A Role for Focal Adhesion Kinase in Vascular Smooth Muscle Cell Proliferation, Migration and Differentiation

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ABSTRACT

Liisa Sundberg Smith: A role for Focal Adhesion Kinase in vascular smooth muscle cell proliferation, migration and differentiation
(Under the direction of Joan M. Taylor, Ph.D.)

Smooth muscle cells (SMC) are involved various vascular diseases, such as atherosclerosis, restenosis following balloon angioplasty and following venous bypass grafts. During the development of these vascular diseases and during normal vascular development, numerous changes occur within the vessel environment that enables SMC to phenotypically modulate between a contractile phenotype and a proliferative phenotype. The remodeling of the extracellular matrix, an increase in growth factors and contractile agonists have all been shown to initiate signaling pathways leading to an increase in SMC proliferation, migration, and differentiation. The tyrosine kinase, Focal Adhesion Kinase (FAK) plays a major role in the integration of the signals transmitted from these extracellular cues. The aim of this dissertation was to determine the SMC functions that are regulated by FAK. To address this goal, I evaluated two prominent signaling pathways; the Ras/Raf/Mek/Erk and Pi3-kinase/Rac/Pak/JNK cascades, both have been shown to be required for cell growth and motility in certain cell types. The data presented herein shows that FAK activity is required for PDGF-, AngII- and adhesion-mediated Rac1 activation. The p21-activated kinases (PAK) are downstream targets of various signaling cascades including PDGF-BB and integrins and are effectors for the GTPases Rac1 and Cdc42. Recently, it was shown that PAK activity is required for maximal activation of the canonical Ras/Raf/Mek/ERK

MapKinase signaling cascade. Therefore, I aimed to determine the role of PAK within the ERK signaling cascade and found that adhesion-dependent activation of PAK may enhance growth factor-stimulated signaling and PAK may serve as a scaffold for the Raf/Mek/Erk signaling cascade in SMC. I also sought to determine if FAK activity plays a role SMC phenotypic modulation. I found that deletion of FAK or inactivation of FAK by FRNK (FAK related non-kinase) expression causes a significant increase SMC-specific gene expression. Additionally, the LIM protein, leupaxin, associates with FAK and can translocate from focal adhesions to the nucleus leading to increased SMC marker gene transcription. These data indicate that FAK may serve as a necessary regulator of SMC phenotypic modulation during vasculogensis, where FAK activation promotes a dedifferentiated proliferative phenotype and FAK inactivation induces a switch to a differentiated contractile phenotype.

To my husband Eric

Whose love and support make me feel I can achieve anything

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LIST OF ABBREVIATIONS

Ab	antibody
AngII	Angiotensin II
Ao	Aorta
ASAP1	actin-related protein
bFGF	basic fibroblast growth factor
Bl	Bladder
BrDU	bromodeoxyuridine
CArG	$CC(AT)_6GG$
CAS	Crk-associated protein
ChIP	chromatin immunoprecipitation
CRIB	Cdc42/Rac interacting binding
Crk	C10 regulator of kinase
CRP	cysteine-rich protein
DMEM	Delbucco’s modified eagle medium
ECM	extracellular matrix
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase

FAT	focal adhesion targeting
FBS	fetal bovine serum
FFSMC	floxed FAK smooth muscle cells
FHL2	four-and-a-half-LIM only protein 2
FN	fibronectin
FRNK	FAK related non-kinase
GAP	GTPase activating proteins
GAPDH	glyceraldhyde-3-phosphate dehydrogenase
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
GFP	green flourescence protein
GFRNK	GFP-FRNK
GRAF	GTPase-activating protein for rho associated wit FAK
GRB2	growth factor receptor-bound 2
GST	glutathione s-transferase
H	heart
Hic5	hydrogen peroxide-induced clone 5
IP	immunoprecipitated
JNK	c-Jun N-terminal kinase
K	kidney
KDa	kilodalton
LC	loading control
LIM	Lin-11, Isl1, MEC-3

LPP	lipoma-preferred partner
Lu	lung
MapKinase	mitogen activated kinase
MEF	mouse embryonic fibroblast
MLCK	myosin light chain kinase
MMP2	matrix metalloproteinase-2
MRTF	myocardin-related transcription factor
NES	nuclear export sequence
PAK	p21-activated kinase
PDGF	platelet-derived growth factor
PI3K	phosphotidyl-inositol-3-kinase
PLC	phospholipase C
Pyk2	proline-rich tyrosine kinase 2
Rho	Ras homology
S1P	sphingosine-1-phosphate
SFM	serum free media
SH2	Src homology domain 2
SH3	Src homology domain 3
SkM	skeletal muscle
SMC	smooth muscle cell
SM-MHC	smooth muscle-myosin heavy chain
SRF	serum response factor
TCF	ternary complex factors

TGF-β	transforming growth factor beta
TK	thymadine kinase
VSMC	vascular smooth muscle cells
WT	wild type
ZRP-1	zyxin related protein 1

CHAPTER I

INTRODUCTION

INTRODUCTION

Even though campaigns promoting cancer awareness and prevention are at the forefront of public attention, cardiovascular disease is still the leading cause of death in the United States. Recently complied data shows that 36.3% of all deaths in 2004 were due to cardiovascular disease [1]. The most common cardiovascular disease is atherosclerosis. Atherosclerosis is characterized by plaque formation in the arterial lumen that can eventually lead to stenosis and thrombosis formation. Endothelial damage triggers an inflammatory response and subsequent accumulation of several cell types to the injured region including, macrophages, mast cells, platelets and smooth muscle cells (SMC) [2]. Damage to the endothelial layer is multifactoral with the leading causes being high blood pressure, high levels of LDL cholesterol and tobacco use. According the American Heart Association, over 72 million people have high blood pressure and 36.6 mil Americans have cholesterol levels over 240mg/dL, both of which will contribute to the 1.2 million cardiac events predicted for 2007 [1]. Several pharmacological agents have been developed to treat high blood pressure and high cholesterol levels. While these drugs are often effective in preventing disease progression, invasive therapies including balloon angioplasty and stent insertion are still necessary to treat vessels with advanced disease. Unfortunately, restenosis occurs in 20 to 30 percent of patients following balloon angioplasty and vein bygrafts. Restenosis results from uncontrolled SMC proliferation and migration, which diminishes the diameter of the newly formed lumen requiring further treatment. Thus, a thorough understanding of the SMC biology and the underlying mechanisms regulating SMC proliferation, migration, and

differentiation could aid in the development of additional therapeutic targets to treat restenosis.

SMC MIGRATION AND PROLIFERATION

Vasculogenesis is initiated by endothelial cell differentiation from angioblasts and the subsequent formation of endothelial tubes comprising the primary capillary plexus. The basal lamina of these tubes, in addition to PDGF secreted from the endothelial cells, attracts SMCs that migrate and proliferate encompassing the endothelial tube and forming the medial layer of the vasculature. PDGF-BB stimulated proliferation and migration has been shown to be the major regulator of vessel maturation, since PDGF-B-null mice die in utero exhibiting vascular hemorrhage due to the absence of mesangial cells within the vessels [3]. While SMC migration and proliferation are essential for vessel formation aberrant regulation of these processes are involved in various vascular pathologies, such as the development of atherosclerosis, restenosis following balloon angioplasty and vascular hypertrophy following vein bypass grafts. The increased SMC accumulation observed in these disease processes and during vascular development is likely due to the activation and upregulation of various signaling components mediating SMC proliferation and migration [4]. During vasculogenesis and vascular injury various cells, including macrophages, platelets, endothelial cells and SMC secrete growth factors (PDGF-BB, IGF-1 and bFGF) and contractile agonists (Ang II, ET-1 and thrombin) that are known stimulators of the canonical MAPKinase and Pi3K/Akt pathways required for cellular growth and migration [5]. However, numerous studies reviewed by Howe et al showed that growth factor mediated

activation of the MAPKinase cascade is an anchorage dependent process requiring additional signaling form the surrounding matrix [6].

Interestingly, in parallel to an increase in secreted agonists, the extracellular matrix (fibronectin, laminin, and collagen) within the vessel remodels during vascular development. During development and following vascular injury, the basement membrane shifts from a quiescent state of low fibronectin and high collagen and laminin levels to an environment with increased fibronectin and decreased collagen and laminin levels [7]. Mouse knockout models have provided additional evidence for the importance of both growth factor and matrix signaling in the developing vasculature. Thus, like the PDGF-B knockout, fibronectin-null mice die in utero due to vascular defects and malformation [3, 8]

Integrins:

Cell adherence to the extracellular matrix (ECM) activates heterodimeric transmembrane receptors, termed integrins. Integrins are composed of various combinations of 18 distinct α and 8 distinct β subunits. The α and β subunits are comprised of an extracellular head, a central transmembrane domain, and a cytoplasmic tail [9]. While integrins are necessary purveyors of inside-out and outside-in signaling, they lack catalytic activity and are dependent upon the recruitment of various adaptor and kinase proteins, such as Syk, Abl, Src family kinases and FAK [10-13].

Integrins are an essential component of the signaling pathways involved in cell migration, proliferation, and differentiation. Various α and β combinations are expressed within the vasculature during different times. β1-containing integrins are highly expressed in vascular smooth muscle cells. α1β1 and α3β1, the collagen ligand receptors are both highly

expressed in contractile SMC and are important for maintaining a differentiated phenotype and tensile strength of the vessel [14, 15]. Expression of α5β1, the fibronectin receptor, is upregulated following vascular injury and mediates phenotypic modulation of SMCs by downregulating SM marker gene expression [16]. Recently, α8β1 integrin expression was also shown to induce phenotypic modulation of SMC by decreasing migratory capacity and increasing the expression of contractile proteins [17].

The vitronectin and osteoponin receptor, αvβ3, is an important regulator of SMC migration. However, in vivo injury models have demonstrated that β3 is essential for SMC migration but not proliferation. Carotid injury performed in β3-/- mice exhibited decreased neointima formation after carotid injury compared to their wildtype littermates. This was attributed to defective migration signaling, since SMCs of aortic explants from β3-/- null mice showed no proliferative defect in response to PDGF-AB [18]. Bendeck et al have proposed that overexpression of αvβ3 stimulates SMC migration by increasing MMP-1 production [19].

FAK:

Once cells attach to the ECM, integrins cluster and rapidly recruit numerous structural and signaling proteins that collectively form a focal adhesion [20, 21]. Focal adhesions are multifarious collections of proteins that are important for actin cytoskeleton stability and signal transmission leading to various cellular processes [22, 23]. One of the essential proteins in this complex is the non-receptor tyrosine kinase, Focal Adhesion Kinase (FAK). FAK is a 125 kDa non-receptor tyrosine kinase, that is comprised of three domains, an N-terminal integrin binding domain, a central kinase domain and a C-terminal, proline

rich, focal adhesion targeting (FAT) domain (Figure 1.1) [24]. After integrin engagement to the ECM, FAK is recruited to sites of adhesion becomes activated and autophosphorylates on tyrosine 397. Tyrosine phosphorylation at this site promotes Src binding [25] which further phosphorylation of FAK and leads to activation of the subsequent downstream signaling cascades leading to cell proliferation, migration, differentiation and survival [26].

Tyrosines 576, 577, 861 and 925 are known targets of Src-dependent phosphorylation sites of FAK. Phosphorylation of Y576 and Y577 that are located in within the central kinase domain of FAK, increases FAK catalytic activity [27]. Y925 has been shown to be important for the association of the Grb2-SOS complex that signals to downstream the MAPKinase cascade [28]. While the precise function of Y861 is not clear at present, studies performed in endothelial cells have shown that VEGF-stimulated Y861 phosphorylation is important for regulating cell migration and anti-apoptotic signaling events [29]. Further work from the Cheresh lab determined that this phosphorylation event heightens the formation of αvβ5/FAK complex, which is essential for VEGF-mediated EC migration [30].

Studies from mass spec analysis have revealed additional phosphorylation sites on FAK. Recently work demonstrates a role for Y407 as an inhibitory phosphorylation site. Located proximal to Y397 within the central kinase domain, Y407 is phosphorylated during a quiescent state and negatively regulates Src-dependent Y397 phosphorylation [31]. These studies support previous data showing that VEGF-stimulated Y407 phosphorylation was mediated by RhoA signaling and Y407 phosphorylation attenuated paxillin and vinculin binding to FAK [32]. The ying yang of Y397 and Y407 activation may aid in our understanding of the role of FAK in focal adhesion turnover and engagement.

Four serine residues within the C-terminus, S722, 732, 843, 910, have been identified as targets of serine/threonine kinases and phosphorylation of these sites may function to antagonistically regulate FAK tyrosine phosphorylation. FAK tyrosine phosphorylation is adhesion-dependent, where as most data indicates that serine phosphorylation is adhesion-independent. Therefore distinctive signaling pathways regulate serine phosphorylation from tyrosine phosphorylation may be involved in how FAK activation is regulated during focal adhesion turnover [31, 33]. Additionally, FAK serine phosphorylation has been thought to be important for regulating cell mitosis, possibly through inhibition of adhesion-dependent signaling complexes [34].

The C-terminus of FAK has been to shown to bind to several focal adhesion proteins. The SH3 domain of p130Cas binds to the first proline rich region designated Site I, while Site II binds the SH3 domains of two GAPs, GRAF and ASAP1 [35-37]. Additionally, the focal adhesion targeting (FAT) domain is necessary for directing FAK to focal adhesions. Numerous other proteins have been identified as FAK C-terminal binding partners, including talin, endophilin A2, and paxillin, [38-40].

FRNK:

The C-terminal portion of FAK is also produced as an independent protein termed FRNK (focal adhesion related non-kinase). Like FAK, FRNK is localized within focal adhesions where it can bind to the same FAK C-terminal binding proteins (Figure 1.1). However, while FAK is ubiquitously expressed, FRNK is selectively expressed in smooth muscle. Taylor et al have previously shown that FRNK protein levels are upregulated during

vascular development and are induced following vascular injury [41]. Interestingly, whereas FAK protein levels remain relatively constant, FRNK protein levels change dynamically with high FRNK expression in the neonatal period and at two to three weeks following balloon injury. The expression pattern correlates with the conversion of SMCs from a synthetic to contractile phenotype that takes place during these times, suggesting that FAK activity is tightly regulated *in vivo.*

The precise mechanism by which FRNK functions in SMCs is unknown. Very few studies have addressed the possibility of FRNK phosphorylation. Ma et al showed that overexpressed FRNK could be serine phosphorylated *in vivo* [42] however, if endogenous FRNK can be serine or tyrosine phosphorylated in an agonist-dependent manner remains to be elucidated. Since FRNK localizes to focal adhesions and inhibits FAK activation, FRNK has been a useful tool to show that FAK activity is necessary for SMC proliferation and migration (Figure 1.2). Additionally, FRNK may function to suppress FAK activity enabling SMC to differentiate to a contractile state; support for this hypothesis is presented in Appendix I.

FAK signaling in vitro:

Several pathological states are correlated with enhanced FAK signaling, including uncontrollable cell growth seen in cancers and vascular diseases. FAK is able to modulate these cellular changes associated with these diseases by functioning as an integrator of various receptors and as a signaling conduit to the Rho family of small GTPases that regulate the cytoskeleton. Two canonical pathways that have been shown to be to be utilized in

adhesion-dependent proliferation and migration: the Pi3K/Rac/Pak/Jnk and Ras/Raf/Mek/Erk pathways.

The Ras superfamily include H-Ras, R-Ras, and Rho family members (Rac, Rho, and Cdc42) are activated by numerous transmembrane receptors such as receptor tyrosine kinases, G-protein coupled receptors, and integrins. Activation leads to a variety of cellular processes including proliferation, differentiation, and migration [43]. GTPases are molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state [44]. In the GTP-bound form, the Ras-related GTPases interact and activate a number of effector molecules that have been implicated in regulating cell cycle progression and/or cell migration such as the serine/threonine protein kinases, lipid kinases, and actin binding/scaffolding proteins [45, 46]. Progress has been made in identifying the specific cellular responses regulated by these effectors, particularly those that are involved in cellular migration. Members of the Rho GTPase family, RhoA, Rac1 and Cdc42, regulate the cytoskeleton through actin reorganization. RhoA activation induces stress fiber formation, active Rac1 leads to lamellipodia protrusions and Cdc42 activation stimulates filapodia formation [47]. FAK is involved in several signaling pathways regulating these proteins. Fibronectin-mediated Rac1 activation was shown to be propagated through either the FAK/Pi3K/SOS pathway or the FAK/Cas/Crk/DOCK180 pathway [48]. To address which pathway is utilized in SMCs, in Chapter II, I show that integrin-, growth factor- and G-protein coupled- receptor –stimulated SMC migration and proliferation is dependent on FAK activation of the small GTPase Rac1.

Additional studies have indicated that signaling through the Rho GTPases is FAK-dependent, which may indicate another mechanism by which FAK regulates cytoskeletal

stability and SM phenotype. Specifically, fibroblast studies have shown that integrin-mediated FAK Y397 activation is necessary for RhoA inactivation allowing for focal adhesion disassembly and cell migration [49). Interestingly, SMC differentiation is dependent on RhoA activity {Mack, 2001 #76] and Taylor et al identified GRAF, a RhoGTPase activating protein that binds to FAK [36], indicating that inhibition of RhoA may be one mechanism by which FAK may regulate SMC differentiation. However, in striated muscle, SK-alpha actin transcription was shown to require FAK-dependent RhoA activation [50], indicating the RhoA may be regulated in a cell-specific fashion. With all of the published evidence showing an intricate signaling network that regulates cell differentiation, a SMC-specific *in vivo* model is needed to detangle the precise function of FAK in these cellular responses.

FAK signaling in vivo:

In vivo examination of FAK has shown that FAK is essential for numerous cellular pathways, since FAK -/- embryos die in utero between day E8.5 and E10 [51]. It has been concluded that cardiovascular abnormalities are the cause of death, for there is no disparity between wildtype and null embryos up until day E7, and post mortum observations showed poor or no heart formation and poor vascular network extension [52]. Studies using FAK-/- embryoid bodies and endothelial cells derived from these mice showed that FAK was not required for endothelial cell differentiation but was necessary for endothelial migration needed for sufficient tubulogenesis [53]. Other studies showed that embryonic fibroblasts derived from FAK-/- embryos displayed in culture an increase in the number of focal adhesions, and an upregulation of the FAK family

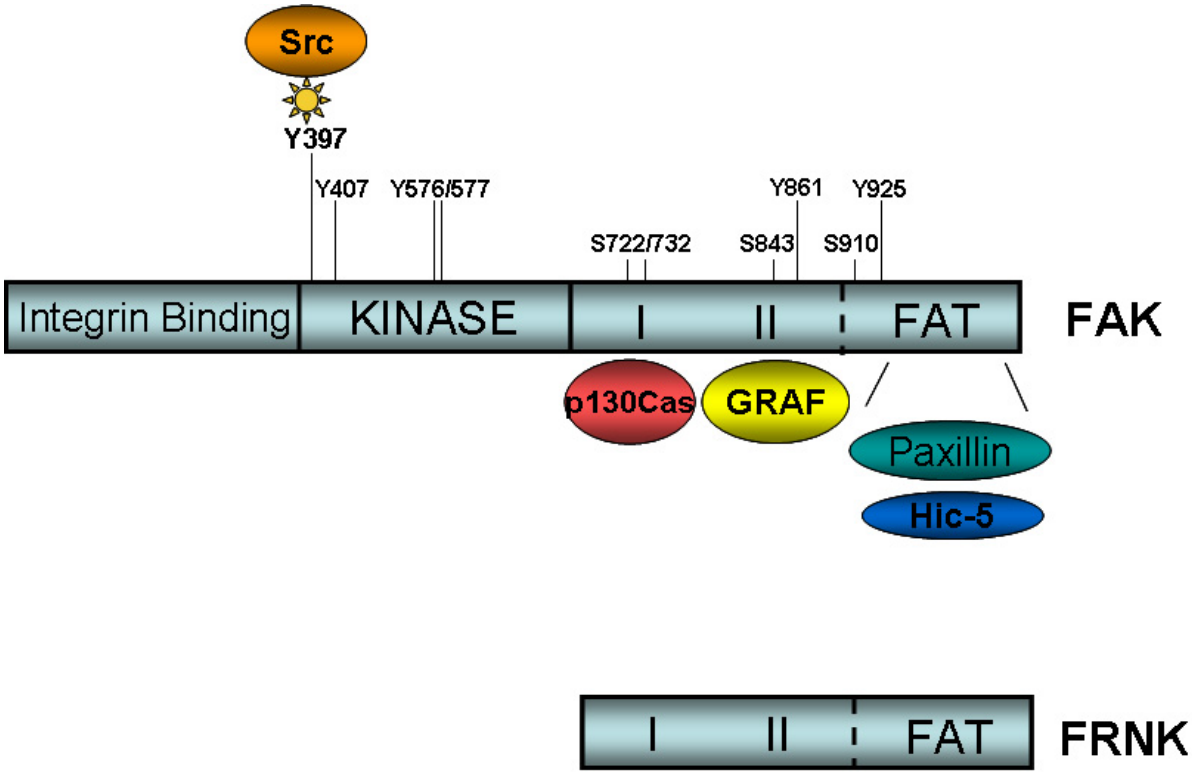


Figure 1.1. Structure of FAK and FRNK. FAK is comprised of an N-terminal integrin binding domain, a central kinase domain and a C-terminal focal adhesion targeting (FAT) domain. There are 6 tyrosine and 4 serine phosphorylation sites identified within FAK. Src-dependent phosphorylation of Y397 is the major site of FAK activity. FRNK, a dominant negative of FAK, is comprised of the FAK C-terminus

member, Pyk2, that allowed for ERK activation [54]. However, this compensation effect was not sufficient to overcome the observed attenuation of migration in these cultured cells. This lethal phenotype of FAK-/- embryos handicaps any advancement in cell-specific understanding of FAK-dependent signaling events, therefore several models have been developed using Cre/LoxP technology. To date, published models include the use of endothelial, heart, brain, and keratinocyte specific FAK deletion models [55-59].

As observed in the original FAK knockout, EC-specific FAK deletion resulted in decreased cell migration, proliferation, and cell survival contributing to embryonic lethality during late embryogenesis [55]. However, EC-specific FAK deletion studies performed in another lab showed that FAK ablation had no effect on EC migration or proliferation, but did result in increased cell retraction and apoptosis causing embryonic lethality during embryogenesis [56]. The disparity observed between the two published EC models could be due the time at which FAK was deleted development. Even though both groups used Tie2-Cre animals, there appears to be variation in the effectiveness of the two Cre transgenes, which may be due to varying transgene copy number between the two Tie-Cre strains. Similar to the FAK-null MEFs, FAK-null keratinocytes exhibited elevated Rho activity and Schober et al found that increased Rho activity resulted in increased MLCK phosphorylation and PAK1 activation. Additionally, these cells exhibited no proliferation defects [59]. Additional disparities at the single cell level could highlight cell-specific regulation of FAK and further studies are required to determine the intricacies of cell-specific FAK signaling.

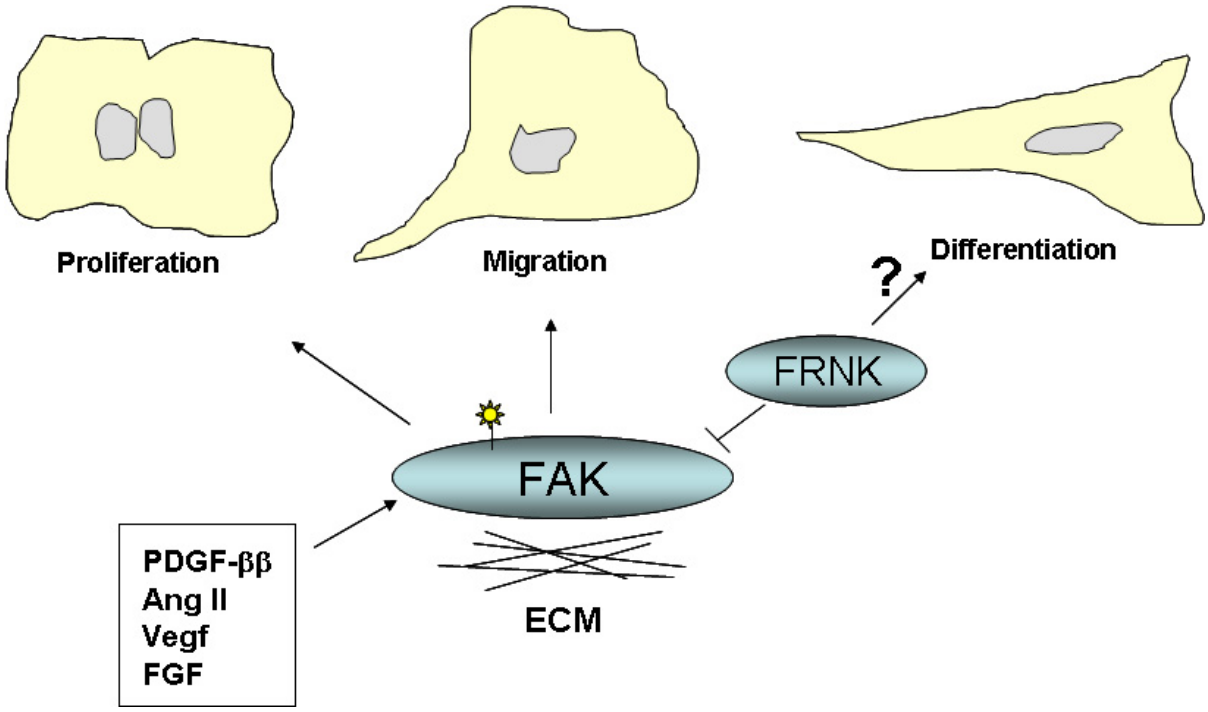


Figure 1.2. FAK activity is required for cell migration and proliferation. Previous data from Taylor et al showed that FAK activity is required for growth factor-, G-protein coupled receptor- and integrin-dependent SMC proliferation and migration. Overexpression of FRNK is able to attenuate FAK-dependent SMC processes and it is hypothesized that this may help regulate SMC phenotypic modulation.

FAK-DEPENDENT SIGNALING PATHWAYS IMPLICATED IN PROLIFERATION AND MIGRATION

MapKinases and Small GTPases:

Ras- and Rac-dependent activation of the MapKinase signaling cascade appears to be the dominant mitogenic pathway utilized by a various agonists [60]. Ras is required for the activation of the serine/threonine protein kinase Raf and the subsequent activation of MEK and ERK kinases [61] whereas Rac is important for the activation of the serine/threonine kinase PAK and the subsequent JNK kinase activation [62]. Once activated, ERK and JNK both translocate to the nucleus and phosphorylate and activate transcription factors necessary for immediate early gene expression and cell growth [63, 64].

Although the Ras-ERK and Rac-JNK pathways were initially thought to be independent parallel pathways, recent studies aimed at deconstructing these pathways have indicated that there is significant cross talk between these mitogenic signaling cascades. Studies using dominant-interfering mutants for Rac showed that activation of Rac was necessary for maximal Ras-dependent ERK activation and nuclear translocation [65, 66]. These data indicated that Ras acts upstream of Rac in the mitogenic signaling cascade. It was later revealed that Ras-dependent activation of Pi3Kinase leads to activation of Rac and its downstream effector PAK [67]. Exactly how Rac signaling feeds back into the Raf/MEK/ERK signaling cascade is highly studied. Li et al have published that Raf activation by Ras can be partially attenuated by overexpressing a dominant negative Rac1 construct [68]. Activation of Raf is a complex process known to involve Ras-dependent plasma membrane association as well as phosphorylation. Interestingly, the carboxy-terminus of activated PAK was recently shown to associate with the catalytic domain of Raf,

phosphorylating Raf Ser-338, which is essential for Raf activation [69]. In addition, PAK can enhance phosphorylation of MEK on a site (S228) that is necessary for maximal MEK activation by Raf. These studies indicate that Pak and Ras co-activate the serine/threonine kinase Raf, and in addition, PAK and Raf co-activate the dual specificity kinase Mek, indicating that the convergence in these two well established pathways likely occurs at the level of PAK [70].

PAK:

The family of p21-activated kinases (PAK) is subdivided into two groups, Group I consisting of PAKs 1-3 (also known as α,γ,β) and Group II consisting of PAKs 4-6 (77). Group I PAKs contain an N-terminal autoinhibitory domain that has been shown to bind and inactivate the C-terminal serine/threonine kinase domain. Originally, autoinhibitory domains were identified in Group II PAKs (reviewed by [71]); however, recent data demonstrated that PAK5 also contains a functionally related C-terminal autoinhibitory domain [72]. PAK contains several interspersed SH3-binding sites necessary for binding to a number of cellular proteins such as the adapter proteins Nck and Grb2, and the chromosomal protein histone H3 [73-75]. In a variety of cell types, PAK1 (the best characterized family member) affects cytoskeleton rearrangement and cell migration through its function as a Rac and Cdc42 effector [47]. GTP-bound Rac and Cdc42 activate PAK1 by binding to the p21-binding domain (PBD) localized within the N-terminal autoinhibitory domain [76]. This interaction dissociates the PAK1 trans-homodimer, exposing the C-terminal kinase domain and enabling subsequent autophosphorylation [71, 77]. Recent studies have shown that integrins are a major upstream activator of PAK. Notably, plating cells on the ECM fibronectin enhanced

the ability of GTP-Rac to activate PAK [78], which mediates β1 integrin-dependent activation of JNK [79]. This signaling cascade could also be dependent on adhesion-activated FAK since Stoletov et al have shown that PAK and FAK are associated within focal adhesions. However, they did not determine whether the observed PAK/FAK association was direct or dependent on the adaptor protein, Nck [80]. Additionally, the adapter protein Grb2 has been shown to mediate PAK1 signaling. Data by Puto et al showed that Grb2 was required for the PAK1/EGFR association utilized for EGF-stimulated migration [74]. Since the binding assays were performed using whole cell lysates, the PAK/EGFR complex formation may also be dependent on FAK localization. FAK can directly bind to the EGFR, therefore EGF-dependent tyrosine phosphorylation of FAK may regulate Grb2 binding to FAK [81], bringing Grb2 proximal to the EGFR. Interestingly, Howe et al demonstrated that inhibition of PAK attenuated integrin-dependent ERK activation, and Eblen et al showed that this was likely due to the ability of PAK to enhance the formation of MEK-ERK complexes in an adhesion-dependent fashion [82, 83]. These results suggest that there is an overlap of pathways between the Raf/MEK/ERK and Pi3K/Rac/PAK cascades. In Chapter III, my studies provide additional evidence of cross-talk between the Raf/MEK/ERK and Pi3K/Rac/PAK signaling cascades. My data shows that ERK and PAK directly associate allowing for ERK-dependent phosphorylation of PAK, indicating that PAK may function as a scaffold for adhesion- and growth factor-dependent activation of the MapKinase cascade.

SMC DIFFERENTIATION

Differentiated SMC constitute the medial layer of large arteries and small arterioles of the vasculature. A fully differentiated SMC is characterized by expression of high levels of SM myosin heavy chain (SM MHC), SM α-actin, SM 22α, SM myosin light chain kinase, telokin, and calponin, all of which are necessary for regulating SMC contractility (see [4] for review). These contractile genes are commonly referred to as SM marker genes. Differentiated SMC maintain tensile strength of the vessel wall in response various vasoactive and constrictive agents to regulate blood pressure and proper tissue perfusion. However, unlike skeletal and cardiac muscle cells, SMC do not terminally differentiate and retain the genetic plasticity that enables them to transition from a highly contractile phenotype to synthetic phenotype. A synthetic phenotype is commonly characterized by low levels of SM contractile gene expression and an increased responsiveness to proliferative and migratory signals. The ability to phenotypically modulate to a more proliferative phenotype in response to various environmental stimuli [4] is critical for proper vessel development and injury repair processes. However, unfettered SMC phenotypic switching can result in various vascular pathologies, such as atherosclerosis and restenosis following balloon angioplasty and following vein bypass grafts.

In addition to vascular pathologies, SMC specification, commitment, and differentiation are essential for proper vasculogenesis and organogenesis during embryonic development. Not much is known regarding SMC specification and commitment, although SMC precursors have been shown to include both neural crest and mesodermal cells [4]. Many labs have contributed to our understanding of SMC differentiation, which has recently

been extensively reviewed by Owens et al [4]. Differentiation appears to be regulated by SRF [84, 85] and myocardin (or myocardin-related) transcription factors [86], which have been shown to regulate expression of CArG-box containing promoters found in most SMC contractile genes, such as SMα-actin, SM22 and SM-MHC [87, 88]. Almost all SM markers are regulated by a SRF DNA binding element termed a CArG box (CC(A/T)6GG). SRF can associate with various cofactors to mediate SRF-dependent gene expression. Known SRF co-factors include the ubiquitously expressed ternary complex factors (TCFs, e.g. Elk-1), GATA-4, Nkx2.5, myocardin family members, and various LIM domain proteins [4, 89, 90]. It is hypothesized that several of these cofactors form a ternary complex with SRF and the CArG element, since most lack the ability to directly bind DNA. Ternary complex formation may promote gene specificity, enhance SRF/CArG binding affinity, and/or result in histone actelyation or deacetylation regulating the DNA configuration and gene transcription.

Myocardin:

Myocardin is a cardiac and smooth muscle specific transcription factor (see [91] for review). Genetic deletion of myocardin results in embryonic lethality at day E11.5 due to the absence of proper smooth muscle cell development within the vasculature [92]. The resulting phenotype of the myocardin knockout highlighted the role of myocardin as being the major regulator of SMC differentiation; however, elaborate studies using myocardin-null ES cells to generate chimeric mice demonstrated that myocardin was not essential for SMC differentiaiton [93]. The authors note that this observation does not explain why the myocardin knockout exhibits defective SMC development. The disparity may be due to differences in *in vivo* and *ex vivo* differentiation models.

Myocardin is composed of several domains that mediate its function. The transactivation domain (TAD) within the C-terminus of myocardin is essential for robust SM marker gene transcription. Interestingly, the central DNA binding domain (SAP) of myocardin is not required for SM22 transcription, suggesting that myocardin may induce transactivation through dimerization with other myocardin family members mediated through the LZ domain (for review see [94]).

The precise mechanism of myocardin transactivation is subject of intense study. Cao et al have demonstrated that myocardin-dependent SM gene transcription is propagated through histone actelyation by an SRF/myocardin/p300 coactivation complex. This complex is necessary for chromatin destabilization, since myocardin does not have HAT activity and p300 expression alone failed to induce SM genes [95]. In addition, Liu et al have found that myocardin stimulated gene transcription is regulated by the forkhead transcription factor FOXO4. They showed that constitutively nuclear myocardin is repressed when bound to FOXO4 and that stimulation of the Pi3K/AKT signaling cascade stimulated the nuclear export of FOXO4 allowing for myocardin-dependent transcription [96]. The ability of myocardin to interact with various transcriptional coactivators and repressors may allow for the variegated SM marker gene expression during SMC phenotypic modulation.

TGF-β signaling:

Selected agonists, including TGF-β and S1P have been shown to induce SMC differentiation. TGF-β, the major activator, has been shown to be sufficient to induce SMC differentiation in various cell lineages, including non-committed fibroblast like cells, 10T1/2's, adipose-derived stem cells, rat peritoneal exudates macrophages, and totipotent ES

cells [97-99]. TGF-β activates a well-characterized signaling cascade initiated by the ligand binding to the membrane bound TGF-β-receptor serine/threonine kinase complex. This complex triggers the subsequent binding and phosphorylation of RSmad (Smad 2 and 3) family members followed by the RSmad-Smad4 complex formation and its nuclear translocation and targeted gene transcription [100]. Several studies aimed to delineate the TGF-β signaling pathway in SMCs have shown that both Smad2 and Smad3 play a role in TGF-β stimulated SM marker gene transcription [99, 101]. Interestingly, even though both Smads are equally expressed in SMC lineages, there is a disparity in which Smad is required for selected SM marker gene transcription. Gene-specificity may be directed through the Smad association with various coactivators. Qiu et al found that Smad3 interacts with the coactivators p300 and myocardin to induce SM22 transcription which may serve to regulate TGF-β dependent SMC differentiation [102, 103]. Of the two known inhibitory Smads, Smad6 and Smad7, Smad7 has been shown to negatively regulate TGF-β signaling in SMC, for overexpression of Smad7 blocked TGF-β stimulation SM marker gene transcription [104].

In contrast to the numerous studies suggesting Smad3 as the main transducer of TGF-β stimulated differentiation, involvement of other signaling pathways have been conflicting. Chen et al determined through the use of pharmacological inhibitors that RhoA, not Pi3K- or ERK- mediated signaling was required for TGF-β stimulated neural crest cell transdifferentiation [105]. On the other hand, Lien et al, also using pharmacological inhibitors, found that TGF-β stimulated SM marker gene transcription in 10T1/2 cells was mediated through the Pi3K/Akt pathway [106]. Lastly, Deaton et al found that TGF-β stimulated SM marker gene transcription was mediated through RhoA/PKN/pp38 MAPK

signaling events in pulmonary aortic SMC [107], although, whether this signaling cascade stimulated SM marker genes in a Smad-dependent, or –independent manner was not examined. The various signaling pathways utilized may suggest cell-type specificity and further studies are required to determine if differentiation state dictated which signaling pathways are activated.

PDGF-BB signaling:

Contractile mechanisms need to be repressed as the cell phenotypically modulates to a more proliferative state. PDGF-BB is thought to not only induce proliferative machinery but to repress pathways utilized for maintaining a differentiated phenotype. It was first observed that quiescent SMC treated with PDGF-BB displayed reduced mRNA and protein levels of SM α–actin [108] and further studies showed that SM22 and SM-MHC were also reduced following PDGF-BB treatment [109]. The reduced levels may be attributed to PDGF-dependent increase of Sp1 levels and its subsequent binding to the G/C repressor element within the SM22 promoter [110]. Another putative mechanism may be through PDGF-BB dependent Elk-1 phosphorylation, which Wang et al showed diminished the affinity of CArG-SRF-Myocardin complex [111]. Recent data showed a decreased association between the myocardin family member, MRTFA and the CArG element following PDGF-BB treatment [112]. PDGF-BB has also been shown to affect SRF nuclear localization, for prolonged growth factor exposure increased cytostolic SRF levels resulting in decreased SM marker gene transcription [113]. Whether SRF nuclear export is the limiting factor regulating the complex formation with myocardin and MRTFA to activate SMC gene promoters or if there is a PDGF-BB-dependent dissociation of the transcriptional

complex that enables SRF nuclear translocation is unknown. Additionally, the cytostolic signaling events regulating SRF return to the nucleus to transactivate CArG containing growth genes, such as c-fos, in response to PDGF-BB is also unknown.

LIM domain proteins:

LIM domain containing proteins have recently been highlighted for their involvement in signal transduction from focal adhesions to the nucleus. These proteins are identified by double zinc-finger like structures anchored by cysteine/histidine residues. These modules were termed LIM domains based on homeodomain commonality observed in LIN-11, Isl1 and MEC-3 proteins and have been shown to facilitate protein-protein interactions [114]. LIM domains are made up of 55 amino acids that vary between the 58 known LIM containing genes [114]. Protein families are segregated by the number of LIM domains, for example, the CRP family (crp1, crp2, crp3) has two, the Zyxin family (zyxin, ZRP-1/Trip6, LPP, Ajuba) has three domains, and the Paxillin family (paxillin, Hic-5, leupaxin) has four domains.

A role for the CRP family has recently been implicated in SMC differentiation. CRP1 is ubiquitously expressed, while CRP2 expression is restricted to arterial SMC and CRP3 is expressed in both skeletal and cardiac muscle [115]. In vitro data have implemented a role for CRP1 and CRP2 in cardiovascular development however, only CRP3 knockout mice die postnatally due to cardiac defects [116]. Due to the high sequence homology among the three family members, a compensation mechanism has been proposed in the CRP1 and CRP2 genetic deletion models. Even though CRP2 is not essential for cardiovascular development, Chang et al have shown that CRP2 plays a role in SMC

differentiation through complex formation with SRF and GATA4 [90]. Additionally, CRP2 expression may be important for regulating SMC phenotype since SMC isolated from CRP2-/- mice exhibit increased PDGF-stimulated Rac1 activation and an increased migratory capacity compared to littermate controls [117].

