FOCAL ADHESION KINASE AND ITS ENDOGENOUS INHIBITOR, FRNK, IN VASCULAR DEVELOPMENT AND INJURY

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ABSTRACT

REBECCA LYNN SAYERS: Focal Adhesion Kinase and its Endogenous Inhibitor,
FRNK, in Vascular Development and Injury
(Under the direction of Joan M. Taylor, Ph.D.)

Development and maintenance of a circulatory system is required for transport of

essential gases and nutrients in vertebrates. Within this work, I have aimed to elucidate

the role of a certain family of adhesion signaling molecules in regulating the responses of

vascular smooth muscle cells (VSMC) that enable circulatory vessels to form and

maintain a differentiated phenotype. Focal adhesion kinase (FAK) is a non-receptor

tyrosine kinase that coordinates signaling from integrins, growth factor receptors, and G-

protein coupled receptors. While FAK is ubiquitously expressed, its variant FAK-related

non kinase (FRNK) is selectively expressed in smooth muscle containing tissues. Our

group has recently demonstrated that over-expression of FRNK in vascular smooth

muscle cells (VSMC) can attenuate PDGF-stimulated proliferation and migration of

vascular smooth muscle cells. FRNK is highly expressed during early postnatal

development (particularly in large arteries) and two weeks following endovascular injury

in the rat femoral artery. Regulation of FRNK in smooth muscles cells is unique in that

FRNK is expressed as a gene within a gene under the control of non-coding sequence

within a FAK intron. This FRNK promoter sequence is void of CArG boxes, making

transcription independent of serum response factor (SRF). Germline deletion of FAK

causes mesodermal defects resulting in embryonic lethality, implicating FAK as a

significant regulator of vascular development. Since FAK$^{-/-}$ embryos die prior to complete formation of the vasculature, we used a conditional approach to delete FAK specifically in cardiac neural-crest derived cells. The goals of this work were to (1) determine the factors that regulate SRF-independent smooth muscle-specific expression of FRNK, (2) use a gene ablation model to elucidate an *in vivo* function for FRNK, and (3) use SMC-specific conditional knockout approach to examine a role for FAK in vascular development. Herein we found that FRNK expression is regulated by TGF-β and extracellular matrix components. Conservation analysis revealed two ~100-150 bp regions of the FRNK promoter that are over 75% conserved between mouse, rat and human. Although we found that FRNK$^{-/-}$ mice exhibit increased phosphorylation of FAK^{Y397} and proliferation our most striking observation was that deletion of FRNK causes decreased expression of smooth muscle α-actin, smooth muscle myosin heavy chain, and smoothelin during postnatal development and in the neointima following carotid artery ligation. Likewise, overexpression of FRNK stimulated smooth muscle promoter activity in serum and enhanced TGF-β mediated increases in smooth muscle promoter activity. To study the role of FAK in development of cardiac neural crest-derived smooth muscle cells, we used a previously described Wnt-1-Cre expressing mouse to conditionally delete FAK in this cell population. We found that deletion of FAK resulted in malformation of the aorticopulmonary septum and that animals did not survive to adulthood. Additional *in vivo* and *in vitro* studies revealed that FAK activity downregulates activity of smooth muscle specific genes. The studies described herein indicate that the adhesion signaling molecule FAK and its truncated family member, FRNK, regulate smooth muscle cell phenotype.

To
William Benjamin Prescott,
John Whitman Prescott,
and
Jane Prescott Thompson,
Scientists

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ACLP   Aortic carboxypeptidase-like protein

AP-1   activator protein 1

APEG1   aortic preferentially expressed gene 1

BCA   Bradford concentration assay

BEC   bovine endothelial cells

BrdU   bromodeoxyuridine

cDNA   complimentary DNA

CNC   cardiac neural crest

CRP2   cysteine-rich protein 2

cSMC   coronary smooth muscle cells

DMEM   Delbucco's modified Eagle medium

DNA   deoxyribonucleic acid

ECM   extracellular matrix

EEL   external elastic lamina

EGF   epidermal growth factor

ERK   extracellular-related kinase

ES   embryonic stem (cell)

FAK   focal adhesion kinase

FBS   fetal bovine serum

FITC   Fluorescein isothiocyanate

FN   fibronectin

FRNK	FAK-related non-kinase
GFP	green fluorescent protein
HRCBP	histidine-rich calcium binding protein
HRP	horseradish peroxidase
IEL	internal elastic lamina
IGF-1	insulin-like growth factor-1
kDa	kiloDalton
LDL	low density lipoprotein
luc	luciferase
MAPK	mitogen-activated protein kinase
Mef2C	myocyte enhancer factor 2c
MG	Matrigel
MK2	MAPK-activated protein kinase 2
mRNA	messenger RNA
MRTF	myocardin-related transcription factor
NF-E2	nuclear factor (erythroid-derived) 2
OFT	outflow tract
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PTA	persistent truncus arteriosis
Pyk	protein tyrosine kinase
RGF-MG	reduced growth factor Matrigel
RIPA	radioimmunoprecipitation assay

RNA	ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
S1P	sphingosine-1-phosphate
SBE	Smad binding element
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH	Src homology domain
SM	smooth muscle
SMC	smooth msucle cell
SM-MHC	smooth muscle-myosin heavy chain
SRF	serum response factor
TBS	tris-buffered solution
TCE	TGF-β control element
TGF-β	transforming growth factor-b
TK	thymadine kinase
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cell
WT	wild type

CHAPTER I

INTRODUCTION

INTRODUCTION

Proper development and maintenance of the cardiovascular system is essential for survival. The circuitry of vessels contained within the human body is responsible for transporting essential gases and nutrients to all cells in the body. This work addresses the specific role of smooth muscle cells in development of the vasculature, maintenance of mature vessels, and response to vascular injury. More specifically, this work aims to elucidate a role for adhesion signaling proteins in regulation of certain vascular smooth muscle cell processes such as proliferation, migration, and differentiation. This chapter reviews smooth muscle cell biology and smooth muscle cell signaling through focal adhesion kinase (FAK) and its variant FAK-related non-kinase, or FRNK.

BIOLOGY OF SMOOTH MUSCLE CELLS

The Developing Vasculature

During mammalian development, the generation of a closed circulatory system is essential for the successful maturation of the embryo. Even during early stages of embryogenesis, prior to commencement of high rates of blood flow, a complete network of vessels must be formed to provide nourishment to the embryo. Indeed, numerous animal studies demonstrate that genetic disruptions causing defective development of the embryonic or extra-embryonic vasculature result in embryonic lethality.

Early in development, endothelial cells derived from the mesenchyme of the embryo begin to align and form a network of tubes. One of the first vascular tubes to form includes cells that will line the dorsal aorta of the animal (1). During angiogenesis, additional vessels sprout from endothelial tubes to begin formation of a vascular plexus.

Eventually, pericytes derived from nearby progenitors within the surrounding mesenchyme, epicardium, or neural crest cell populations are recruited to sites of endothelial tube formation (see below) (2). The initial investment of smooth muscle cells begins forming the contractile muscle layer for the vessel. PDGF-BB (platelet-derived growth factor-BB) and VEGF (vascular endothelial growth factor) signaling leads to further recruitment and proliferation of smooth muscle cells concomitant with increased expression of contractile proteins such as SM α-actin and SM-myosin heavy chain (2). In addition, smooth muscle cells secrete extracellular matrix proteins, including those that will comprise the elastic laminae.

Origin and Heterogeneity of Smooth Muscle Cells

The developmental origin of vascular smooth muscle cells has been a subject of close study. It is difficult to pinpoint when and where the initial steps of smooth muscle cell differentiation begin, since markers for smooth muscle cells are not entirely specific. Evidence has shown that smooth muscle cells, even those within the same vessel, can come from different populations of progenitor cells, contributing to heterogeneity. Vascular smooth muscle cells have been shown to arise from several different lineages including neural crest, secondary heart field, proepicardium, somites, mesangioblasts, mesothelium, and splanchnic mesoderm (3). For example, smooth muscle cells of the coronary arteries are derived from proepicardial cells (4) while neural crest-derived cells form smooth muscle cells of the cardiac outflow tract, as discussed in greater detail below. This diversity bears pathological relevance, since vessels arising from distinct lineages have different responses to identical vascular injury. In addition, smooth muscle

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contractile gene expression appears to be regulated differently in various smooth muscle populations (reviewed by Majesky (3)).

Although smooth muscle cell heterogeneity is attributed in large part to differences in lineage, it is also greatly impacted by the vessel microenvironment. Each vessel, independent of origin, exists in a unique environment, causing smooth muscle cells to respond to extrinsic biological cues in different ways. For example, the specific hemodynamics of a particular vessel affect the ability of smooth muscle cells in that vessel to respond to autocrine or paracrine agents, as differing rates of flow may affect the length of time the vessels are exposed to humoral agents (reviewed by Berk (5)). In addition to the rate of flow, smooth muscle cell behavior is also dependent on the type of flow present in the environment. Vascular flow is generally laminar, with the fastest blood velocity in the middle of the vessel and the slowest against the vessel wall. However, at points of bifurcation, flow becomes disorganized, and is referred to as turbulent. Vessels exposed to turbulent flow are at higher risk for development of atherosclerotic lesions, as discussed later. In addition, the extracellular matrix composition of a particular vessel is important in smooth muscle response to injury. Finally, the presence of specific matrix components, such as fibronectin or laminin, can alter the reactivity of smooth muscle cells to certain proliferative agonists (6-8). The combined diversity of cell origin and environment complicates the study of smooth muscle cells, but must be considered when studying the function of smooth muscle cells in different developmental and pathological paradigms.

Formation of the Cardiac Outflow Tract

A specific example of vascular development relevant to this work is formation of the cardiac outflow tract from cardiac neural crest-derived cells. Cardiac neural crest cells arise from the dorsal neural tube, and are named such not because they only form cardiac tissue, but because ablation of these cells causes cardiac abnormalities (9). Cells derived from this population contribute to parasympathetic nerves of the heart, thymus, thyroid, parathyroid, and most notably for this discussion, smooth muscle cells of the cardiac outflow tract and aorticopulmonary septum (10-12). Migration of these cells from the neural crest to the circumpharyngeal ridge is dependent on expression of the family of Wnt proteins (13). At this site, cardiac neural crest cells are involved in the formation of pharyngeal arches 3, 4, and 6, which eventually form vasculature and glands (9). Cardiac neural crest cells proliferate and migrate to follow mesenchymal cells into the cardiac outflow cushions (14). Migration of these cells is the first step required for formation of the aorticopulmonary septum, followed by differentiation of cells into smooth muscle and myocardium and fusion of the cushions (9). Cells within the outflow tract differentiate into smooth muscle cells, and gene ablation mouse models have indicated that differentiation of smooth muscle cells is required for proper development of the outflow tract. For example, deletion of ALK2 in Wnt1-expressing cells leads to impaired cardiac neural crest migration and expression of smooth muscle genes around the aortic arch (15). Additionally, knockout of MRTF-B, a myocardin-family member discussed below, results in embryonic lethality due to malformation of the aortic arch (16, 17). Since the ability of cardiac neural crest cells to migrate and differentiate into smooth muscle cells is essential for correct development of the outflow tract, further

characterization of the molecules necessary for proliferation, migration, and differentiation must be pursued.

Smooth Muscle Cells in the Mature Vascular Wall

After complete development of the vasculature, the walls of large vessels contain three distinct layers that each carry out a specialized supportive function. The innermost layer, or tunica intima, is a single layer of endothelial cells lining the lumen of the vessel. This layer acts as a selective barrier for the vessel and transmits information from the bloodstream to surrounding cells. Smooth muscle cells form the tunica media and respond to signals from endothelial cells, circulating peptides, and direct nerve stimulation to regulate vascular tone and resistance and maintain mean arterial pressure (5). The outermost layer, the tunica adventitia, consists mainly of adventitial fibroblasts and other connective tissue that supports the vessel.

In a mature vessel, smooth muscle cells within the tunica media have a contractile phenotype and express receptors for a variety of vasoactive stimuli, including catecholamines, prostaglandins, peptides, and steroid hormones. However, unlike striated muscle, smooth muscle cells maintain plasticity following development and can dedifferentiate from their contractile phenotype to a proliferative phenotype. During disease states or following injury, a complement of growth factors and extracellular matrix initiates expression of pro-proliferative genes, which in turn triggers a gene expression profile similar to that seen during development. These gene expression changes lead to proliferation of smooth muscle cells through activation of growth factor receptors and integrins, which is an important factor in response to vascular injury (5).

Smooth Muscle Cells in Vascular Disease

Hypertension:

In 2005, nearly one third of the United States population over age suffered from hypertension (18). Smooth muscle cell growth is thought to contribute to the development of this disease. Changes in pressure within the vessel wall cause the vessel to remodel; in the case of hypertension, the smooth muscle-containing tunica media thickens. Studies have demonstrated chronic hypertension is accompanied by an increase in wall thickness that compensates for additional stress on the vessel (19, 20). Hyperplasia, an increase in cell number, and hypertrophy, an increase in cell size, are two growth mechanisms known to increase medial thickness. Normally, differentiated smooth muscle cells within the vascular wall exhibit a very low proliferation rate. In normotensive rats, smooth muscle cells proliferate at a rate of 0.1% per day, but spontaneously hypertensive rats (SHR) exhibit hyperplasia with a rate increase to 1% per day (21). In chronic hypertension, there can be hypertrophy with or without an increase in DNA. A well-studied mechanism of hypertension is an increase in cell volume due to increased retention of water within the cell. This is generally true in cases of salt-sensitive hypertension, and can usually be reversed. However, hypertrophy with an increase in DNA content is generally irreversible (22, 23). Owens, *et al.* previously showed that in comparison to normotensive controls, SHR have an increase in DNA/cell ratio and an increase in the number of polyploid smooth muscle cells (24). Hyperplasia and hypertrophy can be mediated by autocrine and paracrine growth factors such as PDGF-A and IGF-1, both of which are elevated during hypertension (25-27).

Atheroclerosis:

According to the American Heart Association, 13.9 million Americans suffered from a heart attack or stroke in 2005, and the development of atherosclerosis is a major contributor to this statistic. There are many factors that put humans at risk for atherosclerosis, including elevated cholesterol, hypertension, diabetes, and smoking. Atherosclerosis is defined as a narrowing of a vessel due to the formation of a plaque, or atheroma, consisting of fatty cholesterol deposits. The resulting reduction in lumen diameter increases the risk for heart attack and stroke. Understanding the mechanisms that contribute to atherosclerosis is critical for preventing the poor medical outcomes associated with this condition.

