since the synthetic migratory state is critical in the pathogenesis of atherosclerosis as well as neointimal hyperplasia. We hypothesize that dorsal ruffling is an important process in establishing polarity and initiating migration in the reversion of differentiated to migratory SMC. We show that LARG and P115 are localized to dorsal ruffles and that the depletion of these GEFs differentially effects dorsal ruffle formation as well as SMC migration. These data are a strong foundation for future studies that will focus on the importance of RhoA, LARG, and P115 in the regulation of dorsal ruffles and migration. Further studies will be conducted to determine how LARG and P115 differentially regulate dorsal ruffles and migration as well as to conclusively determine the presence or absence of RhoA in SMC. The combined conclusions of Chapters 1-5 are summarized in figure 6.2

Chapter 6 focuses on the translational perspectives of S1P signaling in arterial vasculature. The realization that GPCRs, like the S1P receptors, are the most common target of manufactured pharmaceuticals, and that S1P could play an important role in vascular regulation requires an investigation of the possible therapeutic use of S1P related agonists in the treatment of CVD. To first show the possibility of efficacy this chapter discusses the recent refinement of *isaria sinclairii* resulting in purification of the compound FTY-720, recently approved for the treatment of multiple sclerosis. S1P as a predictor of coronary artery disease is referenced as well as the protective role S1PR2 plays in the inhibition of neointimal hyperplasia after acute vascular injury. We show that in human atherosclerotic lesions there is a robust increase in S1PR2 expression. Based on this observation

and the observations made concerning the role S1PR2 and its downstream effectors play in regulating SMC differentiation and phenotypic reversion to a migratory state we hypothesize that S1P receptor agonists could be used in clinically relevant ways to inhibit the progression of atherosclerotic plaque, ameliorate pathological neointimal hyperplasia, and in the general regulation of vascular homeostasis.

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