Compared to the CRP family, the zyxin family is widely expressed. Overall, the zyxin family members are important for cell migration through focal adhesion localization. Genetic deletion of zyxin has no effect on viability [118]. Ajuba-null mice were also viable with no observable phenotype. However, cells isolated from Ajuba- null mice displayed impaired migration due to reduced FAK-dependent Rac1 activation [119]. Trip6 can bind to the LPA2 receptor, which is important for its focal adhesion localization, and when localized to focal adhesions Trip6 can associate with FAK [120]. Similar to other family members, LPP localizes to focal adhesions and can translocate to the nucleus, which has been suggested to be mediated through an interaction with VASP [121]. Inhibition of LPP through RNAi decreased SMC migration and LPP expression was reduced in migration-defective FAK-null fibroblasts [122]. However, if LPP expression was directly related to FAK ablation or a due to secondary affect was not studied.

The paxillin family of LIM proteins consists of paxillin, Hic5, and leupaxin. This subfamily has four C-terminal LIM domains and four/five N-terminal LD motifs (Figure 1.3). The focal adhesion protein, paxillin, is a 68 kDa protein that binds numerous other actin-binding proteins, kinases and GTPase activating proteins (GAPs) [123]. Through identification of various binding partners, paxillin has been implicated in cell migration by regulating focal adhesion turnover and matrix adhesion. Genetic deletion of paxillin resulted in embryonic lethality around day E9.5 due to cardiac and somatic defects mimicking those

seen in FAK and FN-/- models [124]. In support of adhesion-dependent defects, paxillin-null ES cells displayed defective cell spreading and reduced FAK activation compared to wt cells [125]. Of interest, several binding assays have mapped LD motifs 2 and 4 of Paxillin to be required for FAK binding [126]. Src- dependent FAK activity (Y397 phosphorylation) has been shown to regulate Paxillin tyrosine phosphorylation [127]. Paxillin phosphorylation is essential for migration since transient expression of a nonphosphorylatable paxillin (Y31FY118F) mutant attenuated robust migration observed in vinculin -/- cells [128]. Vinculin, a focal adhesion adaptor protein, has been mapped to bind to paxillin through LD1, LD2, and LD4 [129] and its binding interferes with FAK/paxillin association thereby regulates cell migration [128]. Thus, unregulated FAK/paxillin-dependent signaling has been attributed to the highly motile phenotypes observed in vinculin knockout models [130]. Additionally, the paxillin/PKL interaction is dependent upon Src/FAK-dependent paxillin tyrosine phosphorylation, which mediates PXL focal adhesion localization [131]. p95PKL has been shown to bind to LD4 of paxillin, forming paxillin/PKL/PIX/PAK complex and stimulating proper cell migration [132, 133]. Another known binding partner, PTP-PEST, negatively regulates paxillin signaling. PTP-PEST binds to the C-terminal LIM domains and dephosphorylates paxillin [134, 135]. This has been proposed to impair cell migration by inhibiting paxillin/Rac1 signaling [135]. The PTP-PEST/paxillin complex may be essential for focal adhesion turnover required for proper migration, since PTP-PEST-/- cells exhibit rapid cell spreading and defective cell migration that has been attributed to increased levels of FAK and paxillin activity in comparison to WT cells [136].

Another paxillin family member is Hic-5. Hic-5, a 55 kDa protein, is structurally homologous to paxillin and has been shown to have several overlapping binding partners,

including, FAK, vinculin, and PTP-PEST [137, 138]. Characteristic of a sub-class of LIM domain proteins, Hic-5 is able to translocate from focal adhesions to the nucleus. Even though very similar in structure, paxillin and Hic--5 have been shown to have independent functions. Hic-5 competes with paxillin for FAK binding. Hic-5 bound to FAK, decreases FAK and paxillin activation resulting in a reduction of cell spreading [139]. Since Hic-5 only shares 57% structure homology with paxillin [140]. Hic-5 may antagonize paxillin-mediated signaling events by recruiting a different subset of signaling proteins to focal adhesions.

Besides regulating cell migration, Hic5 is most commonly known as a transcriptional coactivator. Hic-5 can regulate several hormone-responsive genes through interactions with the glucocorticoid receptor and the androgen receptor [141]. Additionally, Hic5 activates several growth genes including p21 and c-fos [142]. Shibanuma et al have shown that Hic-5 complexes with p300, a gene coactivator, and Smad3 and binds to Sp1 sites located within the p21 gene promoter [143]. Therefore, Hic-5 may function as a transcriptional scaffolding protein mediating the association of promoter-specific coactivators required for transcription.

Leupaxin, the lesser known member of the paxillin family, was first identified by Lipsky et al in leukocytes. Lipsky et al first observed leupaxin expression was highly expressed in lymphocytes compared to undifferentiated bone marrow cells [144]. Similar to Hic-5, leupaxin has four N-terminal LD motifs and four contiguous C-terminal LIM domains. Emerging evidence has shown that Leupaxin has several common binding partners, including Pyk2, FAK, and PTP-PEST, with its paxillin family members [144, 145]. There is little evidence on the localization and function of leupaxin. Published herein is the

first report of leupaxin's enrichment in SMC, cytoplasmic to nuclear shuttling, and function as a SRF-dependent transcription coactivator (Chapter IV).

Figure 1.3. Paxillin family members. The Paxillin family consists of paxillin, Hic-5 and leupaxin. Each has four contiguous LIM domain and five (paxillin) or four (Hic-5 and leupaxin) N-terminal LD motifs. *leupaxin NES is putative and addressed in Appendix II.

SUMMARY

It is becoming increasing clear that there is not one master regulator of SM phenotype. Although SRF is required for CArG-dependent SM gene transcription, it is not specific for SM marker genes, and the mechanism regulating SRF-dependent SM marker gene transcription versus SRF-dependent growth gene transcription is not yet known. Myocardin was initially thought to be the single regulator of SM phenotype through its ability to potently active the SM gene promoters, although knockout models dispersed the notion it was the one essential coactivator. However, the myocardin family, including myocardin, MRTF-A, and MRTF-B could all function in overlapping roles to mediate SM gene expression. Additional loss of function experiments and chimeric models are required to determine precise mechanism of each family member. SM marker genes have also been shown to be regulated by numerous transcriptional coactivators, including LIM domain-containing proteins. These proteins may function in a scaffolding capacity, bringing the required coactivators within close proximity to the transcriptional machinery. Since LIM domain-containing proteins are able to shuttle from focal adhesions to the nucleus, cellular localization of these proteins may be regulated by focal adhesion proteins, such as FAK, in response to various external cues.

Data published by us and others, have demonstrated that FAK is a point of convergence for adhesion-, growth factor- and G-protein coupled receptor- mediated signaling. Since FAK has been shown to activate numerous signaling cascades including the canonical Ras/Raf/MEK/ERK and Pi3K/Rac/PAK/JNK cascades, it can be hypothesized that the specific pathway activated and subsequent gene transcription can be regulated by the activation state of FAK. Therefore, this dissertation focuses on the signaling pathways that

regulate SMC proliferation, migration, and differentiation, highlighting the involvement of Focal Adhesion Kinase. Chapter II demonstrates that FAK is an important point of convergence for adhesion-, G-protein coupled receptor-, and growth factor- dependent signaling. The overexpression of FRNK, a dominant negative inhibitor of FAK, showed that SMC proliferation and migration requires FAK-dependent Rac1 activation. Chapter III illustrates the cross talk between the Ras/ERK and PAK/Rac signaling cascades. Various binding assays showed a novel direct interaction between PAK and ERK suggesting that PAK may function as a scaffold for the Raf/Mek/ERK signaling cascade. Lastly, Chapter IV shows that FAK activity may play role in regulating SMC phenotype. Promoter assays showed that TGF-β-dependent SM marker gene expression is upregulated in FAK-ablated primary SMC cultures. These data also suggest that FAK may regulate SM differentiation through interacting with the LIM domain protein leupaxin. This Chapter is also the first report showing leupaxin can translocate from focal adhesions to the nucleus and bind to transcriptional complexes inducing SM gene transcription.

Review of published data detailing the intricate signaling networks that regulate cell phenotype, growth, and motility, also show conflicting data depending on the utilized cell system. Therefore, a SMC-specific in vivo model is needed to detangle the precise function of FAK in these cellular responses. My studies described herein, using a SMC-specific FAK deletion model to delineate FAK-dependent mechanisms that regulate SMC growth and development contribute to our comprehensive understanding of SMC biology and the putative involvement of integrin-dependent FAK signaling in vascular pathologies.

CHAPTER II

An endogenous inhibitor of Focal Adhesion Kinase blocks Rac1/JNK but not Ras/ERK-dependent signaling in vascular smooth muscle cells.[1]

[1] Data published as: Liisa J. Sundberg, Lisa G. Galante, Heather M. Bill, Christopher P. Mack and Joan M. Taylor. An endogenous inhibitor of Focal Adhesion Kinase inhibits Rac1/JNK but not Ras/ERK-dependent signaling in vascular smooth muscle cells. *J Biol Chem (2003)* 278(32) 29783-91.

ABSTRACT

Humoral factors and extracellular matrix are critical co-regulators of smooth muscle cell (SMC) migration and proliferation. We previously reported that FRNK (FAK Related Non Kinase) is expressed selectively in SMC and can inhibit PDGF-BB- induced proliferation and migration of SMC by attenuating FAK activity. The goal of the current studies was to identify the mechanism by which FAK/FRNK regulates SMC growth and migration in response to diverse mitogenic signals. Transient overexpression of FRNK in SMC attenuated autophosphorylation of FAK at Tyr-397, reduced Src-family dependent tyrosine phosphorylation of FAK at Y576, Y577, and Y881, and reduced phosphorylation of the FAK/Src substrates Cas and paxillin. However, FRNK expression did not alter the magnitude or dynamics of ERK activation induced by PDGF-BB or AngII. Instead, FRNK expression markedly attenuated PDGF-BB-, AngII- and integrin-stimulated Rac1 activity and attenuates downstream signaling to JNK. Importantly, constitutively active Rac1 rescued the proliferation defects in FRNK-expressing cells. Based on these observations, we hypothesize that FAK activation is required to integrate integrin signals with those from receptor tyrosine kinases and G protein-coupled receptors through downstream activation of Rac1, and that in SMC, FRNK may control proliferation and migration by "buffering" FAK-dependent Rac1 activation.

INTRODUCTION

Smooth muscle cell (SMC) proliferation is certainly important during vascular development, but it is clear that increased SMC proliferation and migration are important contributors to the pathogenesis of several important cardiovascular disease states including atherosclerosis, restenosis, and hypertension. A large number of extrinsic cues (growth factors, extracellular matrix, cell-cell interactions, etc.) have been identified that regulate SMC growth and migration (1,2). However, the precise cellular signaling mechanisms involved are not completely understood, and very little is known about how (or if) these signaling pathways are integrated.

The majority of soluble SMC mitogens can be broadly divided into two groups, activators of receptor tyrosine kinases (i.e. platelet-derived growth factor BB homodimer (PDGF-BB), basic fibroblast growth factor, insulin-like growth factor-1 (IGF-1) and activators of G protein-coupled receptors (i.e. angiotensin II (AngII), thrombin, endothelin-1 (ET-1); ref. 2). A large body of evidence indicates that members of both groups activate (to varying degrees) the Ras/Raf/MEK/ERK and Pi3 kinase/Rac/PAK/JNK kinase cascades, PLC, and PKC signaling pathways among others (1). Interestingly, it is becoming clear that the mitogenic responses elicited by many of these factors are dependent upon extracellular matrix (ECM)/integrin interactions. For example, when cultured fibroblasts are held in suspension, PDGF-, EGF- and lysophosphatidic acid-stimulated ERK activity is markedly reduced compared to cells plated on fibronectin (3,4).

Integrin-mediated activation of the non-receptor tyrosine kinase, focal adhesion kinase (FAK) is a critical step in integrin signaling and may be important for growth factor signaling as well. In support of this idea, several mitogens (including PDGF-BB, AngII,

IGF-1, and ET-1) stimulate FAK tyrosine phosphorylation in an adhesion-dependent manner (5-8). In addition, Renshaw et. al. have shown that integrin augmentation of growth factor mediated signaling to ERK2 appears to be dependent upon FAK activation, because the inhibition of MAP kinase activation observed in suspended cells can be rescued by over-expression of activated FAK (9). Furthermore, integrin-mediated autophosphorylation of FAK on Tyr397 leads to recruitment and activation of Src and PI3K and subsequent downstream activation of Ras/ERK or Rac/JNK cascades (10,11).

Interestingly, our lab recently showed that FAK appears to be regulated in a unique fashion in SMC. We reported that the noncatalytic carboxy-terminal domain of FAK, termed FAK related non-kinase (FRNK), is selectively expressed in SMC with very high levels found in the large arterioles. FRNK transcription results from the utilization of an alternative start site within the FAK gene and FRNK expression is independently regulated by a distinct promoter embedded within FAK intronic sequences (12,13). Interestingly, whereas FAK protein levels remain relatively constant, FRNK protein levels in vivo are dynamically regulated with increased FRNK expression in the neonatal period and from two to three weeks following balloon injury. This pattern of expression correlates with the attenuation of SMC proliferation that is known to occur under these circumstances, suggesting that FRNK may be an important regulator of SMC growth (2). In support of this idea, we have shown that overexpression of GFP-FRNK in rat aortic SMC completely prevented the PDGF-BB-induced increase in [^{3}H]-thymidine incorporation and significantly inhibited the mitogenic effects of serum. In addition, GFP-FRNK also significantly inhibited fibronectin-dependent SMC migration toward PDGF-BB (12). Taken together these data suggest that FRNK acts as

an endogenous inhibitor of FAK activity and that its expression in vivo may act to buffer FAK dependent proliferative and migratory signals.

In this report, we sought to identify the mechanism by which FRNK attenuates growth factor and adhesion signaling in SMC. Our data indicate that in contrast to other cell types, FAK/FRNK signaling does not modify agonist-stimulated ERK activity in SMC. Instead, our data reveal that in SMC, Rac1 activation may be a key convergence point in growth factor and FAK-dependent integrin regulated cell proliferation.

MATERIALS AND METHODS

Antibodies and Reagents

The 4G10 phosphotyrosine- specific antibody, an anti-human FAK antibody, and the Rac1 antibody were purchased from Upstate Biotechnology, Inc. The phosphotyrosine specific anti-FAK antibodies (Y397, Y576, Y577, Y861) were purchased from BioSource International. Texas-Red conjugated phalloidin was purchased from Molecular Probes. The phospho-specific and immobilized phospho p44/42 MAP kinase antibody was purchased from Cell Signaling along with the phospho-SAPK/JNK (Thr183/Tyr185) and phospho-ELK-1 antibodies. CAS was detected using a mixture of two polyclonal antibodies generated against the C-terminal domain of CAS (CAS-F and CAS-P; provided by Dr. Amy Bouton, University of Virginia, (14). The monoclonal, Erk1/2 antibody (1B3B9) was generously provided by Dr. Michael Weber (University of Virginia). The anti-flag (M5) antibody, Ang II and fibronectin were purchased from Sigma and PDBF-BB was purchased from Calbiochem.

Adenovirus Production and Expression Constructs

The GFP and GFP-tagged FRNK viruses (replication-defective ψ5 adenovirus) were plaque-purified by cesium-chloride gradients as described previously (12). The cDNA construct encoding an aminoterminal DsRed conjugated L61Rac1 was generated by cloning L61Rac1 into the 5' BamHI and 3' EcoRI restriction sites in the pDsRed2-CI vector (Clonetech).

Cell Culture, Infection, Transfection, and Agonist Treatment

Rat aortic SMC were obtained from 8week rat thoracic aortas by enzymatic digestion as previously described (15). Cells were used from passages 7-21 and were maintained in Dulbecco's modified Eagles medium (DMEM)- F12 (1:1) plus 10% fetal bovine serum and 1% pencillin-streptomyocin. For adenoviral infection, cells were incubated in serum containing media with either GFP or GFP-FRNK (MOI 10) for 12-15 hours prior to treatment. In some experiments, cells were serum starved for 4 hours followed by treatments with PDGF-BB or Ang II for the times indicated. For the adhesion suspension experiments, cells were trypsinized, neutralized in soybean trypsin inhibitor (1mg/ml in PBS), collected by centrifugation and resuspended in serum-free DMEM:F12. Cells were held in suspension or plated on fibronectin-coated (40μg/ml) dishes for the times indicated. In some experiments, SMC were transfected using Gene Jammer transfection reagent (Strategene) following the manufacturers instructions.

Immunocytochemistry.

SMC were plated on Lab Tek II chamber slides (Nalgene; 5,000 cells/cm^2) and infected with virus, as described above. Cells were serum starved for 4 hours (DMEM-F12, 1% pencillin-streptomycin) and then dosed with 20ng/ml PDGF-BB for 45min. Slides were then washed three times with phosphate buffered saline (PBS; calcium and magnesium free) and fixed in 4% paraformaldehyde in PBS for 20 min. Following an additional three washes in PBS, slides were permeabilized for 3 minutes with 4% Triton X-100 in PBS. Detergent was removed by washing three times with PBS and then slides were blocked with blocking solution (5% goat serum, 2% BSA in PBS) for 30 minutes. Cells were incubated for 1 hour

with Texas-Red phalloidin in PBS (1:1000) to visualize filamentous actin. All steps were performed at room temperature.

BrdU and WST-1 proliferation assays

To detect BrdU incorporation, SMC plated on Lab Tek II chamber slides, as described above, were transfected with 0.7 μg pDsRedC1 vector (DsV) or pDsRedC1 tagged RacL61 (DsRacL61) using Gene Jammer transfection reagent. 48 hours following transfection, cells were infected with GFP or GFRNK adenoviruses for 15 hr. Cells were serum starved as described above, then treated with either 10% fetal bovine serum or PDGF-BB (30ng/ml) overnight. Cells were then incubated with 1/1000 dilution of BrdU labeling reagent (Roche) for 1hour at 37^{o} C. Slides were washed 3 times for 5 min with PBS and then fixed with ethanol (50mM glycine, 70%ethananol, pH 2.0) in the dark at –20^{o}C for 20 min. BrdU detection reagent was used following the manufacturers instructions, followed by a 30 min incubation at 37^{o}C with a cascade blue-conjugated secondary antibody (1:1000, Molecular Probes). Slides were washed, mounted with coverslips, and BrdU positive nuclei were visualized by indirect fluorescence microscopy. For the WST-1 experiments, SMC were plated in 96 well culture dishes (2,000 cells/well) and treated as described above, with the exception that the WST-1 tetrazolium salt (Roche) was added to the culture for 2 hrs to monitor cell proliferation as per the manufacturer's instructions. After this incubation period the production of formazan dye was quantitated using a spectrophotometer (450 nm).

Immunoprecipitation and Western Blotting

SMC were lysed in modified RIPA buffer (50mM Hepes, 0.15M NaCl, 2mM EDTA, 0.1% NP-40, 0.05% sodium deoxycholate, pH 7.2) containing 1mM Na_3VO_4, 40mM NaF, 10mM Na_2 pyrophosphate 100μM Leupeptin, 1mM AEBSF, 0.02 mg/ml soybean trypsin inhibitor, and 0.05 TIU/ml aprotinin. CAS and paxillin were immunoprecipitated by incubation of 1 mg of cell extract with 5μg of the appropriate antibody for 2 hours at 4°C, followed by a 1 hour incubation with protein A-Sepharose conjugated beads (Amersham). For paxillin immunoprecipitations, the beads were pre-coupled rabbit anti-mouse Ab (10μg/ml, Jackson Labs). The immune complexes were collected by centrifugation and washed 3 times with RIPA buffer and 2 times with Tris-buffered saline (TBS; 0.2M NaCl, 50mM Tris-HCl, pH 7.4). Proteins were boiled in sample buffer and resolved on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel (PAGE) and transferred to nitrocellulose. Western blots were performed using the appropriate antibodies at a 1/1000 dilution (except anti-ERK, 1B3B9, which was used at 1:10,000). Blots were washed in TBS-T (TBS plus 0.05% Triton-X), followed by incubation with either horse-radish peroxidase conjugated Protein A sepharose (Amersham) or horse-radish peroxidase conjugated rabbit anti-mouse antibody (Amersham) at a 1/2000 dilution. Blots were visualized after incubation with chemiluminescence reagents (ECL, Amersham).

Kinase Activity

Kinase activity was measured by using a p44/p42 MapKinase Assay Kit (Cell Signaling), following the manufacturers protocol. In brief, 250 μg of lysate from PDGF-BB SMC lysate was incubated with 15μg of provided immobilized phospho-p44/p42 MapK antibody and

complexes were rotated overnight at 4°C. Immune complexes were collected by centrifugation and washed 3 times with provided 1X lysis buffer and resuspended in provided 1X kinase buffer containing 200μM ATP and 2μg Elk-1 fusion protein. The reaction was incubated at 30°C for 30 min, and terminated by the addition of 3X SDS sample buffer. Sample proteins were electrophoresed on an 11% SDS-PAGE gel, transferred to a nitrocellulose membrane, incubated with phophos-Elk-1 antibody and processed as described above.

GST-Pull Down Assay

GST-Pak (aa 1-290 of Pak1) was purified from bacterial lysates using glutathione- agarose beads (Pharmacia) as described previously (16). GFP- or GFRNK-infected SMC were serum starved and treated with PDGF-BB or AngII as described above. Cells were lysed in Buffer A (50mM Tris pH 7.6, 500mM NaCl, 0.1% SDS, 0.5% DOC, 1% Triton X-100, 0.5mM $MgCl_2$, plus 100μM Leupeptin, 1mM AEBSF, and 0.05 TIU/ml Aprotinin) and cleared by centrifugation for 10 min at 14000 rpm at 4°C (17). 500μg of lysate was combined with 30μg of GST-PBD and samples were rotated at 4°C for 30 minutes. The complexes were pelleted by centrifugation, and washed 2x times in Buffer B (50mM Tris pH 7.6, 150mM NaCl, 1%Triton X-100, 0.5 mM $MgCl_2$, plus 100μM Leupeptin, 1mM AEBSF, and 0.05 TIU/ml). Samples were boiled in SDS-PAGE buffer, electrophoresed (15% SDS-PAGE gel) and transferred to PVDF (Biorad). Western blotting was performed using an anti-Rac1 primary antibody as described above.

RESULTS

We previously reported that overexpression of GFP-FRNK in cultured aortic SMCs by adenoviral gene transfer resulted in a modest increase in FRNK protein effectively inducing an exogenous FRNK to endogenous FAK ratio of around 3:1. This FRNK:FAK ratio compares favorably with endogenous ratios observed in neonatal vessels and is sufficient to inhibit both FAK activity and PDGF-BB-stimulated proliferation and migration. To begin to dissect the signaling pathways altered by FRNK expression in SMC, we first examined the affect of FRNK overexpression on total cellular phosphotyrosine levels. In adherent cultures of SMC, three major tyrosine phosphorylated bands were attenuated in cells expressing GFP-FRNK (GFRNK) in comparison to GFP-infected or un-infected control cells (Figure 2.1A). The 125 kDa protein is probably FAK since FAK co-migrates with this band, and FRNK overexpression in SMC inhibits auto-phosphorylation of Y397 as previously reported (12). The use of additional phospho-specific antibodies demonstrated that FRNK over-expression in SMC also inhibited phosphorylation of the major Src-dependent phosphorylation sites in FAK namely pY556, pY557 and pY861 (Figure 2.1B). The tyrosine phosphorylated proteins migrating at approximately 130 kDa and 70 kDa likely represent the FAK binding partners p130Cas and paxillin (Figure 2.1C).

Since proliferation and migration of SMC in response to PDGF-BB are at least partially dependent on ERK activation (18-20), and since ERK activation has been reported to be dependent on FAK/p130Cas interactions (21), we tested whether overexpression of GFP-FRNK could attenuate mitogen-stimulated ERK activity. Surprisingly, dose-dependent stimulation of ERK by PDGF-BB was not altered by overexpression of GFP-FRNK (Fig.

2.2A, top panel) even though PDGF-BB-stimulated FAK activation (in the same lysates) was clearly attenuated (Figure.2.2A, middle panel).

Since the time-course of ERK1/2 activation may also regulate cell cycle progression, (22,23) we examined whether FRNK might alter the kinetics of PDGF-BB stimulated ERK activity. As shown in Fig.2.2B and 2.2C, FRNK expression did not alter the kinetics of PDGF-BB-induced ERK activation at time points ranging from 1 min to 3 hr. In addition, we observed no significant difference in the ability of ERK to phosphorylate ELK-1 as assessed by an immune-complex kinase assay that measures direct phosphorylation of a GST-ELK fusion protein (Figure 2.2C). These data likely indicate that in SMC, FRNK inhibits PDGF-BB-stimulated migration and proliferation in an ERK- independent fashion.

Other major signaling pathways known to regulate both proliferation and migration include activation of the small GTPases of the Rho family. Interestingly, Rac1 has been previously shown to be activated by both PDGF receptors and following engagement of certain integrins (24,25). We used a well-defined assay to examine Rac1 activation in SMC using a GST-PAK1 fusion protein to precipitate active (GTP-bound) but not inactive (GDP-bound) Rac1. As shown in Figure 2.3A, this GST-fusion protein effectively precipitated activated L61Rac, but not inactive N17Rac when these constructs were overexpressed in COS-7 cells. Using this assay, we have shown that PDGF-BB induced a rapid transient activation of Rac1 in SMC. PDGF stimulated an approximate 2-3 fold increase in Rac1 activity which peaked between 1 and 3min following stimulation (Figure 2.3B, left panel). As shown in Figure 2.3B (right panel), overexpression of GFP-FRNK slightly inhibited basal activity and markedly attenuated PDGF-stimulated Rac1 activity in SMC. In addition, activation of JNK (a kinase known to be downstream of Rac1 activation) was also attenuated

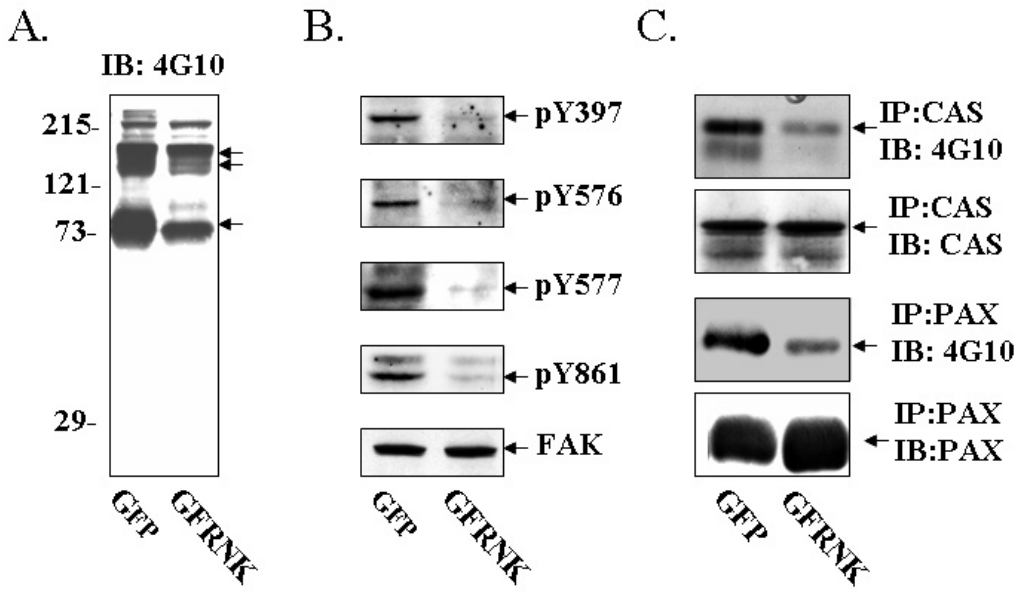


Figure 2.1. FRNK inhibits FAK autophosphorylation and co-localizes with FAK in SMC. Rat aortic SMC were infected with GFP or GFP-FRNK (GFRNK) adenovirus for 12 h. **A.** Extracts (100 μg) were analyzed by SDS-PAGE and immunoblotting using a generic phosphotyrosine antibody (4G10), an antibody to total FAK, or phosphorylation specific antibodies to Y397, Y576, Y577, Y861 of FAK. **B.** Paxillin (Pax) or p130Cas (Cas) were immunoprecipitated (IP) from the GFP or GFP-FRNK infected cell extracts as described under "Experimental Procedures" followed by immunoblotting with anti-phosphotyrosine antibody (4G10) and the appropriate antibody for loading controls.

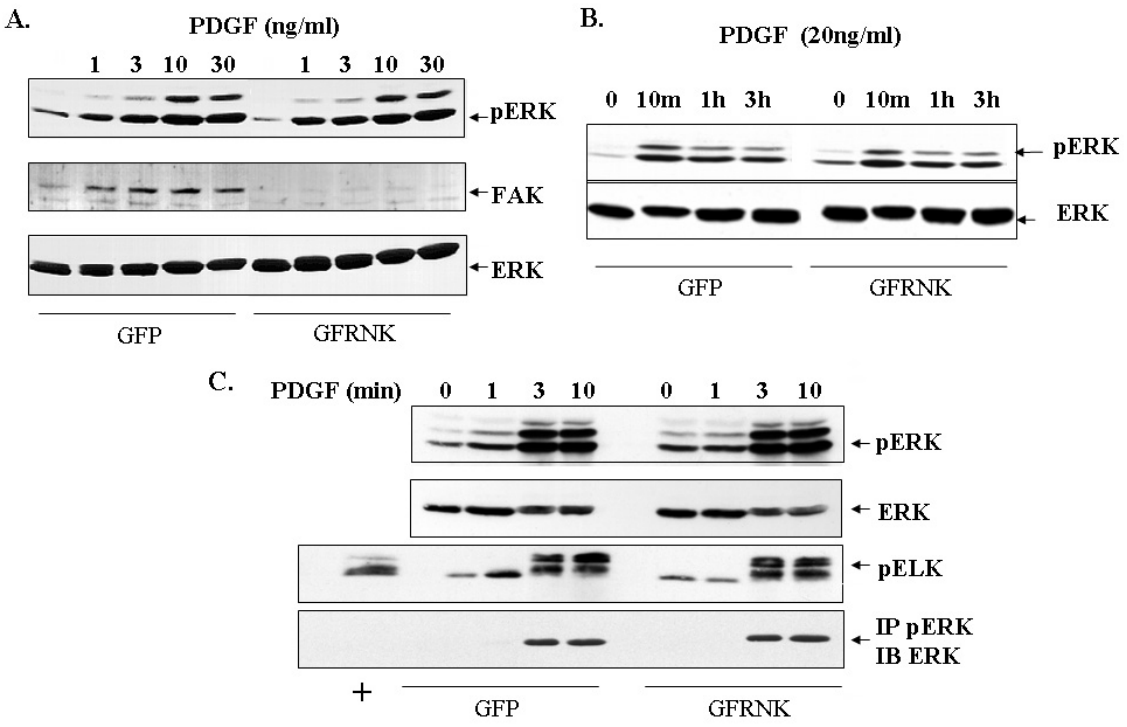
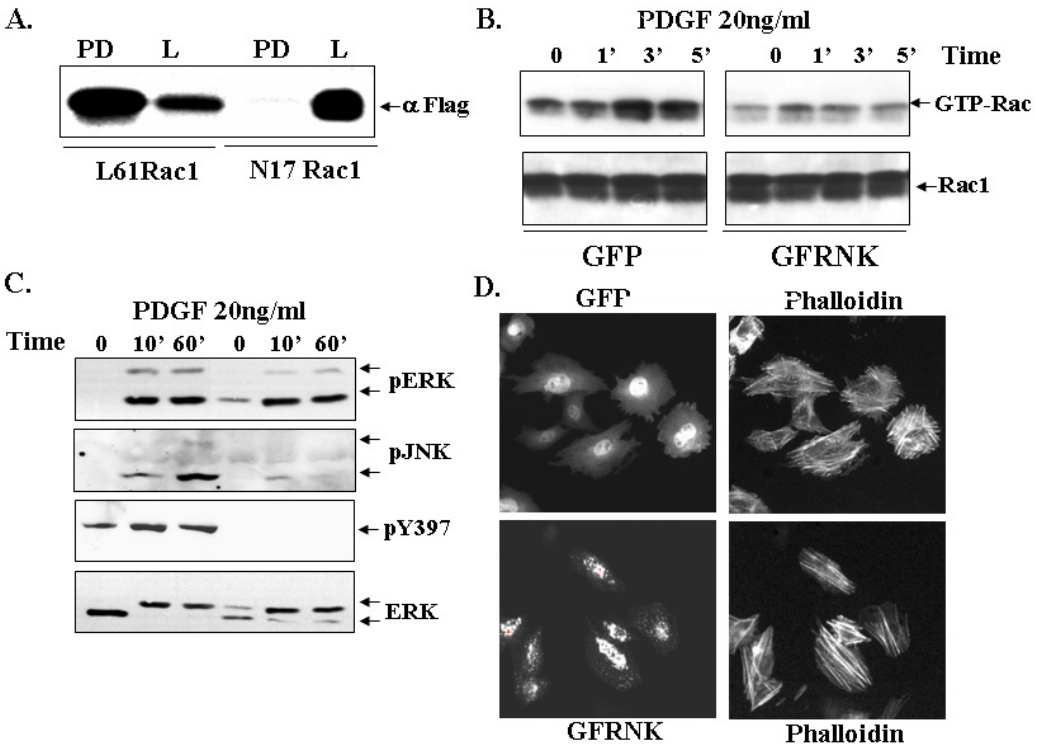


Figure 2.2. FRNK expression does not alter dose- or time-dependent activation of ERK by PDGF-BB in SMC. Rat aortic SMC were infected with GFP or GFP-FRNK adenovirus (10 M.O.I.) for 12 h. Cells were transferred to serum free media and incubated for 4 hr. Cells were treated with PDGF-BB (0-30 ng/ml) for 10 min (**panel A**) or with PDGF-BB (20 ng/ml) for various times as indicated (**panel B**). Lysates were electrophoresed and Western analysis was performed using an anti- ERK or anti- phospho-specific ERK Ab. **Panel C.** ERK was immunoprecipitated from cell lysates with an anti-ERK1/2 antibody, immune complexes were washed and incubated with 2 mg of purified GST-ELK immobilized on glutathione beads in a kinase buffer containing 10 mM MgCl2 and 200 mM ATP for 30 min at 30 o C. Immune complexes were then electrophoresed and Western analysis was performed using a phosphorylation-specific Elk-1 antibody. Control blots indicated that FRNK expression significantly attenuated FAK pTyr as assessed by Western blotting with an anti pTyr397 antibody in each experiment. Data are representative of at least 3 separate experiments.


A.
PD
L
PD
L
α Flag
L61Rac1
N17 Rac1
B.
PDGF 20ng/ml
0
1'
3'
5'
0
1'
3'
5'
Time
GTP-Rac
Rac1
GFP
GFRNK
C.
PDGF 20ng/ml
Time
0
10'
60'
0
10'
60'
pERK
pJNK
pY397
ERK
D.
GFP
Phalloidin
GFRNK
Phalloidin

in the GFP-FRNK expressing cells (Figure 2.3C). Since Rac1 activation stimulates the formation of distinct actin-rich lamellipodia, an important event during cell motility (24,26,27), we examined whether FRNK expression in SMC might alter this process in PDGF-BB-stimulated SMC. Indeed, in GFP-infected cells, PDGF-BB stimulated a reorganization of actin filaments and a resultant ruffled morphology (note the dissolution of stress fibers and formation of actin bundles around the periphery of the PDGF-BB-stimulated cells; Figure 2.3D, top panels). In contrast, PDGF treatment of GFP-FRNK infected cells did not result in noticeable changes in actin rearrangements (Figure 2.3D, bottom panels). Taken together, these data indicate that FRNK expression attenuates the activation of Rac1 and Rac1-dependent signals in SMC.

To determine whether inhibition of Rac1 was the sole determinant for the proliferative defects induced by FRNK, we examined the ability of activated Rac1 to rescue PDGF-BB-stimulated proliferation in FRNK over-expressing cells. To this end, SMC were transfected with either pDsRedC1 vector (DsV) or pDsRedC1 tagged RacL61 (DsRacL61) prior to infection with GFP and GFP-FRNK adenoviruses. Following serum starvation, cells were treated with PDGF and analyzed for their capacity to incorporate BrdU. Consistent with our previous report, FRNK over-expression attenuated PDGF-stimulated BrdU incorporation by approximately 40% to a level comparable to cells in serum free media ($p<0.05$ compared to GFP infected cells; Figure 2.4). Importantly, while Rac1 alone had little effect on BrdU incorporation in GFP expressing cells, over-expression of constitutively active L61Rac1 efficiently rescued the effects of FRNK on cell proliferation ($p<0.01$ compared to FRNK+ DsV). Control experiments indicated that similar amounts of FRNK

were expressed under both conditions (Figure 2.4, upper panel). These data indicate that Rac1 acts downstream of FAK/FRNK to control SMC proliferation.

To determine whether inhibition of Rac1 activity might be a general mechanism by which FRNK attenuates SMC growth, we examined the capacity of FRNK to inhibit Ang II-mediated signal transduction. We found that overexpression of FRNK also inhibits Ang II-stimulated cell growth reducing the proliferative index by 77 +/- 12% after a 24 hour treatment as assessed colorimetrically by monitoring cleavage of the tetrazolium salt, WST-1. These data corroborate studies reported elsewhere (28) and suggest that FRNK can inhibit proliferative signals induced by G protein-coupled receptors as well as receptor tyrosine kinases. Since Ang II has also been shown to stimulate Rac1 activity in SMC (29) and other cell types, we examined whether this affect might also be altered by FRNK. Indeed, as shown in Figure 2.5A, Ang II stimulated a rapid transient increase in Rac1 activity in SMC and this effect was attenuated by ectopic expression of GFP-FRNK. As shown in Figure 2.5B and 2.5C, neither the kinetics of Ang II-stimulated ERK activation nor the concentrations of AngII required for maximal ERK activation were significantly altered by overexpression of GFP-FRNK in SMC. Control experiments indicated that Ang II does stimulate FAK activity and that this activity is blocked by overexpression of FRNK. Thus, FRNK appears to attenuate both AngII and PDGF-BB-dependent mitogenic signaling without effects on ERK activity.

As noted above signaling through many SMC mitogens is dependent on ECM but the precise mechanisms involved in this regulation have not been clearly defined. In certain cell types, fibronectin-binding integrins have been shown to contribute to the activation of Rac1 (30). Thus, we examined we examined whether plating SMC on fibronectin would stimulate

the activation of Rac1 in SMC and if so, whether expression of FRNK might alter integrin-dependent Rac1 activation. As shown in Figure 2.6, plating SMC on fibronectin stimulated a transient activation of Rac1 that peaked at 1 hr and overexpression of FRNK attenuated this response at each time point (Figure 2.6). In contrast, overexpression of FRNK had little effect on fibronectin-stimulated ERK activity. These data indicate that FRNK attenuates FAK-dependent activation of Rac1 in response to PDGF-BB, Ang II, and integrins. Based on these observations, we hypothesize that Rac1 activation may be a key convergence point in growth factor and integrin regulated cell proliferation and migration in SMC, and that FRNK may act to regulate signaling through diverse pathways at this level (see Figure 2.7).