In the development of the atherosclerotic plaque, deposition of fatty acids on the vessel wall results in the growth of large lesions which limit blood flow to vital organs. After initial lesion formation, smooth muscle cells, foam cells, macrophages, and lymphocytes migrate into the lesion and proliferate often leading to the formation of a large plaque. There are many factors promoting formation of plaques, and several proposed mechanisms involve proliferation of smooth muscle cells (reviewed by Fitridge and Thompson (28)). For example, it is well known that incidence of atherosclerosis is directly correlated with levels of low-density lipoprotein (LDL), which induces smooth muscle cell growth (29). In addition, studies indicate that sites of disturbed flow within the vessel, such as areas of bifurcations, are at higher risk for lesion formation, possibly due to the ability of mechanical stress to induce smooth muscle cell proliferation (30). In cultured cells, disturbed flow can also enhance signaling through the PDGF receptor, a potent and positive regulator of smooth muscle cell proliferation and migration (31). As

mentioned previously, hypertension is a risk factor for development of atherosclerosis, possibly by increasing mechanical stress on the vascular wall causing smooth muscle cells to migrate, proliferate, and increase the rate of matrix deposition, contributing to plaque formation (32). The studies mentioned here as well as numerous others have underscored the role that smooth muscle cells play in the development of atherosclerosis.

Restenosis Post-Angioplasty:

As detailed above, development of atherosclerosis causes a narrowing of the arterial lumen, resulting in decreased blood flow to organs. This is of particular concern when blood flow to the heart or brain is compromised, as this can lead to heart attack and stroke. Surgical intervention utilizes balloon angioplasty or stent implantation to mechanically remove blockage within the narrowed artery. Although this increases lumen diameter and improves blood flow to the heart, either of these devices can cause injury to the vessel wall thereby inducing proliferation of smooth muscle cells within the medial layer. This is followed by robust migration of smooth muscle cells into the lumen, forming a neointima. Prior to recent therapeutic advances, 20% of patients who underwent surgery experienced re-growth of cells into the lumen and formation of a neointima (28). This re-growth is clinically referred to as restenosis. This is a significant problem, since further treatment after restenosis is more costly and puts the patient at higher risk repeat angioplasty or bypass surgery (28).

The formation of the neointima is a multi-step process. Injury to the endothelium triggers platelet deposition, resulting in the release of PDGF, a strong mitogen and chemoattractant for smooth muscle cells (33). This is likely responsible for the subsequent increase in the proliferation rate of smooth muscle cells, rising from less

than 1% prior to injury to 10-40% following the injury (34-36). These agonists first induce smooth muscle cells to defifferntiate and proliferate, and then undergo migration outside of the media. After migration, smooth muscle cells again proliferate, thus obstructing the aterial lumen. The neointima, newly populated with smooth muscle cells, is also subject to increased extracellular matrix deposition, resulting in a decline in smooth muscle proliferation. Since smooth muscle cell growth, migration, and response to extracellular matrix are integral processes in neointima formation, it is of clinical importance to understand the adhesion- and growth factor-mediated signaling events behind restenosis.

Regulation of Smooth Muscle Specific Gene Expression

As discussed previously, in response to injury smooth muscle cells retain the ability to return to a proliferative state following differentiation. This phenotypic switching generally manifests as changes in proliferation and expression of smooth muscle marker genes such as SM α-actin, SM-22, and SM-myosin heavy chain. During development, it is the expression of these genes that first classifies a cell as smooth muscle. SM α-actin is the earliest known marker for smooth muscle cells, although during early embryogenesis and cardiac hypertrophy, its expression is not limited to smooth muscle cells (37). SM-22 and SM-myosin heavy chain are more specific markers for smooth muscle cells, but expression is detected later in development. SM-22 appears in smooth muscle cells at E9.5 (38). Expression of SM-22 is also observed transiently in other muscle tissues such as the heart between E9.5 and 12.5 (38). SM-myosin heavy chain is reported to turn on in the developing mouse aorta around E10.5 and in the arch of

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the aorta and outflow tract vessels around E12.5 (39). Expression of all three of these genes continues past development and remains high in mature, contractile smooth muscle cells. Following a vascular injury such as angioplasty or stent implantation, expression of smooth muscle marker genes decreases as smooth muscle cells revert to a proliferative phenotype. In a mouse model of guide wire-mediated injury to the carotid artery, expression of SM α-actin and SM-myosin heavy chain decreased as early as 4 days following injury and returned to nearly normal levels 14 days following injury (40, 41). Since the expression of smooth muscle genes appears to be intricately regulated during both development and disease, much attention has been paid to the mechanisms that regulate these genes.

Expression of most smooth muscle specific genes is dependent on the presence of one or more CArG elements within the promoter (38, 42-47). Mutation of any of the three CArG elements present within the smooth muscle α-actin promoter was shown to ablate expression in a transgenic LacZ reporter mouse (43). This is also true of mutations made to CArG elements within the SM-22α, SM-MHC, and desmin promoters (42, 44, 45, 48). Serum response factor (SRF), a MADS box transcription factor, binds CArG elements to regulate transcription. It is thought that SRF dimerizes upon binding to the CArG element (49). Although the CArG element is necessary for smooth muscle specific expression of these genes, it is likely that a number of DNA response elements are required for combinatorial control of gene expression since SRF is ubiquitously expressed (47). Smooth muscle-specific SRF-dependent gene expression is regulated by binding of coactivators to SRF, including myocardin and mycoardin-related transcription factor-A (MRTF-A). It is also interesting to note that even though CArG-dependent gene

expression is the best described mechanism of smooth muscle differentiation, recent work has shown that myocardin is necessary, but insufficient for complete differentiation of smooth muscle cells (50). Various growth factors have been identified as stimulators of smooth muscle gene expression, one significant example is TGF-β, which is discussed below in detail.

TGF-β in the Vasculature

TGF-β is important in both vascular development and disease. Numerous studies have indicated that alteration of the TGF-β signaling pathway leads to vascular defects during development resulting in embryonic lethality. Mutation of the Type II TGF-β receptor subtype leads to vascular malformations in developing mouse embryos (51, 52) and the Type I TGF-β receptor is required for arterio-venous differentiation (53). Maintenance of TGF-β levels during development also appears to be critical since both TGF-β knockout mice and mice that overexpress TGF-β under the control of a smooth muscle specific promoter have abnormalities in yolk sac vasculogenesis (54, 55). In cultures of both primary smooth muscle cells and 10T1/2 cells (smooth muscle precursor cell line), TGF-β increases expression of smooth muscle α-actin and SM-22 (56, 57). Monc-1 cells (a neural crest cell line), can also be differentiated into contractile cells expressing smooth muscle markers by treatment with TGF-β (58). It has also been suggested that TGF-β may facilitate recruitment and maintenance of smooth muscle cells by controlling media thickness (59). Finally, transcription of TGF-β mRNA increases following endovascular injury, and neutralizing antibodies directed towards TGF-β block

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the progression of neointimal formation, underscoring the pathological significance of this agent (60, 61).

TGF-β stimulates transcription of responsive genes first by binding its own receptors (Type I and Type II) on the cell surface (62). Binding of TGF-β to its Type II receptor stimulates phosphorylation of the Type I receptor (62). Receptor phosphorylation is followed by recruitment of Smad proteins to the receptor. The Smad family of proteins has eight members, Smad1 through Smad8. The Smads are categorized into three groups: receptor Smads, inhibitory Smads, and Co-Smads (63). Receptor Smads (Smads 1,2,3,5) are phosphorylated by the Type I receptor, and can subsequently bind a Co-Smad (Smad 4) in the cytoplasm (64). Inhibitory Smads (Smads 6 and 7) act as dominant negatives and prevent binding of R-Smads and Co-Smads (62). Active Smad complexes translocate to the nucleus and bind DNA response elements to enhance transcription of specific gene products (62). Regulation of transcription can be either positive or negative, as Smad complexes can bind both co-repressors or co-activators in the nucleus (63).

SIGNALING THROUGH INTEGRINS AND FOCAL ADHESION KINASE IN VASCULAR SMOOTH MUSCLE CELLS

Signaling Through Integrins

Communication between a cell and its outside environment is a critical element of proper patterning during vessel development. Integrins, the receptors for extracellular matrix (ECM) proteins, are important for both inside-out and outside-in signaling, in that they both regulate a cell's response to the ECM and they can influence the

polymerization state of the matrix. Integrins are transmembrane proteins forming heterodimers from a variety of α and β subunits. Several integrin subtypes are enriched in vascular smooth muscle cells, such as $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta_1$ (Reviewed by Rupp and Little (65)). Integrins are also necessary for vascular development, as knockout of several integrin subunits (α_5, α_v, and α_4) result in lethality prior to birth due to vascular defects (66-68). ECM components, such as fibronectin, vitronectin, laminin, collagen I and IV, and osteopontin are secreted from vascular cells and form a fibrillar network to which migrating and proliferating cells attach (69). The presence of several of these matrix proteins is necessary for development, as germline deletion of fibronectin, laminin, or collagen IV results in embryonic lethality in mice (70-73).

Binding of an ECM ligand to its appropriate receptor integrin triggers a host of downstream signaling pathways. Following integrin engagement, integrins cluster and cause intracellular phosphorylation of specific proteins. One such protein under close study is focal adhesion kinase (FAK) which is strongly activated by all $\beta_{1,3,5}$ integrin receptors.

Structure and Function of Focal Adhesion Kinase

FAK is a ubiquitously expressed non-receptor tyrosine kinase (125 kDa) present at focal adhesions (sites of cell contact to extracellular matrix). There are four major molecular domains of FAK that determine its function: an N-terminal integrin and receptor binding domain, a kinase domain, a proline rich domain containing binding sites for SH3 domains, and a C-terminal focal adhesion targeting (FAT) domain (illustrated in Figure 1.1) (74). Within FAK, there are six tyrosine residues that can be phosphorylated

(75). Among these six tyrosines, Y397 is critical for activation of FAK (76). This residue is auto-phosphorylated by at least one of several mechanisms, thus creating a binding site for a number of proteins that contain SH2 domains (75). FAK can be activated by signals from numerus extracellular stimuli, including ECM components, growth factors such as PDGF, and G-protein coupled receptor agonists such as angiotensin II (5, 77-80). FAK activation from these upstream signals can lead to subsequent activation of both the Rac-Pak-JNK pathway and the Ras-MEK-ERK pathway (81, 82).

FAK in the Vasculature

Although FAK is ubiquitously expressed, recent evidence has implicated its importance in smooth muscle cells for the processes of vessel development, pathological growth, and contraction. When FAK-deficient mice were generated by inserting a neomycin resistance gene into the beginning of the kinase domain, homozygous mice were unable to survive past embryonic day 8 (83, 84). While FAK-deficient embryos implanted and gastrulated normally, they exhibited growth defects in the anteroposterior axis and poor mesodermal development (84). Although vasculogenesis appeared normal prior to E7.5, the dorsal aorta and enteric arteries failed to develop properly after E8 (84). Our lab and others have shown that *in vitro*, there is evidence that activation of FAK is required for PDGF-BB stimulated migration and proliferation of VSMC, processes that are involved in the vascular injury response and pathological states such as atherosclerosis (78, 85). There is also evidence that implicates FAK in the regulation of VSMC contractility (86). Prior studies indicated that integrins on the cell surface of

VSMC can act as mechanotransducers and sense changes in stretch (87, 88). Additionally, FAK activation by phosphorylation at Y397 through treatment with angiotensin II has been observed specifically in VSMC (79). Furthermore, a role for FAK in regulating contractility is demonstrated that pre-treatment with an antibody to FAK inhibits integrin-mediated increases in Ca^{2+} current (89).

Within this body of literature, a role for FAK in several VSMC processes is emerging, and with it the possibility of unique regulation by its endogenous inhibitor, FRNK, discussed below.

FRNK: An Endogenous Inhibitor of Focal Adhesion Kinase

FRNK Acts as a Dominant Negative to Block FAK Activity

FRNK, a 43 kDa protein, is identical to the carboxy-terminus of FAK and is under the control of an alternative promoter within *fak,* discussed in greater detail below (90, 91). FRNK lacks the kinase domain and integrin and receptor binding sites, but contains proline-rich sites for SH3 domain binding. Since FRNK also still contains the focal adhesion targeting domain (FAT), it is able to localize to sties of focal adhesions (Figure 1.1) (90). It is thought that when FRNK is present at focal adhesions, it is able to compete for proteins that bind the C-terminus of FAK. Our lab, along with others, have demonstrated that FRNK can attenuate FAK activation by inhibiting phosphorylation at Y397 (78). It is interesting to note that following FAK activation by integrin binding to fibronectin, that FRNK is also phosphorylated, likely affecting FRNK's ability to bind other cellular proteins (90). In addition, phosphorylation of FRNK can occur at serine

residues 148 and 151 after activation of protein kinase A (92). More studies are required to address the mechanism by which FRNK regulates FAK activity.

FRNK is Specific to Vascular Smooth Muscle

Previous studies from our laboratory have revealed that FRNK is selectively expressed in vascular smooth muscle cells of large conduit arteries and in vascular and airway smooth muscle cells of the lungs (93). This selective expression of FRNK in VSMC is likely important in vascular injury and during development, since FRNK is upregulated during these processes (93). Specifically, levels of FRNK are low prior to surgeries and increase to robust levels 14 days post-endovascular injury in rat carotid arteries. FRNK levels return to baseline at 21 days, as smooth muscle cell proliferation decreases (93). Increased levels of FRNK protein are also observed between 4 and 14 days following birth in rat (93). Interestingly, these time points correlate with the transition of vascular smooth muscle cells from a proliferative to contractile phenotype, indicating that FRNK may serve to buffer FAK-dependent proliferative signals while enhancing vascular smooth muscle differentiation (94, 95). Indeed, our laboratory has found that ectopic overexpression of FRNK attenuates FAK-dependent PDGF-BB- and angiotensin II-stimulated VSMC proliferation (78).