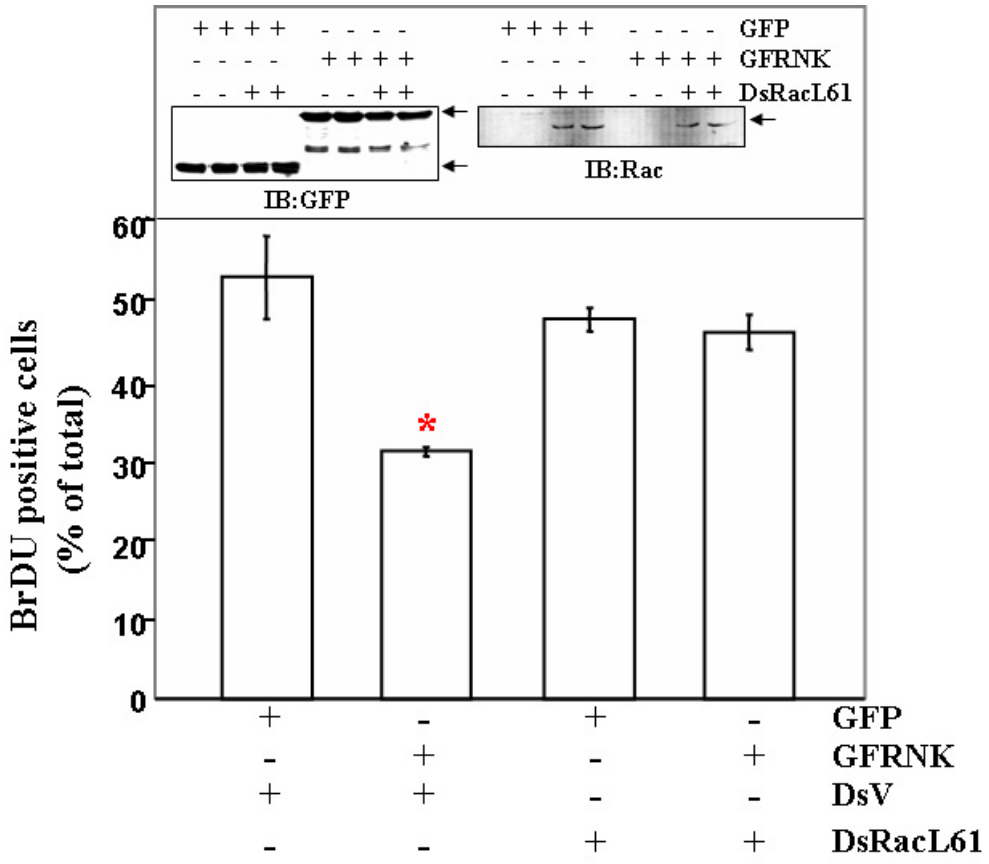


Figure 2.4. L61Rac1 expression overcomes inhibitory effect of FRNK on proliferation. SMC were transfected with pDsRedC1 vector (DsV) or a DsRed fusion protein containing the constitutively active form of Rac1, (DsRacL61). 48 hours after transfection, cells were infected with GFP or GFP-FRNK virus as described above. Cells were serum starved for 4 hours and then dosed overnight with either 30ng/mL PDGF-BB or vehicle control. Cells were labeled with BrdU, fixed and stained for BrdU using a cascade blue-conjugated secondary antibody as described in "Experimental Procedures". The BrdU labeling index for cells positive for GFP and DsRed was determined and data are presented as the percentage of cells labeled positive in the PDGF-BB-stimulated versus un-stimulated wells. The graph represents means +/- SE of three independent experiments in which a total of 180-350 cells were counted for each condition. The percentage of BrdU positive cells in the un-stimulated GFP-FRNK control group was 18.2 +/- 1.9%. Levels of GFP-FRNK were comparable in DsV and DsRacL61 expressing cells (see upper panel).

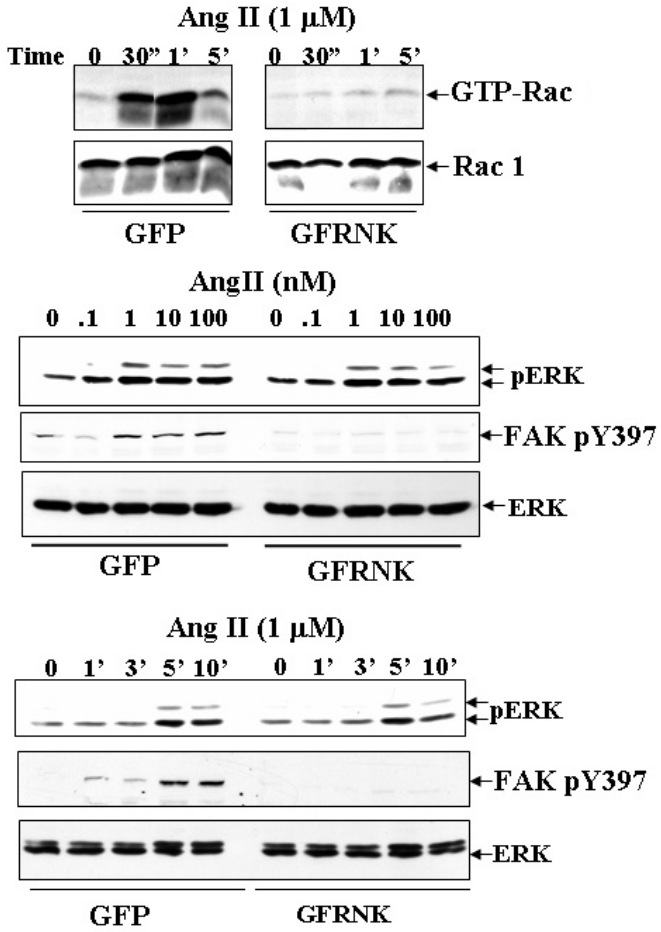


Figure 2.5. FRNK expression attenuates Ang II- stimulated Rac1 but not ERK activity in SMC. Rat aortic SMC were infected with GFP or GFP-FRNK adenovirus (10 M.O.I.) for 12 h. Cells were transferred to serum free media and incubated for 4 hr. Cells were treated with Ang II for various times (panels **A** and **B**) or with various concentrations (panel **C**). **A.** Lysates were incubated with 30 μg of purified GST-PAK as described above. Western blotting was performed using an anti- Rac1 Ab. The bottom panels represent a 10% loading control of total Rac1 in the lysate. **B** and **C**. Lysates were electrophoresed and Western analysis was performed using an anti- ERK or anti- phospho-specific ERK Ab. Control blots indicated that FRNK expression significantly attenuated FAK pY397 in each experiment. Data in panel A are representative of 3 separate experiments and data in panels B and C are representative of at least 4 separate experiments.

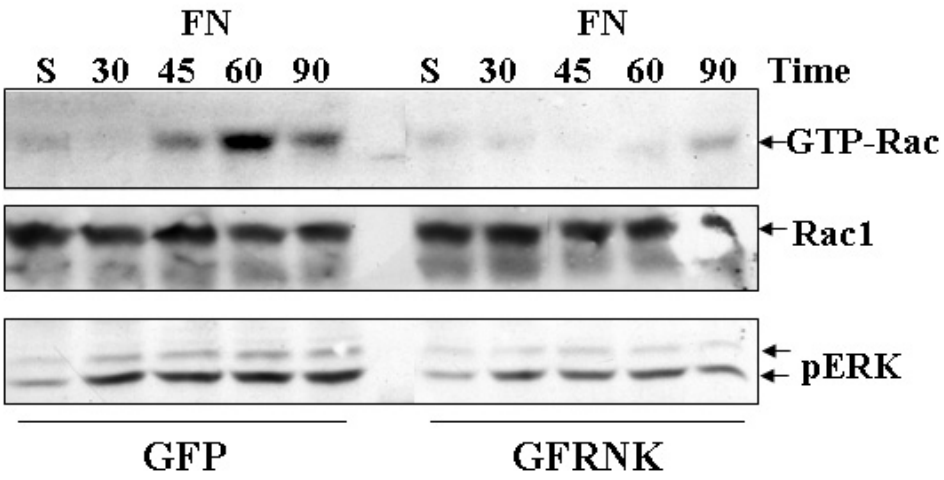
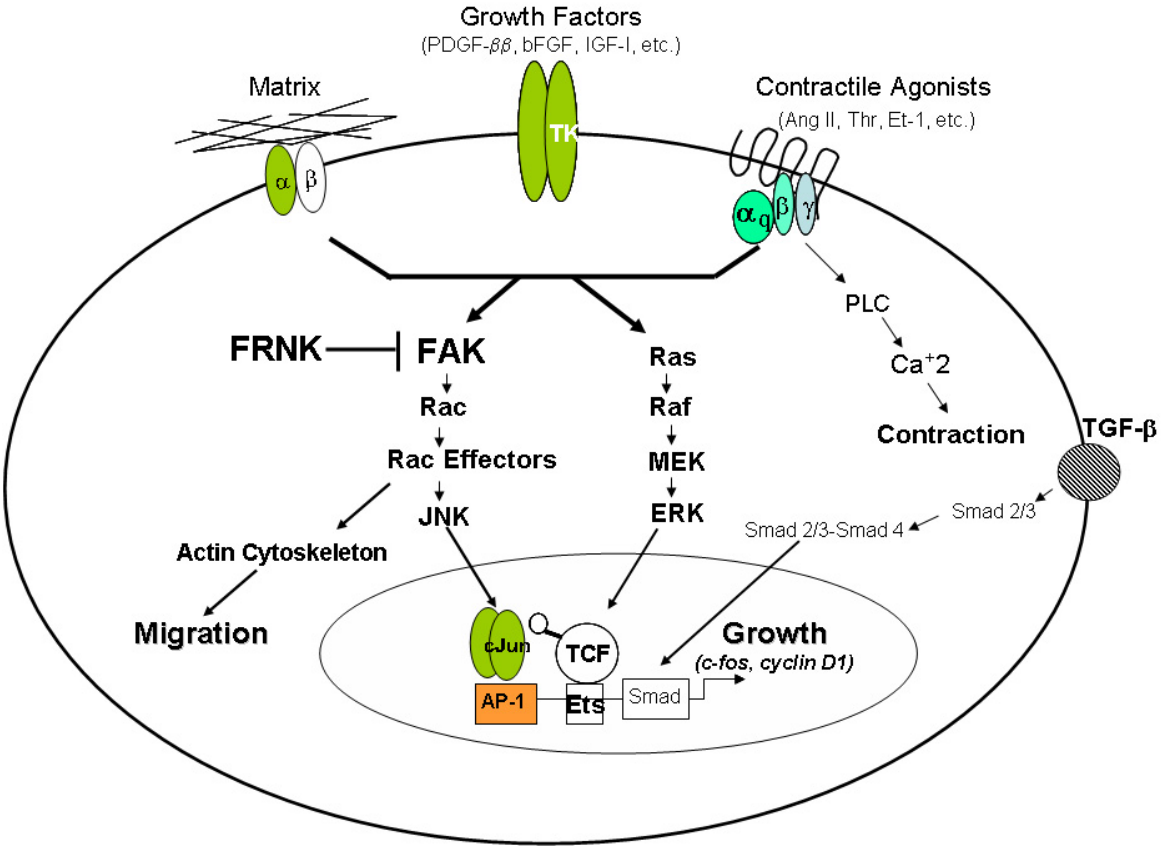


Figure 2.6. FRNK expression blocks FN-stimulated Rac1 activity in SMC. SMC were infected with GFP or GFP-FRNK as described above. Cells were trypsinized, neutralized in soybean trypsin inhibitor, pooled and either held in suspension (S) or plated on fibronectin (FN) for the times indicated. Cells were lysed and incubated with 30 μg of purified GST-PAK as described above. Western blotting was performed using an anti- Rac1 Ab. The bottom panels represent a 10% loading control of total Rac1 in the lysate. Levels of active ERK in lysates were assessed using an anti- phospho-specific ERK Ab. Data are representative of 3 separate experiments.


Growth Factors
(PDGF-ββ, bFGF, IGF-I, etc.)
Matrix
TK
Contractile Agonists
(Ang II, Thr, Et-1, etc.)
α
β
αq
β
γ
PLC
Ca+2
Contraction
FRNK
FAK
Ras
Raf
MEK
ERK
Rac
Rac Effectors
JNK
Actin Cytoskeleton
Migration
TGF-β
Smad 2/3
Smad 2/3-Smad 4
cJun
TCF
AP-1
Ets
Smad
Growth
(c-fos, cyclin D1)

DISCUSSION

Numerous studies have implicated a role for the non-receptor tyrosine kinase, FAK in the regulation of migration and proliferation. FAK is strongly activated by integrin-, growth factor receptor-, and G protein coupled receptor- engagement and thus may serve to integrate downstream signals from a variety of agonists; however the precise mechanisms by which FAK regulates signaling from these diverse pathways have not been clearly defined (11). In this report, we studied the convergence of growth factor and integrin signaling through FAK in the regulation of vascular SMC proliferation. As we previously reported, FAK activity appears to be regulated in a unique fashion in SMC whereby autonomous expression of the carboxyterminal domain of FAK (termed FRNK) can act as an endogenous inhibitor of FAK signaling (12,31,32). In recent studies, we have shown that: 1) FRNK is expressed selectively in SMC with high levels observed in the vasculature; 2) FRNK expression is regulated during development and following vascular injury; and 3) ectopic expression of FRNK in cultured SMC blocked PDGF-BB stimulated migration and proliferation (12). These data indicate that FRNK expression may impart tight regulation on FAK-dependent ECM-mediated signaling events in SMC. Here we report that expression of FRNK in SMC blocks mitogenic signaling from diverse factors by attenuating FAK-dependent co-activation of the Rac/JNK but not the Ras/ERK pathway.

Ras/ERK and Rac/JNK are two of the main pathways known to be key regulators of cell proliferation and migration. Cell adhesion to extracellular matrix regulates Ras/ERK signaling in many cell types and studies have clearly shown that maximal growth factor signaling to ERK is anchorage-dependent (33). Several pieces of evidence highlight a possible role for FAK activation in ERK-dependent growth regulation. First, several

mitogens (including PDGF-BB, AngII, PE, IGF-1, and ET-1) stimulate FAK tyrosine phosphorylation in an adhesion-dependent manner (5-8). Second, at least two pathways have been defined by which FAK can activate ERK activity. One pathway involves adhesion-dependent tyrosine phosphorylation of FAK at residue 925 which directs SH2-mediated binding to the adapter protein GRB2, and recruitment of the GTP exchange factor SOS followed by subsequent activation of the Ras/Raf/MEK/ERK pathway (34,35); and a separate pathway involves the adhesion dependent association of the adapter protein CAS with FAK which can facilitate ERK signaling through the adapter proteins NCK and SOS and subsequent activation of the Ras/Raf/MEK/ERK pathway (21). Finally, overexpression of FAK can rescue growth factor induced ERK activation in non-adherent cells whereas inhibition of FAK function can attenuate activation of ERK in adherent cells (9,36). Indeed, we previously reported that adenoviral infection of GFP-FRNK inhibits PE-stimulated ERK activation in isolated cardiomyocytes (37).

In contrast, we have clearly shown in this report that FRNK expression attenuates FAK signaling in primary cultured SMC (in which FRNK is expressed endogenously) without affect on the time or dose-dependent activation of ERK by both strong (PDGF-BB) or weak agonists (Ang II). Instead, the effects of FRNK on mitogen and ECM-induced proliferation were mediated by inhibition of Rac1/JNK, a conclusion strongly supported by our results demonstrating that L61 Rac1 was sufficient to rescue the FRNK inhibitable component of PDGF-BB-stimulated cell proliferation. Our results do differ somewhat from a previous report in which stable transfection of FRNK into smooth muscle-like cells reduced PDGF-stimulated ERK activity at low but not high concentrations of PDGF (38). The apparent discrepancies between these results may be due to their use of smooth muscle cell

lines immortalized by large T antigen as opposed to primary SMC cultures. Thus, we propose that the mechanisms by which FAK regulates adhesion-dependent growth factor signaling may be cell type specific. Indeed, previous work has shown that in keratinocytes, integrin-dependent ERK activation appears to proceed through SHC- but not FAK-dependent signals (39). Perhaps similar FAK-independent ERK signaling pathways are utilized in SMC. Although most of our data indicates that the effects of FRNK are mediated by inhibition of FAK, we cannot rule out the possibility that FRNK might regulate signaling in a FAK-independent fashion in SMC.

Our results do not completely exclude the possibility that FAK/FRNK may play a role in regulating ERK signaling in SMC in vivo. It is still possible that weak ERK activation induced by limiting amounts of mitogens in the vessel wall may be more susceptible to the effects of FRNK. It is also possible that examination of total ERK activity may not be representative of the relevant functional activity of this enzyme in the cell. For instance, active ERK localizes to focal adhesions as well as the nucleus, suggesting that targeting of this kinase may direct specificity towards downstream targets (40,41). Thus, FRNK may attenuate localization of active ERK, which could be masked in experiments designed to measure total activity in cell lysates. Interestingly, adhesion to the ECM is required for efficient accumulation of active ERK in the nucleus and recent studies point to a role for Rac1 in mediating this process (40,42). Therefore, although FRNK might not affect ERK activation per se, it could affect downstream ERK signaling secondary to its affect on Rac1 activation. Studies to examine this possibility are currently underway.

Our data suggest that in SMC, Rac1 activation may be a key convergence point in growth factor- and integrin-regulated cell proliferation, since FRNK expression attenuated

integrin-, Ang II- and PDGF-BB stimulated Rac1 activation and downstream signaling. Activation of the small GTPase Rac1 elicits membrane ruffling and lamellipodia formation upon growth factor stimulation and this modification of cell morphology is required for cell spreading and migration (24,26,27). In addition, activation of Rac1 is required for cyclin D1 expression and concomitant cell cycle progression (43,44). Rac1, like other low molecular GTPases, is a molecular switch that cycles between the active GTP- and inactive GDP-bound states and is regulated by numerous proteins that facilitate this process. The exchange of GDP for GTP is accelerated by guanosine nucleotide exchange factors (GEFs), while GTPase-activating proteins (GAPs) increase the intrinsic rate of GTP hydrolysis. In the GTP-bound form, Rac1 interacts with and activates a number of effector molecules that have been implicated in regulating proliferation and migration including serine/threonine protein kinases, lipid kinases, and actin binding/scaffolding proteins (45,46). We have shown that FRNK expression attenuates PDGF-BB mediated activation of JNK, a stress-activated protein kinase downstream of Rac1 signaling known to control cell proliferation. In addition, we showed that the proliferative defect in FRNK expressing cells was completely rescued by active RacL61. Taken together, these data provide strong evidence that Rac1 is co-regulated by FAK-dependent integrin and growth factor signaling.

Recent studies corroborate the suggestion that activation of the Rac1 GTPase may be the critical convergence point for mitogen- and integrin-dependent growth and migration in certain cell types. In endothelial cells, mitogens stimulate robust ERK activity when cells are attached to either fibronectin or laminin, but only stimulate proliferation when the cells are plated on fibronectin. This effect was correlated to the ability of fibronectin (but not laminin) to support mitogen-stimulated Rac1 activity, indicating a role for Rac1 in adhesion-

dependent growth responses (30). Two other reports show that β integrins are both necessary and sufficient for adhesion dependent Rac1 activation. Hirsch et. al. showed that fibroblasts derived from mice that express a mutated β_1 integrin display defective activation of FAK, Rac1 and JNK but not ERK and are impaired in G_1-S cell cycle progression (47). In addition, Berrier et al showed that clustering β_1 and β_3 integrin tails on the surface of non-adherent cells activates Rac1 activity (48).

FAK activates a number of signaling molecules that might contribute to integrin-dependent Rac1 (and downstream JNK) activation. Evidence suggests that FAK-dependent signaling through CAS, paxillin, or PI-3Kinase can lead to activation of Rac1 by several different mechanisms. Integrin-mediated activation of Rac1 could proceed through a FAK/(CAS or paxillin)/Crk/DOCK 180 pathway. Indeed, dominant interfering mutants of the SH2/SH3 adapter protein Crk, the adapter/exchange factor DOCK 180 or Rac1 each attenuate Cas- or paxillin- stimulated cell migration (49-52). Recently, a more direct paradigm has been suggested whereby FAK-dependent activation of paxillin could lead to focal adhesion recruitment and activation of the putative Cdc42/Rac guanine nucleotide exchange proteins PIX/COOL (3,30,53). Alternatively, activation of Rac1 could proceed through a FAK/PI3K/SOS pathway. Pi3kinase associates with Tyr397 of FAK upon integrin ligation and one of its catalytic products, phosphatidylinositol-tris-phosphate, binds to and activates the PH-domain containing guanine nucleotide exchange factor, SOS which can activate both Rac1 and Ras (53,54). In endothelial cells fibronectin-stimulated Rac1 activity is dependent on FAK/PI3Kinase/SOS signaling (30); however recent data using Src transformed fibroblasts suggests that a FAK/CAS/DOCK180 pathway might be utilized (55).

Whether the pathways leading from FAK to Rac/JNK are redundant or differentially regulated in a cell-type specific fashion are important questions that remain to be addressed.

ECM is a critical component of the medial layers of the vessel wall which serves to both maintain vascular integrity and to regulate vascular development and remodeling. Results from genetic deletion of various ECM components, integrin receptors, and FAK each result in extraembryonic and embryonic vessel defects leading to lethality at approximately day 8.5-10 of mouse development supporting a role for adhesion signaling in the regulation of growth and migration of vascular SMC (56-59). FAK is co-activated by integrins and autocrine factors and its activation is necessary for proliferation and migration in a number of cell types (11). We have shown that FAK activity may be regulated in a unique fashion in SMC, because FRNK, a dominant-inhibitory form of FAK is selectively expressed in these cells. FRNK overexpression inhibits both AngII and PDGF-BB stimulated migration and proliferation, effects likely due to the ability of FRNK to block Rac1 activation. Interestingly, several SMC mitogens have been linked to Rac1 activation either in SMC (i.e. AngII) or other cell types (i.e. PDGF, ET-1, IGF-1, fibroblast growth factor) and proliferative signals induced by these agonists are attenuated by overexpression of a dominant-interfering mutant for Rac1 (29,60-64). Thus, it is tempting to speculate that enhanced signaling through FAK to Rac1 is permissive for SMC growth during development and for the phenotypic reversion of SMC to a more proliferative state following vascular injury and that expression of FRNK might hold these processes in check.

CHAPTER III

Adhesion stimulates direct PAK1/ERK2 association and leads to ERK-dependent PAK1 Thr212 phosphorylation[2]

[2] Data published as: Liisa J. Sundberg-Smith, Jason T.Doherty, Christopher P. Mack and Joan M. Taylor. Adhesion stimulates direct PAK1/ERK association and leads to ERK-dependent PAK1 Thr212 phosphorylation. *J Biol Chem* (2005) 280(3):2055-64.

ABSTRACT

The Rac1/Cdc42 effector p21-activated kinase (PAK) is activated by various signaling cascades including receptor tyrosine kinases and integrins, and regulates a number of processes such as cell proliferation and motility. PAK activity has been shown to be required for maximal activation of the canonical Ras/Raf/MEK/ERK Map Kinase signaling cascade, likely due to PAK co-activation of Raf and MEK. Herein, we found that adhesion signaling also stimulates an association between PAK1 and ERK1/2. PAK1 and ERK1/2 co-immunoprecipitated from SMC plated on fibronectin and the two proteins co-localized in membrane ruffles and adhesion complexes following PDGF-BB or sphingosine 1-phosphate treatment respectively. Far Western analysis demonstrated a direct association between the two proteins, and peptide mapping identified an ERK2 binding site within the auto-inhibitory domain of PAK1. Interestingly, deletion of a major ERK binding site in PAK attenuates activation of an ERK-dependent serum-responsive element (SRE)- luciferase reporter gene, indicating that association between PAK and ERK is required to facilitate ERK signaling. We also show that ERK2 phosphorylates PAK1 on Thr212 in vitro and that Thr212 is phosphorylated in smooth muscle cells following PDGF-BB treatment in an adhesion and MEK/ERK-dependent fashion. Expression of a phosphomimic variant, PAK T212E, does not alter ERK association, but markedly attenuates downstream ERK signaling. Taken together, these data suggest that PAK1 may facilitate ERK signaling by serving as a scaffold to recruit Raf, MEK, and ERK to adhesion complexes, and that subsequent growth-factor stimulated phosphorylation of PAKT212 by ERK may serve to provide a negative feedback signal to control coordinate activation of ERK by growth factor- and matrix-induced signals.

INTRODUCTION

The Ras superfamily of small GTPases including H-Ras, R-Ras, and Rho family members (Rac, Rho, and Cdc42) among others are activated by numerous transmembrane receptors such as receptor tyrosine kinases, G-protein coupled receptors, and integrins and regulate a variety of cellular processes including proliferation, differentiation, and migration (1). These GTPases are molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state (2,3). In the GTP-bound form, the Ras-related GTPases interact with and activate a number of effector molecules that have been implicated in regulating cell cycle progression and/or cell migration such as the serine/threonine protein kinases, lipid kinases, and actin binding/scaffolding proteins (3,4).

Ras and Rac regulate MAPKinase signaling, and a variety of agonists use Ras-dependent activation of ERK and/or Rac-dependent activation of JNK as dominant mitogenic signaling pathways (5). MAPK activation typically occurs by highly conserved multi-level kinase cascades (i.e. Ste20/Ste11/Ste7/Fus3 in yeast and Raf/MEK/ERK in mammalian cells) eventually leading to MAPK nuclear translocation and transcription factor activation (6,7). Although the mammalian MAPK signaling pathways were initially thought to be independent and parallel, recent studies indicate that there is significant cross-talk between them.

Several lines of evidence indicate that integrins and growth factors promote coordinated activation of the ERK signaling cascade. Integrin-signaling has been shown to be required for maximal activation of either growth factor-stimulated Raf or MEK activation an event likely dependent on the specific integrin receptors engaged in the particular cell type used (8-10). Studies indicate that the Rac effector, p21-activated protein kinase (PAK), a

homologue to the yeast MAP4K, Ste20, may serve as a convergence point between growth factor- and integrin-mediated Ras/ERK signaling. GTP-bound Rac and Cdc42 activate PAK1 in an adhesion-dependent fashion by binding to the p21-binding domain (PBD or CRIB) localized within the N-terminal autoinhibitory domain of PAKs 1-3 (11,12). This interaction exposes the PAK C-terminal kinase domain permitting activation, autophosphorylation, and downstream signaling (13,14). Notably, plating cells on the ECM fibronectin enhances the ability of Rac to activate PAK, and β1 integrins have been shown to transmit signals downstream to JNK through the Pi3kinase/Rac/PAK cascade (15,16).

Interestingly, Howe et al demonstrated that inhibition of PAK attenuated integrin-dependent ERK activation and subsequent studies showed that this was likely due to the ability of PAK to enhance adhesion-dependent Raf and/or MEK activation (17). Activated PAK associates with Raf, and phosphorylates ser-338 and ser-339 within the catalytic domain. Although several phosphorylation sites have been reported to be necessary for Raf activation, phosphorylation of these serines has been shown to be essential for maximal Raf activation by Ras in response to integrin-mediated Pak1 activation (14,18). However, the convergence of integrin signaling at the level of Raf is controversial, and at least one study refutes the necessity of Pak3 for ser-338 phosphorylation in response to EGF (19). Recent studies indicate that PAK also phosphorylates MEK on a site (S298) that is necessary for maximal MEK activation by Raf and that adhesion-dependent PAK signaling enhances association between MEK and ERK (5,20-22). Thus, convergence of these two well-established pathways likely occurs at the level of PAK, which can affect ERK signaling at multiple levels including co-activation of Raf and MEK. Whether the pathways leading from

PAK to ERK are differentially regulated in an integrin-dependent cell-type specific fashion are important questions that remain to be addressed.

Herein, we report that adhesion signaling induces a direct association between ERK and PAK1. Taken together with previous data, our results indicate that PAK may coordinate signaling between Raf, MEK and ERK by acting as a scaffold for these proteins. We also present evidence that ERK2 phosphorylates PAK1 on Thr212, a site just downstream of the ERK binding site on PAK, and that this event may provide negative feedback inhibition of ERK signaling. These data highlight yet another level whereby adhesion signaling may regulate synergy between Ras and Rho family proteins to dynamically regulate the activation state of ERK at distinct regions within the cell.

MATERIALS AND METHODS

Antibodies and Reagents

The phospho-specific ERK1/2 antibody and the anti-PAK1 antibody were purchased from Cell Signaling. The phospho-Tyr15 Cdc2 antibody was purchased from Santa Cruz. The phospho-specific PakT212 antibody, anti-Flag (M5), anti-acetylated tubulin and anti-vinculin antibodies, alsterpaullone and fibronectin were purchased from Sigma. PDGF-BB and UO126 were purchased from Calbiochem. The purified active ERK2 and anti-ERK2 antibody were purchased from UBI. The anti-paxillin antibody was purchased from Transduction Laboratories, and Texas-Red phalloidin was purchased from Molecular Probes.

Expression Constructs

Rat His-PAK1 and His-ERK2 (wild type and kinase defective) constructs were generous gifts from Leslie Parise (UNC) and Melanie Cobb (UTSW) respectively. GFP-PAK1 was made by inserting full length wild type human PAK1 (generous gift from Alan Howe, UVT) into EGFP-C1 vector (Clonetech) cut with Bgl1 +EcoR1, GST-PAK1 (aa 1-290) was constructed as previously described (23). S212E or A and S223A variants were generated by PCR site-directed mutagenesis of GST- or GFP-tagged PAK1 constructs. Forward and reverse complimentary primers corresponding to the following sequence were used: for PAK1 S212A, 5':GAACCACTTCCTGTCGCTCCAACTCGGGACGTGG, for S212E, 5': GTGATTGAACCACTTCCTGTCGAACCAACTCGGGACGTGGC, for PAK1 S223A, 5': GGCTACATCTCCCATTGCACCTACTGAAAATAACACC. Mutations were confirmed by direct sequencing.

Cell Culture and Agonist Treatment

Rat aortic smooth muscle cells (SMC) were obtained from rat thoracic aortas by enzymatic digestion as previously described (24). Cells were used from passages 7-21 and were maintained in Dulbecco's Modified Eagles medium (DMEM)-F12 plus 10% fetal bovine serum and 1% penicillin-streptomycin. In some experiments, cells were serum starved for 4 hours before treatment with PDGF-BB (20 ng/ml) for the times indicated. For adhesion suspension experiments, cells were trypsinized, neutralized in soybean trypsin inhibitor (1mg/ml in PBS), collected by centrifugation and resuspended in serum-free DMEM:F12 plus 1% penicillin-streptomycin. Cells were held in suspension or plated on fibronectin-coated (40μg/ml) dishes for the indicated times. A7r5 (ATCC) smooth muscle cells, were maintained in DMEM plus 10% fetal bovine serum and 1% penicillin-streptomycin.

Protein Purification

His-ERK2 (wild type and kinase defective) and His-PAK1 were purified from bacterial lysates using Qiagen Nickel-NTA (nickel-nitriloacetic acid) agarose according to the manufacturers protocol (The QIAexpressionist^{tm} 5th Ed.). Briefly, cleared lysates in a buffer containing 50mM NaH_2PO_4, 300mM NaCl, 20mM Imidazole, pH 8.0 plus 1mg/ml lysozyme,1 mM Na_3VO_4, 40 mM NaF, 100μM Leupeptin, 1mM AEBSF, and 0.05 TIU/ml aprotinin were combined with 1 ml Nickel NTA-agarose, rotated for 1 hr at 4^{o}C, and then transferred to a chromatography column. The His-ERK Nickel NTA agarose complexes were washed 3x with 50mM Imidazole wash buffer (50mM NaH_2PO_4, 300mM NaCl, 50mM Imidazole, pH 8.0) and eluted with 250mM Imidazole elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM Imidazole, pH 8.0). The His-PAK1 Nickel NTA agarose complex

was washed 3x with 30mM Imidazole wash buffer (50mM NaH_2PO_4, 300mM NaCl, 30mM Imidazole, pH 8.0) and 1x with 40mM Imidazole wash buffer (50mM NaH_2PO_4, 300mM NaCl, 40mM Imidazole, pH 8.0). 25µl of each elution fraction was analyzed by SDS-PAGE to determine the appropriate fractions to pool. Proteins were dialyzed (50mM Hepes, 150mM NaCl and 10% glycerol) overnight at 4 ^{o}C and then aliquoted and stored at –80^{o}C for subsequent experiments.

GST-Pulldown Assay

GST-PAK (aa 1-290 of PAK1, J.Chernoff) was purified from bacterial lysates using gluathione-agarose beads (Pharmacia) as described previously (23). SMC were incubated in serum-free media (DMEM:F12 plus 1% penicillin-streptomycin) and treated with PDGF-BB (20ng/ml) as described above. Cells were lysed in Buffer A (50mM Tris, pH 7.6, 500mM NaCl, 0.1% SDS, 0.5% DOC, 1% Triton X-100, 0.5mM $MgCl_2$, plus protease inhibitors) and 500µg of protein was combined with 30µg GST-PAK1 fusion protein and rotated for 30-60 min at 4^{o}C. The beads were then washed twice with Buffer B (50mM Tris pH 7.6, 150mM NaCl, 1% Triton X-100, 0.5mM $MgCl_2$, plus protease inhibitors) and once with Tris-buffered saline (TBS, 0.2 M NaCl, 50 mM Tris-HCL, pH 7.4). The beads were resuspended in SDS-PAGE sample buffer, boiled for 5 min, electrophoresed on a 12% SDS-polyacrylamide gel, and analysed by Western blotting using either an anti-pERK1/2 or anti-ERK2 antibodies (1:1,000).

In Vitro Kinase Assay

To detect ERK phosphorylation, purified His-ERK2 (kinase dead, 0.1-1µg), and His-PAK1 (0.1-0.5 µg) were incubated with 10µCi of γP-32 ATP in Kinase Buffer (50mM Hepes pH

7.3, 10mM $MgCl_2$, 1mM $MnCl_2$, 5mM NaF, 0.25% Triton 100-X) for 10min at 30 ^oC. To detect phosphorylation of PAK1, freshly purified GST, GST-PAK1(1-290)or GST-PAK1 variants (5μg) were incubated with 10ng of active ERK2 (UBI) in Kinase Buffer in the presence of 50μM ATP with or without [γP^{32}]-ATP (10μCi)) for 10min at 30 ^oC. Samples were resolved by SDS-PAGE. The radioactive gels were fixed in 50% methanol, 10% acetic acid and 20% glycerol for 20min and then rehydrated for 90min in 7% acetic acid 5% methanol and 20% glycerol. The gel was dried for 2 hours at 80 ^oC and then exposed to Kodak XAR autoradiograph film. The gel was then rehydrated in ddH_2O and then stained with 1% Coomassie Blue. For non-radioactive kinase assays, the gel was transferred to nitrocellulose, and analyzed by Western blotting using the phospho-Thr212 antibody.

Western Blotting.

Western blots were performed using the appropriate antibodies at a 1/1000 dilution. Blots were washed in TBST (TBS plus 0.05% Triton-X), followed by incubation with either horse-radish peroxidase conjugated- rabbit anti-mouse antibody or - Protein A sepharose (Amersham) at a 1/2000 dilution. Blots were visualized after incubation with chemiluminscence reagents (ECL, Amersham).

Far Western

Purified GST and GST-PAK1 beads were electrophoresed by SDS-PAGE (12%) and transferred to nitrocellulose. The blot was incubated in 5% powdered milk/TBST + 0.2% sodium azide for 48 hr at 4^oC (to block the membrane and re-nature proteins) the blot was then incubated with purified His-ERK2 (8□g/ml in 5% powdered milk/TBST) overnight at

4°C. The blot was washed 3 times (10min) with TBST and then incubated with India HisProbe-HRP (1:5,000 Pierce) for 1hr at room temperature. The blot was washed with TBST overnight at 4°C before detection with SuperSignal West Pico (Pierce) chemiluminscence substrate.

PAK1 Spot Blot

A membrane containing 12 mer overlapping peptides derived from PAK1 sequence was prepared by SPOT synthesis (ABIMED, generously provided by Leslie Parise). The PAK1 spot blot was blocked with 10% powdered milk in TBS-T, followed by incubation with 2□g/ml purified His-ERK2 in Binding Buffer (20mM Hepes pH 7.4, 150mM NaCl, 1% glycerol, 5% BSA and 0.05% Tween-20), overnight at 4 °C. The blot was washed 3 times (10min) with TBST. The blot was then incubated with anti-ERK2 antibody, followed by incubation with horse-radish peroxidase conjugated Protein A sepharose at a 1/2000 dilution, and visualized by chemiluminscence (ECL, Amersham).

Luciferase Assay

A7r5 cells were transfected with 0.75 μg of SRE-luciferase reporter construct and 0.25μg of the GFP construct variant per well using Superfect (Stratagene) following the manufacturer's protocol. All transfections were done in quadruplicate. Luciferase assays were performed 48 hours post-transfection using Steady-Glo Luciferase Assay Kit (Promega) following the manufacturer's protocol. Relative promoter activity is expressed as the mean +/- standard error relative to total protein.

Immunocytochemistry

A7r5 cells were transfected with the desired GFP-tagged construct using Superfect (Qiagen). After 48 hours, the cells were trypsinized, rinsed in soybean trypsin inhibitor (1mg/ml), centrifuged, washed twice in PBS and resuspended in serum-free DMEM plus 1% penicillin-streptomycin. Cells were counted and plated on fibronectin coated (10μg/ml) slides (Lab-Tek) for 20-90 min. Staining procedure was followed as previously published (25). In brief, cells were fixed with 4% paraformaldehyde, permeabilized with 4% Triton X-100 in PBS, incubated with specified primary antibody for 1 hour at the following concentrations: anti pErk 1:200, anti-vinculin 1:50, anti-paxillin 1:250, anti-acetylated tubulin (1:1000). After washing with PBS, slides were incubated for 1 hour with either Texas Red-conjugated donkey anti-rabbit or donkey anti-mouse antibodies (2μg/ml) or Texas Red-conjugated phalloidin to detect filamentous actin.

Mass-Spectral Analysis

GST-PAK1 (aa 1-290, 10 μg) was electrophoresed on a 14% Pre-Cast Tris/glycine SDS-PAGE gel (Invitrogen). The gel was fixed by soaking in 25% isopropanol/10% acetic acid for 20 min, stained with 0.01% Coomassie in 10% acetic acid overnight, followed by de-staining in 10% acetic acid. Appropriate bands were excised, trypsinized, peptides were eluted and subjected to MALDI-TOF/TOF (ABI 4700 Proteomics Analyzer) and LC-MS (Micromass Q/TOF API-US LC/MS/MS) to define the cleavage site as previously described (26).

RESULTS

We reported previously that inhibition of FAK in SMC by ectopic overexpression of its dominant-interfering form FAK-related non-kinase (FRNK) did not attenuate the magnitude or duration of total cellular ERK activity induced by PDGF-BB, angiotensin II, or fibronectin. However, recent studies indicate that active ERK is localized in several different compartments within the cell including focal adhesions (21,27-29). To determine whether adhesion-dependent FAK signaling might regulate activation of ERK at distinct sites within the cell, we plated GFP-FRNK-transfected SMC on fibronectin for 60 min and stained cells with an antibody that recognizes active (phosphorylated) ERK1/2. As shown in Figure 3.1, ectopic expression of FRNK does dramatically reduce adhesion-stimulated ERK activation in focal adhesions.

In light of our previous data revealing that FRNK expression markedly attenuated integrin-stimulated Rac1 activity, and mounting evidence that the Rac1 effector, PAK regulates ERK activity in vivo in an adhesion-dependent fashion, we examined whether PAK might be involved in regulating ERK activation in focal adhesions. As shown in Figure 2.2A, ectopically expressed GFP-PAK and phospho-ERK1/2 co-localize in membrane ruffles following PDGF-BB stimulation and co-localize within nascent focal adhesions following sphingosine 1- phosphate treatment when SMC are plated on fibronectin. In addition, immunoprecipitation experiments revealed an adhesion-dependent association of endogenous ERK1/2 and PAK1 in SMC. SMC were either continuously grown in serum (denoted A, for attached) or trypsinized and held in suspension (S) in serum free medium and/or plated on fibronectin (FN)-coated plates for the times indicated. As shown in Figure 3.2B, association between PAK and ERK appears to peak after 60 min and surpasses the amount co-

immunoprecipitated in stably adherent cells. Very little of the PAK1-ERK complex was formed in non-adherent cells, as might be expected since activation of PAK is dependent on adhesion signaling (30). Taken together, these data support the hypothesis that PAK1 and ERK1/2 associate in intact cells.

Interestingly, we showed that a GST-PAK1 fusion protein containing the N-terminal 290 amino acids of PAK1 could efficiently precipitate phosphorylated ERK1/2 from SMC lysates, indicating that the N-terminus of PAK is sufficient for ERK binding (Figure 3.3A). Subsequent experiments, in which ERK2 was precipitated from serum starved SMC or SMC treated with 20 ng/ml PDGF-BB for 10 min (to maximally activate ERK), revealed that phosphorylated (active) and un-phosphorylated (inactive) ERK2 are precipitated equally well with the PAK1 fusion protein (Figure 3.3B), indicating that pre-activation of ERK was not required for PAK1 binding.

Previous studies have shown that Raf also binds to PAK, however the binding site for Raf is located within the C-terminus of PAK (in a region not included in our GST-PAK1 construct), thus it was unlikely that ERK was precipitated by an indirect association with Raf and its binding partner MEK. Nonetheless, a number of proteins have been shown to associate with the N-terminus of PAK, including the small GTPases Rac and Cdc42 and the adapter proteins NCK, GRB2, and PKL (31-33). Therefore, to further rule out the possibility of an indirect ERK-PAK1 association, we used a Far Western approach. We probed a membrane containing increasing amounts of GST-PAK1 fusion protein with purified His-ERK2 protein and processed the membrane by Western blotting with an anti-His antibody. As shown in Figure 3.4A, purified His-ERK2 binds directly to GST-PAK1 in a concentration-dependent fashion. In separate experiments, the binding of His-ERK2 to GST-

PAK1 was also detected using ERK-specific antibodies (data not shown). It should be noted that in most preparations of GST-PAK1-290, we observe the full-length product as well as one major putative cleavage product (Figure 3.4B). Interestingly, His-ERK2 also readily associated with the presumptive cleavage product, but did not associate with GST alone (Figure 3.4C). In order to identify the nature of the breakdown product, we isolated the full-length band (denoted P1) and the cleavage product (denoted P2) from a Coomassie Blue-stained polyacrylamide gel and submitted the bands for mass spectral analysis. A combination of MALDI-TOF/TOF and LC-MS data revealed that P1 corresponded to GST fused to N-terminal amino acids 1-290 of PAK1 (as expected) while P2 corresponded to GST fused to PAK1 amino acids 1-55. The PAK sequence from 1-55 of appeared to be sufficient for ERK2 binding, but the observation that ERK2 bound the larger 1-290 fragment slightly better (compare amounts precipitated with 0.5 μg fusion protein in 4A versus 4C) indicates that additional sites within the 56-290 region may also be important.

In order to further map the interaction site on PAK1 for ERK2, a membrane containing immobilized 12mer peptides derived from the amino acid sequence of PAK1 was incubated with purified His-ERK2 and subsequently probed with an anti-ERK2 antibody. As shown in Figure 4D, interactions were detected in three distinct regions corresponding to amino acids 40-54 (site A), 86-94 (site B), and 124-138 (site C) of PAK1. All three binding sites are within the autoinhibitory domain of PAK. Site B is within the CRIB domain that is responsible for binding to Cdc42 and Rac while sites A and C flank this region. Coupled with the result from the previous experiment, these data indicate that amino residues 40-54 within PAK1 are sufficient for ERK2 binding, but that additional interactions within the CRIB domain and autoinhibitory domain may be required for a high affinity interaction.

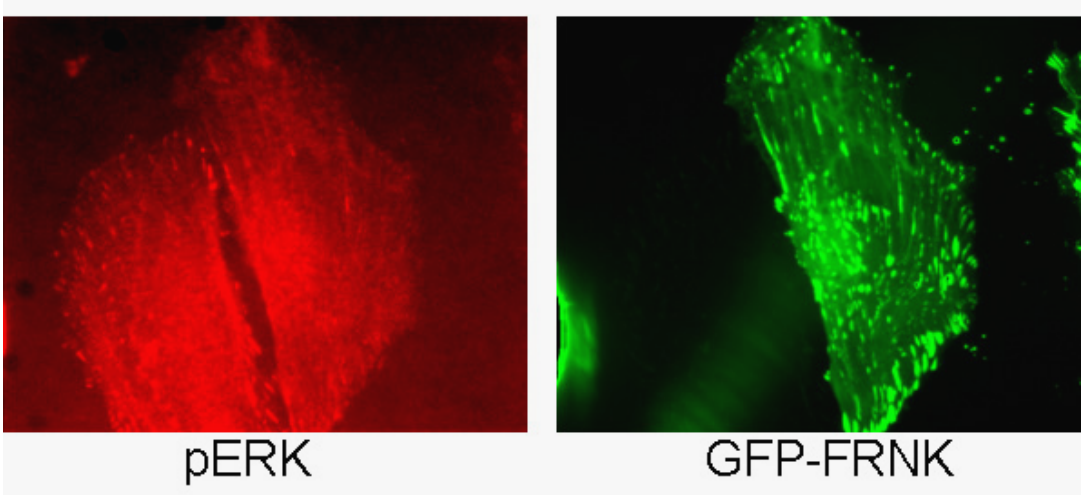


Figure 3.1. **GFP-FRNK expression attenuates ERK activity in focal adhesions.** SMC were transfected with GFP-FRNK and plated on fibronectin-coated chamber slides (10 μg/ml) for 90 min in the presence of serum. Cells were fixed as described in Experimental Procedures and stained with an anti-phosphoErk antibody (Cell Signaling 1:200) followed by a Texas Red-conjugated donkey anti-rabbit antibody (2μg/ml). GFP-FRNK was visualized by direct fluorescence.

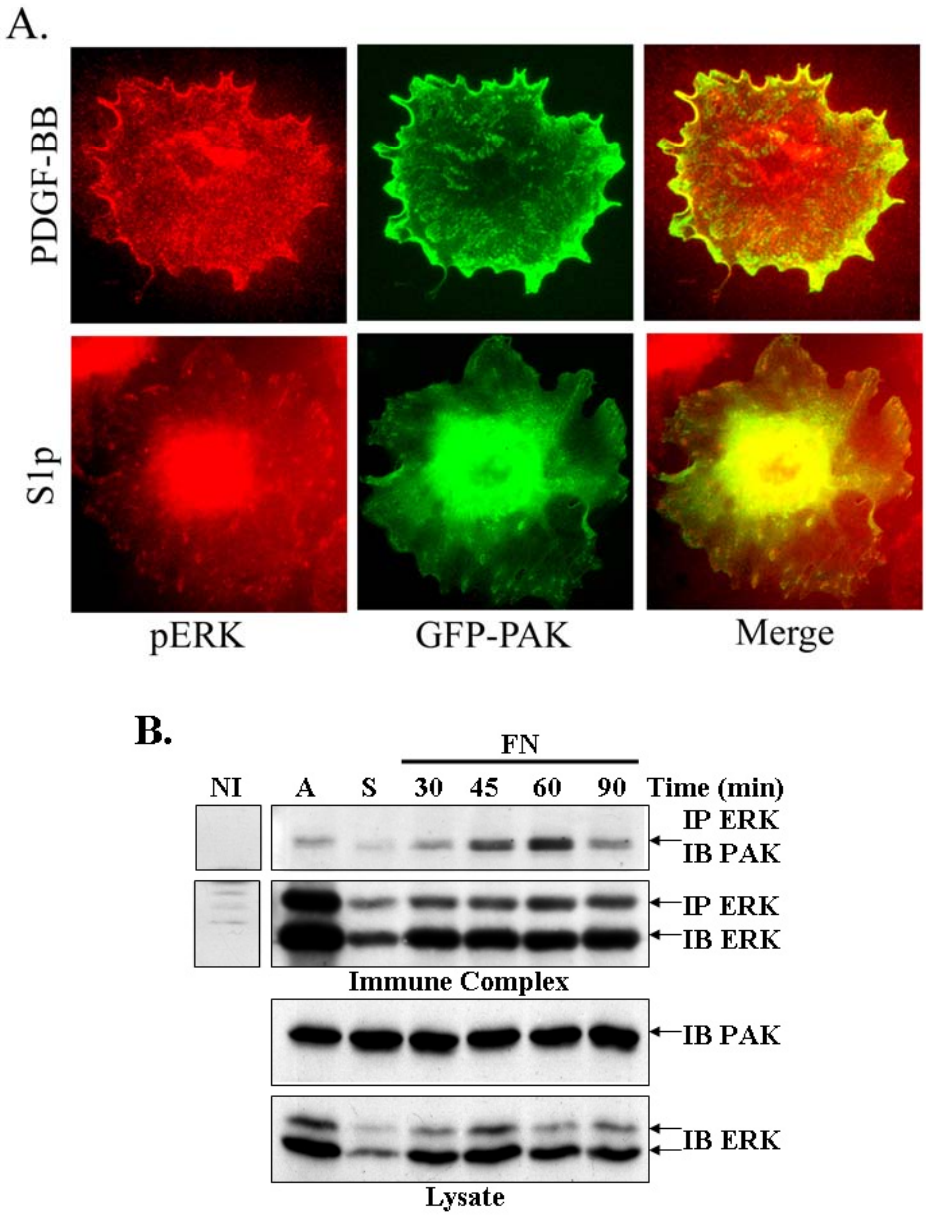


Figure 3.2. ERK1/2 and GFP-PAK1 co-localize and endogenous ERK1/2 and PAK1 associate in SMC plated on fibronectin. **A**. A7R5 cells were transfected with GFP-PAK1 and plated on fibronectin-coated slides in serum free media. After 45 min, cells were treated with PDGF-BB (20ng/ml) or sphingosine 1-phosphate (S1P; 1 μM) for and additional 45 min. Endogenous pErk and ectopically expressed GFP-PAK1 co-localize in membrane ruffles and focal adhesions respectively as indicated by arrowheads. **B**. Rat aortic SMC were either left attached (A), or trypsinized and held in suspension for 90 min (S) or plated on 40μg/ml fibronectin (FN) for indicated times. Cells were lysed and endogenous ERK was immunoprecipitated (IP) using an anti-pERK1/2 antibody as outlined in Experimental Procedures. Western blotting (IB) was performed with anti-PAK1 and anti-ERK antibodies. Bottom panels show a 5% lysate loading control for PAK1 and ERK1/2. Data in Figure 3.2 are representative of three separate experiments.

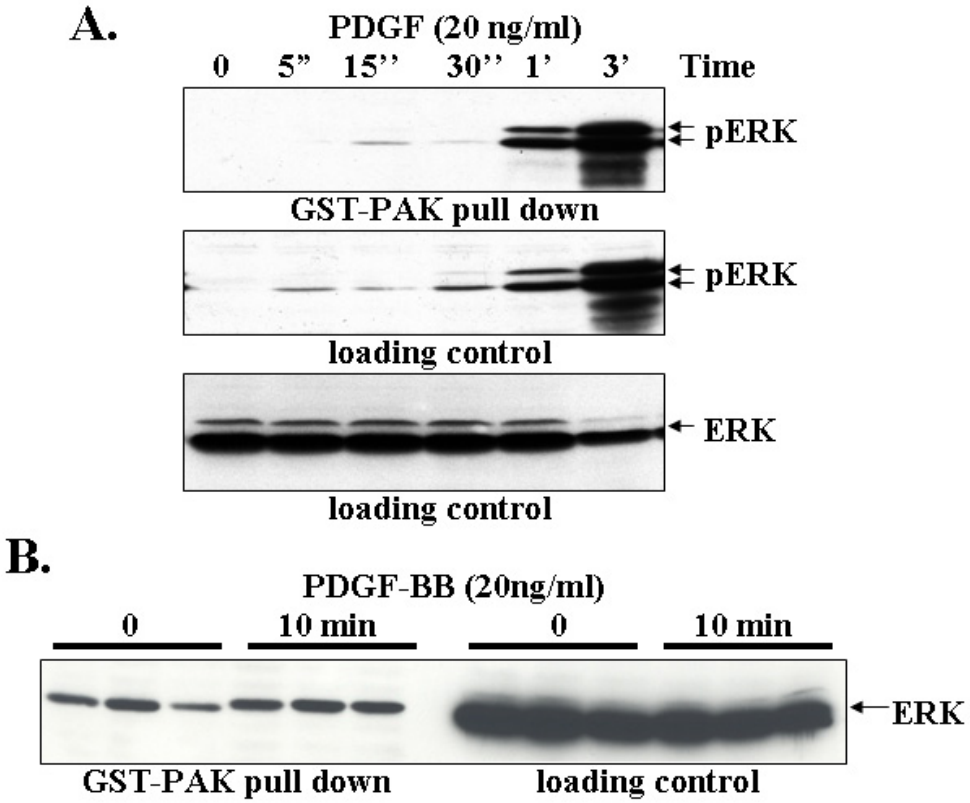


Figure 3.3. ERK precipitates from SMC cell lysate with GST-PAK1 fusion protein. SMC were serum starved for 4 hours before treatment with PDGF-BB (20ng/ml) for the times indicated. Lysates were incubated with 30μg of purified GST-PAK1 (aa 1-290) for 30 min and complexes were precipitated and analyzed by SDS-PAGE as described in Experimental Procedures. **A.** Western blotting was performed using an anti-phosphoERK1/2 antibody (perk, top panel). Middle and bottom panels represent a 10% lysate loading control for phospho-ERK1/2 and total ERK2, respectively. **B.** Pull downs were performed in triplicate for each time point. Western blotting was performed with an antibody recognizing total ERK1/2. Data are representative of three to five separate experiments.

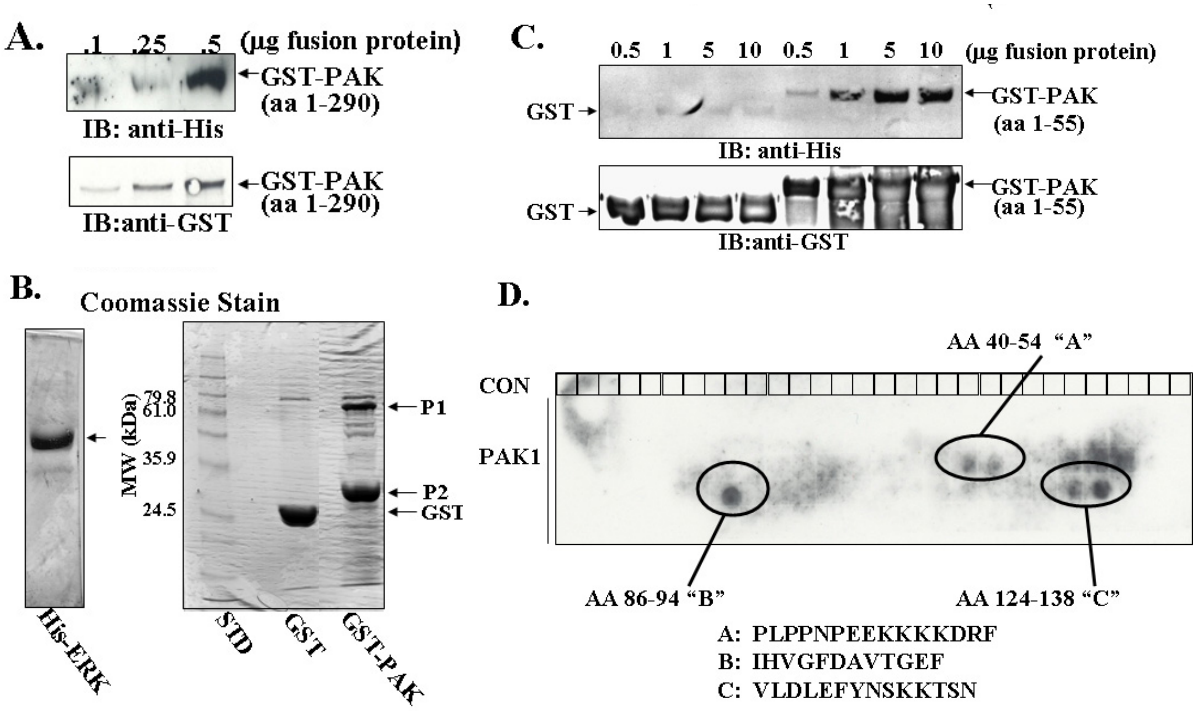


Figure 3.4. ERK2 binds directly to N-terminus of PAK1. **A.** Indicated concentrations of immobilized GST-PAK1 (aa1-290) was electrophoresed, transferred to nitrocellulose, incubated with purified His-ERK2 protein, then probed with anti-His antibody as described in Experimental Procedures (Top). Western blot was striped and reprobed with an anti-GST antibody to reveal the amount of GST-PAK1 loaded at the indicated concentrations (bottom). **B.** His-ERK2 (left), GST, and GST-PAK1 (right) proteins were purified as described in Experimental Procedures. Proteins (5µg) were analyzed on a 12% SDS-acrylamide gel and stained with Coomassie Blue. Mass spectral analysis revealed that the higher molecular weight band in the GST-PAK1 lane (denoted P1) contains GST and aa 1-290 of PAK1 and the lower molecular weight band (denoted P2) contains GST and aa 1-55 of PAK1. **C.** Far Western was performed as described above using GST or GST-PAK1 1-55 as bait and His-ERK2 as a probe. The membrane was probed with anti-His antibody (Top) and then striped and reprobed with an anti-GST antibody to reveal the amount of GST and GST-PAK1 (aa 1-55) loaded at the indicated concentrations (bottom). **D.** PAK1 SPOT peptide membrane was blocked and incubated with purified His-ERK2 protein as described in Experimental Procedures. The membrane was probed with an anti-ERK antibody and binding was detected by chemiluminescence. Three binding sites were identified by chemiluminescence and labeled site A: aa 40-54, B: aa 86-94 and C: aa 124-138. The first row of the grid contains 12 mar random control peptides and the remaining squares contain overlapping 12 mars derived from PAK1 sequence. Data in panels A-C are representative of at least three separate experiments.

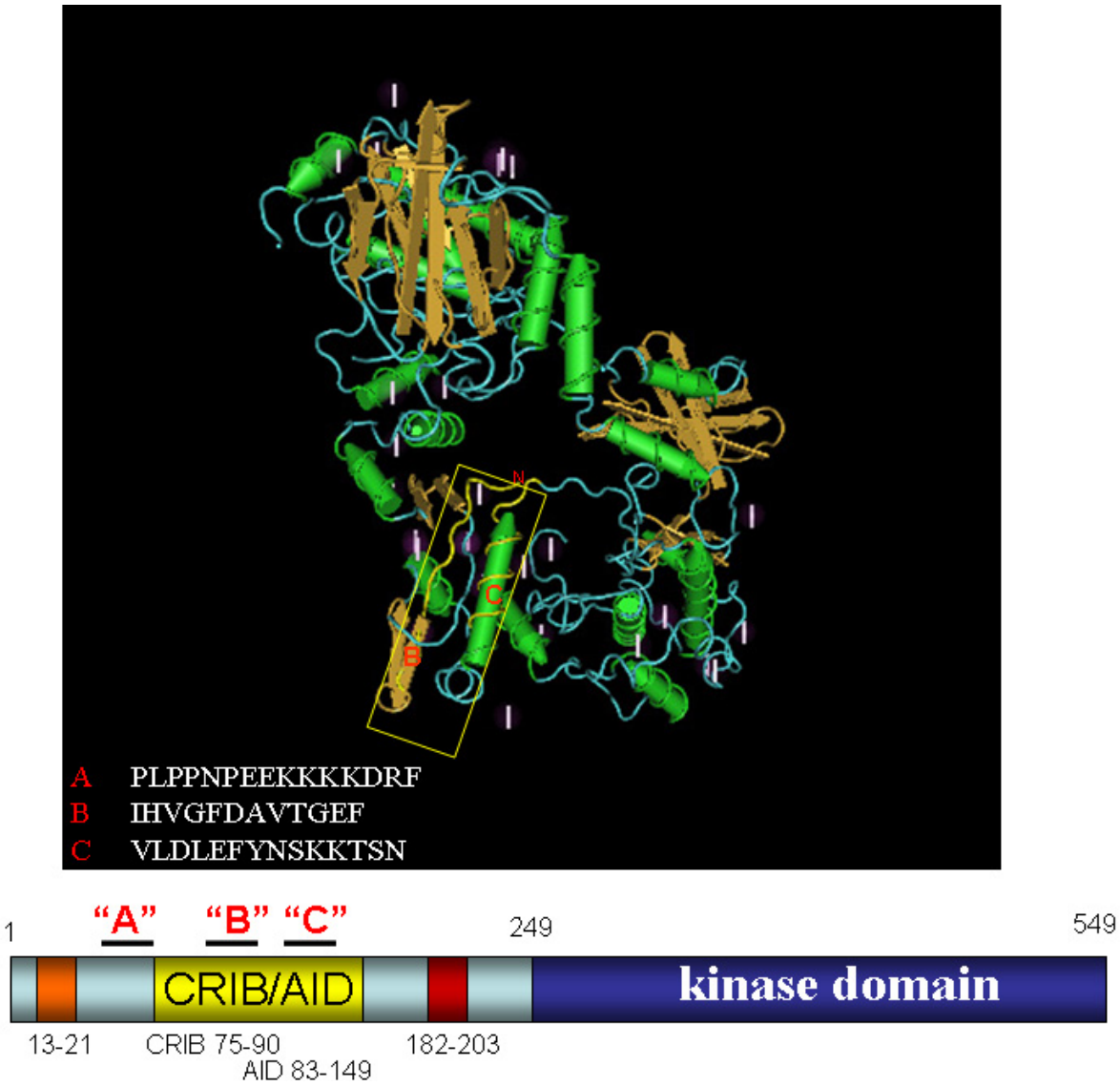
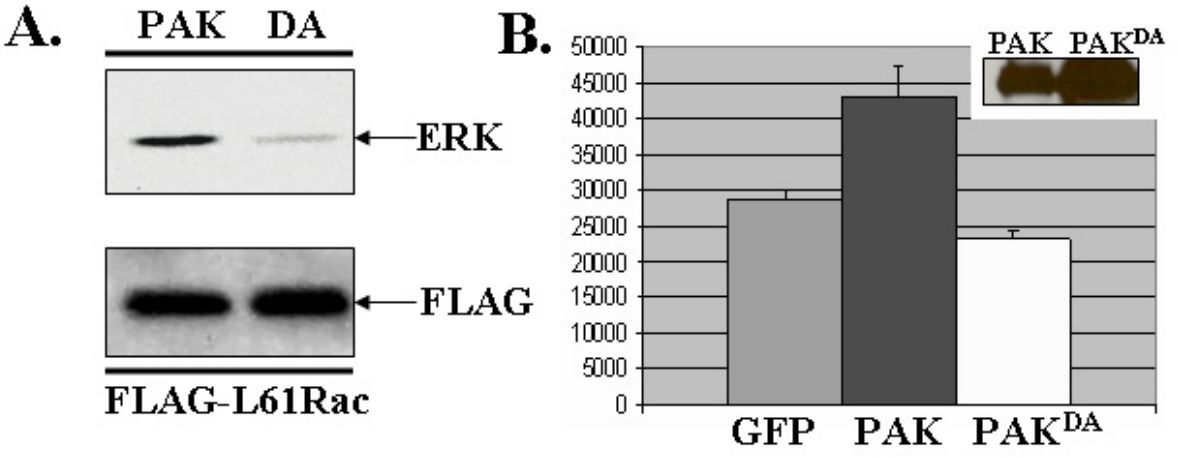


Figure 3.5 PAK crystal structure. The three identified binding sites are near (A) and within (B and C) the CRIB domain in the PAK N-terminus. The crystal reveals that three binding sites (yellow box) form a docking site for ERK binding.

Interestingly, crystal structure analysis of the autoinhibitory domain of PAK1 (aa 70-149) revealed that sites B and C, which form a β-sheet and α-helix respectively, are in close proximity indicating that this region may provide a single docking site for ERK (34, Fig 3.5).

Consistent with the idea that ERK makes multiple contacts within the PAK1 N-terminus, a GST-PAK fusion protein with a deletion of aa 40-54 (GST-PAKdA) greatly reduces, but does not completely block PAK-ERK association (Figure 3.6A). GST-PAKdA and GST-PAK(1-290) did however precipitate comparable amounts of Rac1 from SMC lysates, indicating that the tertiary structure of the CRIB domain in this deletion construct is likely intact (Figure 3.6A, bottom panel). To determine the effect of the PAK-ERK interaction on ERK signaling, we generated a similar deletion (delta aa 40-54) in the context of full length GFP-PAK (GFP-PAKdA). This mutation did not affect PAK localization to focal adhesions (data not shown). We then co-transfected either GFP-PAK or GFP-PAKdA along with a serum-responsive element (SRE)- luciferase reporter construct and measured the activity of this ERK-dependent transgene. In the presence of serum, GFP-PAK expression significantly enhanced SRE-LUC activity, whereas GFP-PAKdA expression did not (Figure 3.6B). These results indicate that PAK/ERK association is important for PAK-dependent ERK signaling.

Since we mapped the binding site of ERK2 to the auto-inhibitory domain of PAK1, we reasoned that ERK binding to PAK might relieve auto-inhibition and activate PAK by a mechanism similar to that observed for Rac or Cdc42. To address this question, we incubated purified kinase-defective ERK2 (KD-ERK2) with purified His-PAK1 and performed an in vitro kinase assay. As shown in Figure 3.7, purified His-PAK1


A.
PAK
DA
ERK
FLAG
FLAG-L61Rac
B.
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
PAK
PAK^DA
GFP
PAK
PAK^DA

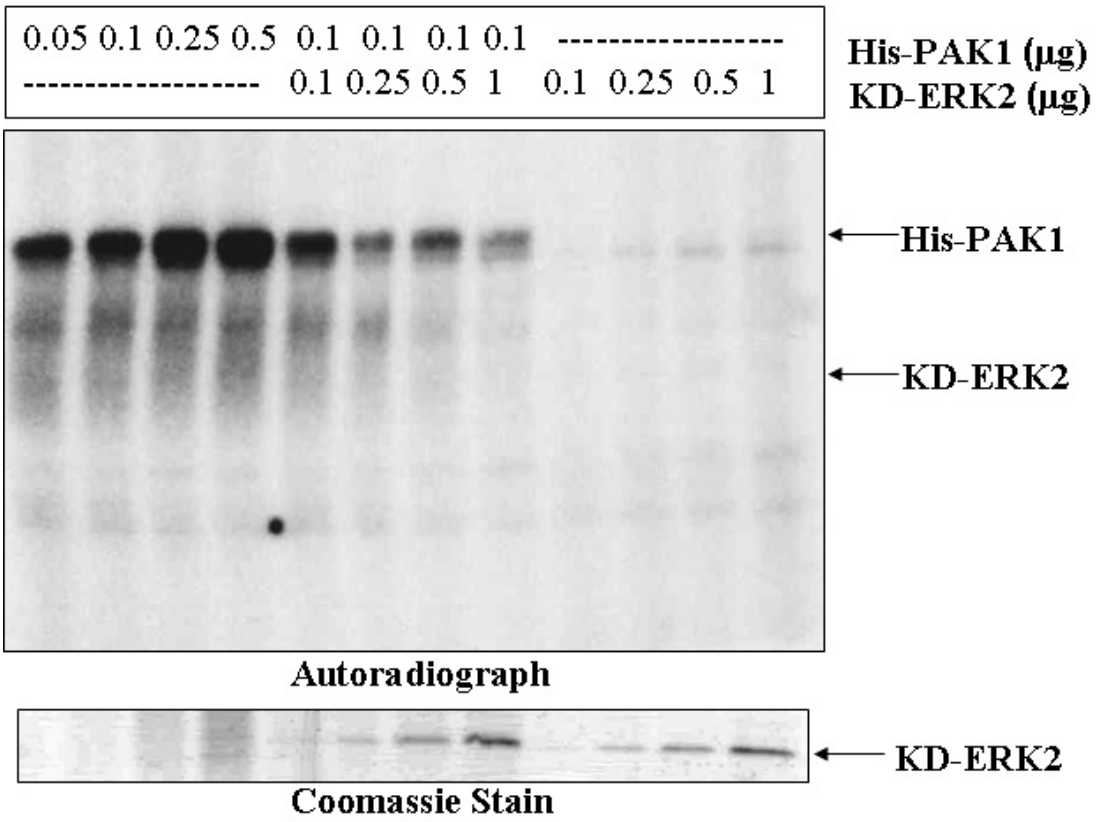


Figure 3.7. PAK1 does not phosphorylate ERK2 in vitro. An in vitro kinase assay was performed with purified His-PAK1 (lanes 1-4), kinase-defective ERK2 (KD-ERK; lanes 9-12) or both (lanes 5-8) in the presence of [γ^{32}P]-ATP for 10min at 30 ^{o}C as described in Experimental Procedures. Samples were examined by SDS-PAGE, and the gel was either dried and exposed to Kodak XAR imaging film for 2hr (top), or re-hydrated and stained with Coomassie Blue R-250 to reveal KD-ERK loading (bottom). Data are representative of two separate experiments.

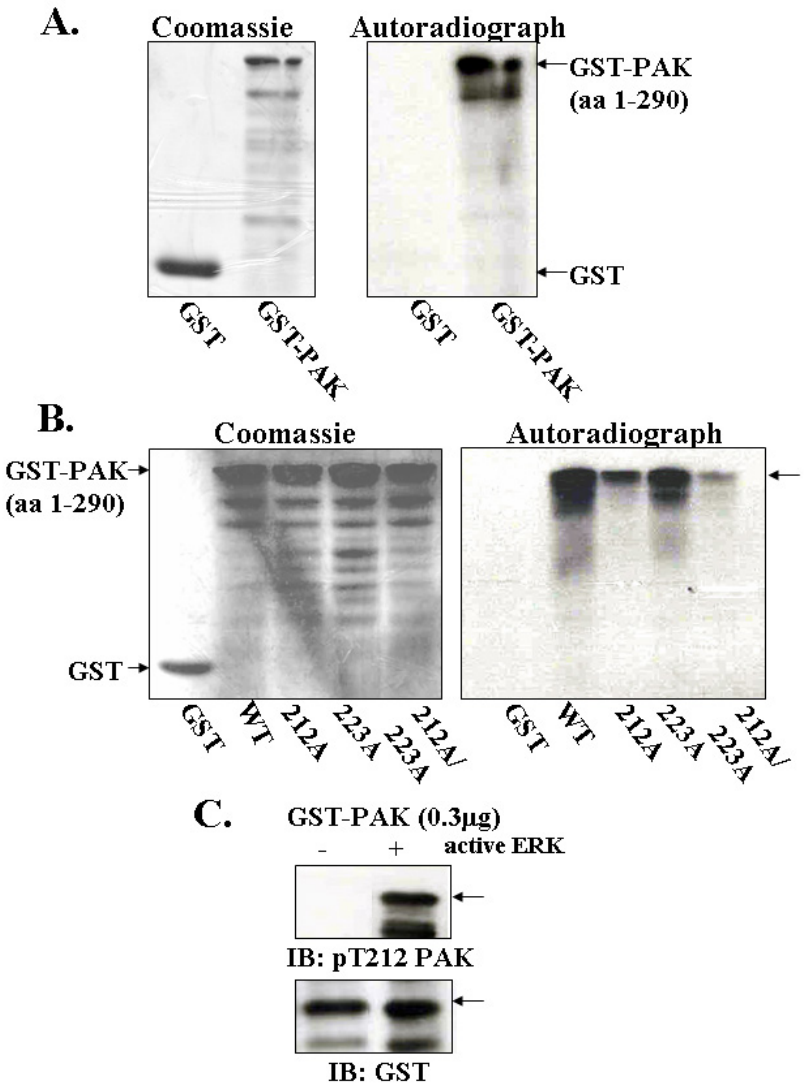


Figure 3.8. ERK2 phosphorylates PAK1 on Thr212 in vitro. **A**. Equal amounts of GST and GST-PAK1 (5µg) were incubated with purified active ERK2 (10 ng) and γ32P-ATP for 10 minutes at 30 ^{o}C as described in Experimental Procedures. Samples were analyzed by SDS-PAGE, the gel was either stained with Coomassie Blue R-250 (left) or dried and exposed to Kodak XAR film for 20 min (right). **B**. An in vitro kinase assay was performed as described above with equal amounts of GST, GST-PAK1 (WT), GST-PAK1 T212A (212A), GST-PAK1 S223A (223A) or GST-PAK1 T212A/S223A (212A/223A). Coomassie stained gel shows equal loading for each variant (left) and the autoradiograph reveals level of ERK2-stimulated phosphorylation (right). **C**. 0.3 µg of GST-PAK1 was incubated with (+) or without (-) active ERK2 (10 ng) and cold ATP (50µM) for 10 minutes at 30 ^{o}C as described in Experimental Procedures. Samples were analyzed by SDS-PAGE, the gel was transferred to nitrocellulose and a Western blot was performed using an anti-pT212 PAK1 antibody (top). The membrane was stripped and re-probed using an anti-GST antibody (bottom) to reveal equal loading. Data are representative of at least three separate experiments.

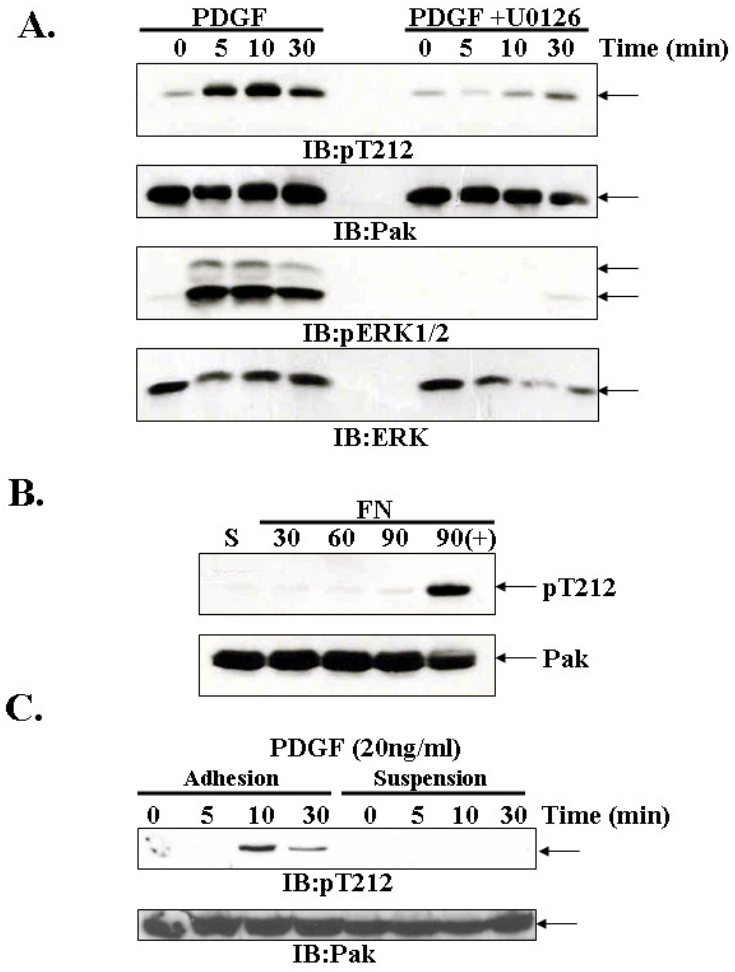


Figure 3.9. PDGF-mediated Thr212 PAK1 phosphorylation in SMC is dependent on both ERK and adhesion signaling. **A**. SMC were serum starved and treated with PDGF-BB (20ng/ml) for times indicated with or without a prior 30 min pretreatment with U0126 (10 μM). Cell extracts were prepared and samples were examined by Western blotting using anti-pT212 PAK1, anti-PAK1, anti-pERK1/2 and anti-ERK2 antibodies. **B.** SMC were either held in suspension (S) or plated on fibronectin for the indicated times or treated with 20ng PDGF-BB for 10 min after plating (+). Western blotting was performed using anti-pT212 PAK1 and anti-PAK1 antibodies. **C.** SMC were either held in suspension or plated on fibronectin for 90 min. Cells were treated with PDGF-BB (20ng/ml) for the times indicated. Western blotting was performed using anti-pT212 PAK1 and anti-PAK1 antibodies.

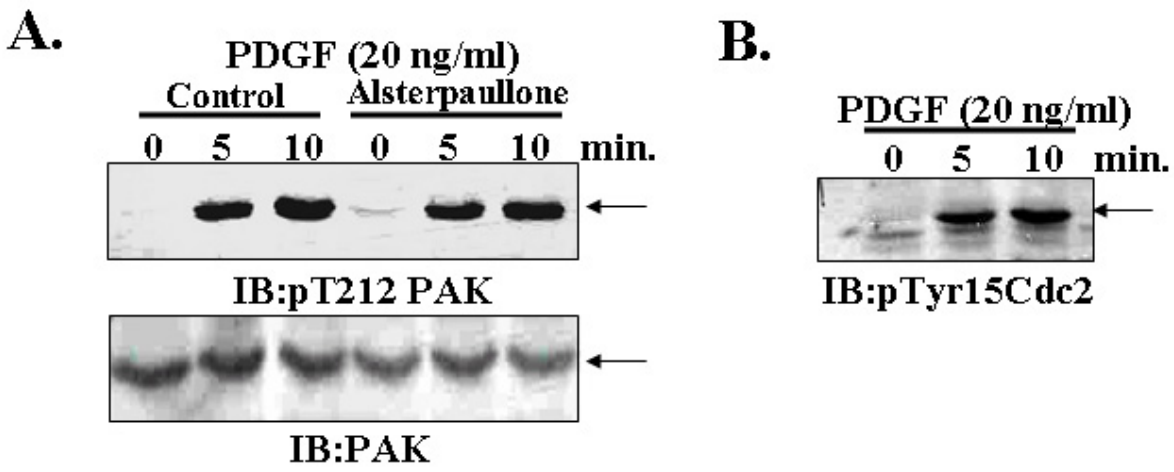


Figure 3.10. **PDGF-BB-stimulated Thr212 PAK1 phosphorylation in SMC is not dependent on Cdc2/Cdk5 activity. A**. Serum starved SMC were pretreated for 3 hours with alsterpaullone (10μM) prior to PDGF-BB (20ng/ml) treatment for the indicated times. Samples were electrophoresed and Western blotting was performed with anti-pT212 PAK1 (top panel) or anti-PAK1 antibodies (bottom panel). **B**. Serum starved SMC were treated with PDGF-BB (20ng/ml) for times indicated. Cell extracts were prepared and samples were examined by Western blotting using an anti-pTyrCdc2 antibody. Data are representative of at least three separate experiments.

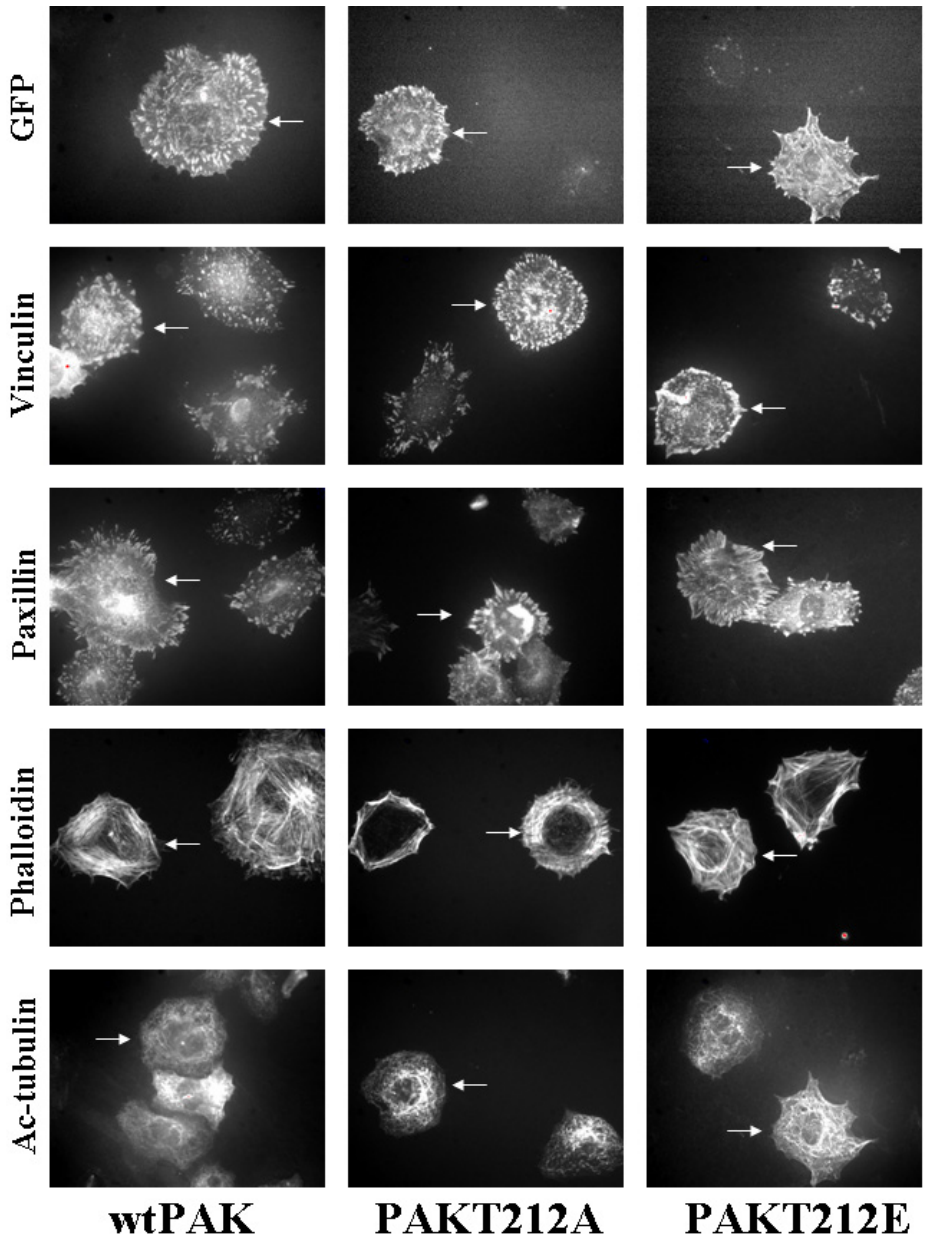


Figure 3.11. PAK1 Thr212 phosphorylation does not alter focal adhesion localization, formation or cell spreading. A7r5 cells transfected with GFP-PAK, GFP-PAKT212A or GFP-PAKT212E were trypsinized and plated on fibronectin-coated chamber slides (10μg/ml) for 1 hour as described in Experimental Procedures. Cells were fixed, permeabilized and either observed by direct fluorescence (GFP, top panel) or stained with anti-vinculin or anti-paxillin antibodies to examine mature and nascent focal adhesions respectively, or phalloidin or anti-acetylated tubulin antibody to examine actin or tubulin polymerization. Arrows indicate GFP-PAK1, GFP-PAK1 T212A or GFP-PAK1 T212E expressing cells identified by direct fluorescence. Data are representative of at least three separate experiments scoring 100-150 transfected cells for each treatment.

autophosphorylates in a concentration-dependent fashion, however titration of KD- ERK2 (up to a 15-fold molar excess over PAK) into the reaction does not enhance PAK1 autophosphorylation. Actually, KD-ERK2 appeared to attenuate PAK activity at the higher concentrations (0.25-1 μg), but the significance of this inhibition is unclear. This experiment also revealed that PAK1 does not phosphorylate ERK2 in vitro as evident by the lack of radioactivity incorporated into KD-ERK2.