FRNK Expression is Regulated by an Alternate Promoter within a FAK Intron

Previous work from Nolan and colleagues has shown that FAK activity is regulated by FRNK in a manner distinct from other tyrosine kinases (91). As illustrated

in Figure 2.1, FRNK is expressed as a gene within a gene. A FRNK-specific leader sequence and a region that exhibits promoter activity exist inside the *fak* intron immediately following the last exon of the kinase domain. Dependent upon organism, this *fak* intron ranges in length from 15 to 21 kb. RNase protection assays demonstrate that there are four FRNK RNA species expressed in abundance in FRNK-expressing cell types (91).

Recently, Hayasaka, *et al.* demonstrated that a construct containing 6 kb of the mouse promoter region (-5338 to +730, relative to first nucleotide of the FRNK 5' leader exon) drives arterial smooth muscle specific expression of a lacZ reporter gene *in vivo* (96). LacZ expression was observed in major conduit arteries, including the pulmonary artery and aorta, and in resistance vessels of the lung, stomach, intestine, brain, and kidney (96). This transgenic mouse also exhibited increased promoter activity in the medial layer of the carotid artery 14 days after endovascular injury (96). Finally, the authors identified a ~100 bp sequence within the FRNK promoter that was necessary for luciferase expression in differentiated P19 cells (96). This sequence is responsive to AP-1 and NF-E2 transcription factors and is identified here in Chapter II as a highly conserved element within the FRNK promoter.

Expression of FRNK is Independent of Serum Response Factor

A search for *cis* elements conserved from chicken to human did not reveal any conserved CArG elements. However, a non-canonical CArG element was identified 75 bp upstream of the mouse FRNK initiation site. A similar variant has been identified in other smooth muscle specific genes, however usually more than one CArG box is

required to confer specificity, (47). My studies described in this chapter indicate that smooth muscle-specific FRNK expression is a CArG-independent event.

Other DNA response elements have been implicated in regulating smooth muscle specific expression in concert with the CArG element. Mutation of the TGF-β control element [TCE; G(A/C)GT(T/G)GG(T/G)GA)] disrupts expression of smooth muscle α-actin and SM-22, as does mutation of E-box elements (CAnnTG motifs) (97-99). Aortic carboxypeptidase-like protein (ACLP) is an example of a smooth muscle-specific gene that is CArG-independent (100). ACLP exhibits an expression profile similar to that of FRNK, with protein levels increasing in VSMC after carotid artery ligation, while canonical smooth muscle genes decrease (100). ACLP is also SRF-independent; rather, its promoter is activated by transcription factors Sp1 and Sp3 (100). VSMC-specific expression of cysteine-rich protein 2 (CRP2) is dependent upon an 800 bp region of its promoter that is CArG-independent (101). Unlike ACLP, this region of the CRP2 promoter appears to be independent of Sp1 and Sp3 (101). Interestingly, CRP2 expression seems to be limited to arterial smooth muscle, much like promoter activity of the 6kb FRNK lacZ promoter mouse (96, 101). Recently, Lin, *et al.* showed that expression of CRP2 in vascular smooth muscle cells is dependent on TGF-β mediated activation of ATF2 (102). Although the histidine-rich calcium-binding protein (HRCBP) is expressed in all three muscle types (skeletal, cardiac, and smooth) it constitutes another example of a smooth muscle-selective protein expressed in a CArG-independent fashion (103). Expression of HRCBP in calciosomes of smooth muscle cells is dependent on a highly conserved myocyte enhancer factor 2 (MEF2) site within the promoter (103). Other CArG-independent smooth muscle genes include Notch3 and APEG1 (104). My

efforts to identify the mechanisms that regulate expression of FRNK are detailed in Chapter II.

STATEMENT OF PURPOSE

Since *in vivo* models have demonstrated that signaling through FAK is essential for development of the vasculature, it is necessary to study the specific FAK-mediated processes that allow proper smooth muscle cell growth. This work provides evidence that FAK signaling is important in regulating smooth muscle cell phenotype *in vivo*.

Prior to this work, little was known about the mechanisms that regulate FRNK expression in smooth muscle cells. Within Chapter II, I describe my analysis of the FRNK promoter, within which I identified matrix components that regulate FRNK expression. In addition, my study of the promoter revealed conserved elements that confer smooth muscle specificity. In addition, since my analysis of the FRNK promoter has indicated that expression of FRNK is dependent on transcription factors other than SRF, this work begins to assess the novel smooth muscle specific transcriptional mechanisms that regulate FRNK expression.

Our lab has previously shown that FRNK is capable of attenuating FAK-dependent migration and proliferation in smooth muscle cells, similar to its behavior when ectopically expressed in other cell types. This work specifically addresses the role of FRNK *in vivo*, which beforehand was largely unknown. Herein, I have tested the hypothesis that FRNK is a negative regulator of focal adhesion kinase, and that expression of FRNK in smooth muscle cells acts to decrease smooth muscle cell

proliferation and migration while concurrently promoting smooth muscle cell differentiation. Indeed, the studies described here utilize *in vivo* and *in vitro* models and show that in the absence of FRNK, smooth muscle gene expression is attenuated

Germline deletion of FAK results in early embryonic lethality, as described previously. Since vasculogenesis is incomplete at this point, an alternative model was needed to characterize the role of FAK in development of the vasculature. Within Chapter IV, I characterized the role of FAK in formation of the cardiac outflow tract and in aorticopulmonary septation. Since FAK has been implicated as a regulator of differentiation in other cell types, we hypothesized that would be true in cardiac neural crest-derived smooth muscle cells.

In general, this study contributes to the understanding of FAK and FRNK in regulating smooth muscle cell processes.
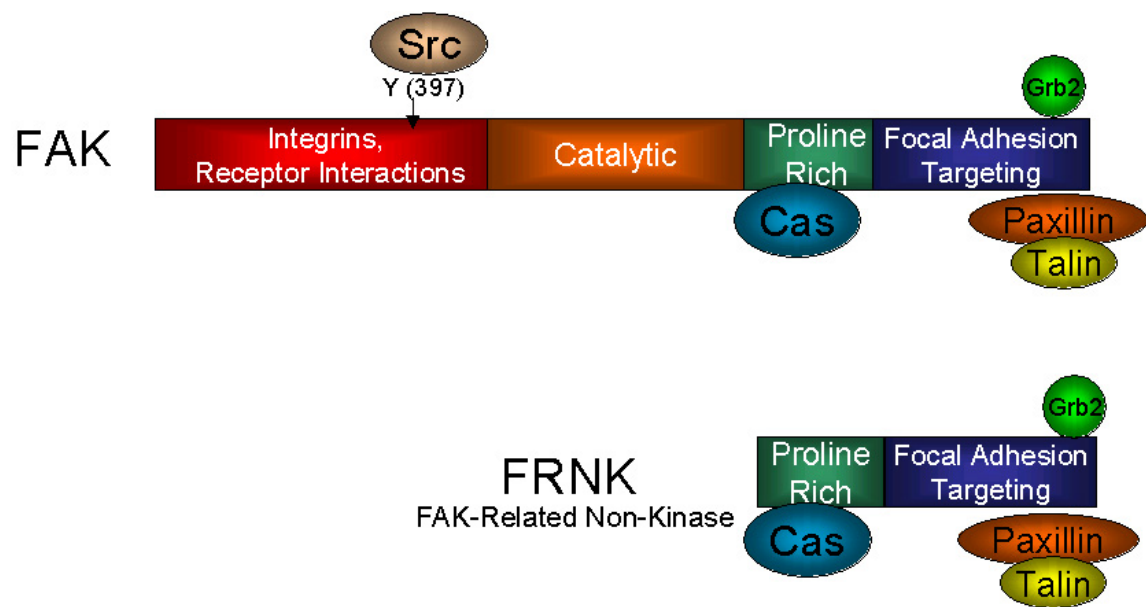
Figure 1.1. Focal adhesion kinase domains.

CHAPTER II

ANALYSIS OF THE FRNK PROMOTER

INTRODUCTION

Previous studies have demonstrated that FRNK is expressed as a unique protein product from the *fak* gene, under the control of an internal promoter (91). Within exons 21 and 22 of the *fak* intron (15 to 21 kb, dependent on species) immediately following the last exon of the kinase domain, there is a FRNK-specific leader sequence only observed in FRNK cDNA. Adjacent to this leader sequence is a 5' non-coding region exhibiting promoter activity. Our group recently showed that FRNK expression is limited to smooth muscle tissues and is regulated temporally both during development and following injury in the vasculature (93). Nolan, *et al.* showed that a 2.4 kb region of the chicken *fak* intron is capable of directing luciferase reporter expression in chicken embryonic fibroblasts (91). Likewise, Hayasaka and colleagues demonstrated that a construct containing 6 kb of the mouse promoter region (-5338 to +730, relative to first nucleotide of the FRNK 5' leader exon) drives arterial smooth muscle specific expression of a LacZ reporter gene *in vivo* (96). The authors observed LacZ expression in major conduit arteries including the pulmonary artery and aorta and in resistance vessels of the lung, stomach, intestine, brain, and kidney (96). This promoter-reporter transgenic mouse also exhibits increased LacZ expression in the medial layer of the carotid artery 14 days post-endovascular injury (93, 96). These results were consistent with our own *in vivo* studies and demonstrate that a 6kb region of the *fak* intron is required for smooth muscle-specific expression of FRNK.

The study of smooth muscle specific gene expression has most closely examined the role of serum response factor (SRF) in regulating transcription of contractile genes. Expression of genes such as SM α-actin and SM-myosin heavy chain require the binding of SRF to one or more CArG elements within their promoter, as mutation of the CArG

motif ($CC(AT)_6GG$) results in disruption of expression (38, 42-47). In the smooth muscle α-actin gene, mutation of any of the three CArG elements present within the promoter resulted in no activity in a transgenic LacZ reporter mouse (43). This is also true of mutations made to CArG elements within the SM-22, SM-MHC, and desmin promoters (42, 44, 45, 48). Recent data indicates that smooth muscle-specific, SRF-dependent gene expression is likely regulated by binding of coactivators to SRF, including myocardin and mycoardin-related transcription factors-A and –B (MRTF-A and -B) (105-107). It is also interesting to note that even though CArG-dependent smooth muscle specific expression is well described, recent work has determined that myocardin, while required for complete maturation of smooth muscle cells, is not sufficient for smooth muscle cell specification (50). Therefore, elements responsible for smooth muscle-specific expression are still at large.

Other DNA response elements have been implicated in regulating smooth muscle specific expression in the absence of the CArG element. Independent of SRF binding, the ACLP promoter is activated by transcription factors Sp1 and Sp3 (100). In addition, cysteine-rich protein 2 (CRP2) is also CArG-independent and exhibits expression patterns similar to those seen with FRNK (101). VSMC-specific expression of CRP2 is dependent upon an 800 bp region of its promoter and unlike ACLP, this region of the CRP2 promoter also appears to be independent of Sp1 and Sp3 (101). Interestingly, CRP2 expression seems to be limited to arterial smooth muscle, much like the 6kb regions of the FRNK promoter (96, 101). Even though a collection of CArG-independent mechanisms for smooth muscle-specific gene expression has emerged from

the literature, our preliminary evidence has indicated that these mechanisms do not play a role in regulation of FRNK promoter activity.

For several reasons, we wanted to explore further what regulates FRNK expression: 1) FRNK is highly expressed during postnatal development and following injury and 2) FRNK is selectively expressed in smooth muscle cells with the absence of conserved CArG elements. Thus, we used the FRNK promoter to determine potential regulators. In addition to identifying matrix proteins and growth factors that regulate FRNK expression levels, here we have also identified highly conserved promoter elements and characterized their response to the differentiation agonist TGF-β. Finally, we used a recently described mammalian-cell based assay to screen a mouse cDNA library for potential regulators of the FRNK promoter (108).

MATERIALS AND METHODS

Cell Culture, Transfections, and Reporter Assays

Aortic vascular smooth muscle cells were cultured from rats using enzymatic digestion (34). Aortas from 6-8 rats were dissected and stripped of adventitial fat. Following enzymatic digestion, the adventitia and endothelium were stripped from each vessel. After a second enzymatic digestion, cells were maintained in Delbucco's modified eagle medium with F12 supplemented with 10% fetal bovine serum and 0.5% penicillin/streptomycin and used from passage 8-16. Cos, NIH-3T3, 10T1/2, and A7r5 cells were obtained through ATCC and were maintained in Delbucco's modified eagle medium supplemented as above. SRF$^{-/-}$ cells were a generous gift from Albert Nordheim and maintained on 0.1% gelatin coated plates in DMEM plus 15% ES Qualified FBS, 50U/mL penicillin, 50μg/mL streptomycin, 0.1mM β- mercaptoethanol, and 1000U/mL Leukemia Inhibitory Factor (LIF).

Matrigel (Becton-Dickinson) was diluted 1:3 in ice cold phosphate buffered saline, applied to pre-chilled plastic tissue culture plates, and allowed to polymerize at 37°C for one hour. Fibronectin (Sigma) was diluted in phosphate buffered saline to 10μg/mL and coated on plates overnight at 4°C. Cells were treated with TGF-β (Chemicon) at 1 ng/mL.

For overexpression, constructs were transfected into cells using GeneJammer (Stratagene), SuperFect (Qiagen), or TransIT-LT1 (Mirus) transfection reagent according to the manufacturer's instructions. Following transfection, cells were lysed in Glo Lysis Buffer and assayed using Steady Glo Luciferase Assay system (Promega) as directed by the manufacturer.

Real-Time Quantitative RT-PCR

Cells were lysed in TRIzol (Invitrogen) and total RNA was isolated accoroding to the manufacturer's protocol. Expression was measured using the ABI Prism 7700 or 7500 TaqMan system. Primers and fluorescent probes were designed for the following genes: FRNK (Forward: GCTGCATTCTGAGGCGTTTA, Reverse: CAGGATTGTGCACCACCAG, Probe: AGCCAGGACTGAGACGCCGCC) and FAK (Forward: GAAAGCAGTAGTGAGCCAACC, Reverse: GAGACTGTCCACTATCTTCTG, Probe: CTCCATGCCTGATAATACTGGCCCAG). 18S primers and probes were a generous gift from Hyung-Suk Kim.

Western Analysis

To examine protein levels, cells were lysed in RIPA buffer with protease and phosphatase inhibitors as previously described (78). Protein concentration of lysates was determined by using a colormetric BCA assay (Pierce). Lysates were electrophoresed on an 11% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with an antibody directed towards the c-terminus of FAK (Upstate).