In terms of the reciprocal phosphorylation event, PAK1 contains two consensus sites for ERK phosphorylation, PVTP (T212) and PISP (S223) just downstream of the defined ERK2 binding site. To determine whether ERK2 phosphorylates PAK1 directly, we performed an in vitro kinase assay in which a GST-PAK1 fusion protein containing the two putative phosphorylation sites (aa 1-290) was incubated with purified active ERK2 and γ^{32}P-ATP. Figure 3.8A shows that the N-terminal GST-PAK1 fusion protein, but not GST alone, was efficiently phosphorylated by ERK2 in vitro. Based on this observation, we mutated each of the consensus amino acids to alanine individually and in combination to determine if these sites were phosphorylated by ERK2 in vitro. As shown in Figure 3.8B, the T212A mutation dramatically reduced ERK-mediated phosphorylation. The S223A mutation had only a slight effect on its own or in combination with T212A. To confirm these data, ERK2-mediated phosphorylation of Thr212 was measured using a phospho-threonine 212-specific antibody. Results shown in Fig 3.7C provide further evidence that PAK Thr212 is a major target for ERK phosphorylation.

To determine whether Thr212 is phosphorylated by ERK in cells, we treated SMC with PDGF-BB for various times and examined the phosphorylation of PAK1 Thr212 (pThr212) by Western Analysis. As shown in Figure 3.9A, PDGF-BB stimulated a time-

dependent increase in pThr212 that lagged slightly behind activation of ERK, with an observed peak in phosphorylation around 10 minutes (Figure 3.9A). Pretreatment of SMC with the MEK inhibitor, UO126, almost completely blocked PDGF-BB-stimulated PAK1 phosphorylation, indicating that this response is mediated by ERK signaling. Interestingly, although adhesion signaling can stimulate association between ERK and PAK, plating on fibronectin alone for 30-90 min is not sufficient to induce pThr212 (Figure 3.8B). Nonetheless, adhesion is required for PDGF-BB stimulated pThr212, since PDGF-BB treatment of SMC held in suspension did not alter pThr212 levels (Fig 9C).

Recent reports demonstrated that Cdc2/Cdk5 can phosphorylate PAK1 Thr212 in a cell cycle-dependent manner (35-38). To rule out the possibility that these cyclin-dependent kinases are involved in PDGF-BB-stimulated Thr212 phosphorylation, we exposed SMC to the Cdk1 inhibitor alsterpaullone prior to PDGF-BB stimulation and examined the level of Thr212 phosphorylation. As shown in Figure 3.10A, the cyclin-dependent kinase inhibitor did not diminish Thr212 phosphorylation. Interestingly, PDGF-BB inhibited Cdc2/Cdk5 activity in SMC as measured by the inhibitory phosphorylation of Cdc2-Tyr15 (Figure 3.10B) further suggesting that adhesion-dependent agonist-stimulated phosphorylation of PAK1 Thr212 in SMC is dependent upon ERK and not Cdc2/Cdk5.

Irrespective of the kinases involved, phosphorylation of PAK1 Thr212 has been implicated in the regulation of post-mitotic cell spreading and microtubule organization (35-37). In addition, phospho-ERK1/2 and PAK1 co-localize in focal adhesion structures in SMC, so we hypothesized that PAKT212 phosphorylation might regulate focal adhesion formation. To this end, we transfected GFP-tagged variants of PAK1 including a non-phosphorylatable Thr212 (T212A) and one that mimics phosphorylation, T212E, into A7R5

SMC that were then plated onto fibronectin for 20-90 minutes. No change in the rate of cell spreading was observed between GFP-PAK1, GFPPAKT212A, or GFP-PAKT212E expressing cells (data not shown). Furthermore, each of the variants localized in focal adhesions and had no effect on the organization of nascent or mature focal adhesions as assessed by paxillin and vinculin staining, respectively. Organization of actin microfilaments and acetylated tubulin were also indistinguishable between wild type GFP-PAK1 and the phosphorylation variants (Figure 3.11).

Because PAK potentiates ERK activation, we sought to determine whether PAKThr212 phosphorylation might regulate ERK-PAK association and/or ERK-dependent signaling. GST-PAK, GST-PAK pre-treated with purified active ERK2 and γ^{32}P-ATP, and GST-PAKT212E each precipitated similar amounts of ERK from SMC lysates, indicating that pT212 does not attenuate ERK-PAK association (data not shown). To determine the possible effects of T212 phosphorylation on downstream signaling, we examined the effect of GFP-PAK and GFP-PAK T2312E expression on transactivation of the ERK-dependent SRE- luciferase reporter gene. As shown in Figure 3.12, in contrast to GFP-PAK which stimulated serum-induced luciferase activity, GFP-PAK T212E expression markedly attenuated this response. Based on the observations presented in this report, we hypothesize that Rac-dependent adhesion signaling activates PAK and induces an association between PAK and components of the ERK signaling cascade to facilitate ERK signaling, and that subsequent growth-factor stimulated phosphorylation of PAKT212 by ERK may serve to provide a negative feedback signal to control coordinate activation of ERK by growth factor- and matrix-induced signals (see Figure 3.13).

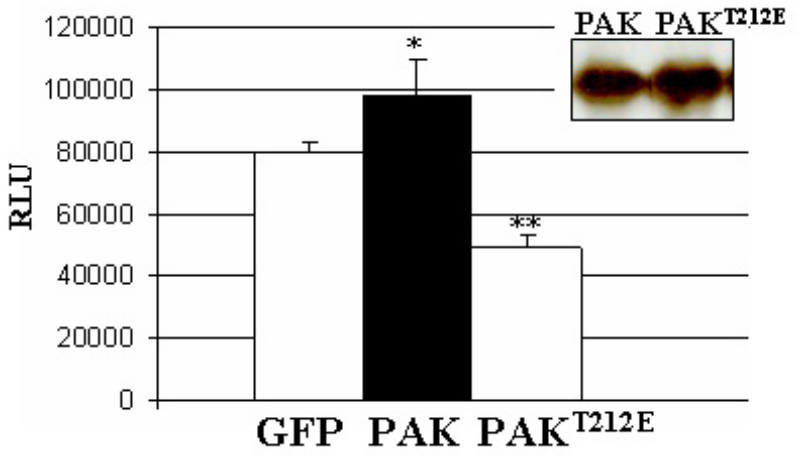


Figure 3.12. **Thr 212 phosphorylation suppresses SRE activation.** A7r5 cells were transfected with an SRE-luciferase reporter plasmid along with either GFP-C1 vector control, GFP-PAK1, or GFP-PAK212E. 48 hours following transfection, cells were lysed and analyzed for luciferase activity as described in Experimental Procedures. The inset shows equivalent expression levels of the GFP-PAK1 and GFP-PAK212E constructs. Data was normalized to total cellular protein. The graph represents means +/- S.E. of four separate experiments. The single and double asterisks indicate significant increases or decreases from control GFP-transfected cells respectively ($p<0.05$).

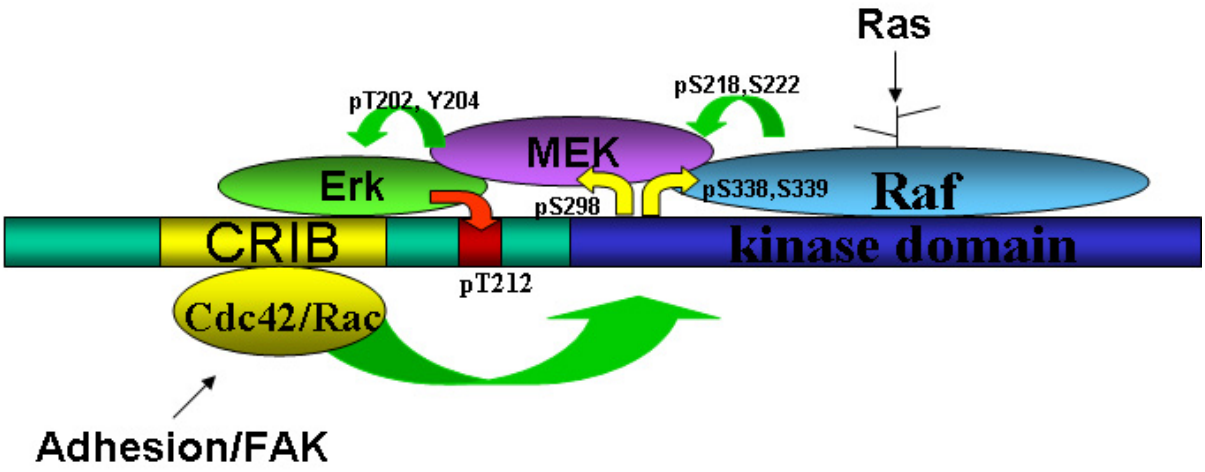


Figure 3.13. **Adhesion-dependent PAK-ERK association facilitates ERK-signaling.** Numerous extrinsic factors stimulate Ras activity and thereby initiate activation of the canonical Raf/Mek/Erk cascade wherein Raf phosphorylates Mek on serines 218 and 222, then Mek phosphorylates Erk on Thr202 and Tyr204 (green arrows). Adhesion signaling through Focal Adhesion Kinase (FAK) activates the smGTPase Rac1, which binds to CRIB domain of Pak1, enabling Pak1 activation. Our studies reveal that activated PAK associates with ERK through N-terminal sequences and past studies reveal that C-terminal sequences of Pak direct an interaction with Raf and mediate phosphorylation of Raf on serines 338 and 339 (yellow arrow). Although Mek has not been demonstrated to interact in a direct fashion with Pak, PAK can phosphorylate Mek on Ser282 (yellow arrow) and enhance association between Mek and ERK. Each of these events facilitates ERK-dependent signal transduction. Following growth factor stimulation, ERK phosphorylates PAK on Thr 212 resulting in a negative feedback loop to depress downstream signaling (red arrow).

DISCUSSION

PAK kinases regulate various cellular processes such as proliferation, migration, contraction, and apoptosis. PAK1 is a Rac/Cdc42 effector that coordinates actin-based cellular protrusions, an important step in persistent directional migration (39,40). Recent studies reveal that PAK1 activity is also essential for maximal activation of the mitogenic Raf/MEK/ERK cascade (40). Indeed, PAK can synergize with Ras to activate Raf and can synergize with MEK to activate ERK (5,14,17,18,20-22). Herein we report that ERK2 associates with PAK1 in an adhesion-dependent manner through sites mapped within the N-terminal auto-inhibitory domain of PAK. In addition, we show that a PAK deletion variant that does not readily associate with ERK has a reduced capacity to enhance ERK-dependent transactivation of an SRE-reporter construct. These data are consistent with previous findings that adhesion-dependent Rac activation is required for efficient accumulation of active ERK in the nucleus (41,42). The ability of PAK to bind (and phosphorylate) Raf, phosphorylate MEK and, as demonstrated in this study, bind to ERK, indicates that PAK may serve as a scaffold to recruit members of the Raf/MEK/ERK complex to focal adhesions and subsequently facilitate signaling through the ERK pathway.

In yeast, Ste5, which regulates activation of the Fus3 MAP Kinase mating pathway is a classic example of a high fidelity signaling scaffold, because it interacts with each member of the cascade (Ste20, Ste11, Ste7, and Fus3) (43,44). To date, a protein that acts in an analogous fashion to Fus3 in the ERK cascade has not been identified. However, Kinase Suppressor of Ras (KSR) and MEK- binding partner 1 (MP-1) are putative ERK scaffolding molecules because they bind directly to both MEK and ERK (specifically MEK1 and ERK1 in the case of MP-1) (45,46). Herein, we have shown that PAK (the Ste 20 homologue or

MAP4K of the ERK pathway) interacts with ERK2 through a region within the N-terminal auto-inhibitory domain. Because PAK has also been shown to interact with Raf within sequences in the C-terminus, it may serve as an additional potential scaffold for the ERK cascade. Even though there is no evidence of a direct PAK-MEK interaction, it has been shown that upon Raf binding to the C-terminus of PAK, MEK becomes phosphorylated at S298, a site required for maximal MEK activation (14,18). Furthermore, Slack-Davis et. al. showed that adhesion induces PAK-dependent phosphorylation of MEKS298, and Elben et. al. have shown that PAK activation was required for adhesion dependent association of MEK1 with ERK (21,22). Based on these reports, is possible that PAK1 could function to target Raf, MEK and ERK to nascent focal adhesions to impart restricted activation of the ERK cascade at the leading edge of the cell during migration.

We identified three distinct ERK2 binding sites that mapped within the N-terminal autoinhibitory domain of PAK1: aa40-54, aa86-94 and aa124-138 (denoted A, B and C respectively). Although the reported crystal structure of the complex between the N-terminal autoregulatory fragment (aa 70-149) and the C-terminal kinase domain (aa 249-545) of PAK1 does not contain the most proximal A binding site, the structure reveals that the β-sheet comprising the "B" binding site packs tightly against the α-helix comprising the "C" binding site, indicating that the binding sites for ERK2 that we defined may indeed constitute a single binding pocket within PAK1. Interestingly, the "A" binding site in PAK1 which we have shown is sufficient for ERK binding does contain a cluster of basic residues similar to the "D-domain" found in other ERK binding partners including MEK, MAPK phosphatase, and ribosomal S6 kinase (47,48). This motif has been shown to function independently (47,48), corroborating our Far Western data revealing that PAK aa 1-55 was sufficient for

directing the ERK2-PAK1 interaction and our findings that a GST-PAK fusion protein with a deletion of aa 40-54 had a markedly reduced capacity to precipitate ERK from SMC lysates compared to the full N-terminal construct. Future mutagenesis experiments will aid in determining the relative contribution of the defined sites to direct high affinity binding to ERK. Notably, the 12 amino acid core of each of the defined binding sites in PAK1 are highly conserved across the Group I PAK family members (100%, 100%, and 75% identical for A, B and C respectively) thus it is likely that ERK may interact with PAK2 and PAK3 as well as PAK1. In fact, a recent report by Shin et. al. highlights the possibility that ERK1 and PAK2 may indeed associate in cells in a FGF-dependent fashion as determined by co-immunoprecipitation (49). In addition, the authors reported that a GST-ERK1 fusion protein could precipitate exogenously expressed PAK2 from cell lysates, although a direct association between the two proteins has yet to be demonstrated (49).

The location of the ERK2 binding site indicates the likelihood that ERK association with full-length PAK in vivo may require pre-activation of PAK by Rac and/or Cdc42. Recent structural studies have indicated that the inactive autoinhibited conformation of PAK1 is an asymmetric dimer whereby the auto-inhibitory domain of one molecule associates tightly with the kinase domain of another (34). Binding of GTP-loaded Cdc42 or Rac with the CRIB domain disrupts the dimer, unfolds the protein, and exposes the auto-inhibitory domain allowing for subsequent protein-protein interactions (34). Indeed, the association of PAK1 and ERK following cell adhesion mimicked the time course we observed for Rac activation following plating cells on fibronectin (23). Thus, it is possible that recruitment of ERK to PAK may be dependent on integrin-dependent Rac activation.

We also show that adhesion-dependent association of PAK and ERK can provide an additional means of regulation by driving phosphorylation of PAK by ERK. The time course of PAK1 phosphorylation, inhibition of phosphorylation by UO126 and the fact that ERK2 directly phosphorylated PAK1 in vitro on the same site that is also phosphorylated after PDGF-BB treatment of SMC all support the possibility that PAK1 is an in vivo substrate for ERK. Interestingly, although the data presented in this report are consistent with T212PAK being an in vivo target for ERK in SMC, other reports have clearly shown that Thr212 is also a target for Cdc2/Cdk5 in cells undergoing mitosis (35-38). Two lines of evidence rule out the possibility that PAK1 is a target for Cdc2/Cdk5 following PDGF-BB-treatment of SMC. First, pre-treatment of SMC with alsterpaullone (a potent pharmacological inhibitor of Cdc2/Cdk5) had no effect on PDGF-BB-stimulated Thr212 phosphorylation, whereas treatment with the MEK inhibitor U0126 almost completely inhibited the response. Second, PDGF-BB-treatment of SMC caused a phosphorylation-dependent inactivation of Cdc2, similar to what has been reported following ERK activation in Xenopus extracts (50). Thus, at least in the context of adhesion-dependent growth factor signaling, phosphorylation of PAK1 in SMC appears to be directed by ERK and not Cdc2/Cdk5.

Unlike the ERK2 binding sites that are conserved in PAK1-3, Thr212 is unique to PAK1. This consensus site is however conserved in all mammalian forms of PAK1, indicating that phosphorylation of the site may regulate an important function. Notably, a previous detailed temporal analysis of pThr212 *in vivo* revealed that high levels were observed in the embryonic forebrain, lung, kidney, intestine, and skin, but that pThr212 was virtually undetectable in adult tissues (51). In terms of function, previous studies indicate that the kinase activity of the PAK1 variants T212A and T212E were indistinguishable from

wild type PAK1 (36). Previous reports also indicate that Cdc2-mediated pT212 altered the rate and extent of postmitotic spreading of murine fibroblasts (37) and regulated microtubule dynamics and overall morphology in neurons (35,36). We show herein that ectopic expression of PAKT212A or PAKT212E in SMC did not alter focal adhesion formation, microtubule formation, or the rate of cell spreading, although the consequence of PAK1 phosphorylation on focal adhesion turnover or directed cell migration has yet to be determined. However, PAKT212E did attenuate serum-stimulated ERK-dependent transcription, indicating that phosphorylation of this site might provide a negative feedback inhibition to limit adhesion and growth factor-stimulated ERK signaling. The precise mechanism of this inhibitory response is not yet clear. We have determined that pThr212 within the context of GST-PAK1 (1-290) does not alter the association of ERK and PAK in vitro, indicating that ERK can still bind pT212PAK. However, Thr212 is located within the core of a canonical SH3-binding motif, thus it is feasible that phosphorylation of this site could modulate association with a binding partner that could in turn regulate ERK phosphorylation or nuclear translocation. Experiments to address this question are presently underway.

In summary, we have shown that ERK2 binds within the N-terminus of PAK1 and when activated by adhesion-dependent growth factor signaling, phosphorylates PAK1 on Thr 212. Our present data, together with previous reports that illustrate a role for PAK in the regulation of the ERK cascade, indicate that PAK1 may function as a scaffold for this canonical pathway. Why would the ERK pathway require so many scaffolds? One possible explanation may be that scaffolding controls local activation of this seemingly promiscuous pathway. For example, KSR has been shown to shuttle from the cytoplasmic membrane to

the plasma membrane following growth factor treatment, and p14, a partner of the putative scaffold, MP1, targets MP1-MEK1 and ERK1 to late endosomes/lysosomes (52-54). In the case of PAK, association of the complex could lead to the targeting of active ERK to focal adhesions. Since association of ERK with PAK may be dependent on adhesion-induced activation of Rac, one might speculate that adhesion-stimulated PAK-dependent targeting of ERK to these dynamic sites might regulate directional lamellipodial extension during cell migration. Notably, studies have shown that ERK activation enhances focal adhesion turnover by enhancing the rate of focal adhesion disassembly (55). This effect could be due to the ability of ERK to phosphorylate and regulate several focal adhesion proteins such as paxillin, MLCK, and calpain II (56-58). We have also shown that coordinate signaling through adhesion and growth factors regulates phosphorylation of PAK on Thr212, and provide evidence that this event may decrease subsequent ERK-dependent signaling. It is likely that ERK signaling would have to be tightly regulated in a temporal fashion in order for directed cell migration to occur, thus negative feedback phosphorylations such as Thr 212 may prove important in controlling this dynamic cellular process.

Chapter IV

Inactivation of focal adhesion kinase promotes smooth muscle cell differentiation: A role for the nuclear shuttling of the LIM protein, leupaxin.[3]

[3] To be submitted as: Liisa J. Sundberg-Smith, Lee E. Mangiante, Hilary E. Beggs, Louis F. Reichardt[4], Christopher P. Mack, and Joan M. Taylor. Inactivation of focal adhesion kinase promotes smooth muscle cell differentiation: A role for the nuclear shuttling of the LIM protein, leupaxin. *Mol Cell Biol*

ABSTRACT

Smooth muscle cell (SMC) differentiation is a dynamic process that must be tightly regulated for proper vascular development and to control the onset of vascular disease. Extensive studies indicate that autocrine factors and extracellular matrix (ECM) are critical co-regulators of vascular SMC growth and differentiation during development and following vessel injury. The non-receptor tyrosine kinase, Focal Adhesion Kinase (FAK) is strongly activated by integrin/matrix interactions and growth factor receptor engagement, and the observation that *fak*$^{-/-}$, fibronectin$^{-/-}$, and $\alpha_5^{-/-}$ mice die between E8.5-10 with notable defects in vessel integrity suggests that FAK plays an important role in vascular maturation. Herein, we found that deletion of FAK in SMC enhanced TGF-β-stimulated SM marker gene expression, but did not affect PDGF-BB or EGF-stimulated proliferation. These data suggest that enhanced FAK activity limits SM differentiation and that tight regulation of FAK activity is likely important for proper SMC phenotypic modulation during development and following vascular injury. We identified leupaxin as a binding partner for FAK in SMC and show that leupaxin is enriched in SMC, leupaxin associates with SRF, and that leupaxin enhances SRF-dependent expression of SM marker genes. In addition, we show that leupaxin undergoes focal adhesion-nuclear shuttling and that FAK activity attenuates the nuclear accumulation of this LIM domain containing SRF co-factor. Our data indicate that sequestration of leupaxin to focal adhesion plaques is one mechanism by which activated FAK might limit SMC marker gene expression and we suggest the possibility that extrinsic signals may regulate the SMC gene profile by modulating the activation state of FAK.

INTRODUCTION

The investment of newly formed endothelial cell tubes with differentiated SMC is a very important process during vascular development and requires intricate regulation of SMC motility, growth, and differentiation. Mature medial SMC express high levels of the SMC differentiation marker genes (ie. myosin heavy chain (SM-MHC), SM α-actin, SM22α among others) that contribute to the regulation of SMC contractility (see [4] for review). SMC never terminally differentiate and can transition to synthetic phenotype characterized by low levels of SMC contractile gene expression and responsiveness to pro-growth and migratory signals. This unique plasticity is critical for proper vessel development, blood pressure homeostasis, and injury repair (1), but can also contribute to the development of various vascular pathologies, including atherosclerosis, restenosis, and vascular hypertrophy following vein bypass grafts (2). Thus, defining the signaling mechanisms that regulate SMC growth and differentiation will be important for understanding the processes that modulate vascular development and is critical for the design of agents that might regulate aberrant SMC responses in diseased vessels.

The transcription mechanisms that regulate SMC differentiation are starting to become clear. Serum response factor (SRF) binding to conserved CArG ($CC(A/T)_6GG$) promoter elements is required for the expression of most SMC differentiation marker genes. It is well known that SRF activity is regulated through interactions with additional cell-type-selective and ubiquitous transcription factors, and the potent SRF co-factors of the myocardin family are particularly important activators of SMC-specific gene expression. Interestingly, several LIM domain proteins including CRP1, CRP2, and FHL2 interact with SRF to

regulate SMC differentiation marker gene expression [4, 89, 90, 114] most likely by altering SRFs interactions with additional transcription factors.

The identification of the signaling pathways that regulate SMC-specific transcription mechanisms will be very important for our understanding of the control of SMC phenotype. TGF-β, which promotes SMC differentiation [97-99], and PDGF-BB, which promotes phenotypic modulation, are important regulators of SMC phenotype and genetic ablation of these genes resulted in defective vasculogenesis (12, 13). TGF-β-mediated nuclear localization of SMAD2 and SMAD3 can stimulate SMC-specific transcription [99, 101, 104], and a TGF-β control element (TCE) has been identified in a number of SMC-specific promoters. Several studies have also shown that SMADs interact with SRF, myocardin, and p300 to enhance SMC-specific transcription [102, 103]. PDGF-BB stimulates SMC proliferation by activating Ras/ERK signaling, and this pathway can also limit SMC differentiation by competitive inhibition of myocardin-SRF binding by the ERK-activated TCFs.

Extensive evidence indicates that extracellular matrix signaling is also an important regulator of SMC growth and differentiation. Enhanced levels of medial fibronectin (FN) (which supports proliferation) are produced early in the developing vasculature, whereas the basement membrane components collagen type IV and laminin (which promote differentiation) are more prominent in the mature vessel [13,14]. In addition, deletion of either FN, the α_5 integrin FN receptor, or focal adhesion kinase (FAK) (the kinase that mediates α5-dependent signaling) each results in extraembryonic and embryonic vessel defects leading to lethality in the mouse from E8.5 to E10 [18,19] [53]).

Although a direct role for FAK in vascular smooth muscle growth and development has yet to be examined, FAK is known to play critical role in matrix signaling by supporting the formation of multi-protein signaling complexes that lead to downstream activation of a number of signaling molecules that been previously implicated in the regulation of SMC phenotype including ERK, JNK, and the small GTPases, Rac and Rho [146]. Importantly, our lab has recently showed that FAK activity is regulated by the SMC-specific expression of FRNK (FAK-Related Non Kinase), a carboxyterminal variant of FAK that acts as an endogenously expressed dominant negative. While FAK protein levels remained relatively constant during vascular development, FRNK expression in SMC is significantly increased in the post-natal period and two to three weeks following vessel injury [147]. These results suggest that FAK activity is tightly controlled in SMC especially during the transition from the synthetic to contractile phenotype.

The aim of the present study was to determine whether FAK activation plays a direct role in the phenotypic modulation of SMC. We present evidence that FAK deletion (by homologous recombination) in SMC enhances TGF-β stimulated differentiation but does not effect cell survival or PDGF-mediated SMC growth. Our data also indicate that the effects of FAK on SMC phenotype are mediated, at least in part, by regulating the cytoplasmic-nuclear shuttling of the LIM protein leupaxin, which interacts with SRF to enhance SMC differentiation marker gene expression. These data highlight the possibility that extrinsic signals can regulate the SMC gene profile by modulating the activation state of FAK.

MATERIALS AND METHODS

Antibodies and Reagents:

The phospho-specific ERK1/2 antibody was purchased from Cell Signaling. The anti-Flag (M2), anti-vinculin and SMα-actin antibodies, and leptomycin B were purchased from Sigma. PP2, PDGF-BB and TGF-β were purchased from Calbiochem. The N-term specific anti-FAK antibody, C-term specific anti-FAK antibody, and anti-ERK2 antibody were purchased from Upstate. The anti-phospho Y397FAK antibody was purchased from BioSource and the Texas-Red phalloidin was purchased from Molecular Probes. The anti-GFP antibody was purchased from Clontech. The anti-leupaxin antibody was a generous gift from Dr. Don Staunton, ICOS. Ad5CMV Cre Adenovirus was purchased from the University of Iowa Gene Transfer Vector Core, and Ad5CMV LacZ adenovirus was purchased the University of North Carolina-Chapel Hill Viral Core. Both viruses were expanded using Puresyn's Adenopure adenovirus purification kit according to manufacturer's protocol. The Pyk2 antibody was purchased from Cell Signaling.

Constructs:

Leupaxin and Hic5 human cDNA clones were purchased from Open Biosystems. Leupaxin was directionally cloned into the Flag vector using EcoRI and Xho1. To generate GFP-leupaxin, Flag-leupaxin was cut with EcoRI and XbaI and directionally cloned into the GFP-C1 vector (Clonetech). Murine Flag-Trip6 was a generous gift from Mary Beckerle (University of Utah). The promoter reporter constructs: SM α-actin (from -2560 to +2784), SM22 (from -450 to +88) and SM-MHC (from -4200 to +11600) and SM α-actin mutant luciferase constructs used have been previously described [148 1568]. Flag-FAK, Flag-

FAKY397F and Flag-FRNK were a generous gift from Dr. Tom Parsons (University of Virginia) and were previously described [149]. PBSK-SuperFAK was a generous gift from Dr. Michael Schaller (University of North Carolina, [150]). To generate Flag-SuperFAK, PBSK-SuperFAK was cut with BamHI and Xho1 and directionally cloned into the Flag vector. GST-FAK variant constructs were generated as previously described [151].

Cell Culture and Agonist Treatment

Aortic smooth muscle cells were isolated from either Wistar Rats or *fak*$^{flox/flox}$ mice, a generous gift from Dr. Louis Reichardt and Dr. Hilary Beggs, UCSF. In brief, thoracic aortas were stripped of the endothelial and adventitial layers by microdissection. The SMC in the media were isolated by enzymatic digestion in buffer containing trypsin and collagenase as previously described [152]. Each of our preparations are routinely tested for expression of smooth muscle-specific markers (by immunohistochemistry) and the ability to drive smooth muscle specific expression of reporter constructs. Only the cell lines that are deemed at least 85% pure by these measurements are utilized for further experimentation. Cells are grown in DMEM: F12 (1:1) supplemented with 10% FBS and 1% penicillin-streptomycin and are used between passages 5 and 18. 10T1/2 cells (ATCC) were maintained in DMEM plus 10% fetal bovine serum and 1% penicillin-streptomycin. Human coronary SMC (Cambrex) were maintained in Clonectics SmGM media containing 5% FBS, insulin, hEGF, hFGF and gentamicin.

Promoter Assays:

Cells were transfected using either Superfect (Qiagen) or Trans-IT (Mirus) transfection reagents according to manufacturer's protocol. Luciferase activity was detected by the Steady-Glo luciferase assay reagent (Promega).

Western Blotting:

Western blots were performed using the appropriate antibodies at a 1/1000 dilution. Blots were washed in TBST (TBS plus 0.05% Triton-X), followed by incubation with either horse-radish peroxidase conjugated- rabbit anti-mouse antibody or - Protein A sepharose (Amersham) at a 1/2000 dilution. Blots were visualized after incubation with chemiluminscence reagents (ECL, Amersham).

GST-Pulldown Assays:

GST-FRNK was purified from bacterial lysates using gluathione-agarose beads (Pharmacia) as described previously [153]. hCSMC were lysed in RIPA (50 mM Hepes, 0.15 M NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 0.05% sodium deoxycholate, pH 7.2) containing 1 mM Na_3VO_4, 40 mM NaF, 10 mM Na_2 pyrophosphate, 100 μM leupeptin, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.02 mg/ml soybean trypsin inhibitor, and 0.05 trypsin inhibitory units/ml aprotinin and 500μg of protein was combined with 1õμg of GST, GST-CTII, GST-CTI or GST-CTIm fusion protein and rotated for 60 min at 4°C. The beads were then washed twice with the lysis buffer and once with Tris-buffered saline (TBS, 0.2 M NaCl, 50 mM Tris-HCL, pH 7.4). The beads were resuspended in SDS-PAGE sample buffer, boiled for 5 min, electrophoresed on an 11% SDS-polyacrylamide gel, and

analysed by Western blotting using either an anti-leupaxin (1:1000) or anti-GST antibodies (1:10,000). To generate GST-leupaxin, a 3' Sma1 site was inserted into GFP-leupaxin by PCR and was then cut with EcoR1 and Sma1 and was directionally cloned in the PGEX vector (clontech). Flag-SRF was *in vitro* translated with S-35 using TNT T7 translational kit (Promega) according the manufacturer's directions. Translation reaction was diluted 1:10 in lysis buffer and rotated with 30ug of either GST or GST-leupaxin beads for 1 hr, washed 4 times with lysis buffer and then boiled for 5 minutes. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and exposed to film.

Immunoprecipitation Assays

Cos-7 cells were transfected with FL-SRF and GFP-Leupaxin DNA (7.5μg each) for 48 hrs. using Mirus Transfection Reagent according to manufacturer's protocol. Cells were then lysed in RIPA plus inhibitors. 5μg of antibody (Flag:M2 or GFP) was incubated with 500μg of protein for 2 hrs and then rotated overnight with either Protein-A Sepharose (PAS) beads (Sigma) or precoupled Rabbit anti-Mouse PAS beads. Beads were washed three times with RIPA plus inhibitors and once with 1x TBS and then Complexes were electrophoresed on an 11% SDS-polyacrylamide gel and western blotting was performed with either anti-Flag or anti-GFP (both 1:1000) antibodies.

Immunocytochemistry:

Cells were processed for immunocychemistry using previously published methods [147]. In brief, cells were fixed with 4% paraformaldehyde, permeabilized with 4% Triton X-100 in PBS, incubated with specified primary antibodies for 1 hour at the following concentrations: anti-vinculin (1:1000), anti-FAK (N-term 1:250), or anti-leupaxin (1:300) Abs. After

washing with PBS, slides were incubated for 1 hour with Texas Red-conjugated donkey anti-mouse antibodies (2μg/ml) or Texas Red-conjugated phalloidin to detect filamentous actin.

Chromatin immunoprecipitation Assays:

HCSMC were cross-linked for 20 min with 37% formaldehyde. Cells were was once with cold PBS and then lysed in PBS and spun for 5 min at 1500rpm. Pellets were lysed in Chip lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCL plus aprotinin, leupeptin and pepstatin) on ice for 10 min. DNA was sheared by sonication (10 pulses 20 times). 100μg of DNA was immuprecipitated with 5μg Leupaxin Ab along HS-RαM-PAS beads (precoupled) overnight. Beads were washed twice with Abcam CHIP wash buffer (.1% SDS, 1% triton X-100, 2mM EDTA pH 8.0, 150mM NaCl, 20mM Tris-HCl), once with Promega LiCl Immune Complex Wash Buffer (.25M LiCL, 1% NP-40, 1% deoxycholate, 1mM EDTA pH 8.0) and one time with Abcam Final Wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA pH 8.0, 500 mM NaCl, 20mM Tris-HCl). Complexes were eluted with Elution buffer (1% SDS, 100mM $NaHCO_3$) for 15min at RT and then reverse-crosslinked overnight with 16μl 5M NaCl and 5μl Proteinase K (20mg/ml) at 65^{o}C. DNA was purified using Phenol:Chloroform. PCRs were performed with the following primers: SMA 5' CAGTGGATGCAGTGGAAGAGACCAGGC, 3' CCTCCCACTTGCTTCCCAAACAAGGAG, SM-MHC 5' CCACTCGGCACCATATTTAGTCAGGGGAGA, 3' CGGGCGGGAGACAACCCAAAAAGGGCAGG. c-fos specific primers were used according to previous published reports (owens, jci) PCR products were separated on a 2% agarose gel.

RESULTS

To examine the role of FAK in SMC, aortic SMC were isolated from *fak*$^{flox/flox}$ mice [57] using standard procedures and were infected with either a Cre-expressing virus (FAK null) or a Lac-Z expressing adenovirus (control). Incubation of *fak*$^{flox/flox}$ SMC with Cre adenovirus for 72 hr resulted in a significant depletion of FAK protein, while levels of FRNK, and the FAK homologue, Pyk2 were not significantly affected (Fig 4.1A). Importantly, the FAK-null SMC maintained a well spread phenotype and exhibited similar organization of vinculin-containing focal adhesions and actin filament distribution when compared to FAK-containing control SMC (Fig 4.1B).

Since FAK has been shown to be a major regulator of mitogenesis in a variety of cell types, we first tested its effects on SMC proliferation. As shown in Figure 4.2A, FAK was not required for SMC growth with control and FAK-null cells demonstrating similar growth rates under serum-free conditions and in cells stimulated with PDGF-BB and EGF. Accordingly, mitogen-stimulated ERK activity and cyclin D protein levels were similar in Cre- and LacZ- treated cells (Fig 4.2B, 4.2C), indicating that FAK was not required for activation of the major mitogenic Ras/ERK signaling pathway. Consistent with our findings that deletion of FAK was well tolerated, we observed no significant difference in cell survival as measured by caspase 3/7 activity (Fig 4.2D). FAK-null SMC did exhibit a marked decrease in PDGF-BB-stimulated chemotaxis, an event likely due to impaired lamellipodial stability in these cells (manuscript in preparation).

To determine whether FAK deletion had a deleterious effect on other focal adhesion components, we examined the expression levels of paxillin and vinculin in LacZ- and Cre-treated SMC maintained in 10% serum. While paxillin levels were similar between FAK-null

and control SMC, we observed a consistent increase in vinculin levels in FAK-null SMC (Fig 4.3A), a finding that supports previous studies in FAK-null mouse embryo fibroblasts (31). Since vinculin is a well-known SRF-target gene, we hypothesized that other SRF-dependent SMC differentiation marker genes would also be up-regulated in the FAK-null SMC. Indeed, SM-MHC levels were also significantly enhanced in FAK-null SMC (Fig 4.3A). We next serum starved the SMC and asked whether TGF-β-induced SM marker gene expression was altered in FAK-null SMC. As shown in Fig 4.3B, TGF-β induced a more marked up-regulation of SM α-actin protein levels in the Cre-treated compared to LacZ treated SMC. To test whether this effect was due to enhanced SMC-specific transcription, we also examined the responsiveness of an SM22 promoter/luciferase reporter. As shown in Figure 4.3C, SM22 activity in control SMC was induced 2-fold by TGF-β, but by 10-fold in FAK-null cells. A similar enhancement in TGF-β induced expression of the SM α-actin and SM-MHC promoters was also observed in FAK-null SMC (Fig 4.3C middle, bottom panels). To test the possibility that FAK inactivation enhanced TGF-β-mediated SMAD activation, we measured the phosphorylation of Smad 2 and Smad 3 in control and FAK-null SMC using phospho-specific antibodies. Results shown in Figure 4.3D indicate that FAK deletion did not significantly affect this parameter. Taken together, our findings provide evidence that FAK activity does not directly regulate SMC growth but does limit TGF-β-stimulated SMC differentiation by a mechanism downstream or parallel to SMAD activation.

Since FAK is a multifunctional protein that associates with a number of adapter molecules, we reasoned that the effects of FAK deletion on SM differentiation may be secondary to effects on additional focal adhesion proteins. To identify SMC proteins that might mediate the effects of FAK on SMC transcription, we performed a yeast two-hybrid

screen in a mouse aortic SMC library using the C-terminal protein-binding module of FAK as bait. Interestingly, the vast majority of the clones identified from this screen were LIM-domain containing proteins including Hic5, zyxin related protein 1 (zrp-1/TRIP6) and leupaxin. Since the LIM domain proteins CRP-1 and CRP-2, and FHL2 have recently been shown to play a role in the regulation of SMC marker gene transcription [89, 90], we examined whether expression of any of the aforementioned FAK binding partners would alter SMC differentiation marker gene transcription in pluripotent 10T1/2 cells. Expression of leupaxin but not Hic5, or zrp-1(TRIP6) induced a significant increase in SM22 and SM α-actin (Fig 4.4A) promoter activity in control and TGF-β treated 10T1/2 cells, and further experiments showed that leupaxin enhanced SMC reporter activity in a dose-dependent fashion (Fig 4.4B). Importantly, transient overexpression of leupaxin in 10T1/2 cells markedly enhanced endogenous SM α-actin protein levels (Fig 4.4C) and also enhanced SMC reporter activity in primary SMC cultures (Fig 4.4D). Collectively, these data strongly support a role for this LIM containing protein in the regulation of SMC differentiation.

Leupaxin is a member of the paxillin family (along with Hic5) that was previously reported to be preferentially expressed in spleen, thymus, and other lymphoid tissues as well as several cultured hematopoetic cell lines [144]. Our identification of leupaxin in the aortic SMC yeast two-hybrid library suggested that it was also expressed in SMC. To our knowledge, an antibody that recognizes murine leupaxin has not been developed. Thus, we used an antibody specific for human leupaxin to demonstrate [144], that leupaxin was also strongly expressed in SMC isolated from human coronary vessels (HCSMC) (Fig 4.5A). Importantly, this antibody does not recognize the leupaxin family members paxillin or Hic 5

(Fig 4.5B). We also used semiquantitative RT-PCR to confirm that leupaxin message was expressed in mouse aorta and in our isolated aortic SMC preparations (data not shown).

Leupaxin was previously shown to interact with the FAK homologue, Pyk2, and we used GST pull-down assays to confirm that it interacted with the C-terminus of FAK. Somewhat surprisingly, we found that leupaxin did not utilize the same binding site on FAK as paxillin and Hic5. As shown in the top panel of Figure 4.5C, GST fusion proteins containing the entire FAK C-terminus (GST-CTII) or the more C-terminal FAT domain (GST-CTI) efficiently precipitated paxillin from HCSMC lysates, while a FAT construct containing a mutation in the paxillin/Hic5 binding site (GST-CTIm) did not. In contrast, leupaxin was efficiently precipitated from the same cell lysates by the GST-CTII, but not by either of the GST-CTI fusion proteins indicating that leupaxin association with FAK requires sequences N-terminal to the FAT domain. In addition, the finding that paxillin was equally precipitated with fusion proteins that do or do not bind leupaxin (from lysates containing leupaxin) may imply that leupaxin and paxillin may not compete for FAK binding.

Several LIM domain proteins have been shown to reside in both the cytoplasm (within focal adhesions) and the nucleus where they can act as transcriptional co-factors [114]. Thus, we sought to determine whether leupaxin was present in both of these compartments and whether FAK activity regulated leupaxin localization. We first examined endogenous leupaxin localization by immunofluorescence in HCSMC and found that leupaxin was predominantly localized within focal adhesions (Fig 4.5; top panel). In order to determine whether leupaxin can undergo regulated cytoplasmic/nuclear shuttling, we treated HCSMC with the CRM1-dependent nuclear export inhibitor, leptomycin B (LMB) for 30min (Fig 4. 6; bottom panel). LMB treatment induced a dramatic redistribution of leupaxin to the

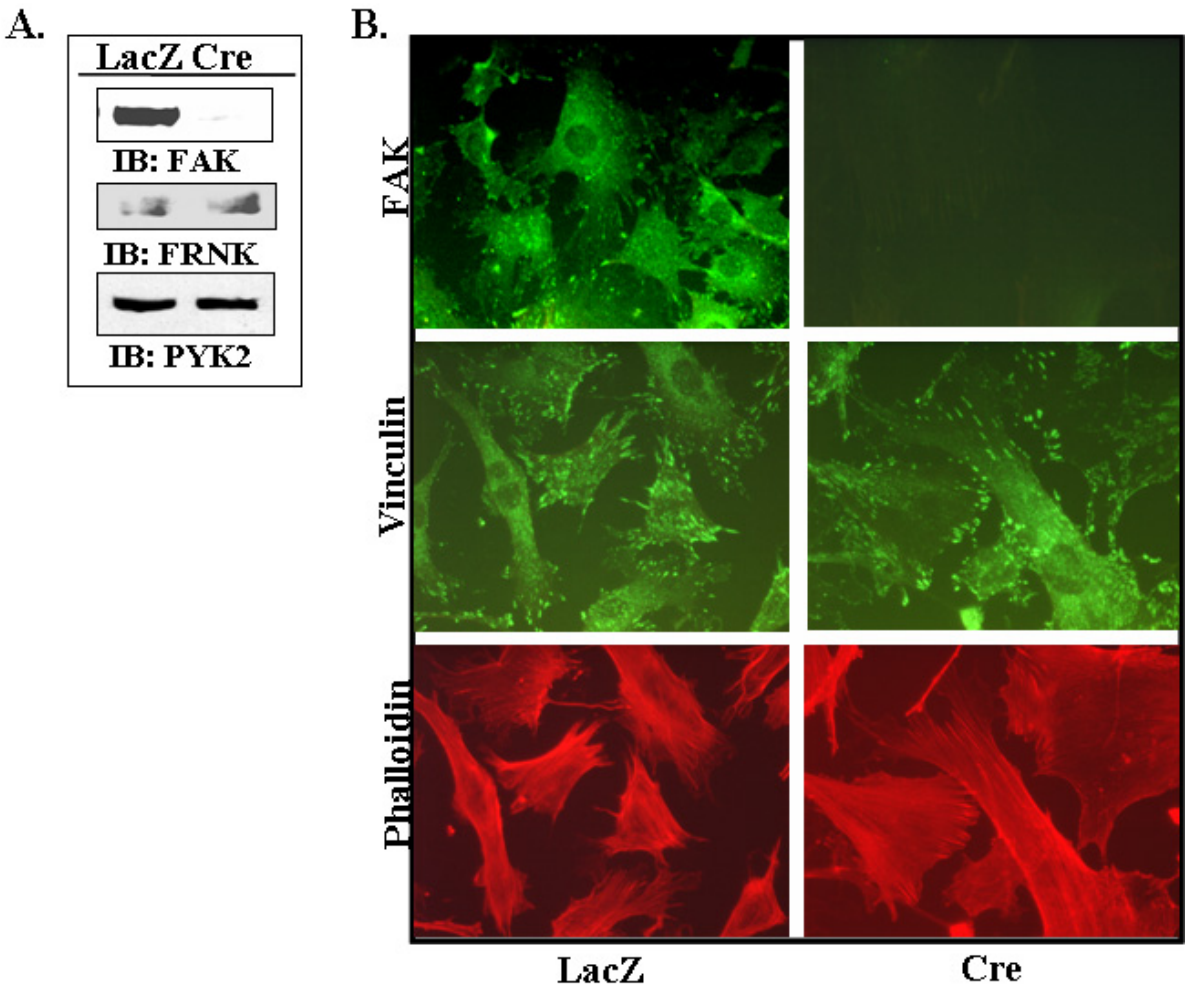


Figure 4.1. Characterization of FAK-null SMCs. A. *fak*$^{flox/flox}$ SMC were infected with either LacZ or Cre adenovirus for 72 hours, cells were lysed and Western blotting was performed using either anti-C-terminal FAK (that recognizes both FAK and FRNK), or anti-Pyk2, specific antibodies. **B**. *fak*$^{flox/flox}$ SMC were infected with either LacZ or Cre adenovirus for 72 hrs prior to fixation. Cells were permeablized, blocked, and stained with phalloidin and either anti-FAK (N-terminal specific) or anti-vinculin specific antibodies and processed as described in the Experimental Procedures section.

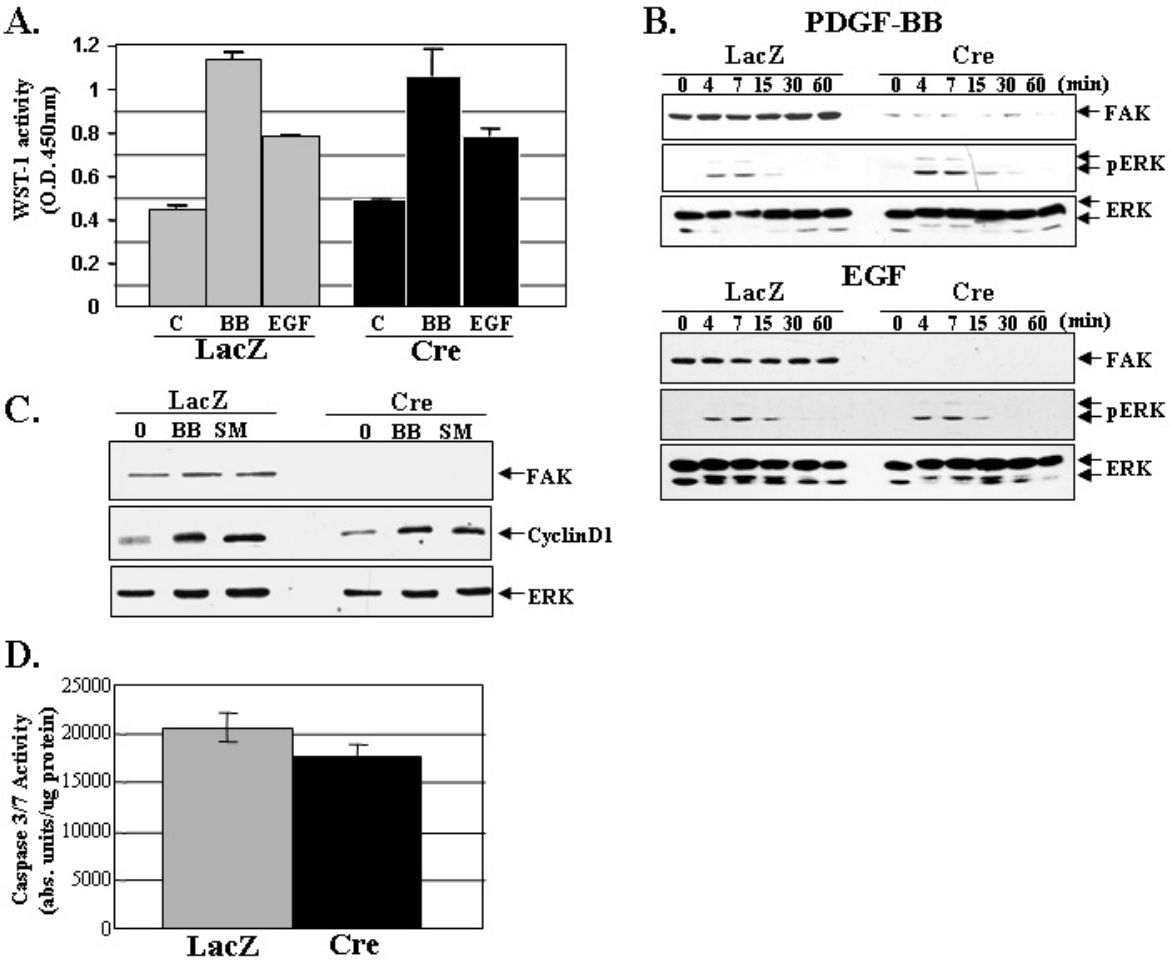


Figure 4.2. FAK depletion does affect SMC growth or growth factor-stimulated ERK activation A. *fak*$^{flox/flox}$ SMC were infected with either Cre or LacZ adenovirus for 72 hrs. Cells were then serum starved for 24 hrs prior to treatment with either PDGF-BB (20ng/ml) or EGF (100 ng/ml) for 48 hrs prior to determining WST-1 activity **B**. Cre or LacZ infected *fak*$^{flox/flox}$ SMC were serum starved for 24 hrs prior to treatment with either PDGF-BB (20ng/ml) or EGF (100 ng/ml) for the times indicated. Cells were lysed, electrophoresed and Western blotting was performed with anti- FAK, anti- active pERK1/2 and anti-ERK antibodies. **C**. *fak*$^{flox/flox}$ SMC were infected with either Cre or LacZ adenovirus for 72 hr. Cells were then serum starved for 4 hrs prior to treatment with either 10% serum (SM) or PDGF-BB (20ng/ml) for 24 hrs. Cells were lysed, electrophoresed and western blotting was performed with anti- FAK, anti- Cyclin D1 and anti-ERK antibodies. **D**. Caspase 3/7 Activity was measured in serum starved Cre or LacZ infected *fak*$^{flox/flox}$ SMC. Data are representative of at least three individual experiments.

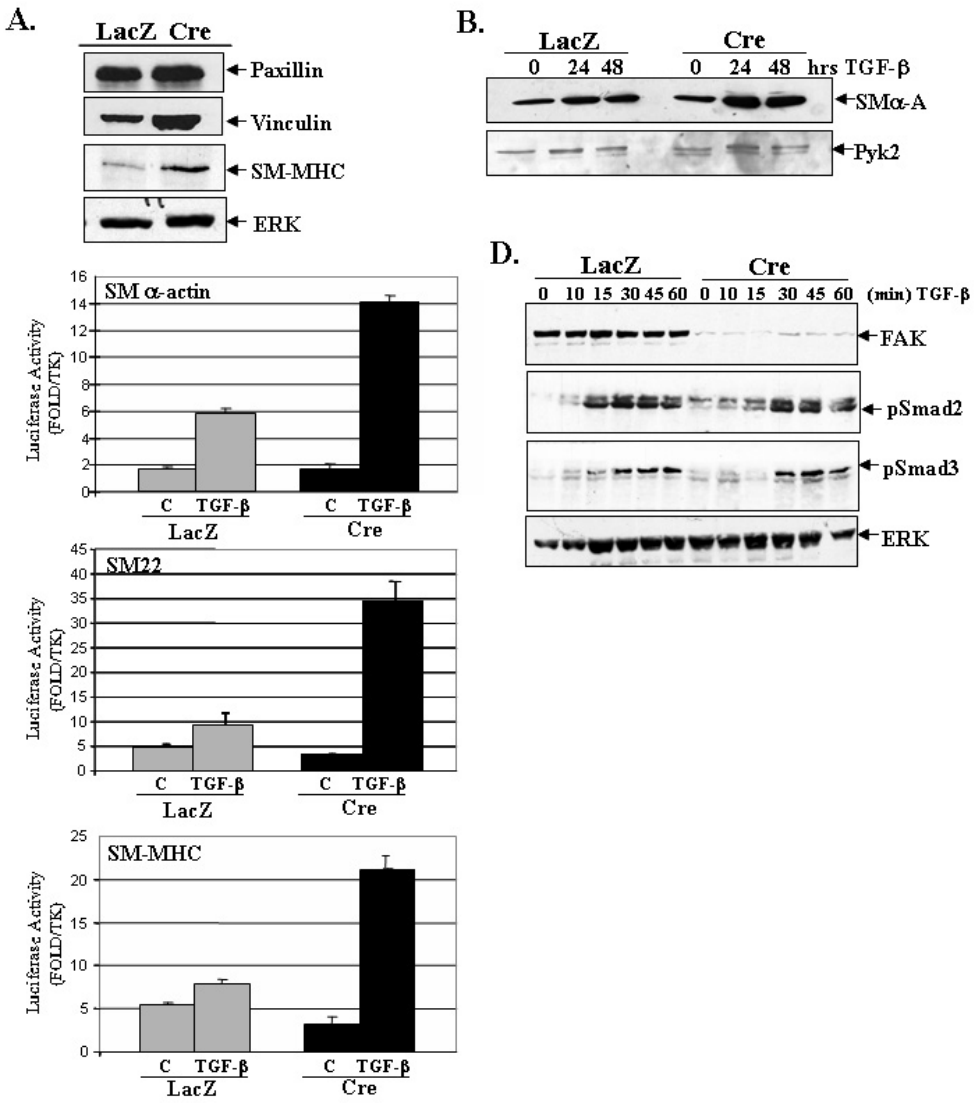


Figure 4.3. FAK depletion enhances TGF-β dependent SM differentiation. A. *fak*$^{flox/flox}$ SMC were infected with either LacZ or Cre adenovirus for 72 hours, cells were lysed and Western blotting was performed using either anti-paxillin, anti-vinculin, anti-SM-MHC, or anti-ERK specific antibodies. **B.** *fak*$^{flox/flox}$ SMC were infected as mentioned above. Cells were serum starved for 24hrs prior to TGF-β treatment for times indicated. Western blotting was performed with anti-SMα-actin and anti-Pyk2 (loading control) antibodies. **C**. *fak*$^{flox/flox}$ SMC were transfected with SM22- SM α-actin, or SM-MHC luciferase reporter constructs. 6 hrs post transfection cells were infected with Cre or LacZ adenoviruses. 24 hrs following infection, cells were serum starved for 24 hrs prior to treatment with TGF-β (1ng/ml) overnight. Cells were lysed and luciferase activity was measured. **D.** *fak*$^{flox/flox}$ SMC were infected as mentioned above. Cells were serum starved for 24hrs prior to TGF-β treatment for times indicated. Western blotting was performed with anti-phosphoSmad2, anti-phosphoSmad3, anti-FAK and anti-ERK antibodies.

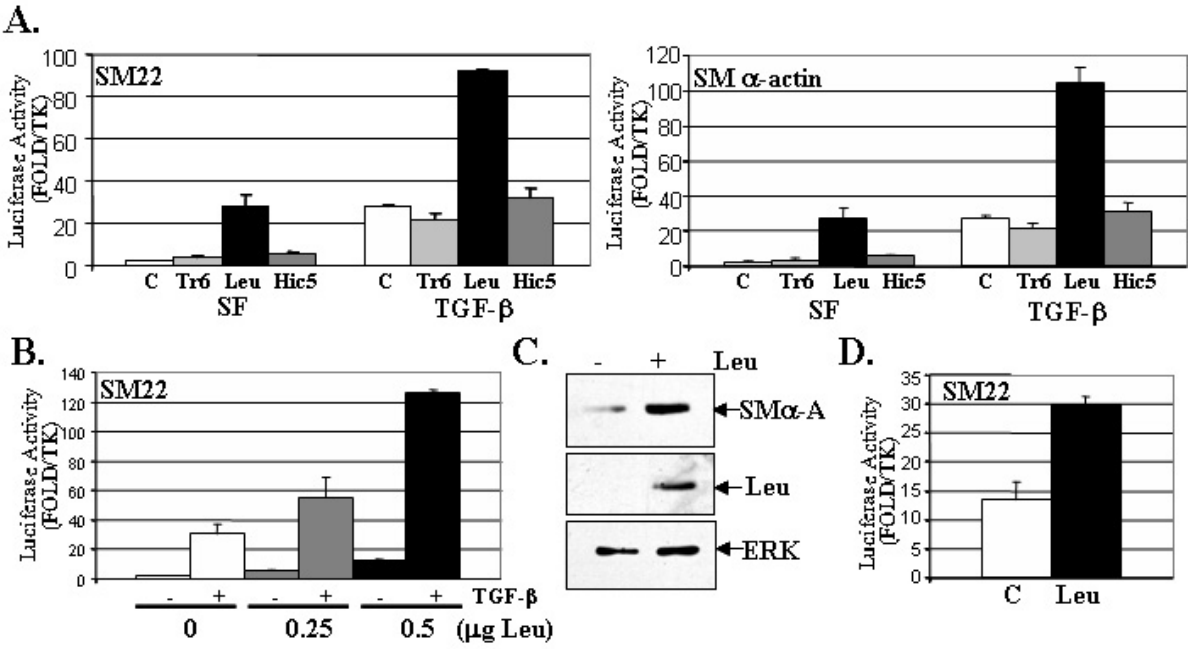


Figure 4.4 Expression of the FAK binding partner leupaxin stimulates SM marker gene transcription. **A**. 10T1/2 cells were co-transfected with either SM22 or SMα-actin-luciferase construct (0.5μg) and 0.5μg of either vector control (C), Flag-Trip6 (Tr6), Flag-leupaxin (Leu) or Flag-Hic5. 24 hrs following infection, cells were serum starved for 24 hrs prior to treatment with vehicle or TGF-β (1ng/ml) overnight. Cells were lysed and luciferase activity was measured. **B**. 10T1/2 cells were co-transfected with the SM22-luciferase construct with either 0, 0.25 or 0.5μg of leupaxin. Total DNA was normalized with empty vector. Cells were serum starved 24 hrs prior to treatment with vehicle or TGF-β overnight. Cells were lysed and luciferase activity was measured. **C**. 10T1/2 cells were untransfected (-) or transfected with Flag-leupaxin (+) and serum starved for 48hrs before Western blotting was performed with either anti-SMα-actin, anti- leupaxin or anti-ERK antibodies. **D.** Rat aortic SMC were co-transfected with the SM22-luciferase construct with along either empty vector (C) or leupaxin (leu; 0.3 μg). Luciferase activity was measured 48 hrs following transfection. All promoter measurements are presented as fold over a minimal TK promoter and all data represent three separate experiments.

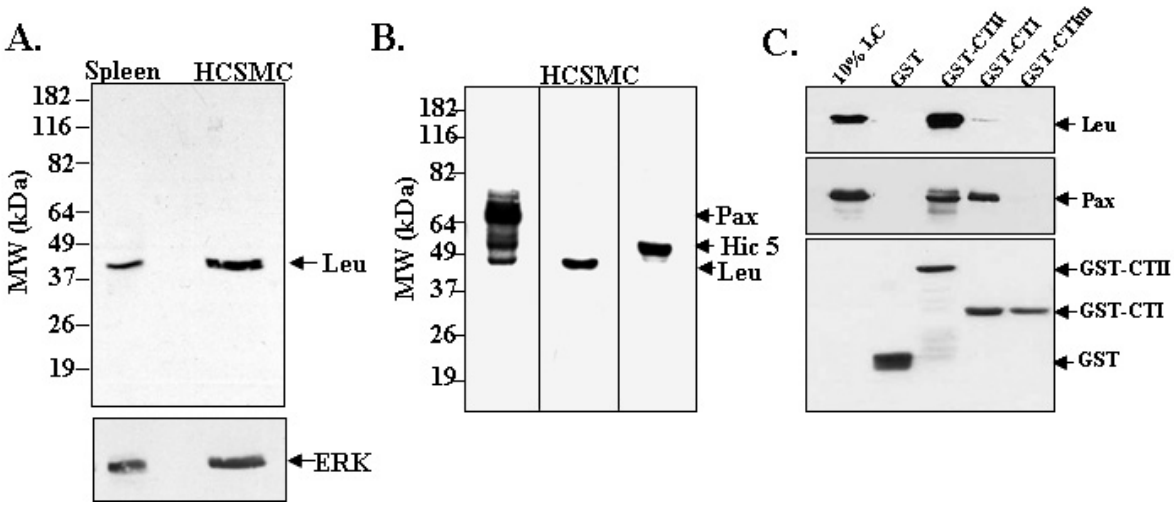


Figure 4.5. Leupaxin is enriched in SMC and endogenous leupaxin interacts with FAK in HCSMC. A. Expression of leupaxin protein in spleen (positive control: human spleen lysate from Clontech; 25 μg) and human coronary smooth muscle cells (HCSMC; 25 μg) was determined by Western blotting using an anti-leupaxin specific antibody. **B.** Equal concentrations of HCSMC lysate (25 μg) was electrophoresed and Western blotting was performed with anti-paxillin, anti-leupaxin and anti-hic5 antibodies. **C**. HCSMC lysates were incubated with 30μg of purified GST, GST-CTII, GST-CTI, or GST-CTIm for 1 hr, complexes were precipitated and analyzed by SDS-PAGE as described in Experimental Procedures and Western blotting was performed using anti-leupaxin, anti-paxillin and anti-GST specific Abs.

nuclear compartment. These data indicate that leupaxin can undergo nuclear-cytoplasmic shuttling and that leupaxin likely contains a functional CRM-1-dependent nuclear export sequence.

Since the available leupaxin antibody reacts with human, but not mouse leupaxin, we generated a GFP-tagged leupaxin construct to track leupaxin shuttling in real time in our mouse SMC lines and to determine the consequence of FAK deletion on leupaxin translocation. Results from co-transfection experiments demonstrated that GFP-leupaxin activated the SM22 promoter to a similar extent as Flag-tagged leupaxin, indicating that fusion to GFP did not significantly disrupt leupaxin function (data not shown). We found that GFP-leupaxin localized almost exclusively to focal adhesions in control cells maintained in 10% serum (Fig 4.7A). However, even in the absence of leptomycin B, nuclear localization of GFP-leupaxin was evident in approximately 30% of the FAK-null SMC that were cultured under the same conditions (Fig 4.7A, right panel). To determine whether FAK regulates the rate of leupaxin translocation, we identified FAK-containing and FAK-null SMC in which GFP-leupaxin was predominantly cytoplasmic and performed time-lapse imaging following LMB treatment. As shown in Fig 4.7C and 4.7D, LMB-induced leupaxin nuclear accumulation was evident much earlier in FAK-null SMC (90% of cells exhibited nuclear localization within 7.5 min) as compared to FAK-containing SMC (in which significant leupaxin accumulation required approximately 20 min).

We also used a pharmacological approach to confirm that leupaxin nuclear shuttling was regulated by FAK/Src activity. To this end, we treated SMC with PP2 (10 μM), which reduced autophosphorylation of FAK on Y397 in HCSMC maintained in 10% serum (Fig 4.7B; [154]). As shown in the bottom panel of Figure 4.7C, LMB-induced endogenous

leupaxin nuclear accumulation was much more rapid in PP2 treated cells than control cells. The use of the leupaxin antibody in LMB and PP2-treated HCSMC revealed similar effects on endogenous leupaxin localization (data not shown). Further supporting the functional significance of FAK-regulated leupaxin shuttling, we found leupaxin was more effective in promoting SMa-actin transcription in Cre infected cells versus LacZ. Additonally, we show that over-expression of a constitutively active FAK variant (termed SuperFAK; [150]) attenuated the leupaxin nuclear accumulation observed in FAK-null SMC (not shown) and partially reversed leupaxin-mediated SMC-marker gene up-regulation (Fig 4.7E). Collectively, these studies indicate that leupaxin nuclear translocation is regulated by changes in FAK activity and that sequestration of leupaxin to focal adhesion plaques is one mechanism by which activated FAK might limit SMC differentiation marker gene expression.

We hypothesized that leupaxin might regulate SMC-specific gene expression by interacting with SRF. As shown in Figure 4.8A, a GST-leupaxin fusion protein precipitated in vitro transcribed 35S-labeled SRF, and GFP-leupaxin and Flag-SRF were shown to co-associate in Cos-7 lysates by reciprocal co-immunoprecipitations (Fig 4.8B). We next performed ChIP assays in HCSMC to determine whether endogenous leupaxin could be found associated with the CArG-containing regions of the SMC differentiation marker gene promoters. Our results show that leupaxin associated with the CArG-containing regions of the SM α-actin and SM-MHC promoters but not with the c-fos promoter in HCSMC maintained in 10% serum (Fig 4.8C). Furthermore, leupaxin did not associate with the SMα-Actin promoter under serum-starved conditions, but could be induced by TGF-β (Fig 4.8D). When taken together with results from gel shift experiments demonstrating that leupaxin did

not associate directly with the SM α-Actin CArGs (data not shown), these results indicate that leupaxin likely associates with the SMC-specific promoters in an SRF-dependent fashion and that this interaction is facilitated by TGF-β. Since TGF-β enhanced leupaxin association with the SM promoters, we wondered whether TGF-β treatment might negatively regulate FAK activity. Recently Lim et al showed that FAK is negatively regulated by phosphorylation of Y407, an event likely due to stabilization of the closed inactive conformation of FAK [31]. Interestingly, we found that treatment with TGF-β led to rapid and prolonged phosphorylation of Y407-FAK in SMC (Fig 4.8E). Thus, repression of FAK-dependent signals may play a role in the promotion of SM marker gene expression following TGF-β treatment.

The studies described above indicate that leupaxin and SRF can associate in cells and that leupaxin interacts with SRF-CArG binding elements on SM promoters. To test whether activation of SMC-specific transcription by leupaxin required SRF, we co-expressed leupaxin with a SM α-actin promoter that contains mutations in all three CArG boxes. As shown in Fig 4.9A, leupaxin activated the wild type SM α-actin promoter, but not the promoter containing the triple CArG mutation. We also examined the ability of leupaxin to regulate SMC-specific transcription in SRF-/- ES cells. Importantly, expression of leupaxin in SRF-/- ES cells did not enhance SM22 reporter gene expression, but this ability could be rescued by re-expression of SRF (Fig 4.9B). These data strongly support the idea that leupaxin cooperates with SRF to stimulate SMC differentiation in a CArG-dependent manner. We propose a model that leupaxin plays a dual role in SM function; aiding to coordinate multiprotein complexes in focal adhesions and in the nucleus and that leupaxin localization is regulated by FAK/Src activity.

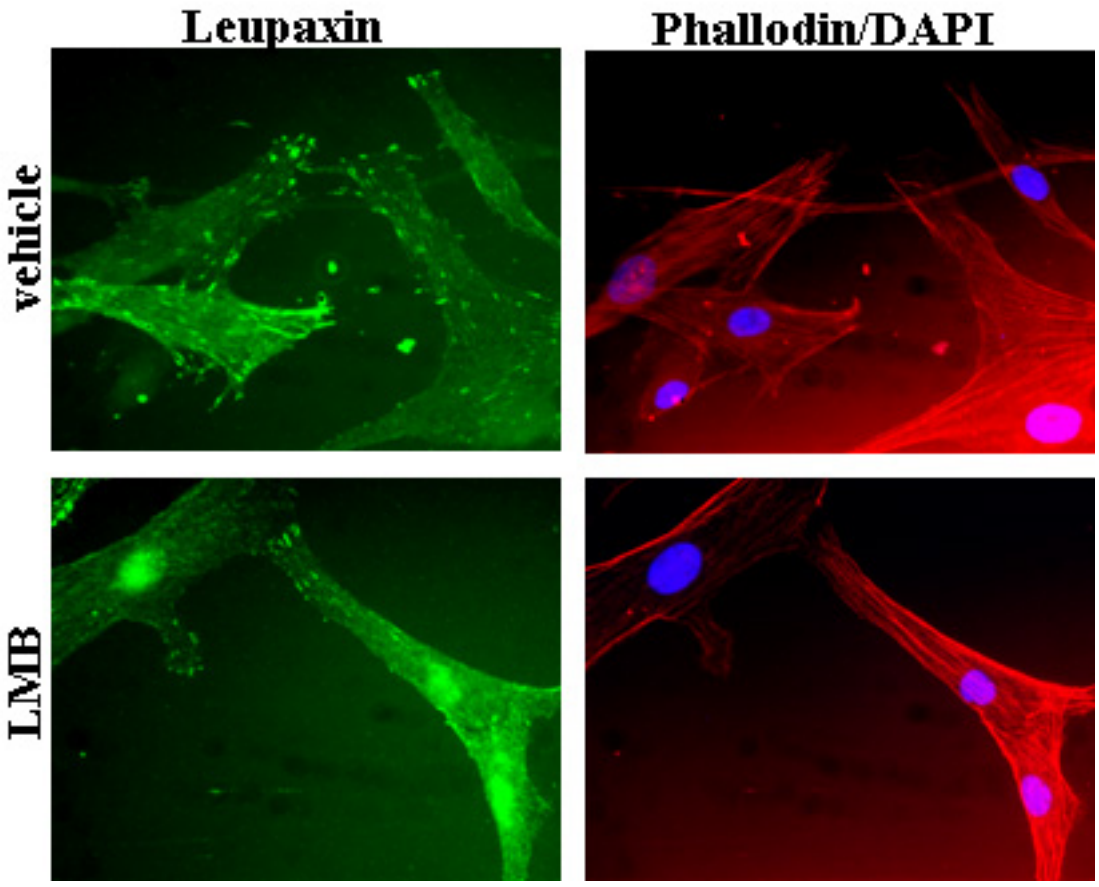


Figure 4.6. Endogenous leupaxin is localized within focal adhesions.
HCSMC were either untreated (top panel) or treated with Leptomycin B (LMB; 5ng/ml): 30 min (bottom panel) prior to fixation. Leupaxin localization was determined by immunofluorescense using FITC-conjugated secondary antibodies (left panel, green). Cells were co-stained with phalloidin (red) and DAPI (blue) to visualize the actin cytoskeleton and nuclei respectively (right panel).

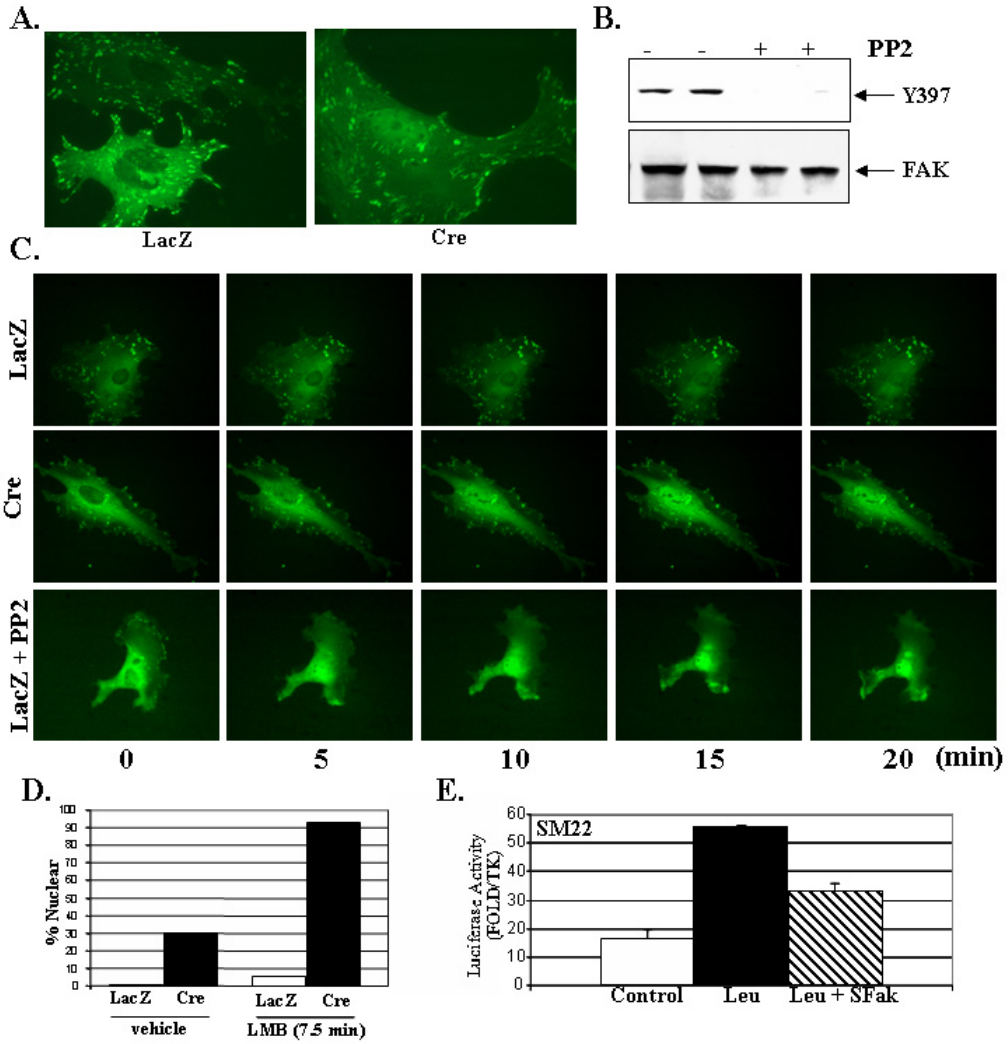
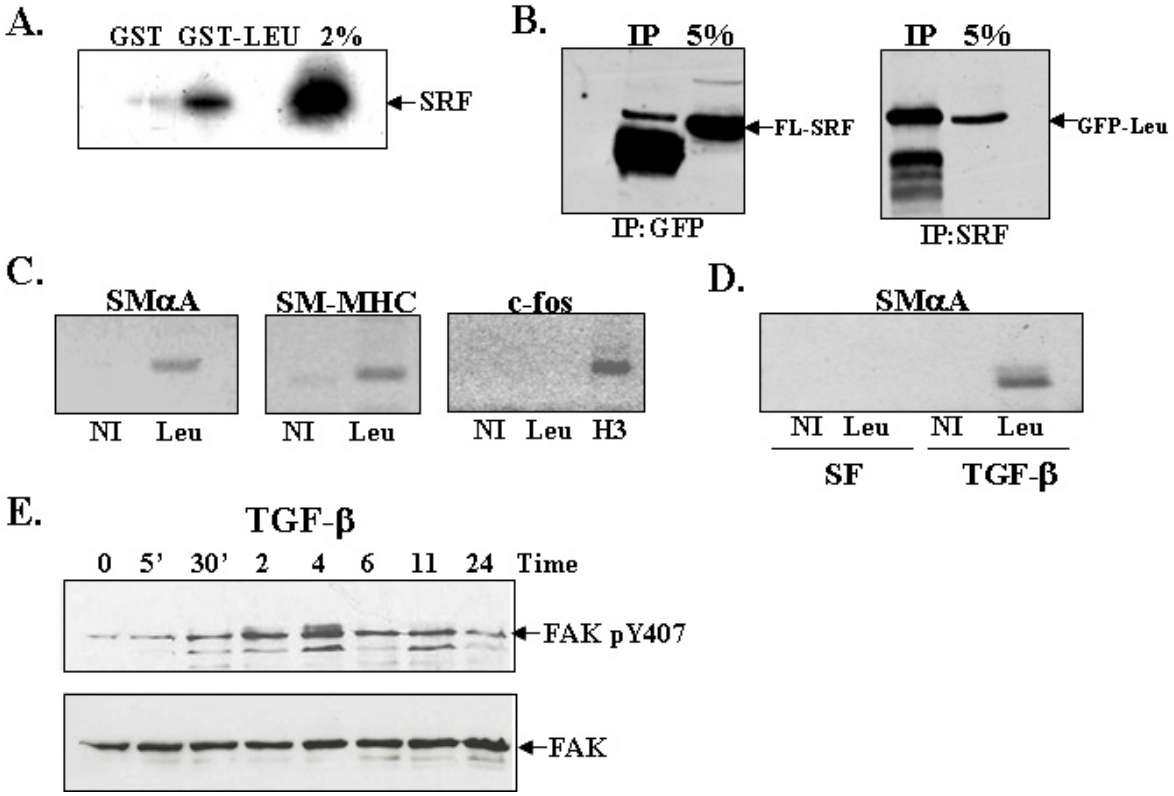


Figure 4.7. FAK activity regulates leupaxin nuclear localization. **A.** *fak*$^{flox/flox}$ SMC were infected with LacZ or Cre adenovirus as described previously. 24hrs following infection, cells were transfected with GFP-leupaxin for 48hrs. **B.** HCSMC were treated with vehicle (-) or PP2 (+) for 15 min and Western blotting was performed with anti-Y397 FAK and anti-FAK antibodies. **C.** LacZ or Cre infected *fak*$^{flox/flox}$ SMC adenovirus were transfected with GFP-leupaxin and serum starved for 24 hrs prior to treatment. Cells were imaged at time 0 and then at 1 min increments following LMB treatment (top and middle panel) or LMB and PP2 treatment (bottom panel). Images captured at 0, 5, 10, 15, 20 minutes are shown. **D**. LacZ and Cre *fak*$^{flox/flox}$ SMC were transfected with GFP-leupaxin for 48 hrs (vehicle) and then treated with LMB for 7.5 min. Cells were counted to score percentage nuclear localization Bar graph represents the % of cells with visible nuclear localization (n=150-200 cells/treatment). **E.** 10T1/2 cells were co-transfected with the SM22-luciferase promoter and either empty Flag vector (Control), leupaxin (0.25μg) or leupaxin and Flag-SuperFAK (SFAK; 0.25μg each). Luciferase activity was measured 48 hr following transfection. Data are representative of three individual experiments.


A.
GST GST-LEU 2%
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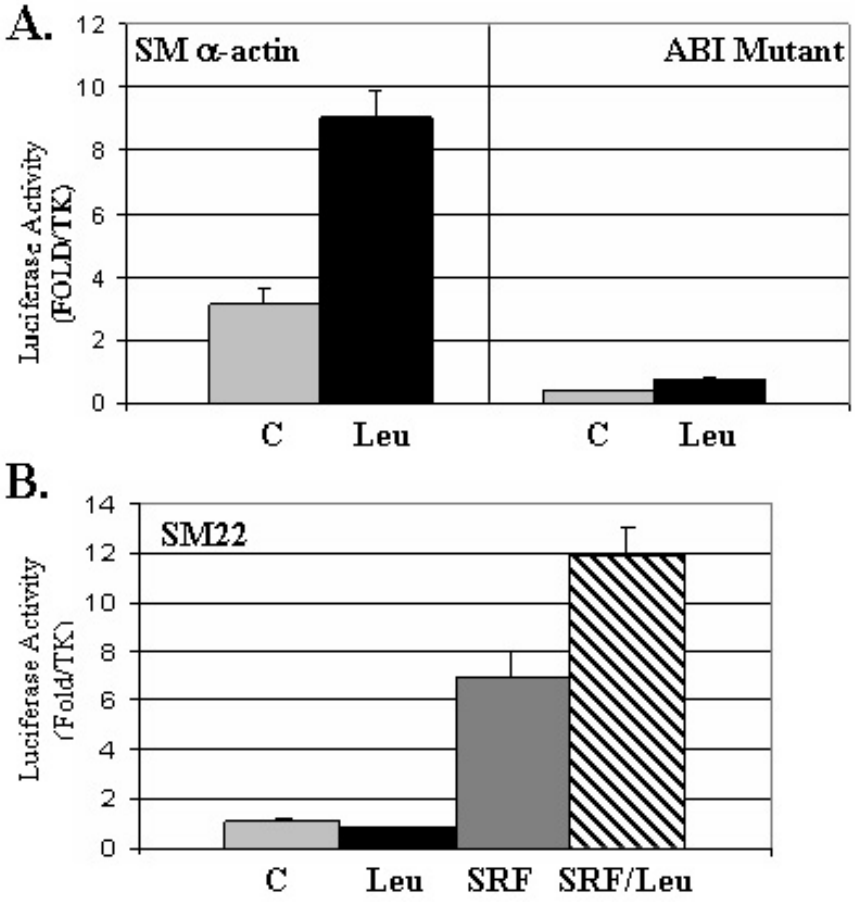


Figure 4.9. **Leupaxin regulation of SMC gene transcription is SRF and CArG-dependent. A.** 10T1/2 cells were transfected with the SMα-Actin promoter (left panel) or a triple CArG mutant SM α-actin promoter (ABI Mutant, right panel) along with either empty vector (C) or leupaxin (Leu; 0.4μg). 48 hrs following transfection luciferase activity was measured. **B.** SRF-/- ES cells were co-transfected with the SM22-luciferase construct along with empty vector (C), leupaxin (leu), SRF or SRF plus leupaxin. Luciferase activity was measured 24 hrs following transfection. Data are representative of three individual experiments.