Generation of Constructs

To generate deletion mutants, PCR mutagenesis was utilized. Briefly, forward and reverse primers were designed starting approximately 20 bp preceding and following the deletion site, excluding the deletion site. PCR was performed using cloned *Pfu* and guidelines from the Stratagene Quickchange mutagenesis kit. To generate TK-luciferase

and β-galactosidase constructs, primers were designed to add either *BglII* or *KpnI* 5' or 3' overhangs to PCR amplicons corresponding to the DNA region of interest. TK-luciferase or *NotI*LacZ was cut at the multiple cloning site with *BglII* and *KpnI* and re-ligated with the PCR amplicons.

Mammalian Cell Screen

Our screen of mammalian cells for activation of PeakA/B-luciferase was modified from an assay previously described by Chang, *et al* (109). We obtained an E13.5 mouse cDNA library from Invitrogen and amplified the library on approximately one hundred 150mm plates. Colonies were allowed to grow overnight at 25°C and were subsequently washed off the plates and pooled in LB broth. DNA was prepared by using a GigaPrep kit (Sigma). Following resuspension, DNA was divided into approximately 900 aliquots. 300 ng of each aliquot was singly transfected in to Cos cells plated in 24 wells along with 150 ng of PeakA/B-luc and 50 ng of SV40-β-gal (internal control). Each well was assayed 24 hours following transfection for luciferase and β-gal activity (first round). After normalization to β-gal activity and empty vector, those aliquots which fell in the top 10th percentile for activity were retransfected in triplicate. From this round, ten aliquots were chosen that exhibited the most significant increase in promoter activity (second round). These pools were retransformed, and DNA was isolated by alkaline lysis and isopropanol precipitation from 24 separate pools of approximately 20 colonies for each aliquot. This DNA (third round), was subsequently transfected into Cos cells and assayed as described above. Those pools that exhibited significant activity were retransformed. Individual colonies were selected, cultured, lysed for isolation of DNA.

The DNA from these individual colonies was transfected into Cos cells and assays were performed as above. The 95 colonies with the most significant increase in promoter activity were selected for high-throughput DNA sequencing (UNC Genome Anlaysis Facility). The sequences were then compared to the entire mouse genome (using NCBI Blast) for identification.

RESULTS

FRNK promoter activity is specific to smooth muscle cells and independent of activation by serum response factor

Previous work showed that vascular specific expression of FRNK is under the control of a *fak* intron, diagrammed in Figure 2.1A (91, 96). To investigate what factors regulate transcription of FRNK, we utilized a promoter-reporter construct in which 6kb of the FRNK promoter drives expression of luciferase. Since we previously reported that FRNK expression is specific to smooth muscle containing tissues in the rat (93), we chose to investigate the activity of this promoter in cell types with varying degrees of likeness to primary cultured smooth muscle cells. In comparison with other cell types, primary cultured vascular smooth muscle cells (VSMC) exhibited promoter activity over twenty-fold that of 3T3 fibroblasts (Figure 2.1B). Sequence analysis revealed that the FRNK promoter did contain a nearly conserved CArG element. To test whether the FRNK promoter activity was dependent upon binding of SRF, we measured the activity of the FRNK promoter in SRF$^{-/-}$ embryonic stem cells. In contrast to the smooth muscle α-actin promoter, the activity of the FRNK promoter was not upregulated in response to re-expression of wild type SRF (Figure 2.1C).

Expression of FRNK is regulated by components of the extracellular matrix

Since we established that FRNK expression is limited to smooth muscle tissues and is regulated during development and injury, we sought to identify distinct factors that control expression. In addition to testing the effects of various growth factors on FRNK expression (TGF-β, Angiotensin II, PDGF, described in chapter III), we also looked at

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the ability of extracellular matrix proteins to regulate FRNK expression. After 24 hours, VSMC plated on Matrigel had significantly lower levels of FRNK promoter activity in comparison with the uncoated control (Figure 2.2A). Likewise, we also observed decreased levels of FRNK mRNA in VSMC plated on either type of Matrigel for 24 hours (Figure 2.2B). Using an antibody specific for the C-terminus of FAK that detects both FAK and FRNK, we found that VSMC plated on Matrigel exhibited decreased levels of FRNK protein starting at only 12 hours post-plating. By 48 hours, FRNK protein was almost completely absent (Figure 2.2C). In subsequent experiments, we sought to identify the specific elements within the extracellular matrix that were responsible for the observed decrease in FRNK expression. Promoter assays in which cells have been plated on specific ECM components revealed a small, yet consistent, decrease in FRNK promoter activity in response to laminin, but no response to collagen IV or fibronectin (data not shown).

The FRNK promoter contains two highly conserved regions that are necessary and sufficient for smooth muscle-specific activity

Our earlier studies demonstrated robust changes in FRNK expression during development and following vascular injury. Although our initial experiments revealed extrinsic modulators of FRNK expression, we have not yet identified a transcription factor capable of increasing FRNK expression to the level seen following vascular injury. Therefore, we employed a method of conservation analysis to identify regions of the FRNK promoter that may have particular importance in regulating smooth muscle-specific expression of FRNK. Using VISTA, a web-based sequence analysis tool, we aligned the FAK intron that contains the FRNK-specific exon from mouse, rat and human

(110, 111).   This analysis revealed two regions over 75% conserved (termed Peak A and Peak B) that are contained within the 6kb region of the FRNK promoter under study (Figure 2.3A). Peak A is approximately 220 bp in length and appears as a doublet, while Peak B is only 130 bp in length.   As expected, high levels of conservation were also observed between the FRNK-specific exon and the first exon that FRNK and FAK have in common. An additional conserved region was identified further away from the FRNK start site that is not contained within the 5kb promoter region of interest (data not shown).

To determine if these highly conserved regions were necessary for regulation of FRNK expression, I designed a series of promoter-reporter constructs in which the conserved regions identified from the VISTA screen were deleted from our 6kb promoter fragment (Figure 2.3B). In addition, we also generated promoter-constructs in which the highly conserved regions were used to drive expression of luciferase on their own. Likewise, we generated a construct in which both regions were cloned together in tandem into one promoter-reporter (Figure 2.3B).

To test whether these conserved elements were necessary for smooth muscle specific FRNK promoter activity *in vitro*, I transfected NIH-3T3 fibroblasts, bovine endothelial cells, and primary cultured aortic VSMC with each of the aforementioned constructs (Figure 2.4A).   In NIH-3T3 cells, the FRNK promoter had relatively low activity which was mostly unchanged with deletion of either Peak A, Peak A1, Peak A2, or Peak B. In contrast, smooth muscle cells showed robust activation of the wild type FRNK promoter, and this activity was significantly reduced by deletion of any one of the conserved regions. Bovine endothelial cells had relatively low levels of wild type FRNK

promoter activity, but with deletion of Peak A1, Peak A2, or Peak B, promoter activity was significantly increased (Figure 2.4A).

I also demonstrated that these conserved elements can drive luciferase expression on their own in VSMC. Transfection of constructs containing each element combined with a minimal promoter (TK) resulted in luciferase activity 6-12 fold over control. Combination of Peak A and Peak B in the Peak A/B constructed resulted in promoter activity that was additive of each region alone (Figure 2.4B).

Since the Peak A/B construct appeared to be sufficient for VSMC-specific expression in culture, we next wanted to determine if that was true in an *in vivo* model. We generated transgenic *Xenopus laevis* that expressed dsRed under the control of PeakA/B. Expression during early stages prior to smooth muscle investment was obsereved in the heart and somites (data not shown). To test whether these elements were sufficient for vascular-specific expression in a mammalian model, we generated several lines of transgenic mice containing a transgene that contained Peak A and B in tandem driving expression of β-galactosidase (Figure 2.4C). Unfortunately to date, we have been unable to show that any of the 7 founder lines we generated expresses the transgene, indicating that these elements are likely insufficient for complete promoter activity in the vasculature.

Conserved FRNK promoter elements are not responsive to TGF-β

Other studies on the FRNK promoter (described in Chapter III), have shown that in 10T1/2 cells, FRNK promoter activity is increased by approximately 2-fold in response to treatment with TGF-β. Although this response is relatively modest,

endogenous mRNA and protein exhibited greater response to TGF-β, possibly due to additional TGF-β responsive elements outside of the 6kb promoter construct. To test whether or not the conserved elements we identified were responsible for this response, we transfected the deletion constructs and minimal promoter constructs into 10T1/2 cells and treated these cells with TGF-β (1 ng/mL). 24 hours following treatment, we observed the expected two-fold increase in wild type promoter activity in response to TGF-β (Figure 2.5A). Although it was not significant, activity of the mutant promoters still appeared to increase (Figure 2.5A). In addition, we also transfected the minimal promoter constructs containing Peak A and/or Peak B and found that on their own, none of these constructs showed any response to TGF-β (Figure 2.5B). Taken together, these data indicate that the TGF-β responsive elements within the FRNK promoter are not located within the conserved regions we identified. At this point, our data does not clearly indicate whether this response is dependent on Smad activity or on other known downstream pathways such as p38 or ATF2.

An important element lies within the 5' end of the FRNK promoter

We also chose to look for responsive elements by making serial truncations of the FRNK promoter (Figure 2.6A). In VSMC, we found that deletion of the first 1000 bp of the 5kb FRNK promoter resulted in no significant change in promoter activity, but that subsequent truncations caused a steady decline in luciferase expression (Figure 2.6B). It will be interesting to test whether any of the truncations cause a differential response to TGF-β.

***Identification of possible activators of the conserved elements of the FRNK promoter
using a luciferase-based mammalian cell screen***

Our studies revealed that the conserved regions of the FRNK promoter that we
identified from the VISTA screen were necessary and sufficient for smooth muscle
specific activity *in vitro*, but that these elements were not responsive to TGF-β. In an
attempt to determine what transcriptional coactivators or upstream signals are responsible
for the inherent promoter activity of these conserved elements, we screened a cDNA
library made from E13.5 mouse embryo for proteins that resulted in increased activation
of Peak A/B-luciferase. Figure 2.7 shows representative results from the screen.
Following *sib*-selection of individual clones, we sequenced positive scoring clones. A
table of identified clones that activated the promoter is shown in Table 2.1.

Our analysis of the FRNK promoter has provided several findings regarding its specific activity in smooth muscle cells. Previous work indicated that FRNK expression was differentially controlled in smooth muscle cells during vascular development and also in the response to guide-wire mediated injury of the carotid (93). Prior analysis of the FRNK promoter yielded a 6kb region that resulted in vascular specific expression (91, 96). In this study, we were able to (1) identify specific factors that down-regulate FRNK expression, (2) isolate small, highly conserved regions of the FRNK promoter that are necessary and sufficient for *in vitro* smooth muscle-specificity, and (3) screen for potential activators of the FRNK promoter.

As discussed previously, the expression of most smooth muscle genes has been well described, and is thought to be under the control of SRF binding to CArG boxes embedded within the promoters. The FRNK promoter does contain an unconserved CArG-like element and other non-consensus CArG boxes have shown to be responsive to SRF. We found that re-expression of SRF in SRF$^{-/-}$ embryonic stem cells did not induce the FRNK promoter, while SRF-dependent activity of the SM a-actin promoter increased by over 10-fold. This confirmed that indeed, the FRNK promoter did not rely on this mechanism of smooth muscle-specific gene expression.

In 2003, Walker, *et al.* published results indicating that both Matrigel and perlacan, a heparin sulfate proteoglycan had the ability to upregulate levels of FRNK promoter activity and FRNK protein levels (112). The authors observed increases in FRNK protein in smooth muscle cells plated on Matrigel for four hours in comparison to cells plated on fibronectin (112). This group also looked at the activity of a portion of the

FRNK promoter and found that in comparison to the control, it was more active in A10 smooth muscle cells plated on perlecan or Matrigel (112). However, the construct used in their experiment was not the entire portion shown by Hayasaka, *et al.* to direct vascular specific expression, but a smaller 2.4 kb portion that exhibits promiscuous activity in fibroblasts (91, 96). In our study, we looked at levels of FRNK promoter activity, mRNA, and total protein in response to plating on Matrigel in comparison to tissue culture plastic. We found that in general, plating smooth muscle cells on Matrigel for an extended amount of time resulted in decreased FRNK promoter activity and expression (Figure 2.2). Preliminary data from luciferase reporter assays also indicate that this may be due to the high concentration of laminin present in Matrigel (data not shown). Our results support our hypothesis that FRNK expression is regulated by the environment of smooth muscle cells in vessels. The composition of Matrigel (mostly laminin and collagen IV) mimics the environment of a mature, quiescent vessel. We have proposed that FRNK expression is upregulated during injury and development to act as a negative regulator of proliferative signaling through FAK. In a mature vessel, FRNK expression would likely be unnecessary, since smooth muscle cells are mostly quiescent and their rate of proliferation is too low to require regulation. Therefore, we were not surprised that primary cultured smooth muscle cells (with a tendency to be highly proliferative), gradually showed decreased expression of FRNK protein in response to the matrix environment. This hypothesis is consistent with other results discussed herein that show increased promoter activity, mRNA, and protein levels in response to TGF-β. TGF-β is an agonist involved in differentiation of smooth muscle cells and is highly expressed in the vasculature during development and following vascular injury. Early vascular

development and injury repair are distinct times in which vascular smooth muscle cells are encouraged by extracellular cues to proliferate and it is after these times that we hypothesize FRNK is being turned on to act as a negative regulator of smooth muscle cell proliferation, perhaps to aid in transition to a mature, contractile phenotype.

Although our findings that FRNK promoter activity is modulated by extracellular matrix and growth factors are significant, they do not fully detail the regulation of the FRNK promoter. For this reason, we chose to analyze the full intronic region of the *fak* gene containing the FRNK promoter to identify the most highly conserved regions. Other groups have taken a similar approach and have identified novel CArG-independent mechanisms of smooth muscle-specific expression (103). We identified two separate regions that were over 75% conserved between mouse, rat, and human (Figure 2.3). Among these two regions, Peak B had been previously identified by truncation analysis of the promoter by Hayasaka, *et al.* (96). In addition, they also showed that mutation of this region attenuated binding of AP-1 and NF-E2 enhancers (96). Our studies have demonstrated that the two elements we identified, Peak A and Peak B, are necessary and sufficient for smooth muscle specific promoter activity *in vitro* (Figure 2.4). We found that deletion of either element had a dramatic effect on promoter activity. When we looked at the inherent promoter activity of each of these regions individually in smooth muscle cells, we found that stringing Peak A and B together in tandem to drive luciferase expression resulted in an additive increase in activity (Figure 2.4). Interestingly, we also found that deletion of certain portions of the conserved elements resulted in increased promoter activity in bovine endothelial cells, indicting that these regions may have repressor activity in non-smooth muscle cell types (Figure 2.4A).