DISCUSSION

We found that deletion of FAK in SMC enhanced TGF-β-stimulated SM marker gene expression, but did not affect PDGF-BB or EGF-stimulated proliferation. These data suggest that enhanced FAK activity limits SM differentiation and that tight regulation of FAK activity is likely important for proper SMC phenotypic modulation during development and following vascular injury. We identified leupaxin as a binding partner for FAK in SMC and show that leupaxin associates with SRF and enhances SRF-dependent expression of SM marker genes and that FAK regulates the nuclear-cytoplasmic shuttling of this LIM domain containing SRF co-factor. Thus, these studies indicate that leupaxin nuclear translocation is regulated by changes in FAK activity and that sequestration of leupaxin to focal adhesion plaques is one mechanism by which activated FAK might limit SMC marker gene expression.

The cell culture model we have described herein, provides a direct comparison of SMC with or without FAK. The well spread phenotype of our FAK-null SMC differs from previously described embryonic FAK-null fibroblasts which were more round and less spread than wild type fibroblasts, possibly due to enhanced Rho A activation [51, 81]. However, recent studies that utilized a similar Cre/LoxP approach as ours (or siRNA) to knock down FAK expression in fibroblasts also did not reveal morphological changes following FAK depletion [155]. Collectively, these data suggest that the conventional FAK-/- cells may have acquired mutations that regulate cell morphology. Interestingly, the conventional FAK-/- cells express high levels of Pyk2, while Pyk2 levels are comparable in our Cre or LacZ treated SMC cultures and were also reported to be similar in the previously described Cre and LacZ treated $fak^{flox/flox}$ fibroblasts [155]. We did however observe a

consistent increase in vinculin protein levels in the absence of FAK, similar to what was observed in FAK-null mouse embryo fibroblasts (31). Since vinculin is expressed in an SRF-dependent fashion, this finding supports our hypothesis that SRF-dependent gene transcription is elevated in FAK-null cells [156].

In support of the idea that FAK plays an active role in regulating smooth muscle cell phenotypes, FAK-null mouse embryonic fibroblasts were recently reported to exhibit a myo-fibroblast appearance as assessed by high levels of SM α-actin containing stress fibers relative to control fibroblasts [157]. In addition, recent studies have provided evidence for a role of FAK in promoting striated muscle cell differentiation. Clemente et. al. showed that the induction of differentiation of C2C12 cells into myotubes is accompanied by a transient inhibition of FAK activity from 0-2 hrs followed by later increase in FAK activity that lasted up to 5 days. Interestingly, ectopic expression of a FAK^{Y397F} variant that reduced FAK activity in C2C12 cells lead to enhanced skeletal muscle marker gene expression, although FAK inactivation ultimately blocked myotube formation [158]. Thus, the dynamic regulation of FAK activity is essential for differentiation of C2C12 myoblasts into myotubes. A role for FAK in the promotion of cardiogenesis was suggested by studies in which stable expression of FRNK in ES cells was shown to induce cardiac α-myosin heavy chain and sarcomeric myosin expression [154]. Previous studies from FAK-null ES cells revealed that the absence of FAK did not preclude hematopoetic differentiation or differentiation of cells into all three germ layers [159], but no mention was made of the relative contributions of FAK-null cells to mesenchymal compared to non-mesenchymal lineages. Since numerous reports suggest that FAK activity is required for osteoblast differentiation [160] it is possible that limiting FAK activity might enhance SRF-dependent muscle cell differentiation at the

expense of cartilaginous cell differentiation. Further careful examination of the multi-lineage potential of $FAK^{-/-}$ ES cells using a combination of *in vitro* and chimeric approaches should aid in determining which cell types are restricted and which are promoted by FAK activation.

Although FAK inactivation strongly enhanced TGF-β stimulated SM marker gene expression, TGF-β treated FAK-null and FAK-containing SMC had comparable activation of the canonical Smad pathway. We postulate that FAK inactivation enhances SMC differentiation, at least in part, by promoting nuclear localization and subsequent SRF-dependent transcriptional activation of SM marker genes by the LIM domain-containing protein leupaxin. Since TGF-β inactivates FAK and stimulates leupaxin association with the CArG, the regulation of leupaxin could be one mechanism by which FAK and TGF-β -induced signals converge. However, we cannot rule out the possibility that FAK activity limits TGF-β SM gene transcription by altering alternative signaling pathways such as the RhoA/PKN/pp38 MAPK and Pi3K/Akt pathways that have been implicated in mediating TGF-b stimulated SM marker gene expression[105-107].

Leupaxin is an understudied 43 kDa protein that is structurally similar to the focal adhesion adapter proteins paxillin and Hic-5. Although originally reported as having a lymphoid-restricted expression pattern [144], we show that leupaxin is also highly expressed in aorta and in cultured smooth muscle cells. In addition, the NCBI Gene Expression Omnibus Database shows that while 134 leupaxin EST transcripts per million (TPI) were found in human lymph node, 78 TPI were found in aorta, in support of our findings that leupaxin expression is enriched in smooth muscle cells.

Although little is known regarding leupaxin's cellular function, some parallels can be drawn from studies on its family members paxillin and Hic-5, since leupaxin shares

considerable structural homology with these proteins. Paxillin, Hic-5, and leupaxin are each comprised of four N-terminal LD motifs and four C-terminal LIM domains that mediate protein-protein interactions [123, 140]. The most widely studied family member, paxillin, was originally identified as a major tyrosine phosphorylated protein in vSrc transformed cells [129], and was subsequently shown to be an adapter protein involved in the regulation of integrin-mediated signal transduction and cell migration [161]. Paxillin associates with the cytoskeletal proteins, vinculin, and tubulin and numerous signaling molecules such as the tyrosine kinases FAK, Pyk2, and Src, the tyrosine phosphatase, PTP-PEST, and the adapter protein Crk that can lead to activation of the small GTPases Rac1, Rap1A, and Ras and activation of Map Kinase signaling pathways [123]. In spite of the ability of Hic 5 to also localize to focal adhesions and associate with many of the same binding partners as paxillin, Nishiya *et. al.* showed that Hic-5 expression inhibits integrin-dependent growth and cell spreading by competing with paxillin for FAK binding (35). This competitive function is thought to result from the known lack of integrin-dependent tyrosine phosphorylation of Hic-5 and consequent lack of association of Hic-5 with the SH2-containing protein Crk that links FAK activation to downstream GTPases and MapKinases [139]. Leupaxin was previously shown to bind to Pyk2 and co-localize with cortical F-actin in JY8 lymphoblasts and to bind to FAK and localize within podosomes of osteoclasts [144, 145]. Herein we show for the first time that leupaxin localizes within nascent focal contacts and mature focal adhesions in smooth muscle cells. Although the binding sites for FAK and Pyk2 within leupaxin have not yet been determined, the interaction is likely mediated through one of the conserved LD structures that direct paxillin-FAK interactions. Leupaxin does contain the critical YXXP Crk binding site in it's amino terminus (present in paxillin) but whether leupaxin is tyrosine

phosphorylated at this site following integrin ligation and whether leupaxin associates with Crk (or other paxillin binding partners) are questions for future studies.

Paxillin and Hic-5 were previously shown to undergo nucleocytoplasmic shuttling and to affect gene expression. Similar to our findings with leupaxin, treatment with the nuclear export inhibitor, leptomycin B causes retention of paxillin and Hic-5 in the nucleus, providing evidence that these proteins normally cycle between focal adhesions and the nucleus [162, 163]. Although the precise mechanisms whereby the paxillin family members traffic into and out of the nucleus remains unclear, the conserved LD motifs in these proteins resemble conserved leucine-rich nuclear export sequences. Both paxillin and Hic-5 bind to steroid receptors and have been shown to co-activate androgen, glucocorticoid, and progesterone response genes [164, 165]. Whether leupaxin can also regulate these transcriptional targets in addition to the SMC promoters is not yet known. However, in spite of their ability to regulate steroid hormone induced gene expression, we found that forced expression of Hic-5 or paxillin (unpublished observations) did not influence SM marker gene expression, while leupaxin promoted a consistent increase in SM-MHC, SM α-actin, and SM22 promoter activity. These data indicate that the nuclear functions of the paxillin family members are unique. Interestingly several other LIM domain proteins including CRP1/2, LPP, and FHL2 also cycle between focal adhesions and the nucleus and these proteins like leupaxin influence SRF-mediated gene transcription [89, 90, 122]. We showed that leupaxin nuclear localization was enhanced following FAK inactivation and that FAK expression limits leupaxin-mediated transcription of SRF-dependent target genes. It remains to be determined whether FAK activity may also regulate SM differentiation through cytoplasmic retention of other LIM containing SRF co-factors.

Several questions remain regarding the function of LIM proteins in the nucleus. The fact that so many LIM domain proteins undergo nuclear-cytoplasmic shuttling and that several of these proteins regulate SM marker gene expression suggests that there might be some redundancy in function. Selective expression of the varying LIM domain proteins may impose one means of divergent regulation. For example, LPP is expressed in synthetic but not highly differentiated SMC expressing smoothelin [122] whereas CRP2 is expressed in differentiated SMC and has been shown to be down regulated when SMC exhibit a synthetic proliferative phenotype [166]. Thus, it is likely that different LIM proteins can facilitate coordinated transcriptional regulation at different stages of development. Since each LIM domain has the capacity to binding to select partners it is also tempting to speculate that these adapter proteins might recruit a different set of co-activators to the transcriptional machinery to impart tight regulatory control. In support of this idea, the N-terminal LIM domain of CRP directs binding to SRF, while the C-terminal LIM domain interacts with GATA 4 [90]. Herein we present evidence that leupaxin interacts directly with SRF and can associate with SRF-bound CArG elements within SM promoters. We have not observed a functional interaction between GATA4, SRF, and leupaxin in our studies (data not shown), but it is possible that leupaxin associates with additional SRF-cofactors. Since TGF-β stimulates leupaxin association with CArG regions of the SM promoters, it will be of particular interest in the future to determine whether leupaxin associates with Smad 2 or 3.

Although reported to affect a wide variety of cellular processes a principal function for FAK in numerous cell types is its ability to modulate integrin and growth factor receptor stimulated cellular migration [155]. Direct evidence for the role of FAK in modulating fibronectin-dependent motility was previously shown using FAK-/- fibroblasts [167]. We

have also shown that FAK activity in SMC is essential for PDGF and FN-stimulated migration [147]. Taken together with our studies presented herein that reveal FAK activity also affects SMC phenotypic modulation, we postulate that tight regulation of SMC phenotype, by modulating FAK activity (or the intrinsic shifting of FAK/FRNK expression) could mediate a balance between SMC migratory and contractile capacities necessary for proper vasculogenesis during development and following vascular injury. Indeed, our recent data indicates that SM marker genes are markedly down-regulated in FRNK-/- vessels following injury, indicating that endogenous FRNK expression (and FAK inactivation) is required for appropriate re-differentiation of neointimal SMC (manuscript in preparation). Interestingly, FAK is aberrantly active in many solid tissue cancers and enhanced FAK activity correlates with poor prognosis, an event thought to be a result of enhanced metastatic potential in cells with unchecked FAK activity [168]. Our data suggest that FAK could be playing an additional role in this setting, perhaps by modulating the differentiation state of cells to a phenotype that might make them more responsive to migratory signals.

CHAPTER V

CONCLUSIONS

Smooth muscle cells (SMC) are involved various vascular disease, such as atherosclerosis, restenosis following balloon angioplasty and vein bypass grafts. During these vascular diseases and also during normal vascular development, numerous changes occur within the vessel environment that enables SMC to dedifferentiate from a contractile phenotype to a synthetic proliferative phenotype. The remodeling of the extracellular matrix, an increase in growth factors and contractile agonists have all been shown to initiate signaling pathways leading to an increase in SMC proliferation, migration, and differentiation. The tyrosine kinase, Focal Adhesion Kinase (FAK) plays a major role in the integration of the signals transmitted from these extracellular cues. Our laboratory has previously shown that integrin- and growth factor-mediated SMC proliferation and migration are dependent on FAK activity. Very little is understood about the role of FAK in embryonic development for FAK knockout embryos died between day E8.5 and E10.5 due to general cardiovascular defects [52]. Since extensive hemorrhage was observed, the vascular defect in FAK -/- embryos may be due in part to defective SMC recruitment or maturation. There is very little is known about the role of FAK in SMC development and how the absence of FAK that could result in the observed cardiovascular phenotype. The overall aim of this dissertation was to determine the role FAK in SMC vascular signaling by identifying the downstream targets of FAK that lead to SMC migration, proliferation, and differentiation through the utilization of in vitro and in vivo models.

FAK has been established as an important signaling conduit for integrin-, G-protein couple receptor-, and growth factor-stimulated cell migration and proliferation. The signaling pathways responsible for transmitting the proliferative or migratory cues have been conflicting in the literature and appear to vary depending on the cell model system in which

the studies were performed. Since FRNK, a dominant negative inhibitor of FAK is endogenously and selectively expressed in SMC, we hypothesized that tight regulation of FAK activity and downstream signaling is essential in SMC and that this signaling pathway may be distinct. We showed that FRNK expression attenuated PDGF-stimulated migration and proliferation by dampening the Rac/JNK pathway. Additionally, we were able to show that FAK inactivation by FRNK overexpression lead to a more differentiated phenotype characterized by an increased expression in the contractile genes, SM22, SM-MHC and SM α-actin.

To better understand the role of FAK in SMC cellular processes we next generated a primary cell line that allowed us to examine SMC-specific deletion of FAK by utilizing Cre/loxP technology. This system has highlighted several differences between SMC specific FAK responses and those documented in FAK-null fibroblasts and FAK-null endothelial cells. Overall, FAK is essential for cell migration, regardless of the cell system. FAK may function within focal adhesions as an important integrator of extracellular cues and as a signal transducer responsible for recruiting the necessary proteins for focal adhesion assembly and disassembly powering cell motility. Studies are underway to identify the downstream signaling targets of FAK that are required for proper SMC migration.

While we have previously shown by FRNK overexpression, that FAK activity is necessary for SMC proliferation, we found that in FAK-null SMC the proliferative response was unadulterated. These data indicate that proliferation may be regulated at the level of FRNK. Previous studies by Taylor et al have shown that FRNK expression is dynamically regulated during development and following vascular injury and its expression tightly correlates with SMC phenotypic modulation to a more contractile state [41]. Even though

we have shown that FAK-ablation results in increased SM marker gene transcription, we also found that FRNK expression induced SM marker gene expression. However, the upregulation we observed in the absence of FAK was dependent on TGF-β stimulation whereas FRNK expression alone was sufficient to increase gene transcription. The precise mechanism by which FRNK regulates SMC phenotypic modulation, by the promotion of differentiation and repression of proliferation still needs to be deciphered. There are several putative mechanisms. One, FRNK expression may regulate the localization of SMC specific transcription factors, including the myocardin family member, MRTF-A. It is easy to hypothesize that FRNK expression might increase the propensity of MRTF-A nuclear localization and increase MRTF-A/SRF dependent SM gene transcription. Additionally, FRNK may function to inhibit PDGF-dependent SM marker gene repression, by activating the Pi3K/Akt pathway diminishing the FOXO4/myocardin complex and enabling myocardin to bind SRF and activate the SM marker genes. Further studies are required to determine how FRNK may function within SMC. Utilizing inducible FRNK expression/repression SMC model systems in FAK- containing and FAK-null SMC may aid determining FRNK signaling in vivo.

Herein, we have shown that the expression of the FAK binding partner, leupaxin can generate an increase SM marker gene transcription. Like other LIM containing proteins, we found that leupaxin can translocate from focal adhesions to the nucleus where it can regulate gene transcription. We show that leupaxin can bind to SRF and transactivate SM marker genes when it is localized within the nucleus. While leupaxin nuclear localization was increased in the absence of FAK, we found that FRNK expression had no effect on leupaxin cytoplasmic/nuclear shuttling (unpublished observations). The possibility of FRNK

expression regulating leupaxin protein levels was not tested. Very little is known regarding leupaxin expression. However, based on initial observations, where Lipsky et al noted that leupaxin expression was increased in mature leukocytes compared to undifferentiated bone marrow cells [144]. Leupaxin expression could be limited to differentiated SMC. Antibody limitations have prevented studies to determine endogenous leupaxin expression during murine development. Future studies using in situ hybridization or the generation of a leupaxin-LacZ transgenic mouse model might provide insight into leupaxin expression.

As mentioned above we found the FAK activity diminished the ability of leupaxin to induce SM marker gene transcription. We hypothesized that active FAK may sequester leupaxin within focal adhesions preventing nuclear translocation. Based on leupaxin binding partners identified so far, such as FAK and PTP-PEST [145], focal adhesion localized leupaxin may play a role in cell migration. Studies to address this possibility are necessary.

A better understanding of the role of FAK/FRNK signaling within the vasculature remains to be elucidated. The utilization of a SMC-specific model system will help in our understanding and aid in the development of therapeutic targets to treat and prevent various vascular diseases.

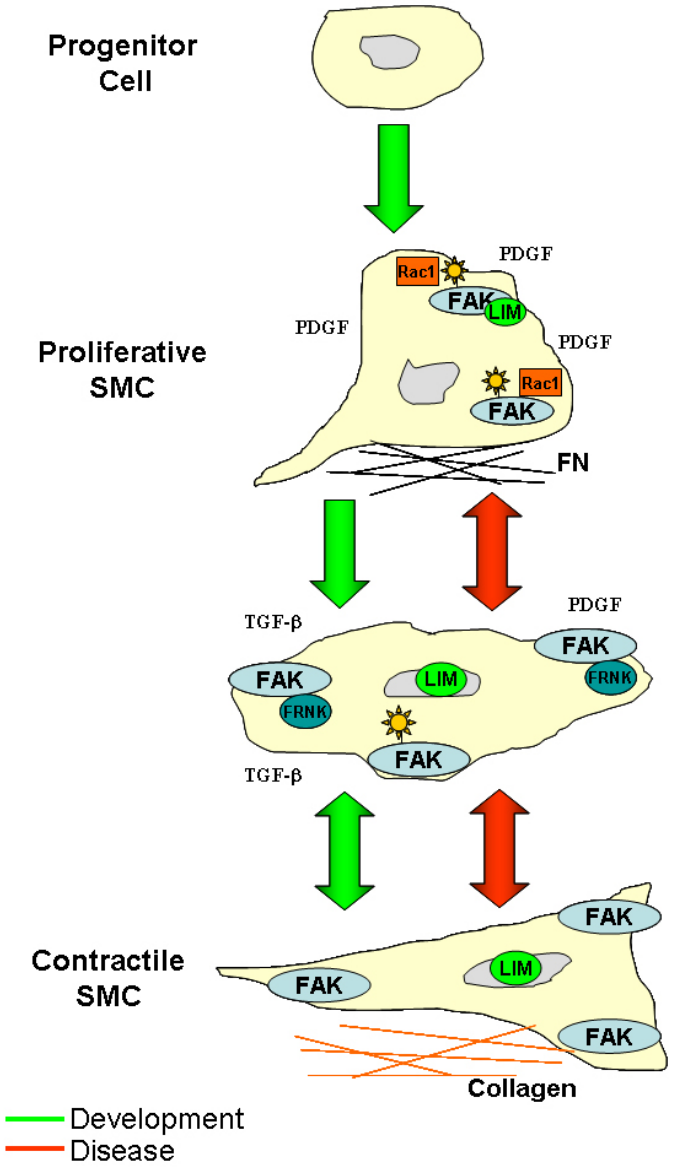


Figure 5.1 Model for FAK/FRNK signaling in vessel development. During vascular development, the surrounding microenvironment is enriched with FN and secreted PDGF. We postulate that this environment induces high levels of FAK activity and an increase in FAK-dependent Rac1activation that contributes to the highly motile phenotype. TGF-β is induced during vessel maturity leading to an upregulation of FRNK expression. This leads to diminished FAK activity and the subsequent translocation of LIM containing proteins, such as leupaxin, from focal adhesions to the nucleus where they can promote differentiation. The ECM within the medial layer of the adult vasculature is rich in collagen, maintaining SMC in a quiescent state. Vascular injury increases FN expression and PDGF secretion resulting in SMC dedifferentiation and the reactivation of FAK-dependent signaling pathways.

APPENDIX I

FRNK expression induces smooth muscle differentiation

ABSTRACT

Adult medial vasculature SMC have the unique ability to phenotypically modulate from the usual differentiated contractile state to a dedifferentiated synthetic state. This synthetic state allows for an increased proliferation and migration capacity generally needed for repair of vascular injury. However, SMC differentiation is a dynamic process that must be tightly regulated to thwart the onset of vascular disease. Our lab previously reported that a specific focal adhesion kinase (FAK) inhibitor termed FRNK (FAK related non-kinase) is selectively expressed in large arterioles when SMC are transitioning from a synthetic to contractile phenotype during vascular development and during vascular remodeling following vascular injury and that FRNK inhibits FAK-dependent SMC proliferation and migration. Herein, we sought to determine if modulation of FAK activity by FRNK influenced the differentiation state of SMC. Ectopic expression of FRNK increased the expression of SM marker genes. In addition, FRNK increased TGF-β-stimulated transcription of SM marker gene promoters. By utilizing SMC isolated from a Myc-FRNK transgenic mouse line, we were able to show that FRNK promotes SM marker gene transcription *in vivo*. These data indicate that dynamic regulation of FRNK expression during development and following vascular injury functions to mediate SMC phenotypic modulation required for proper vasculogenesis.

INTRODUCTION

The microenvironment within the vessel during vasculogeneis and various vascular pathologies is constantly shifting to maintain vessel integrity and tensile strength. Vascular SMC retain an innate plasticity that enables them to respond to the fluctuation in circulating autocrine factors and remodeling extracellular matrix. This plasticity enables SMC to dedifferentiate from a contractile phenotype to a synthetic proliferative phenotype. Various autocrine factors and extracellular matrix cues have been shown to play a role in regulating SMC phenotype. Focal Adhesion Kinase (FAK) has been shown to be an important regulator SMC proliferation and migration and I recently showed that FAK expression regulates SMC differentiation ([41], Chapter IV).

Although a direct role for FAK in vascular smooth muscle growth and development in vivo has yet to be examined, germline deletion of FAK results in general mesodermal defects and embryonic lethality between E8.5-10 (similar to both fibronectin$^{-/-}$, and $\alpha_5^{-/-}$ mice) [52]. Interestingly, our lab recently showed that FAK activity is regulated in a unique fashion in SMC, whereby a separate protein comprising the carboxyterminus of FAK, termed FRNK (FAK Related Non Kinase) that is selectively expressed in SMC with very high levels found in the large arterioles. FRNK transcription results from the utilization of an alternative start site within the FAK gene and FRNK expression is independently regulated by a distinct promoter embedded within FAK intronic sequences [147, 169]. Whereas FAK protein levels remain relatively constant during vascular development, FRNK protein levels are dynamically regulated. Arterial FRNK expression is greatly increased after birth and from two to three weeks following vessel injury [147]. The timing of FRNK expression indicates that FAK activity is tightly controlled when SMC are transitioning from a synthetic to

contractile phenotype. Additionally, data has shown that FRNK can function as a dominant-interfering mutant for FAK. The aim of this study was to determine whether modulation of FRNK protein levels plays a direct role in the phenotypic state of SMC. Herein we present evidence that ectopic expression of FRNK and overexpression of FRNK in SMC in vivo enhances TGF-β-stimulated differentiation.

MATERIALS AND METHODS

Constructs and Reagents

The promoter reporter constructs: SM α-actin (from -2560 to +2784), SM22 (from -450 to +88) and SM-MHC (from -4200 to +11600) and SMα-Actin mutant luciferase constructs used have been previously described [148 1568]. GFP and GFP-FRNK adenovirus were generated as previously described [147]. Flag-FAK, Flag- FAKY397F and Flag-FRNK were a generous gift from Dr. Tom Parsons (University of Virginia) and were previously described [149]. The anti –Myc and anti-total FAK antibodies were purchased from Upstate. The anti-Flag (M2) antibody was from Sigma.

Cell Culture and Agonist Treatment

Aortic smooth muscle cells were isolated from either Wistar Rats or *fak*$^{flox/flox}$ mice or MycFRNK transgenic mice. In brief, thoracic aortas were stripped of the endothelial and adventitial layers by microdissection. The SMC in the media were isolated by enzymatic digestion in buffer containing trypsin and collagenase as previously described [152]. Each of our preparations are routinely tested for expression of smooth muscle-specific markers (by immunohistochemistry) and the ability to drive smooth muscle specific expression of reporter constructs. Only the cell lines that are deemed at least 85% pure by these measurements are utilized for further experimentation. Cells are grown in DMEM: F12 (1:1) supplemented with 10% FBS and 1% penicillin-streptomycin and are used between passages 5 and 18. 10T1/2 cells (ATCC) were maintained in DMEM plus 10% fetal bovine serum and 1% penicillin-streptomycin.

Promoter Assays

Cells were transfected using Trans-IT (Mirus) transfection reagents according to manufacturer's protocol. Luciferase activity was detected by the Steady-Glo luciferase assay reagent (Promega).

Western Blotting

Western blots were performed using the appropriate antibodies at a 1/1000 dilution. Blots were washed in TBST (TBS plus 0.05% Triton-X), followed by incubation with either horse-radish peroxidase conjugated- rabbit anti-mouse antibody or - Protein A sepharose (Amersham) at a 1/2000 dilution. Blots were visualized after incubation with chemiluminscence reagents (ECL, Amersham).

Immunocytochemistry

Cells were processed for immunocytochemistry using previously published methods [147]. In brief, cells were fixed with 4% paraformaldehyde, permeabilized with 4% Triton X-100 in PBS, incubated with an anti-Myc antibody (1:250) for 1 hr. After washing with PBS, slides were incubated for 1 hour with Texas Red-conjugated donkey anti-mouse antibodies (2μg/ml).

RESULTS

Since the timing of FRNK expression during development and following vascular injury correlates with the conversion of SMC from a synthetic to contractile phenotype, we asked whether FRNK expression altered SMC differentiation. We utilized a well-characterized SMC promoter-reporter assay in 10T1/2 cells using constructs containing the CArG-region of SMC promoters. As shown in figure A1.1A, the ectopic expression of FRNK by adenoviral infection, resulted in a two to four-fold increase in serum- induced SM22, SM α–actin and SM-MHC promoter activity. Since our previous data showed FAK deletion in SMC resulted in increased SM marker gene transcription, we reasoned that FRNK expression likely promotes SMC differentiation by attenuating FAK activity and relieving FAK-dependent repressive signals (Fig A1.1C).

Previous data published from our lab demonstrate a role for FAK in regulating vascular SMC migration and proliferation and we recently determined that SMC depleted of FAK display an increase in SM marker genes compared to FAK-containing SMC (Chapter IV). Therefore, we hypothesized that FAK activity might regulate SM marker gene transcription. To determine if FAK activation limits SM marker gene expression we ectopically expressed a constitutively active FAK variant (termed SuperFAK; ref. [150]). This variant has enhanced FAK activity as assessed by autophosphorylation of FAK Y397 (Western blot, Fig A1.1C). We performed promoter assays experiments examining SuperFAK expression in 10T1/2 cells. SuperFAK significantly reduced SM22- and SM-αActin reporter gene expression, in support of the hypothesis that FAK activity may attenuate SM marker gene transcription (Fig A1.1B). We performed additional SMC promoter-reporter assay in primary SMC and found that the ability of FAK variants to

stimulate SMC marker gene transcription is related to their efficacy to inhibit endogenous FAK activity (FRNK>autophosphorylation-deficient ^{Y397F}FAK>FAK>SuperFAK; Fig A1.1C). Thus, we have determined that there is an inverse correlation between FAK activity and SMC differentiation.

Selected agonists, such as TGF-β [170] and S1P [148], are potent stimulators SMC differentiation and have been shown to be sufficient to induce SMC differentiation in non-committed fibroblast like cells, 10T1/2. To determine if FAK/FRNK signaling regulated SMC differentiation in an agonist-dependent manner, we performed promoter assays in 10T1/2 cells that were infected with GFP or GFP-FRNK adenovirus. As shown in figure A1.2A, FRNK overexpression resulted in a 9 and 2-fold increase in SM22 and SM α-actin promoter activity, respectively, over GFP adenoviral controls. However, TGF-β but not S1P treatment further enhanced FRNK-dependent SM gene transcription. Since, FRNK attenuates FAK activation; we then asked if FAK activity had an effect on agonist-dependent stimulation. Transient expression of a constitutively active FAK variant inhibited TGF-β-dependent SM22 transcription markedly, but did not inhibit S1P-induced transcription (Fig A1.2B). These data indicate that FRNK expression alone can induce SMC marker gene expression in SM precursor cells and that TGF-β stimulated SM marker gene transcription is negatively regulated by FAK activation.

We created a transgenic mouse model that would allow us to determine the effectiveness of FRNK as a regulator of SM phenotype in vivo. In these mice, the Myc-FRNK transgene is under control of the SMα-Actin promoter. Aortic SMC isolated from these mice show that Myc-FRNK is properly localized within focal adhesions (Fig A1.3A, left panel) and that Myc-FRNK is expressed three-fold compared to endogenous FRNK

levels (Fig A1.3A, right panel: courtesy of Rebecca L. Thompson). Since transient FRNK overexpression in SM precursor cells exhibited an increase in SM marker gene transcription, we aimed to determine if Myc-FRNK SMC exhibited a more differentiated phenotype characterized by increased SM marker gene expression. SM promoter-luciferase assays showed an increase in TGF-β stimulated SM marker gene transcription in Myc-FRNK SMC compared to Wt ($fak^{flox/flox}$) cells (FigA1.3B). These data indicate that FRNK may function to inhibit FAK activity enabling the transition to a more differentiated phenotype.

DISCUSSION

FAK activity is tightly regulated in SMC by selective and dynamic expression of FRNK, a dominant interfering mutant that attenuates FAK activity. We previously reported that FRNK attenuates SMC proliferation and migration by regulating Rac1-dependent signaling [153]. Herein we have found an additional function for FAK/FRNK signaling in SMC. We found that forced expression of FRNK enhanced SM marker gene expression in cultured cells. We also created a mouse model where FRNK is constitutively but moderately overexpressed (2-3 fold over endogenous) in SMC in order to determine the role FRNK in vivo. SMC isolated from these mice corroborate our in vitro findings and displayed a significant increase in SM marker gene expression compared to WT cells. These data suggest that tight regulation of FAK activity by FRNK is likely important for proper SMC phenotypic modulation during development and following vascular injury.

Data presented here shows that FAK/FRNK regulation of SMC differentiation appears to be specific for TGF-β-dependent signaling and not S1P. S1P-stimulated SM22 transcription was not affected by FAK activity or FAK inactivity by FRNK overexpression. Lockman et al have shown that S1P-stimulated SMC differentiation is mediated through the RhoA signaling cascade [148]. While conventional FAK-null displayed a rounded phenotype attributed to increased RhoA activity, our FAK-null SMC show no defects in cell spreading indicating that RhoA activity might not be enhanced in our cells. Therefore, S1P-dependent RhoA activation and subsequent SM marker gene transcription appears to be independent of FAK/FRNK signaling.

Interestingly, both ectopic expression of FRNK and deletion of FAK in SMC led to synergistic induction of SM marker genes following TGF-β treatment. However, when we

expressed FRNK in 10T1/2 cells, while FRNK expression alone enhanced SM gene expression, no difference was observed between FRNK expressing and control cells in the fold induction of the marker genes by TGF-β. There are two possible explanations. One, there may be a difference in ectopic FRNK expression levels compared to that in vivo, where overexpression by adenoviral infection may result in maximal FRNK expression that is beyond that observed following vascular injury or during development. Gross FRNK expression may sequester proteins away from active sites of focal adhesions and alter focal adhesion dynamics. Alternatively, since recent data from our lab indicates that TGF-β induces FRNK protein levels in SMC, TGF-β upregulation of FRNK expression could intiate a positive feedback loop to regulate the phenotype of SMC. This positive feedback loop may explain the observed increase in both TGF-β treated FAK-null SMC and Myc-FRNK SMCs but not in 10T1/2 cells. TGF-β may directly regulate FRNK expression resulting in FAK inactivation and SMC differentiation (FigA1.3). Studies to determine the mechanism of FRNK regulation are currently underway.

Additional studies are also required to delineate the precise signaling pathway governed by FAK/FRNK during SM phenotypic modulation. For example, FRNK expression may potentiate differentiation by activating the Pi3K/AKT pathway. In support for this possibility, Liu et al have demonstrated the role of this pathway in regulating SM marker gene expression. They found activation releases FOXO4 from the nucleus freeing myocardin to bind the transcriptional machinery. Taken together with our studies presented herein that reveal FAK activity also affects SMC phenotypic modulation, we postulate that tight regulation of SMC phenotype, by intrinsic shifting of FAK/FRNK expression could

mediate a balance between SMC migratory and contractile capacities necessary for proper vasculogenesis during development and following vascular injury.

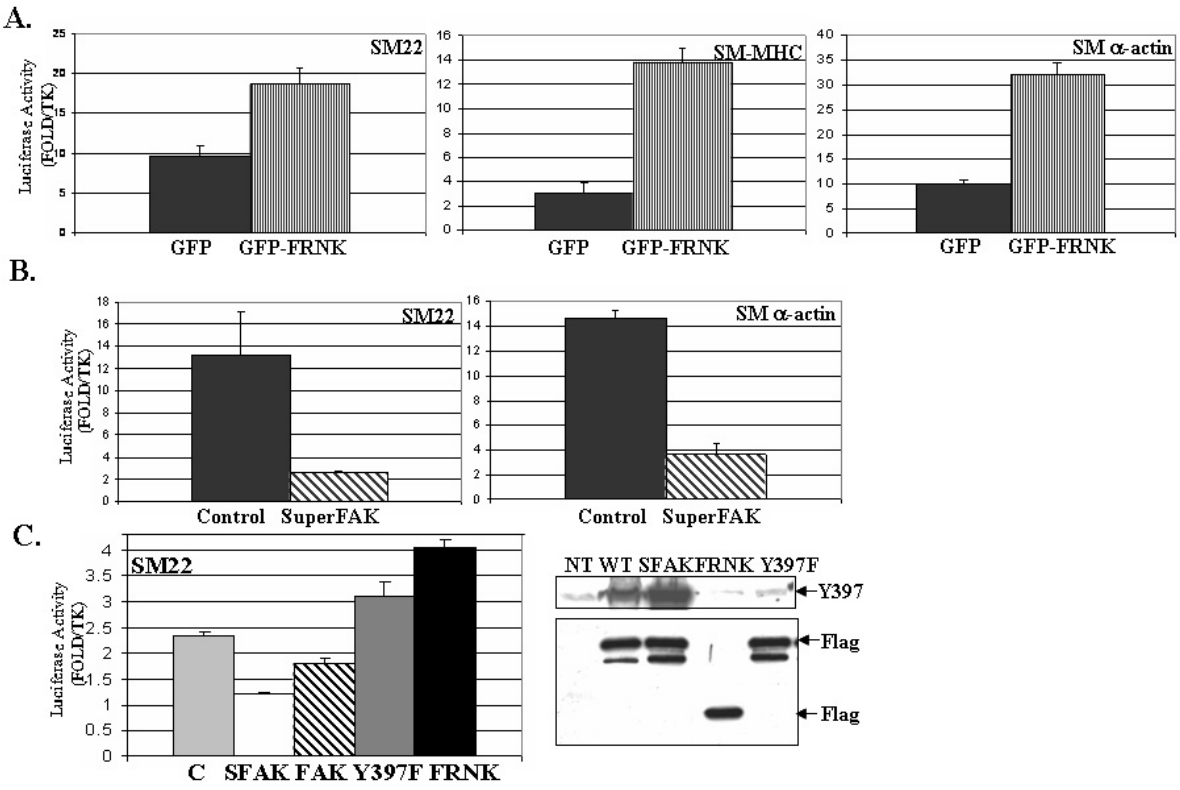


Figure A1.1 SM marker gene expression is modulated by FAK/FRNK signaling. A. 10T1/2 cells were transfected with SM22, SM-MHC or SM α-actin-luciferase reporter plasmids. Cells were transfected 24 hrs following transfection with either GFP or GFP-FRNK adenovirus for 24 hrs prior to luciferase. **B**. 10T1/2 cells were co-transfected with SM22 or SMα-Actin luciferase reporter and either empty vector (control) or Flag-SuperFAK. Luciferase activity was measured 48 hrs following transfection. **C.** Rat Aortic SMC were co-transfected with SM22-luciferase reporter construct and with either empty vector (C), Flag-SuperFAK (SFAK), FAK, ^{Y397}FAK or FRNK in the presence of serum. Luciferase activity was measured 48 hrs following transfection. Cells not transfected (NT) or transfected with FAK (WT), Flag-SFAK, Flag-FRNK or ^{Y397}FAK were lysed and subjected to Western analysis using anti-Flag or an activation state-specific FAK Y397 Ab. Data are representative of at least three separate experiments.