We next strove to determine if these elements regulate *in vivo* expression of FRNK. Our initial attempts at answering this question have been unsuccessful thus far. To study whether these elements were sufficient to drive vascular specific expression of LacZ in the mammal, we generated a transgenic promoter reporter mouse model. The transgene we designed contained Peaks A and B in tandem directing expression of LacZ. Although we had seven mouse lines that contained the transgene, we were unable to determine if any of these founder lines actually expressed LacZ or if we were unable to detect LacZ. More intricate experimental design will be required to develop additional transgenic models that will more directly answer this question. Specifically, an approach that determines whether these elements are necessary for smooth muscle-specific expression of FRNK will likely be successful. Future experiments will involve generating transgenic mice that contain the entire 6kb FRNK promoter driving LacZ expression with each conserved region deleted from the promoter.

During our study of the promoter, TGF-β emerged as a growth factor that increases FRNK expression. Our results indicated that deletion of either conserved element within the FRNK promoter does not alter the response to TGF-β. Our preliminary studies suggest that Activity of the promoter also appears to be independent of the Smad signaling pathway (data not shown). Analysis of the FRNK promoter for other published consensus sites for alternative downstream targets of TGF-β has not yielded any possibilities for this mechanism. To delineate other regions of the FRNK promoter that is are smooth muscle-specific, we truncated the FRNK promoter from the 5' end approximately 1 kb at a time. With deletion of the first 1950 bp, we did see a

significant decrease in promoter activity. Ongoing studies include further analysis of this 950 bp region to determine whether it is responsive to TGF-β.

Since our studies have not revealed what mechanism regulates the conserved regions within the FRNK promoter, we undertook a major effort to uncover novel regulators of these elements. We utilized a mammalian cell line and a widely used and highly repeatable assay system to screen an E13.5 mouse cDNA library for potential coactivators and enhancers of the FRNK promoter. From this screen, we were able to generate a list of genes that activated identified conserved elements (Table 2.1). However, we were surprised that within the list of known genes from our screen, no known transcription factors were identified. One caveat of this study was that all assays were performed in Cos cells, a cell line that most likely exhibits transcriptional signaling pathways differing from vascular smooth muscle cells. There were several unknown genes in the screen that repeatedly activated the Peak A/B construct nearly 20-fold. As more information in the mouse genome becomes available, it will be valuable to research the database for possible matches for these genes.

In conclusion, our analysis of the FRNK promoter has developed multiple paths for future study of smooth muscle-specific regulation of FRNK. This work has shown that regulation of the FRNK promoter is quite complex and not only involved regulation by known matrix proteins and growth factors, but possibly contains unique, unstudied elements that may lead to the discovery of novel mechanisms of CArG-independent smooth muscle-specific gene expression.

Table 2.1: Selected enhancers of Peak A/B-luc identified from screen.

Name of Gene	Fold Increase
ARP2 actin-related protein 2 homolog	>7
Isocitrate dehydrogenase 3 (NAD+) alpha	>14
Secret frizzled-realted protein 2	>17
RIKEN cDNA 2610203C20 gene	>13
LMBR1 domain containing 1	>18
Tubby candidate gene	>18
Suppressor of zeste12 homolog	>15
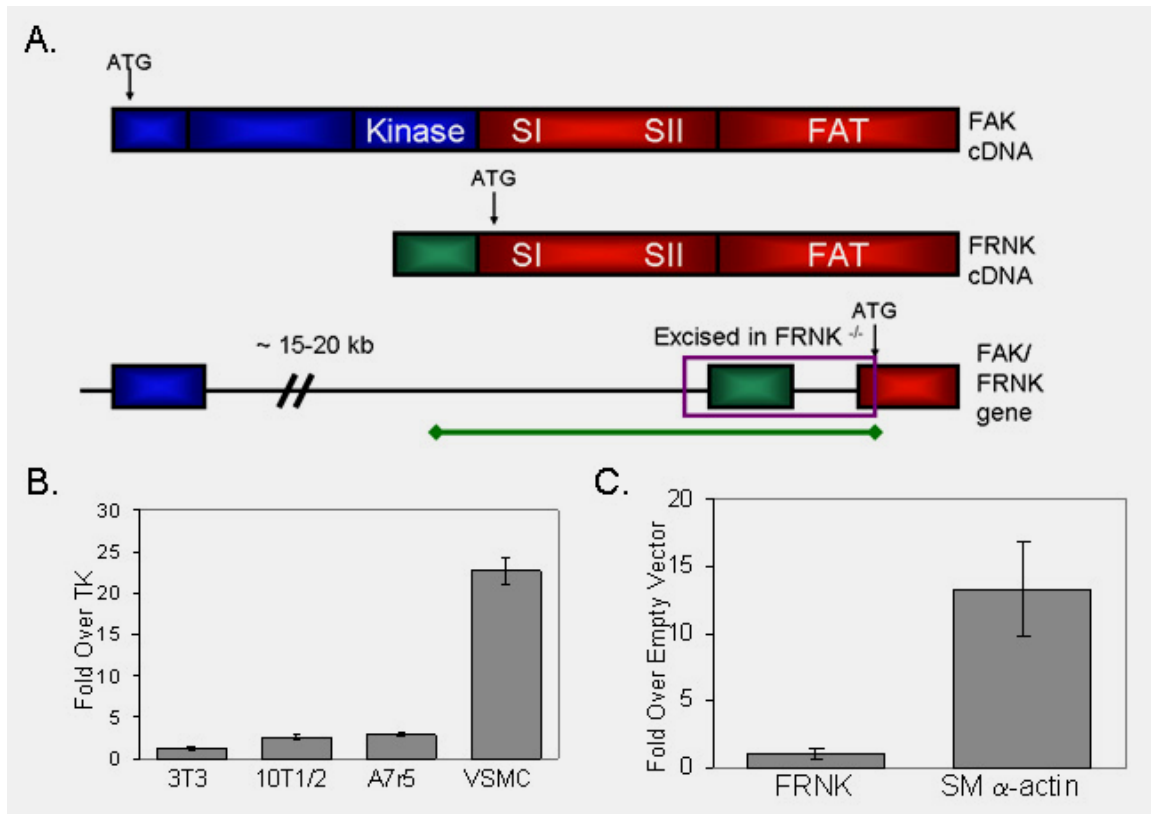
Figure 2.1. An intronic region of FAK contains smooth muscle-specific, CArG-independent FRNK promoter activity. A. Schematic showing that a FRNK specific exon lies within an intron of the FAK gene. The blue regions indicate those exons contained by FAK alone, the green region indicates the single FRNK-specific exon, and the red region indicated the portion of the gene that FAK and FRNK share. A 6kb region containing the FRNK-specific exon (shown at bottom of panel) is sufficient to regulate smooth muscle-specific expression *in vivo*. A reporter plasmid expressing luciferase under the control of the before-mentioned 6kb piece is used to assay promoter activity in VSMC. **B.** Activity of the 6kb FRNK promoter-luciferase construct in NIH-3T3 fibroblasts, 10T1/2 smooth muscle precursor cells, A7r5 cells, and primary cultured vascular smooth muscle cells (VSMC) normalized to minimal promoter activity. **C.** Activity of the FRNK promoter or smooth muscle α-actin promoter in SRF$^{-/-}$ cells in response to addition of recombinant SRF. Data is representative of at least 3 separate experiments and is expressed as the mean +/- SEM.
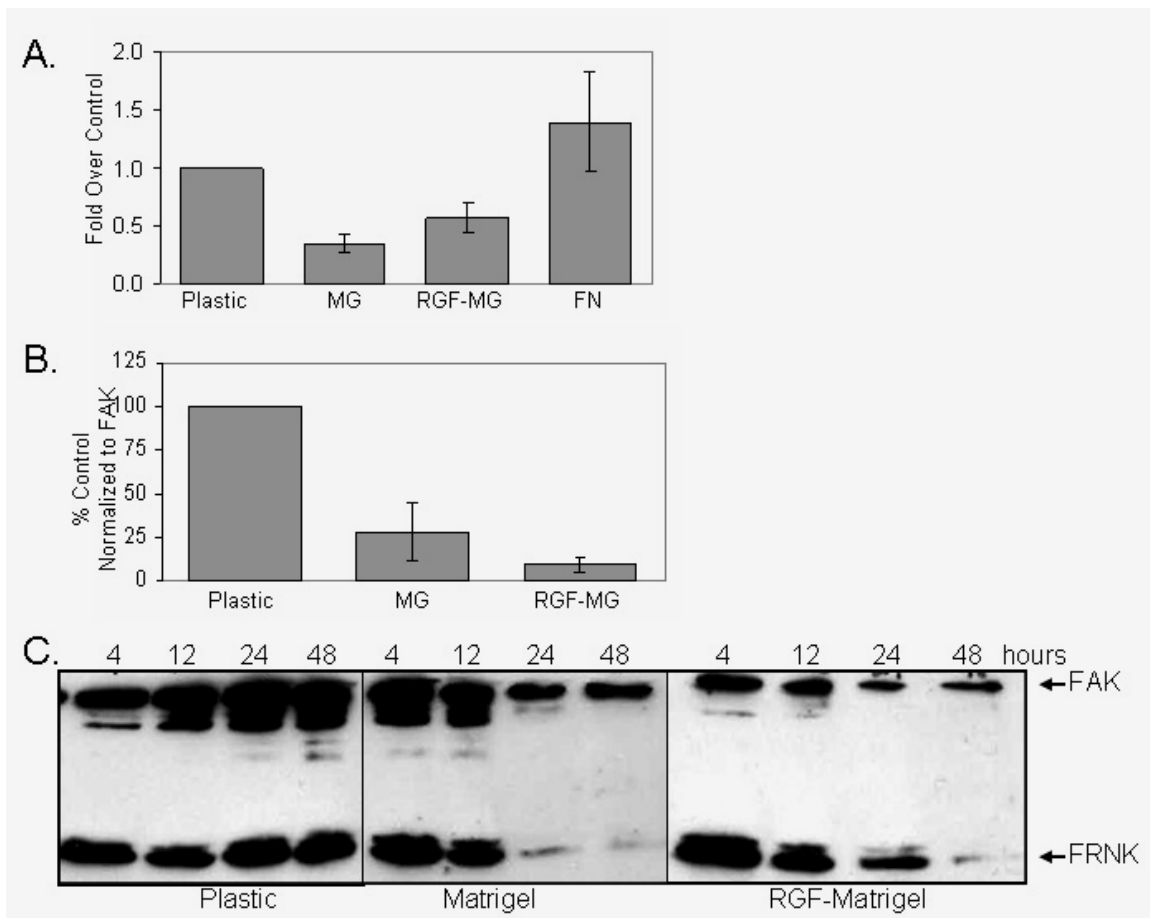
Figure 2.2. VSMC plated on Matrigel exhibit reduced expression of FRNK. A. Primary VSMC were transfected with FRNK-luc for 24 hours and subsequently trypsinized and plated on tissue culture plastic, Matrigel (MG), reduced growth factor Matrigel (RGF-MG), or fibronectin (FN) for 24 hours in serum-free media. Cells were assayed for luciferase activity and data was normalized to control (plastic). **B.** VSMC were maintained in serum-free media plated on either plastic, Matrigel, or growth factor reduced Matrigel for 24 hours. Total RNA was isolated from resuspended cells and 250 ng was subjected to real time quantitative RT-PCR with primers and probes specific for the FRNK specific exon. **C.** VSMC were harvested and plated for 4, 24, and 48 hours on plastic dishes or dishes pre-coated with Matrigel or reduced growth factor Matrigel. The cells were lysed in RIPA buffer and protein content was determined. 100 μg of protein was analyzed by gel electrophoresis followed by Western blotting with a C-terminal FAK-specific antibody.

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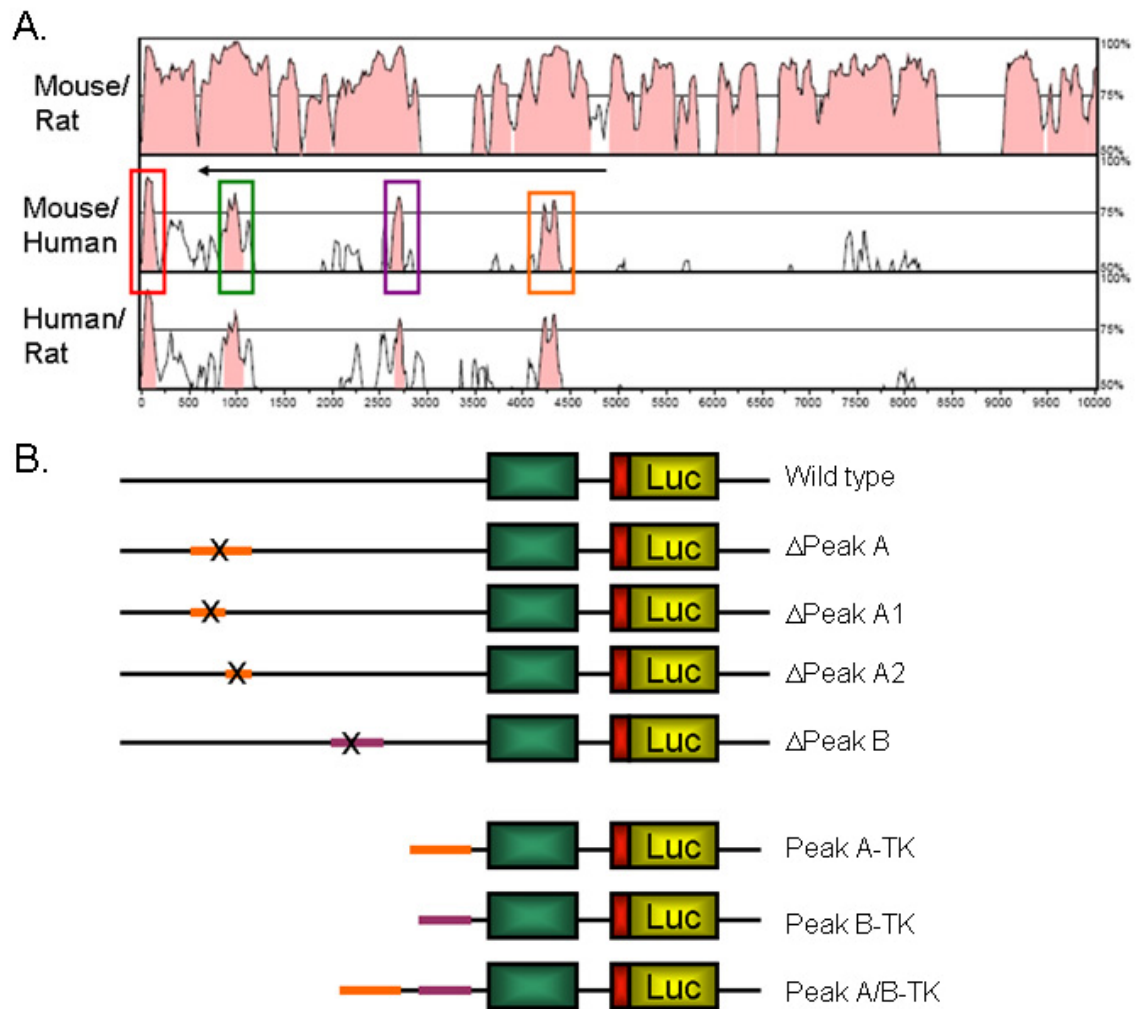
Figure 2.3. The FRNK promoter has two highly conserved regions. A. VISTA screen for homology within the FRNK promoter between mouse, rat, and human. The region outlined in red is the exon common to both FRNK and FAK, green indicates the FRNK-specific exon, and regions in orange and purple are peaks over 75% homologous in human/mouse and human/rat alignments. Pink shading indicates >75% homology. **B.** FRNK promoter constructs with deletion/addition of Peaks A and B.
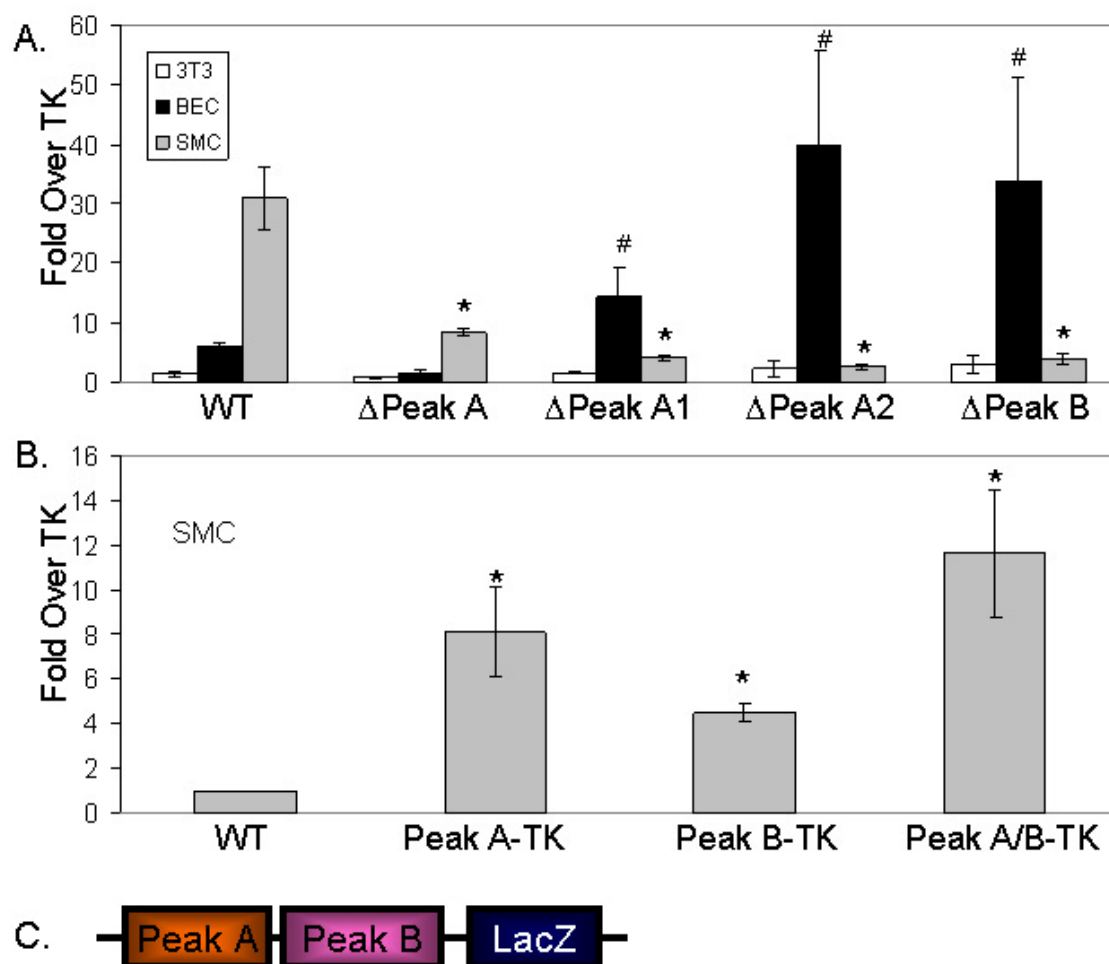
Figure 2.4. Conserved regions of FRNK promoter are necessary and sufficient for smooth muscle specific FRNK-promoter activity *in vitro*. A. NIH-3T3, bovine endothelial cells (BEC), or primary cultured vascular smooth muscle cells (SMC) were transfected with wild type or constructs with deletions of conserved regions driving luciferase expression. Cells were maintained in media containing 10% FBS and assayed for promoter activity 48 hours following transfection. Data is normalized to activity of a minimal promoter (TK). **B.** Smooth muscle cells were transfected with constructs contained the 100-220 bp conserved regions either alone or in tandem. Luciferase activity was determined 48 hours following transfection as described above. **C.** Diagram of construct used to generate transgenic promoter-reporter mouse line.
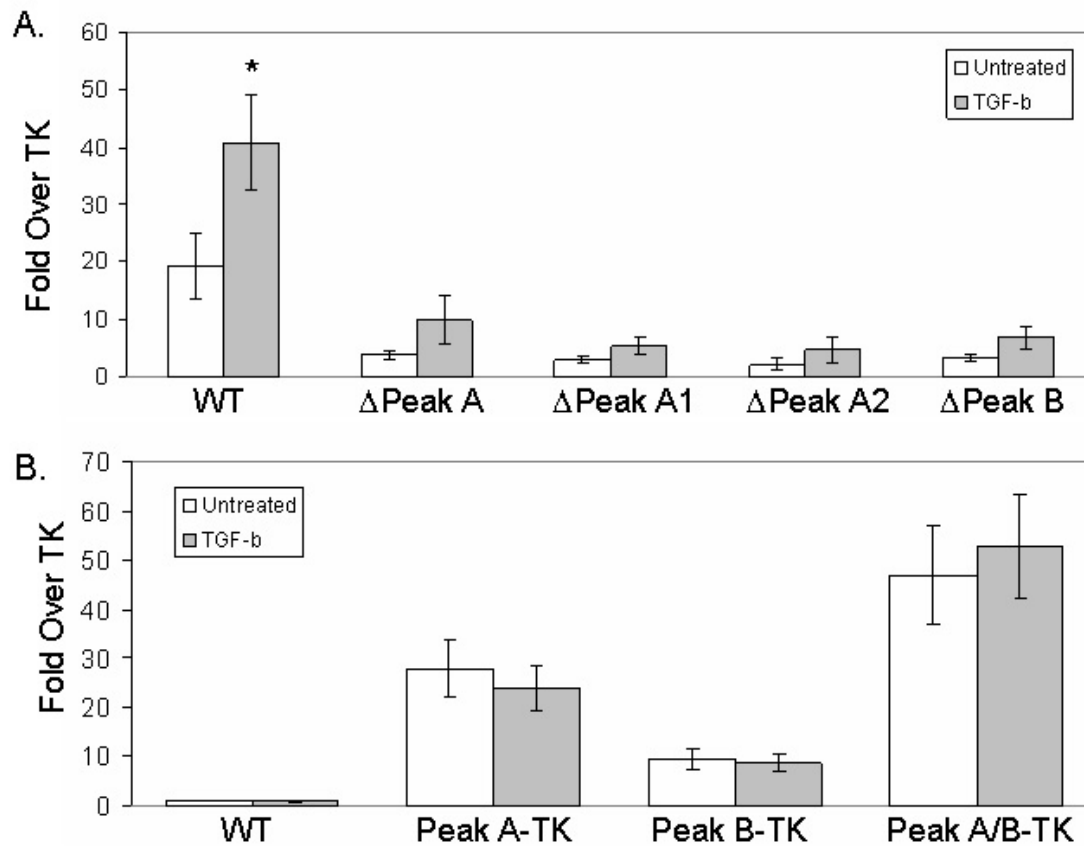
Figure 2.5. **Conserved regions of FRNK promoter do not contain elements responsive to TGF-β.** **A.** 10T1/2 cells were transfected with either wild type or deletion constructs. 24 hours following transfection, cells were served in DMEM containing 0.2% FBS for 24 hours. Cells were starved once more with 0.2% FBS for four hours and subsequently treated with TGF-β (1 ng/mL) for 24 hours. Cells were assayed for luciferase activity and data is reported as relative activity compared to a minimal promoter (TK). **B.** 10T1/2 cells were transfected with constructs containing the conserved regions of the FRNK promoter and were subsequently starved, treated with TGF-β, and assayed as described above.
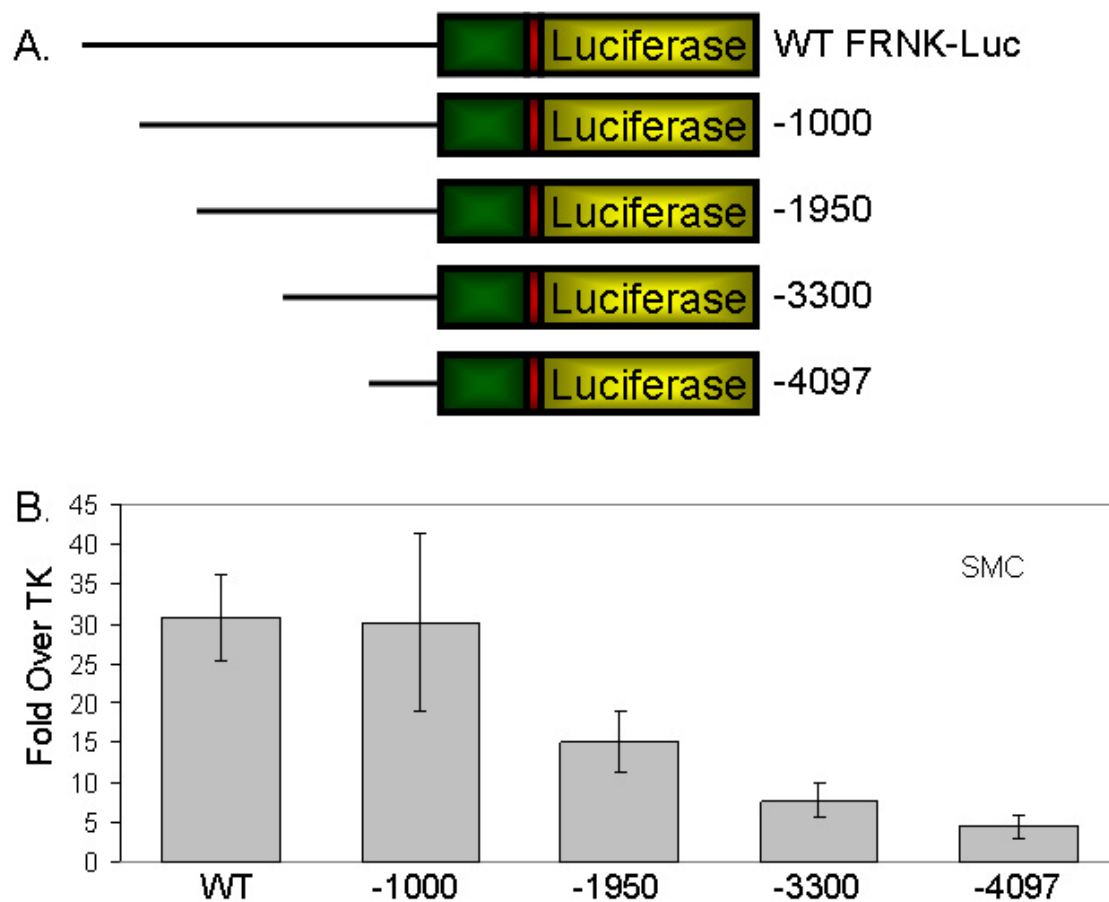
Figure 2.6. A region directing smooth muscle-specific expression lies within the 5' end of the FRNK promoter. A. Diagram showing serial truncation of the FRNK-luciferase promoter construct. **B.** Primary cultured VSMC were transfected with wild type and truncated luciferase constructs and maintained in 10% FBS for 48 hours. Cells were assayed for luciferase activity. Data is presented as fold over no treatment for each construct.
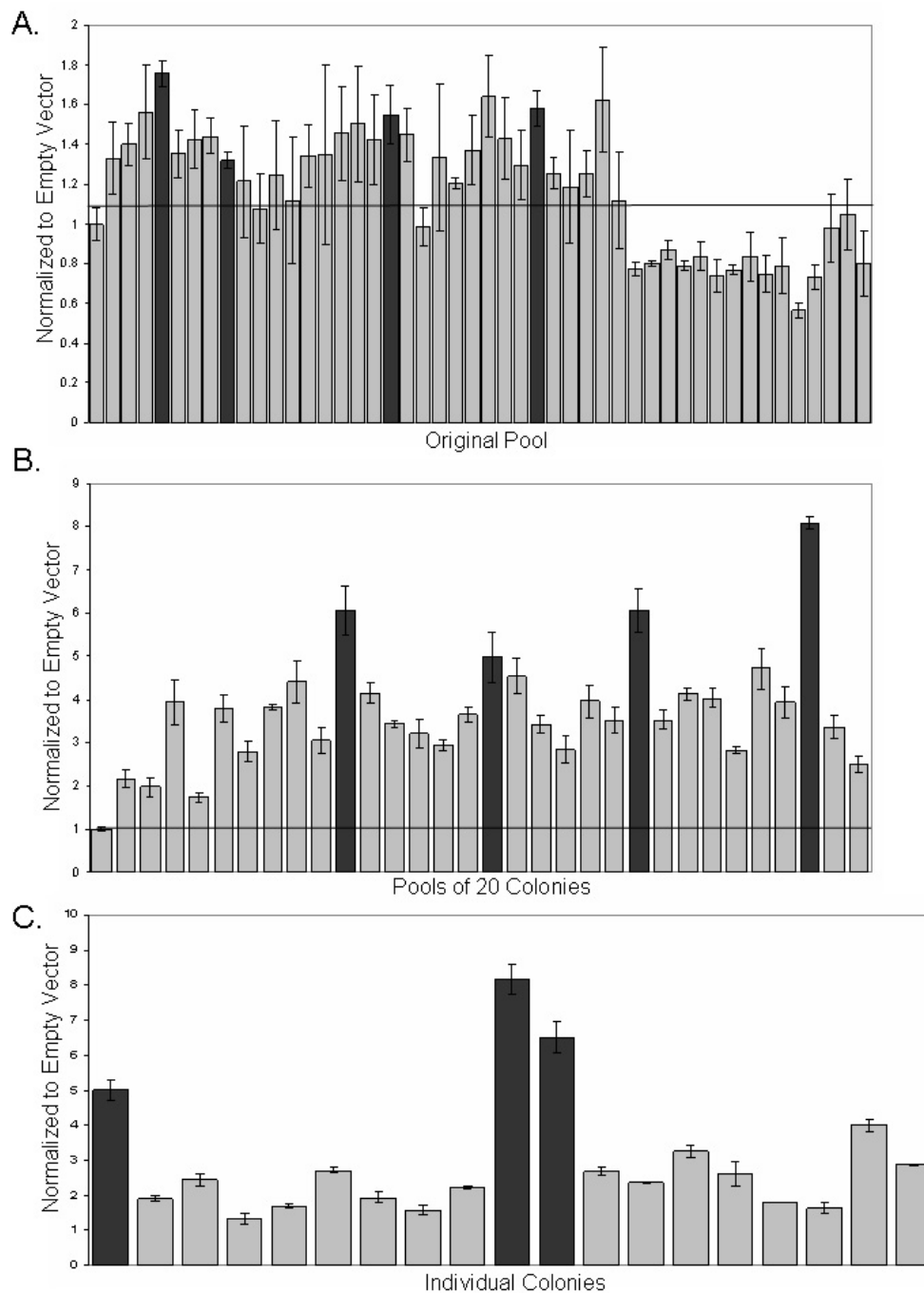
Figure 2.7. Luciferase based screen in mammalian cells for activators of the FRNK promoter. **A.** Shown is example profile from pools of >100 colonies from E13.5 mouse library co-transfected with PeakA/B-luciferase in cos cells. **B.** Peak A/B-luciferase was cotransfected with pools of 20 colonies sib-selected from original library pools (example profile). **C.** Luciferase activity of individual colonies *sib*-selected from pools of 20 colonies. All data is normalized to activity of empty vector control (example profile). Bars containing darker filling indicate those pools or colonies that were picked for further study.