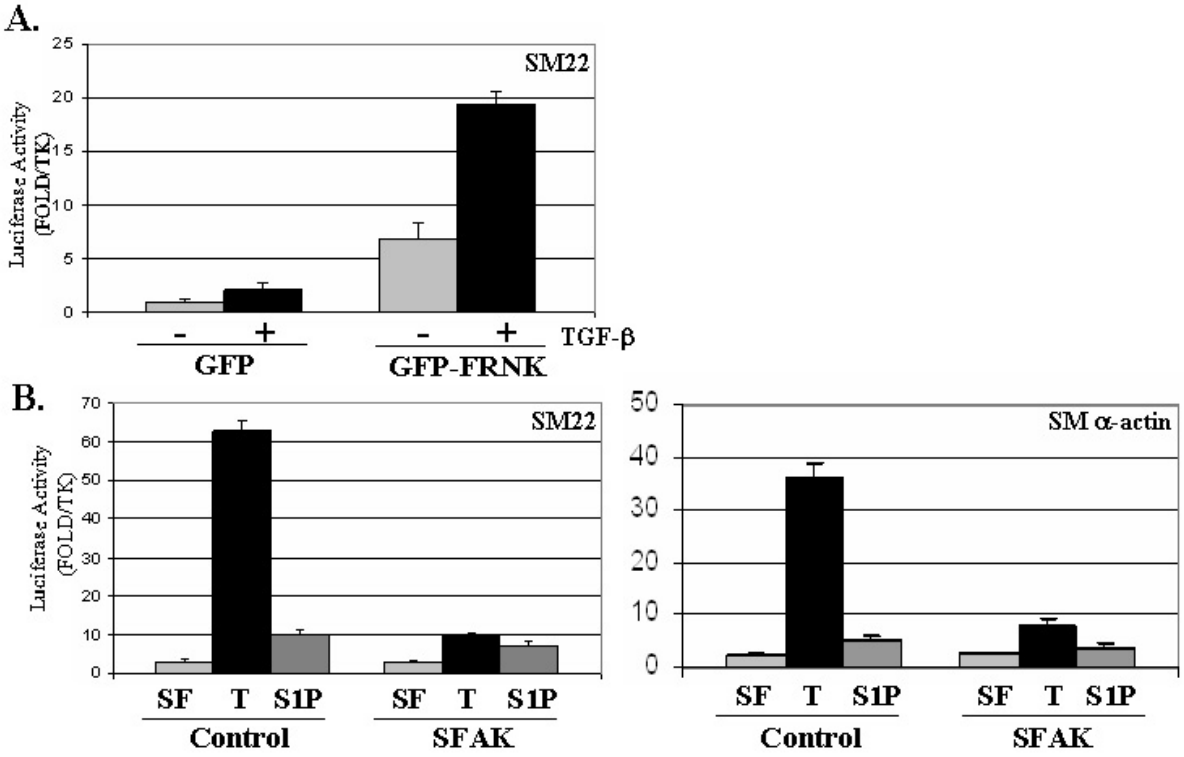
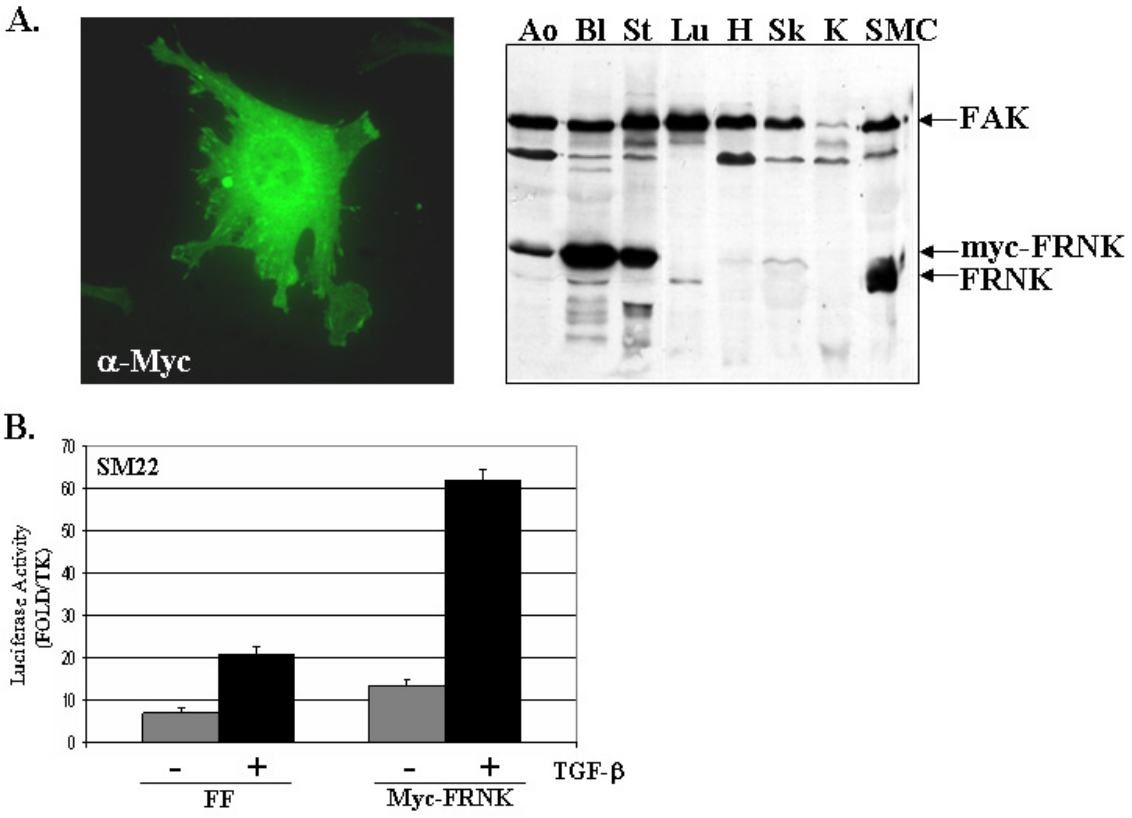


Figure A1.2 FAK/FRNK signaling regulates TGF-β stimulated SM marker genes. A. Cells were infected 24 hrs following transfection (SM22 or SM α-actin promoters) with either GFP or GFP-FRNK adenovirus for 24 hrs prior to luciferase detection or serum starved for 6 hrs and treatment with TGF-β overnight prior to detection. Data displayed as fold GFP-FRNK/GFP. **B**. 10T1/2 cells were co-transfected with SM22, or SM α-actin-luciferase reporter plasmids with either empty vector (Flag) or Flag-SuperFAK (SFAK). Cells were serum starved (SF) for 24hrs prior to treatment with either TGF-β (T) or S1P for 24hrs. Data are representative of at least three (A) or two (B) separate experiments.



$^{flox/flox}$ (FF) and Myc-FRNK SMC were transfected with the SM22-luciferase promoter reporter construct for 24 hrs. Cells were serum starved 24 hrs prior to TGF-β treatment. Luciferase activity was determined 24 hrs following agonist treatment.

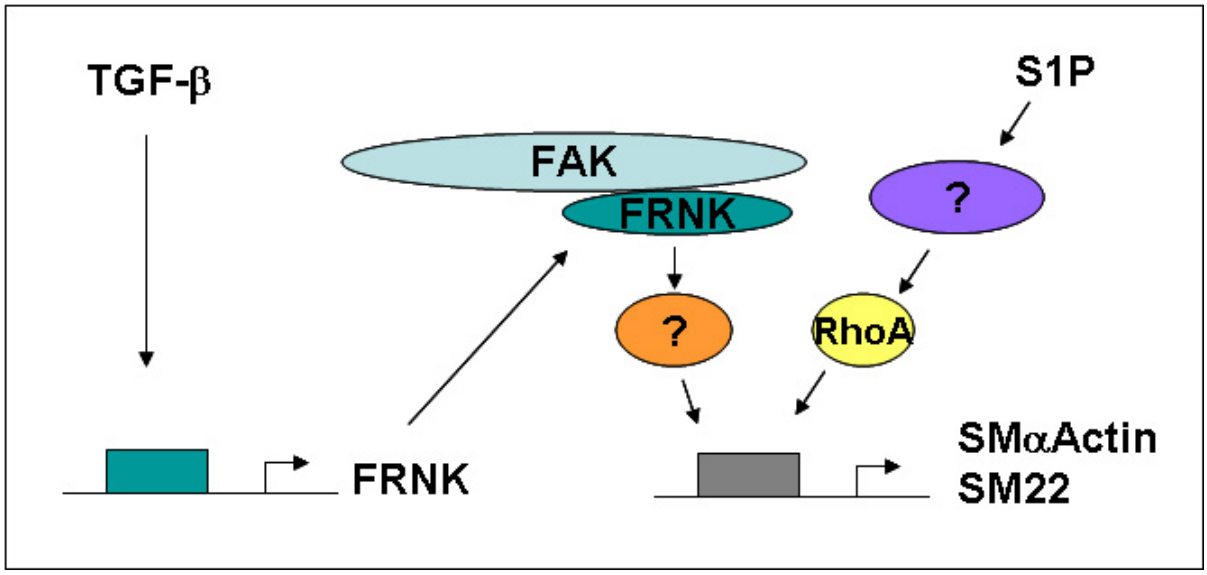


Figure A1.4 Proposed Model of FAK/FRNK regulation of SMC differentiation

Appendix II

Structural analysis of the LIM domain protein Leupaxin

ABSTRACT

LIM containing proteins are characterized by the presence of double zinc-finger like structures that facilitate protein-protein interactions. A subset of LIM proteins have been shown to localize within both focal adhesions and to undergo cytonuclear shuttling. The paxillin family of LIM proteins includes paxillin, hic-5 and leupaxin. We have previously shown that leupaxin is able to translocate to the nucleus where can stimulate SM marker gene transcription through interaction the transcriptional cofactor SRF. However, very little is known regarding the function of the multiple C-terminal LIM domain and N-terminal LD protein binding motifs contained with leupaxin. Therefore, we aimed to perform a structure-function analysis to determine the importance of each leupaxin binding motif. By generating proteins containing various non-functional LIM domains we found that LIM3 is necessary for leupaxin focal adhesion localization, while deletion of LD3 is dispensable for leupaxin localization. Additionally, we identified a putative NES sequence in the extreme N-terminus. Leucine to alanine mutations in the identified NES consensus motif resulted in a 50% increase in leupaxin nuclear localization. Collectively, these structure studies have provided insight towards understanding the function of this complex adapter protein.

INTRODUCTION

LIM domain containing families of proteins play a role in signal transduction from focal adhesions to the nucleus. These proteins are characterized by double zinc-finger like structures that mediate protein-protein interactions. These modules were termed LIM domains based on homeodomain commonality observed in LIN-11, Isl1 and MEC-3 proteins [114]. Over 58 genes encoding LIM containing proteins have been identified and segregated into protein families based on the number of LIM domains, for example, the CRP family has 2, the Zyxin family has 3 domains, and the Paxillin family has 4 domains [114].

The paxillin family members include paxillin, Hic-5 and leupaxin. Hic-5 and leupaxin share 57% and 37% homology with paxillin, respectively [140]. In addition to 4 C-terminal LIM domains, paxillin, Hic5 and leupaxin contain several N-terminal LD motifs, that are also important for facilitating protein-protein interactions. As previously observed with paxillin and Hic-5, we have recently shown that leupaxin is able to undergo cytoplasmic to nuclear translocation (Chapter IV). While the importance of paxillin in regulating cell migration and the role of Hic-5 in gene transcription have been delineated, very little is known about the role of leupaxin. We have previously shown that once in the nucleus leupaxin binds to the transcription factor SRF, and coactivates SM marker gene transcription (Chapter IV). Herein, I have sought to dissect the mechanism of leupaxin function by analyzing the importance of both the LD motifs and LIM domains. By determining the consequence of the loss of function of each protein-protein interaction domain, may help identify putative leupaxin binding partners and an understanding of leupaxin function within the cell.

MATERIALS AND METHODS

Constructs and Reagents

The GFP antibody was purchased from Clontech and Leptomycin B was from Sigma. The SU6656 Src inhibitor was from Calbiochem. GFP-leupaxin was constructed as previously described (Chapter IV). LDD mutations and mLIM C/H to A mutations and Y to E/F point mutations were made by PCR mutagenesis using the primers detailed in Table A.1. To generate GST-leupaxin, GFP-Leupaxin was cut with EcoRI and SmaI and directionally cloned into the GST vector. GST-leupaxin mutations were made using the same primers as the GFP mutations. The promoter reporter constructs: SMα-Actin (from -2560 to +2784), SM22 (from -450 to +88) and SM-MHC (from -4200 to +11600) and SMα-Actin mutant luciferase constructs used have been previously described [148 1568]. Flag-FAK, Flag-FAKY397F and Flag-FRNK were a generous gift from Dr. Tom Parsons (University of Virginia) and were previously described [149]. PBSK-SuperFAK was a generous gift from Dr. Michael Schaller (University of North Carolina, ref 15). To generate Flag-SuperFAK, PBSK-SuperFAK was cut with BamHI and Xho1 and directionally cloned into the Flag vector.

Cell Culture and Agonist Treatment

Aortic smooth muscle cells were isolated from either Wistar Rats or *fak*$^{flox/flox}$ mice, a generous gift from Dr. Louis Reichardt and Dr. Hilary Beggs, UCSF. In brief, thoracic aortas were stripped of the endothelial and adventitial layers by microdissection. The SMC in the media were isolated by enzymatic digestion in buffer containing trypsin and collagenase as previously described [152]. Each of our preparations are routinely tested for

expression of smooth muscle-specific markers (by immunohistochemistry) and the ability to drive smooth muscle specific expression of reporter constructs. Only the cell lines that are deemed at least 85% pure by these measurements are utilized for further experimentation. Cells are grown in DMEM: F12 (1:1) supplemented with 10% FBS and 1% penicillin-streptomycin and are used between passages 5 and 18. 10T1/2 cells (ATCC) were maintained in DMEM plus 10% fetal bovine serum and 1% penicillin-streptomycin.

Promoter Assays

Cells were transfected using Trans-IT (Mirus) transfection reagents according to manufacturer's protocol. Luciferase activity was detected by the Steady-Glo luciferase assay reagent (Promega).

Western Blotting

Western blots were performed using the appropriate antibodies at a 1/1000 dilution. Blots were washed in TBST (TBS plus 0.05% Triton-X), followed by incubation with either horse-radish peroxidase conjugated- rabbit anti-mouse antibody or - Protein A sepharose (Amersham) at a 1/2000 dilution. Blots were visualized after incubation with chemiluminscence reagents (ECL, Amersham).

Immunocytochemistry

Cells were processed for immunocychemistry using previously published methods [147]. In brief, cells were fixed with 4% paraformaldehyde.

In Vitro Kinase Assay

To detect leupaxin phosphorylation: Flag-SuperFAK was transiently expressed in COS7 cells. 48 hrs following transfection, cells were lysed in RIPA plus inhibitors (SU6656 was added 45 min prior to lysing). Flag-SuperFAK was immunoprecipitated from cell lysates with 5 μg of anti-Flag (M2) antibody for 2 hrs followed a 2 hr incubation with pre-coupled Rabbit-αMouse Protein-A Sepharose beads. Beads were washed twice with RIPA and resuspended in 2x Kinase buffer (50mM Hepes pH 7.3, 10mM $MgCl_2$, 1mM $MnCl_2$, 5mM NaF, 0.25% Triton 100-X,). GST, GST-leupaxin, and GST-leupaxin Y22F were prepped as previously described for GST-PAK beads. Freshly purified GST constructs (5μg) were incubated with 5 μl of immunoprecipitated SuperFAK and 10μCi of γP-32 ATP in Kinase Buffer plus 25μM cold ATP for 10min at 30 ^{o}C. Samples were resolved by SDS-PAGE. The radioactive gels were fixed in 50% methanol, 10% acetic acid and 20% glycerol for 20min and then rehydrated for 90min in 7% acetic acid 5% methanol and 20% glycerol and then incubated in Amplify reagent (Amersham) for 30 min prior to exposure to film.

Mutation	Primer
mLIM1	5' GCT CTA GGG CAA TCA TGG GCT CCT GAG GCT TTT GTC GTC ACT CAT GCC AAA GAA GAG ATT GGC
	3' GCC AAT CTC TTC TTT GGC ATG AGT AGC GAC AAA AGC CTC AGG AGC CCA TGA TTC CCC TAG AGC
mLIM2	5' GCA ATG AAC CAG ACC TGG GCC CCA GAG GCC TTC TTC GCC TCT CAC GCC GGA GAG GTG TTT GTG C
	3' G CAC AAA CAC CTC TCC GGC GTG AGA GGC GAA GAA GGC CTC TGG GGC CCA GGT CTG GTT CAT TGC
mLIM3	5' GCC ATG GAC ACT GTC TGG GCC CCA GAG GCC TTT GTT GCG GGG GAC GCC TTC ACC AGT TTT TCT ACT GGC
	3' GCC AGT AGA AAA ACT GGT GAA GGC GTC CCC CGC AAC AAA GGC CTC TGG GGC CCA GAC AGT GTC CAT GGC
mLIM4	5' GG TAC AAG TTC GCC CCT GAG GCC TTT GTG GCC GCT TTC GCC CTG ACA CAG TTG TCG AAG GGC
	3' GCC CTT CGA CAA CTG TGT CAG GGC GAA AGC GGC CAC AAA GGC CTC AGG GGC GAA CTT GTA CC
LDΔ1	5' GGA TGT AGG ACA ATG GAA GAG GAA CGC TCC ACC C
	3' G GGT GGA GCG TTC CTCTTC CAT TGT CCT ACA TCC
LDΔ2	5' CCA GAA AGG AGA CTA ACT CTA TTC AGG
	3' CCT GAA TAG AGT TAG TCT CCT TTC TGG
LDΔ3	5' CGT CAG CAG CTG CTC AGA CTG AGA TGC AGG
	3' CCT GCA TCT CAG TCT GAG CAG CTG CTG AGC
LDΔ4	5' GGA TCA CAA GGC CTC CGA GCA GGA ATT GC
	3' GCA ATT CCT GCT CGG AGG CCT TGT GAT CC
LEUY22F	5' GGA CAG TGA TGA ATT TTC CAA CCC AGC
	3' GCT GGG TTG GAA AAT TCA TCA CTG TCC
LEUY22E	5' GGA CAG TGA TGA AGA GTC CAA CCC AGC
	3' GCT GGG TTG GAC TCT TCA TCA CTG TCC
mNES	5' GGA AGA GGC AGA TGC CGC AGC GGA ACG CTC CAC CGC TCA GG
	3' CCT GAG CGG TGG AGC GTT GGG CTG CGG CAT CTG CCT CTT CC

Table A2.1. Leupaxin primers

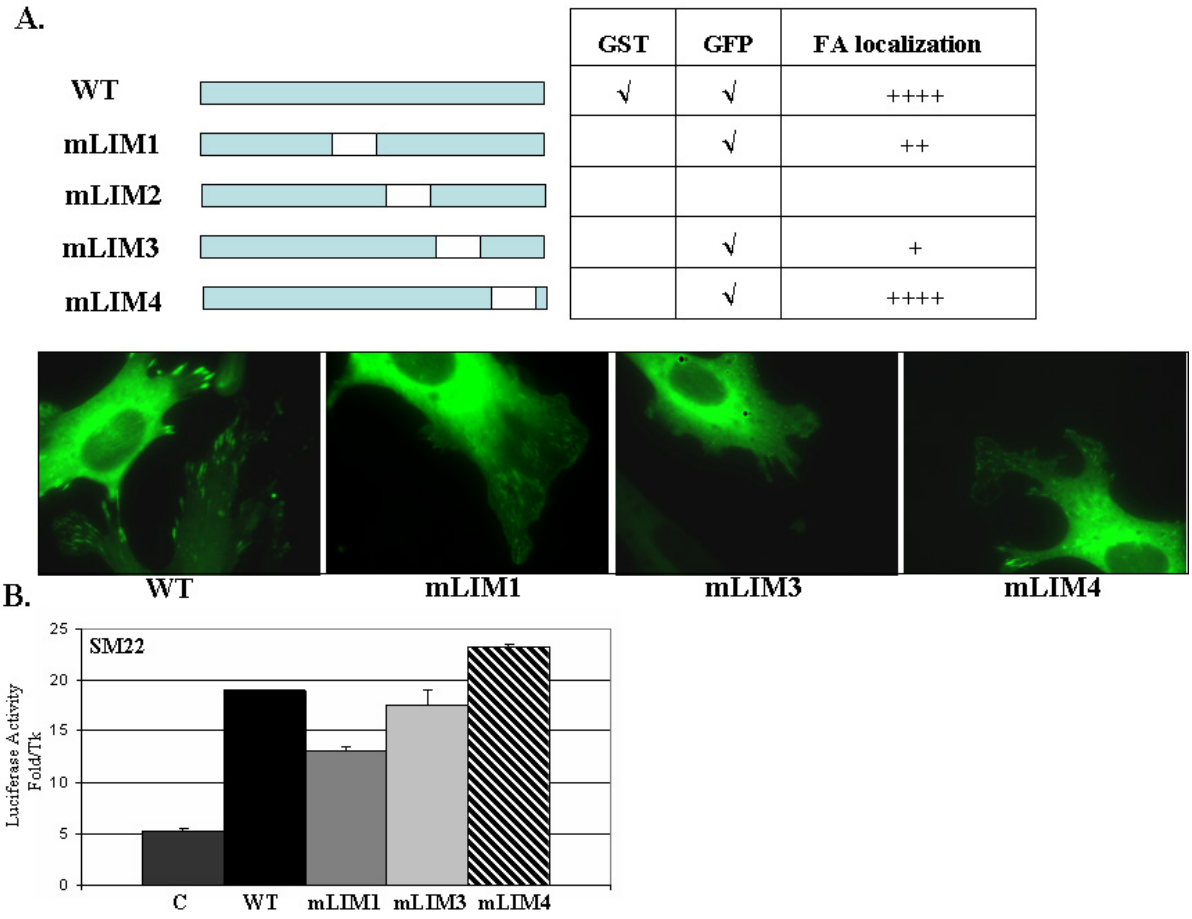
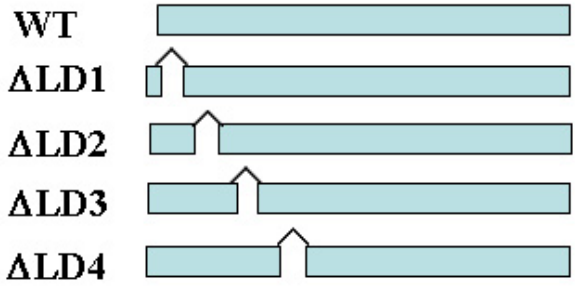
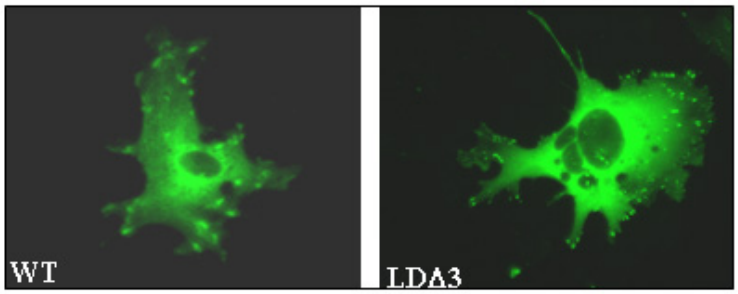


Figure A2.1. Leupaxin focal adhesion localization is mediated through LIM domain 3. A. Schematic of WT and mutant LIM (mLIM) constructs created. GFP- tagged constructs were transfected into 10T1/2 cells and focal adhesion (FA) localization was scored: strongest (++++) moderate (++) and minimal to none (+). **B**. Rat Aortic SMC were co-transfected with SM22-luciferase promoter construct and either empty vector (C), GFP-leupaxin (WT), GFP-mLIM1, GFP-mLIM3 or GFP-mLIM4. Luciferase activity was measured 48 hrs following transfection. Data represents the average of three separate experiments.

A.

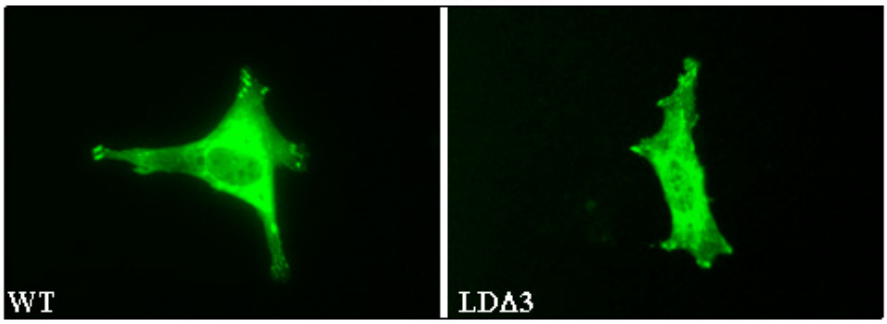


GST	GFP	FA localization
√	√	++++
√		
√		
√	√	++++



B.

20min LMB



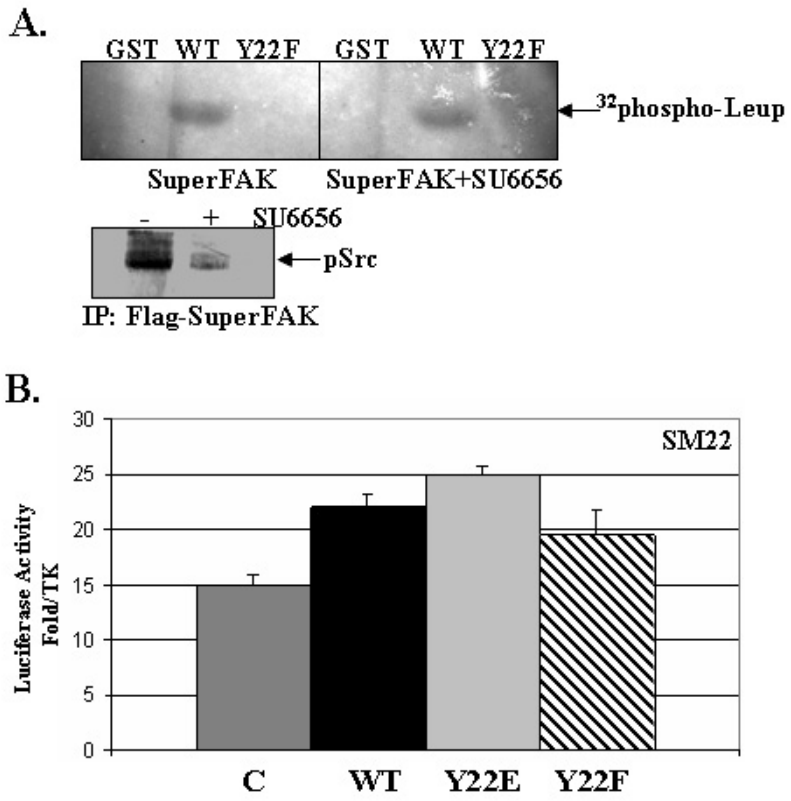


Figure A2.3 Leupaxin Y22 is a target of FAK phosphorylation. A. An in vitro kinase was performed by incubating immunoprecipitated SuperFAK (no treatment, left panel or treatment with SU6656) with either GST, GST-leupaxin or GST-leupaxinY22F and ^{32}p-ATP. The rest of the IP complex was electrophoresed and western blotting was performed to determine Src activity in the immune complex. **B**. SMC were co-transfected with SM22-luciferase promoter report construct and with either empty vector (C), GFP-leupaxin (WT), GFP-Y22E, or GFP-Y22F. Luciferase activity was measured 48 hrs following transfection. Data represent 2 (A) and 3 (B)

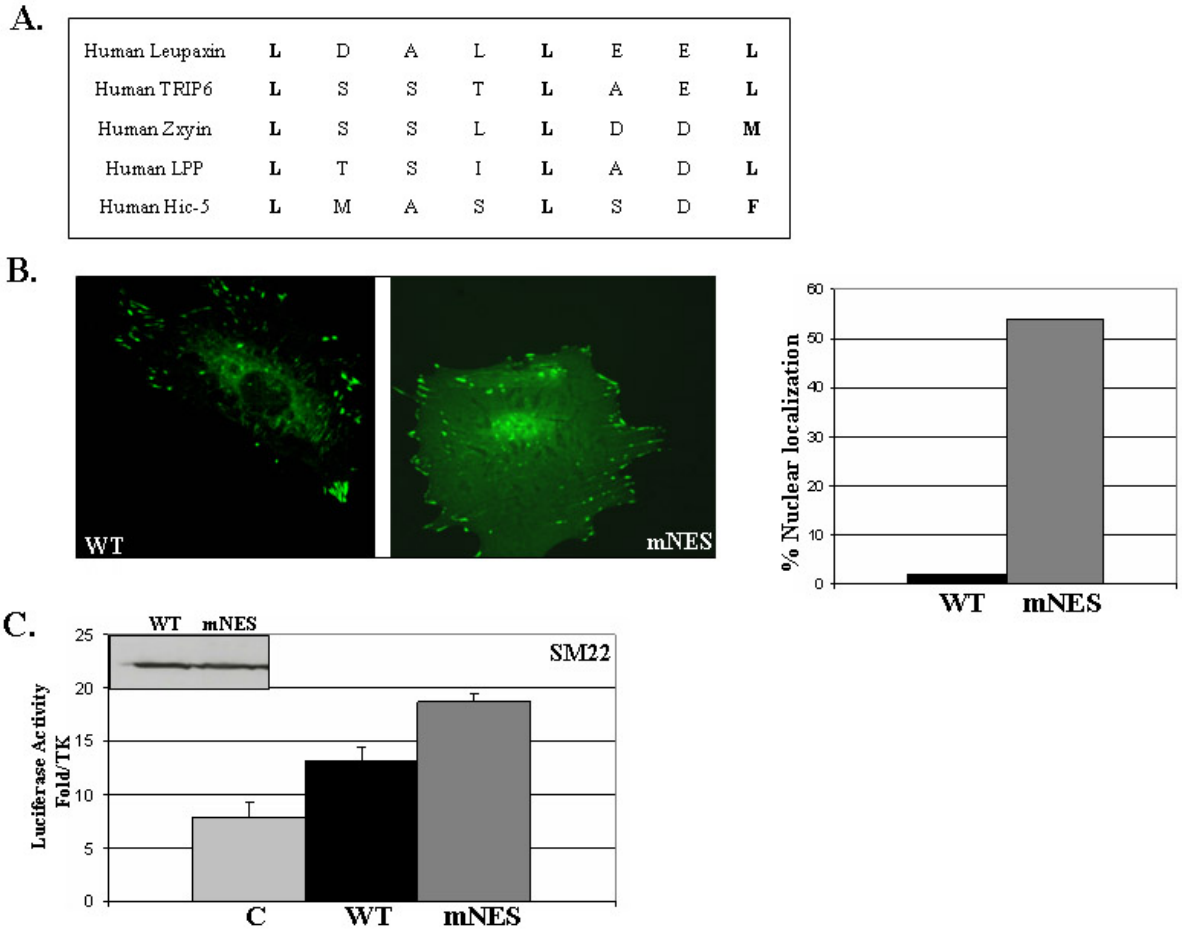


Figure A2.4 Leupaxin contains a putative NES motif. **A.** Alignment of assumptive leupaxin NES with other known NES sequences within the LIM domain containing proteins, TRIP6, Zxyin, LPP and Hic-5. Highlighted leucines are required for conserved NES and were mutated to alanines (mNES). **B**. GFP-leupaxin (WT) and GFP-mNES were transfected into 10T1/2 cells to determine nuclear localization. Over 50% of mNES transfected cells showed nuclear localization compared to WT (graph). **C**. SMC were co-transfected with SM22- luciferase reporter and either empty vector (C) or GFP-leupaxin (WT) and GFP-mNES. Luciferase activity was measured 48 hrs following transfection. Western blot probed with an anti-GFP antibody shows equal expression of both constructs (inset). Luciferase data is an average of three separate experiments.

RESULTS

Like its other family members, paxillin and Hic-5, leupaxin localizes to focal adhesions. Brown et al have shown that LIM3 of paxillin is essential for mediating paxillin focal adhesion localization. We aimed to determine which of the four C-terminal LIM domains of leupaxin might facilitate leupaxin localization. Each LIM domain of leupaxin was individually disrupted by mutating the four central cysteine or histine residues responsible for Zn^{+} binding. Each mutated LIM construct was expressed in 10T1/2 cells to determine localization. As shown in figure A2.1A WT leupaxin is highly localization within focal adhesions. Disruption of LIM1 (mLIM1) shows a reduced expression within focal adhesions while mLIM4 expression localized as well as WT leupaxin. As observed in paxillin, mutation of LIM3 dramatically impaired leupaxin focal adhesion targeting. Next, we determined if LIM domain mutations had an effect on leupaxin-dependent SM marker gene transcription. Promoter assays were performed in SMC (Fig A2.1B). As anticipated, mLIM4 transactivated the SM22 promoter as well as WT leupaxin. Even though mLIM3 was unable to localize to focal adhesions, it was able to induce SM22 transcription, suggesting that binding partners regulating cytoplasmic localization differ from those responsible for nuclear localization. While mLIM1displayed reduced focal adhesion localization it also exhibited a slight fold decrease in SM22 activation, however, it was still able to activate nearly 3-fold over empty vector. These data indicate that while LIM3 facilitates focal adhesion localization none of the LIM domains examined are singularly required for mediating transcriptional coactivation.

The greatest disparity in structure homology between the paxillin family members is within the N-terminus [123]. Therefore, we aimed to determine the importance

of the four LD motifs within the N-terminus of leupaxin. LD motifs are classified by the consensus sequence, LDXLLXXL. Primers were designed to individually delete each LD motif. As shown in Fig A2.2A, deleting LD3 did not disrupt leupaxin localization within focal adhesions. Therefore, we hypothesized that LD3 might be important for nuclear localization. However, treatment with the CRM1-dependent inhibitor, leptomycin B, induced nuclear accumulation of both WT leupaxin and LDΔ3 leupaxin. These data indicate that LD3 does facilitate the cellular localization of leupaxin.

Leupaxin shares several binding partners with the other paxillin family members, including Pyk2, PTP-PEST, and FAK [144, 145]. Additionally, we have previously shown that FAK can regulate leupaxin cytoplasmic/nuclear shuttling for inactivation of FAK or loss of FAK resulted in an increase in leupaxin nuclear translocation (Chapter IV). We hypothesized that FAK might regulate leupaxin localization through direct phosphorylation. To address this question, we ectopically expressed a constitutively active FAK mutant (SuperFAK), immunoprecipitated the kinase and then incubated with purified GST- leupaxin and γ^{32}P-ATP to perform an in vitro kinase assay. As shown in Figure A2.3A, purified GST-leupaxin, but not GST alone, was efficiently phosphorylated by FAK in vitro. We identified a tyrosine kinase phosphorylation target consensus YXXP site in the N-terminus of leupaxin and based on this observation, we mutated the tyrosine amino acid to phenylalanine to determine if this site was the target of FAK phosphorylation. As shown in Figure A2.3A, the Y22F mutation inhibited FAK-dependent leupaxin phosphorylation. Since SuperFAK was immunoprecipitated from whole cell lysates, we wanted to make sure that FAK was directly phosphorylating leupaxin and that phosphorylation was not induced by the FAK binding partner Src (which could be present in the FAK IP). To eliminate the possibility of Src-

dependent phosphorylation, cells were pretreated with the Src-specific inhibitor, SU6656, prior to SuperFAK immunoprecipitation. In this case, we found that Src inhibition had no effect on leupaxin phosphorylation in vitro (Fig A2.3A, right panel), indicating that Y22 was likely a specific target for FAK. Since we hypothesized that FAK phosphorylation may be one mechanism by which FAK regulates leupaxin localization, we asked if leupaxin Y22 phosphorylation would affect leupaxin nuclear localization and subsequent SM marker gene transcription. To this end, we generated a phospho-mimetic mutant (Y22E) leupaxin. This mutant did not differ from WT leupaxin in its ability to enhance SM22 promoter activation (Fig A2.3B) or translocate to the nucleus (unpublished observations).

We have previously shown that leupaxin can undergo cytoplasmic/nuclear shuttling through treatment with the CRM1-dependent inhibitor, leptomycin B. CRM1 is a nuclear export protein that binds to leucine-rich nuclear export sequences (NES). Several LIM domain containing proteins have identified NES sequences, such as Hic-5, LPP and Trip6 [121, 162, 171]. Therefore, we examined the leupaxin sequence for the presence of a leucine-rich NES and found a putative sequence in the extreme N-terminus, aa4-11. In comparison to other known NES sequences, we found that these amino acids contain the three highly conserved leucines required for a functional NES (Fig A2.4A, bold). To determine if this sequence was indeed a functional NES, we mutated the leucines to alanines (mNES). We observed an increase in nuclear localization in mNES leupaxin compared to WT leupaxin expression, where over 50% of the cells showed some nuclear accumulation of mNES leupaxin (Fig A2.4B). Since mNES showed an increased nuclear accumulation, we asked if this increase translated to an upregulation in SM marker gene transcription. Leupaxin mNES did exhibit a significant (albeit moderate) increase in SM22 gene

transcription compared to WT (FigA2.4C). These data indicate that the ability of leupaxin to enhance SM marker gene expression correlates with its nuclear localization.

DISCUSSION

Leupaxin is one of three members of the paxillin family of LIM domain containing proteins. We have previously shown that leupaxin can effectively shuttle from focal adhesions to the nucleus where it can interact with the transcriptional coactivator, SRF and induce SM marker gene transcription (Chapter IV). Other labs have identified a few binding partners, including, FAK, Pyk2 and PTP-PEST [144, 145]. However, very little is known regarding the function of the four LD motifs and four LIM protein-protein interaction domains that comprise leupaxin. Herein, we have shown that LIM3 is important for facilitating leupaxin localization to focal adhesions. We also show that leupaxin is directly phosphorylated by FAK on tyrosine 22 and lastly, we have identified a putative CRM1-dependent NES sequence within the N-terminus. Collectively, these data may aid in our understanding of leupaxin-dependent mechanisms.

The three paxillin family members, paxillin, hic-5, and leupaxin, all contain four C-terminal LIM domains. The leupaxin LIM3 shares a 71% sequence identity with paxillin, therefore it is not surprising that like paxillin, that we found that LIM3 is important for facilitating leupaxin focal adhesion localization [126, 140]. However, it is still unknown how paxillin is targeted to focal adhesions, and it could be hypothesized that leupaxin and paxillin are targeted through similar interactions. In addition, even though disruption of the individual LIM domains, 1,3 and 4, did not alter leupaxin stimulated gene transcription it does not eliminate the potential of these LIM domains to mediate an interaction with the transcriptional machinery. Since leupaxin has four C-terminal LIM domains, individual mutations may not be sufficient to disrupt leupaxin's association with transcriptional coactivators, such as SRF. Therefore, different LIM mutation combinations might be

required to effectively determine the role of leupaxin in regulating SM marker gene transcription.

The disparity between paxillin and leupaxin N-terminal sequence suggests divergent roles within the cell. The sequence coding LD2 and 3 of leupaxin only share 19% homology with paxillin [140]. We showed that the loss LD3 has no effect on focal adhesion localization or nuclear translocation of leupaxin. These data indicate that LD3 is dispensable for both focal adhesion localization and nuclear shuttling. However, this does not preclude the importance of an interaction between LD3 and an unknown binding partner that could regulate another undetermined function of leupaxin.

Leupaxin does contain a consensus YXXP Crk binding site in its amino terminus (present in paxillin) that is tyrosine phosphorylated by active FAK, indicating that leupaxin may be regulated in an adhesion-dependent fashion. However, if phosphorylated leupaxin associates with Crk (or other paxillin binding partners) are questions for future studies. Other LIM domain proteins have been implicated in regulating cell migration, therefore, it could be hypothesized that leupaxin localization within focal adhesions might play a role in propagating migratory signals. We had originally hypothesized that FAK-dependent leupaxin phosphorylation would sequester leupaxin within focal adhesions based on our previous studies showing that FAK activation diminished the ability of leupaxin to stimulate SM22 gene transcription. However, we found that leupaxin Y22 phosphorylation was able to properly translocate to the nucleus where it could induce SM marker gene expression. Our phosphorylation studies were determined through in vitro kinase assays and the consequence of leupaxin phosphorylation may be agonist-dependent or cell-type specific, so further studies are necessary to decipher FAK-dependent leupaxin responses.

Like paxillin and Hic-5, we have previously shown leupaxin can undergo nucleocytocplasmic shuttling and affect gene expression. We found that treatment with the nuclear export inhibitor, leptomycin B causes retention of leupaxin in the nucleus, providing evidence that leupaxin normally cycles between focal adhesions and the nucleus (Chapter IV). Leptomycin B inhibits CRM1-dependent export by blocking CRM1 binding to leucine-rich nuclear export sequences (NES) on target proteins. We identified a putative NES sequence in the extreme N-terminus of leupaxin. While mutations to this sequence resulted in a robust increase in leupaxin nuclear entrapment, it was not observed throughout the cell population. NES studies have created a hierarchy of conserved NESs based on rate of nuclear export [173] and subsequent experiments revealed that the strongest NES shuttles at the greatest rate with the protein localization being almost exclusively cytoplasmic. However, nuclear localization of constructs containing strong NESs was induced following agonist treatment [172]. These reports may suggest that leupaxin nuclear localization might be agonist-driven. Similar findings were shown for Hic-5, where mutation of the NES did not result in 100% Hic-5 localization, only following treatment with H_20_2 was Hic-5 100% nuclear. One might predict that a SM differentiation agonist, such as TGF-β would induce leupaxin nuclear shuttling. Additionally, leupaxin nuclear export may be regulated by another mechanism, such as a KNS shuttling element, which is not regulated by CRM1 and therefore not inhibited by leptomycin treatment [173]. The presence of another shuttling motif may explain why leupaxin can still be observed within focal adhesions following leptomycin treatment. Further analysis of leupaxin sequence is required to determine the presence of other consensus export sequences.

Many questions remain to be answered. Hic-5 has been shown to activate various gene promoters, including c-fos [142] and interactions with various coactivators may dictate gene-specific activation. The SM marker genes, SM22, SM-MHC, SM α-actin, and the growth gene c-fos all contain CArG-dependent promoters requiring transactivation by SRF. We have previously shown through ChIP assays, that leupaxin can associate through SRF with the CArG elements of SM-MHC and SM α-actin but not c-fos. The underlying mechanisms regulating the discrepancy between SRF-dependent activation of the contractile SM marker genes and SRF-dependent growth genes are poorly understood. However, it could be postulated that LIM containing proteins, such as leupaxin, may function as a scaffold and regulate gene activation through the binding of various coactivators specific for each promoter. Further work identifying leupaxin binding partners may assist in our studies aimed to determine the processes regulating SMC phenotypic modulation.

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