CHAPTER III

FRNK EXPRESSION PROMOTES SMOOTH MUSCLE CELL MATURATION DURING VASCULAR

DEVELOPMENT AND FOLLOWING VASCULAR INJURY[1]

ABSTRACT

Objective--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. Our lab previously reported that a specific focal adhesion kinase (FAK) inhibitor termed FRNK (FAK Related Non-Kinase) is specifically expressed in large arterioles when SMC are transitioning from a synthetic to contractile phenotype and that FRNK inhibits FAK-dependent SMC proliferation and migration. Herein, we sought to determine whether FRNK expression modulates SMC phenotypes *in vivo*.

Methods and Results-- We present evidence that $FRNK^{-/-}$ mice exhibit attenuated SM marker gene expression during post-natal vessel growth and following vascular injury. We also show that FRNK expression is regulated by TGF-β and that forced expression of FRNK in cultured cells induces serum- and TGF-β-stimulated SM marker gene expression, while FRNK deletion or expression of a constitutively activated FAK variant attenuated SM gene transcription.

Conclusions--These data highlight the possibility that extrinsic signals regulate the SMC gene profile, at least in part, by modulating the expression of FRNK and that tight regulation of FAK activity by FRNK is important for proper SMC differentiation during development and following vascular injury.

INTRODUCTION

While medial smooth muscle cells (SMC) found in the mature vessel are fully differentiated and express high levels of SM contractile proteins, these cells do not terminally differentiate and can transition to a synthetic phenotype characterized by low levels of SM 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 processes (47, 113).

A number of secreted growth factors (i.e. platelet derived growth factor (PDGF-BB), sphingosine 1-phosphate (S1P), transforming growth factor-β (TGF-β) and contractile agonists (angiotensin II, endothelin-1 and thrombin) have been shown to regulate SMC phenotype *in vitro* and *in vivo* (58). In addition to levels of circulating factors, studies have also shown that the extracellular matrix (ECM) that surrounds SMC in the vessel wall can impart control over SM phenotypes (114). Indeed, genetic ablation of either fibronectin or the α_5 integrin fibronectin receptor results in embryonic lethality associated with impaired SMC investment of both embryonic and extraembryonic vessels (115, 116). Although the precise signaling mechanisms by which these diverse agonists and ECM regulate SMC transcription have not been completely delineated, several studies indicate that many (but not all) SM-specific genes (particularly contractile genes) depend on the presence of an serum response factor (SRF) DNA binding element termed a CArG box (CC(A/T)6GG) and many of these aforementioned intrinsic factors alter SRF or SRF-cofactor activity (58, 117-120).

One of the major proteins involved in the integrin intracellular signaling cascade is the non-receptor protein tyrosine kinase, focal adhesion kinase (FAK), which is

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strongly and rapidly activated by various aforementioned growth factors and by ligation of all β_1, β_3 or β_5 containing integrins (121). Although a direct role for FAK in vascular growth and development has yet to be examined, germline deletion of FAK phenocopies the lethal defects observed in fibronectin^{-/-} and α_5 integrin^{-/-} embryos. 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 acts as a dominant-interfering mutant for FAK 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 (122, 123). Whereas FAK protein levels remain relatively constant during vascular development, FRNK protein levels are dynamically increased in neonatal vessels and in adult vessels two weeks following injury, when SMC are transitioning from a synthetic to contractile phenotype.

The aim of this study was to determine whether FRNK expression plays a direct role in the phenotypic modulation of SMC *in vivo*. Herein we present evidence that FRNK^{-/-} mice exhibit repressed SM marker gene expression during post-natal vessel growth and following vascular injury. These data highlight the possibility that extrinsic signals regulate the SMC gene profile by modulating the dynamic expression of FRNK.

MATERIALS AND METHODS

Generation of Mice

Mice with germline deletion of FRNK were generated by homologous recombination as previously reported and were backcrossed to the C57/Bl6 strain at least 6 times before experimentation (124). Animal procedures were approved by our accredited American Association for Accreditation of Laboratory Animal Care committee.

Cell Culture, Transfections, and Reporter Assays

Aortic vascular smooth muscle cells (SMC) were cultured from mice or rats using enzymatic digestion as previously described (125). Cells were maintained in Delbucco's modified eagle medium with F12 supplemented with 10% fetal bovine serum and 0.5% penicillin/streptomycin and used from passage 8-16. 10T1/2 cells were obtained through ATCC and were maintained in Delbucco's modified eagle medium supplemented as above. Chick proepicardial cells were a generous gift from Mark Majesky. Cells were induced to differentiate into coronary SMC (cSMC) by serum-starvation for 7 days as previously described (126). For reporter assays, cells were transfected with appropriate constructs using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's instructions. Following transfections, cells were lysed in Glo Lysis Buffer and assayed using Steady Glo Luciferase Assay system (Promega) as directed by the manufacturer. To determine FRNK half-life, SMC were treated with cyclohexamide (Sigma) for indicated times.

Immunocytochemistry and detection of BrdU incorporation in cultured cells

Cells were processed for immunocytochemistry using previously published methods (122). In brief, cells were fixed with 4% paraformaldehyde, permeabilized with 4% Triton X-100 in PBS, incubated with SM α-actin (1:100) for two hours. After washing with PBS, slides were incubated for 1 hour with FITC-conjugated donkey anti-mouse antibodies (2 μg/ml) or Texas Red-conjugated phalloidin to detect filamentous actin.

To measure proliferating cells, BrdU (Sigma, 30 μg/mL) was administered to SMC grown on chamber slides. After 4 hrs, cells were fixed in 4% paraformaldehyde and stained using a BrdU detection kit (Invitrogen).

Transwell Assays

SMC were trypsinized and resuspended in DMEM:F12 containing 1% penicillin/streptomycin and 0.1% bovine serum albumin. Approximately 15,000 cells were plated on transwell filters (8 μm pore size) precoated with FN. After 18 hours, the cells were fixed in 4% paraformaldyhyde and the remaining cells in the upper chamber were removed with a cotton swab. Migrated cells were stained with crystal violet and counted.

Western Analysis

To examine protein levels, lysates from cells or tissues were prepared by lysing in RIPA buffer with protease and phosphatase inhibitors as previously described (78). Protein concentration was determined by using a colorimetric BCA assay (Pierce).

Lysates were electrophoresed on an 11% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with antibodies for c-terminal FAK (Upstate), SM α-actin (Sigma), FAK pY397 (BioSource), SM-myosin heavy chain (generated by U. Groeschel-Stewart), and SM-22 (generous gift from Mario Gimona).

Real-Time Quantitative RT-PCR

Tissue or cells were lysed in TRIzol (Invitrogen) and total RNA was isolated accoording to the manufacturer's protocol. Expression was measured using the ABI Prism 7700 TaqMan system. Primers and fluorescent probes were designed for the following genes: FRNK (Forward: GCTGCATTCTGAGGCGTTTA, Reverse: CAGGATTGTGCACCACCAG, Probe: AGCCAGGACTGAGACGCCGCC), FAK (Forward: GAAAGCAGTAGTGAGCCAACC, Reverse: GAGACTGTCCACTATCTTCTG, Probe: CTCCATGCCTGATAATACTGGCCCAG), SM-22 (Forward: TGCAGTGTGGCCCTGATGT, Reverse: TGCTCAGAATCACGCCATTCT, Probe: AGATCGTGGGCGCCTGGGCT), and SM α-actin (Forward: CGCTGTCAGGAACCCTGAGA, Reverse: CGAAGCCGGCCTTACAGAG, Probe: CAGCACAGCCCTGGTGTGCGAC). 18S primers and probes were a generous gift from Hyung-Suk Kim. Primers and probes for smoothelin were used as described previously (127).

Animal Procedures

Genotypes were obtained from tail snip DNA using PCR analysis for primers specific to FRNK (124).

Conscious blood pressure was measured in mice aged 13 weeks using a tail cuff detection system (Hatteras). Over a period of 20 minutes, 20 measurements were taken every day for 6 consecutive days. Data is presented as an average of measurements for all days.

To measure unconscious blood pressure, mice were anaesthetized with isofluorane, and the left carotid artery was exposed. A pressure transducer (Millar) was inserted through the carotid into the aorta to measure aortic pressure. Phenylephrine was administered through a catheter inserted into the jugular vein. Between doses of phenylephrine, each animal was allowed to recover its blood pressure to resting levels.

For injury studies, mice aged 9-10 weeks were anesthetized using isofluorane and a suture was tied around the left common carotid artery just below the bifurcation as previously described (41). Animals were allowed to recover and were sacrificed at various times following injury. For RNA studies, a 3mm portion of the carotid was dissected out from 1 mm below the site of ligation (left), or 1mm below the bifurcation (right). For histology, the carotids were removed *en bloc* and sectioned serially from 2-3 mm away from the site of injury.

In situ *hybridization*

Tissues from 1 week post-natal C57black6 mice were harvested, fixed and embedded in paraffin. Serial sections (12 μm) were hybridized in the absence or presence of a FRNK-specific digoxingenin-labeled antisense RNA probe O/N at 42° C. Sections were washed and incubated with an alkaline phosphatase-conjugated anti-

digoxigenin antibody (Roche; 1:1,000 O/N at 4°C). Slides were developed using NBT/BCIP as substrates (purple staining) and counterstained with methylene-green (1%).

BrdU incorporation, Immunohistochemistry and Trichrome/Elastin Stain

Pregnant mothers were injected with BrdU (30 mg/kg) 1 day prior to giving birth. Wild type (+/+) or FRNK$^{-/-}$ (-/-) pups were sacrificed at postnatal day 4 and aortas were removed, paraffin embedded, and sectioned. An antibody to BrdU was used to detect proliferating cells within the media. Positively stained area was quantified with ImageJ software.

For general tissue immunohistochemistry, tissue from mouse pups or post-surgical animals was harvested, formalin-fixed, and paraffin-embedded. Sections (8-9 μm) were dehydrated, permeabilized, blocked, and incubated with an antibody for SM α-actin (Sigma). Antibody binding was detected with HRP-linked secondary antibody. Sections were developed with diamino benzidine and subsequently counterstained with methyl green. For morphometric measurements, a modified Masson's trichrome/Verhoeff stain was used to localize elastin. We used ImageJ (NIH) to measure circumference of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL), along with area of the media and lumen.

The 41/43 kDa FRNK protein is selectively expressed in the vasculature from approximately E12.5 onward with highest levels observed in large arterioles and lung of neonatal rats (post-natal day 4-14) (Figure 3.1A) (93, 124). As previously reported (and depicted in Figure 3.1B), FRNK expression is regulated by a promoter embedded within the *fak* gene and *frnk* transcription initiates from a non-coding exon located approximately 300 bp upstream of the FRNK translational start site in the mouse gene. (93, 123). Since FRNK shares the same amino acid sequence as the C-terminus of FAK, we have been unable to develop a specific antibody that recognizes FRNK (but not FAK) for immunohistochemical analysis of FRNK expression patterns. However, since the *frnk* non-coding exon is selectively protected in RNA isolated from SMC and SMC-containing tissues (93), we developed an *in situ* probe directed against this unique sequence to examine FRNK expression patterns *in vivo.* Our *in situ* analysis of tissues harvested from post-natal day 7 mice revealed that FRNK is expressed throughout the media of large arterial vessels, but is not expressed in either the endothelium or adventitial layer (Figure 3.1C, top). Some visceral SMC staining was apparent as well, with high levels observed in the lung (Figure 3.1C) and bladder (not shown). FRNK expression in the lung was localized to the smooth muscle lining of the bronchi in addition to vessels (Figure 3.1C, middle and bottom left), similar to the staining pattern observed for SMα-actin (Figure 3.1C, middle and bottom right). FRNK was also detectable (albeit in low levels) in coronary arteries (data not shown) and coronary SMC cultures derived from explanted pro-epicardial organs (Figure 3.1D) (126). Further analysis of FRNK expression in the developing mouse vasculature using quantitative RT-

PCR revealed a dynamic up-regulation of FRNK mRNA in aorta between 7 and 10 days post-natal, corroborating our previous Western analysis (Figure 3.1E). Since our previous studies revealed that ectopic expression of FRNK attenuated FAK activity and blunted PDGF-stimulated growth and motility of SMC, we theorized that FRNK might function to regulate SMC phenotype in post-natal vessels. FRNK$^{-/-}$ mice were recently generated by a strategy that resulted in deletion of a 1kb fragment that included the non-coding exon and 700 bp of upstream *fak* intronic sequence (see schematic in Figure 3.1B). Although these mice were born in the expected Mendelian frequency and showed no gross phenotype, they were not specifically examined for defects in SMC differentiation marker gene expression or growth (124). 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 first examined the proliferation index of medial SMC in post-natal vessels from wild type or FRNK$^{-/-}$ mice. As shown in Figure 3.2A, medial SMC in post-natal FRNK$^{-/-}$ vessels exhibit a significantly higher index of BrdU incorporation in comparison to littermate control vessels. Importantly, SMC tissue from postnatal FRNK$^{-/-}$ pups reveal lack of FRNK protein and higher levels of active pY397FAK, in comparison to wild type littermate controls, while total FAK protein levels are unaltered (Figure 3.2B).

We next analyzed the dynamic expression of the contractile genes, SMα-actin, SM-22, and smoothelin in the aorta of post-natal control and FRNK$^{-/-}$ mice by quantitative RT-PCR. Expression of these contractile genes is enhanced approximately 6-10 fold from 4 day post-natal to adult vessels in wild type mice (data not shown). However, as shown in Figure 3.2C, FRNK$^{-/-}$ vessels contain significantly fewer transcripts of these

genes in the 2-3 week period of vessel development, when FRNK protein is most highly abundant in wild type vessels (93).

Since the depletion of FRNK from developing vessels appeared to prolong the synthetic phenotype of SMC in the developing vasculature, we reasoned that FRNK$^{-/-}$ mice might exhibit defective vascular homeostasis. However, we found that FRNK$^{-/-}$ mice did not exhibit significant changes in mean systolic blood pressure (as assessed by tail cuff measurements) or pressor responses (as assessed by intra-aortic catheterization) following phenylephrine treatment in comparison to wild type littermate controls (Figure 3.3A, B). In accordance with these data, medial thickness of FRNK$^{-/-}$ adult arterioles (aorta or carotids) was not significantly different from that of littermate control vessels, indicating that the lag in SM differentiation observed in the neonatal vessels was normalized during vessel maturation (Figure 3.3C). Indeed, no significant differences in SM22, SMα-actin or smoothelin expression were observed in adult aorta (see data for 8 week animals in Figure 3.2C) or carotid vessels isolated from wild type or FRNK$^{-/-}$ mice (Figure 3.4A).

Since we previously reported that FRNK expression is relatively low in adult vessels, but is dramatically up-regulated by 14 days following catheter-induced vessel injury, we postulated that FRNK might also regulate SMC phenotypic switching during restenosis. Numerous studies have revealed that contractile gene expression is dynamically regulated during following vascular injury induced by either vessel ligation or surgical endothelial denudation. Typically, reduced SMC marker gene expression is apparent within 3-7 days following injury followed by a burst of re-expression returning after approximately 2-3 weeks following injury (40, 41). To examine a possible involvement of FRNK in the

regulation of SMC re-differentiation following vascular injury, we performed quantitative RT-PCR for SM marker genes in wild type and FRNK$^{-/-}$ carotid arteries 7-21 days following carotid ligation. As shown in Figure 3.4A-C, significant decreases in re-expression of SMα-actin, SM-22, and smoothelin (but not β-actin) were observed in FRNK$^{-/-}$ vessels in comparison to littermate controls, indicating that the recovery of SM differentiation was defective in injured FRNK$^{-/-}$ arteries. Notably, no significant differences in SM gene expression were observed in the control un-ligated right carotid artery at the time points examined (data not shown). Concomitantly, immunohistochemical analysis for SMα-actin revealed a striking difference between wild type and FRNK$^{-/-}$ vessels 14 days after ligation. While, robust SMα-actin staining was observed throughout the media and neo-intima of wild type vessels, SMα-actin expression in the neoinitma of FRNK$^{-/-}$ vessels was markedly reduced (Fig 3.4D). While we anticipated that this phenotype might lead to a more exacerbated injury response, morphometric analysis at this time point revealed no significant differences in the extent of remodeling induced by ligation of wild type and FRNK$^{-/-}$ vessels, likely due to the fact that the ligation induces a very strong proliferative response in wild type C57Black6 mice (Figure 3.5). Indeed, by 21 days after ligation full occlusion of injured vessels was observed in both wild type and FRNK$^{-/-}$ mice. Collectively, these data provide strong support for the hypothesis that up-regulation of FRNK expression following vessel injury is required for appropriate SMC maturation that occurs subsequent to injury repair.

The studies described above suggested a role for FRNK in promoting SMC differentiation. To directly explore this possibility, we performed SMC promoter-reporter assays in 10T1/2 cells, a multi-potential SMC precursor line that has been shown

to markedly up-regulate SMC-specific gene expression upon stimulation with serum, S1P, or TGF-β (57, 128). While FRNK is not expressed in detectible levels in 10T1/2, forced expression of FRNK in these cells inhibited FAK activity and resulted in a two to four-fold increase in SM22, SMα-actin and SM-MHC promoter activity (Fig 3.6A). We reasoned that FRNK expression likely promotes SMC differentiation by relieving FAK-dependent repressive signals. To determine whether FAK activation limits SM marker gene expression we ectopically expressed a constitutively active FAK variant (termed SuperFAK (129)) that leads to enhanced FAK activity as assessed by phosphorylated Y397FAK levels. As shown in Figure 3.6B, SuperFAK expression in 10T1/2 cells significantly reduced SM22 and SMα-actin reporter gene expression (Fig 3.6B). Similarly, ectopic expression of FRNK and FAK variants in primary rat aortic SMC revealed an inverse relationship between FAK activity and SM gene expression (i.e. low FAK activity correlates with high levels of SM markers, Fig 3.6C). Notably, aortic SMC isolates from FRNK^{-/-} mice consistently exhibited enhanced FAKpTyr, and lower levels of the contractile proteins SM22, SMα-actin, and SM-MHC than SMC isolates from aged matched (and passage-matched) control mice (Fig 3.6D). Expression of FRNK does not effect transcription of myocardin (Figure 3.6E). Staining FRNK^{-/-} SMC revealed that deletion of FRNK results in lower, yet detectable levels of SM a-actin expression (Figure 3.7A). Re-expression of FRNK also decreased proliferation and migration of FRNK^{-/-} SMC (Figure 3.7A, B). Taken together, our findings provide compelling evidence that FAK activity limits SMC differentiation, and up-regulation of FRNK promotes differentiation by attenuating FAK activity.

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We next sought to identify factors that can modulate dynamic FRNK expression during vascular morphogenesis. We first utilized a FRNK promoter reporter construct to define factors that regulate FRNK transcription. A fragment comprising approximately 6kb of sequence upstream of the FRNK ATG (-5388 to +876) was previously shown to drive SM-specific expression of LacZ *in vivo* in a pattern reminiscent of FRNK expression (124). As shown in Figure 3.8A, we found that the corresponding FRNK promoter attached to a luciferase reporter (FRNK-Luc) exhibited high activity in cultured SMC in comparison to 10T1/2 cells (consistent with the levels of FRNK expressed in these two cell types). Although FRNK is expressed in a SM-restricted fashion, careful analysis of the FRNK promoter region from chicken, mouse and human sequence did not reveal any conserved CArG elements, known to direct SRF-dependent transcription. Indeed, co-expression of SRF and FRNK-Luc in SRF$^{-/-}$ ES cells did not alter basal FRNK-Luc promoter activity in these cells, while expression of a SMα-actin-Luc construct was induced approximately 15-fold (Figure 3.8B). Furthermore, when expressed in SMC, the FRNK-Luc construct was un-responsive to over-expression of the myocardin family of SRF co-factors, which stimulated strong activation of the SMα-actin-Luc reporter (Figure 3.8C). Endogenous FRNK transcription was not stimulated by overexpression of Flag-Myocardin, whereas SM-22 expression was increased approximately 75-fold (Figure 3.8D). Collectively, these data provide strong support for FRNK being expressed in a non-SRF/CARG dependent fashion as previously suggested (104).

We next screened a number of cytokines/growth factors known to be released following vessel injury for their ability to increase FRNK promoter and protein levels in

cultured rat aortic SMC. We found that TGF-β induced a marked increase in FRNK-Luc activity, FRNK mRNA (as assessed by quantitative RT-PCR) and protein levels compared to vehicle treated cells (Figure 3.9A-C), while other agonists including the potent SMC mitogens PDGF-BB, S1P, angiotensin II, thrombin, and basic-fibroblast growth factor had no effect (data not shown). Since TGF-β induces SM differentiation, we wondered whether FRNK expression might contribute to the TGF-β response. To this end, we next examined TGF-β stimulated SM gene expression in our cell culture models. We found that TGF-β induced a more robust induction of SM22 reporter activity in FRNK expressing compared to vector-transfected control 10T1/2 cells (Figure 3.9D). In addition, we found that TGF-β-induced expression of endogenous SM22 was significantly decreased in FRNK$^{-/-}$ SMC in comparison to matched wild type SMC isolates (Figure 3.9E). Collectively, these data provide support for a model whereby TGF-β induces FRNK expression and that FRNK, in turn, contributes to TGF-β induced SMC differentiation by dampening FAK-dependent signals.

FRNK, a dominant interfering mutant that attenuates FAK activity, exhibits selective and dynamic expression in SMC. We previously reported that FRNK attenuates SMC proliferation and migration by regulating FAK/Rac1-dependent signaling (78). Herein we have found an additional function for FAK/FRNK signaling in SMC. We present evidence that FRNK deletion (by homologous recombination) represses SM gene expression during post-natal vessel growth and following vascular injury. We also show that FRNK expression is regulated by TGF-β and that forced expression of FRNK induces SM marker gene expression in cultured cells grown in serum and enhances TGF-β-stimulated SM marker gene expression. Conversely, FRNK deletion or expression of a constitutively activated FAK variant attenuated SM gene transcription. These data suggest that enhanced FAK activity is permissive for SMC growth and migration, but limits SM differentiation, and that tight regulation of FAK activity is likely important for proper SMC phenotypic modulation during development and following vascular injury.

FAK is activated by a process that involves dimerization and intermolecular phosphorylation of tyrosine 397 *in trans* (130). Phosphorylation of Y397 results in subsequent recruitment of the tyrosine kinase Src (and/or Fyn), which phosphorylates and further activates FAK (and phosphorylates certain FAK binding partners) (82). FRNK likely attenuates FAK activity by inducing the formation of FRNK/FAK heterodimers that are incapable of Y397 phosphorylation and Src binding. In support of this notion, expression of wild type FAK (and Src) can rescue FRNK-dependent inhibition of FAK (and paxillin) phosphorylation, while expression of a phospho-deficient FAKY397F cannot (131). Our findings indicating that FAK activity is enhanced in tissues and cells

derived from FRNK$^{-/-}$ mice strongly support the hypothesis that the dynamic regulation of FRNK expression can impart specific spatial and temporal control of FAK activity *in vivo*. In this regard, it is interesting to note that FAK protein is extremely stable with a reported half-life exceeding 20 hr (132), while FRNK protein turnover is rapid (Figure 3.1F). We speculate that transient FRNK expression is particularly important in the vasculature, where direct apposition of SMC with extracellular matrix could lead to high levels of FAK activation and un-controlled SM growth. It is feasible, however, that FRNK has additional FAK-independent functions, and future studies will examine this possibility.

In support of the idea that FRNK/FAK signaling plays an active role in regulating smooth muscle cell phenotypes, FAK$^{-/-}$ 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 (133). Interestingly, we recently found that FAK-deficient SMC (like FRNK over-expressing SMC) exhibit enhanced TGF-β stimulated SM marker gene expression (un-published observations). In addition, recent studies have provided evidence for a role of FAK in promoting striated muscle cell differentiation as the dynamic regulation of FAK activity was found to be essential for differentiation of C2C12 myoblasts into myotubes (134). Also, 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 (135). Since SRF plays a critical role in the regulation of contractile gene expression in each of these muscle types, it will be of future interest to determine whether limiting FAK activity regulates SRF activity and/or co-factor recruitment.

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Since FRNK exhibited a striking SMC-restricted expression pattern, we sought to explore the mechanisms underlying its transcriptional regulation. Interestingly, we found that the FRNK promoter does not contain a canonical CArG box, is not affected by SRF deletion, and is un-responsive to over-expression of the myocardin family of potent SRF co-factors. Thus, FRNK belongs to a sub-class of smooth muscle specific genes that are regulated in a CArG-independent fashion including aortic carboxypeptidase-like protein (ACLP), cysteine-rich protein 2 (CRP2), and histidine-rich calcium-binding protein (HRCBP) (100, 101, 103). Interestingly, TGF-β, a strong activator of SMC differentiation, led to significant up-regulation of FRNK expression. Thus, future exploration of the TGF-β-dependent mechanisms that control FRNK expression may lead to identification of additional transcription factors that regulate SMC phenotypes.

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 (82). Direct evidence for the role of FAK in modulating fibronectin-dependent motility was previously shown using FAK$^{-/-}$ fibroblasts, endothelial cells, neurons, and keratinocytes (136-139). We previously showed that FRNK expression in SMC attenuated FAK-dependent PDGF-stimulated chemotaxis (93) and our recent studies reveal that FAK$^{-/-}$ SMC exhibit a similar defect (un-published observations). Since FRNK appears to play a dual role in SM function; aiding to block SMC growth and migration and to promote SMC differentiation, FRNK may function as a toggle in the regulation of SMC phenotypes.
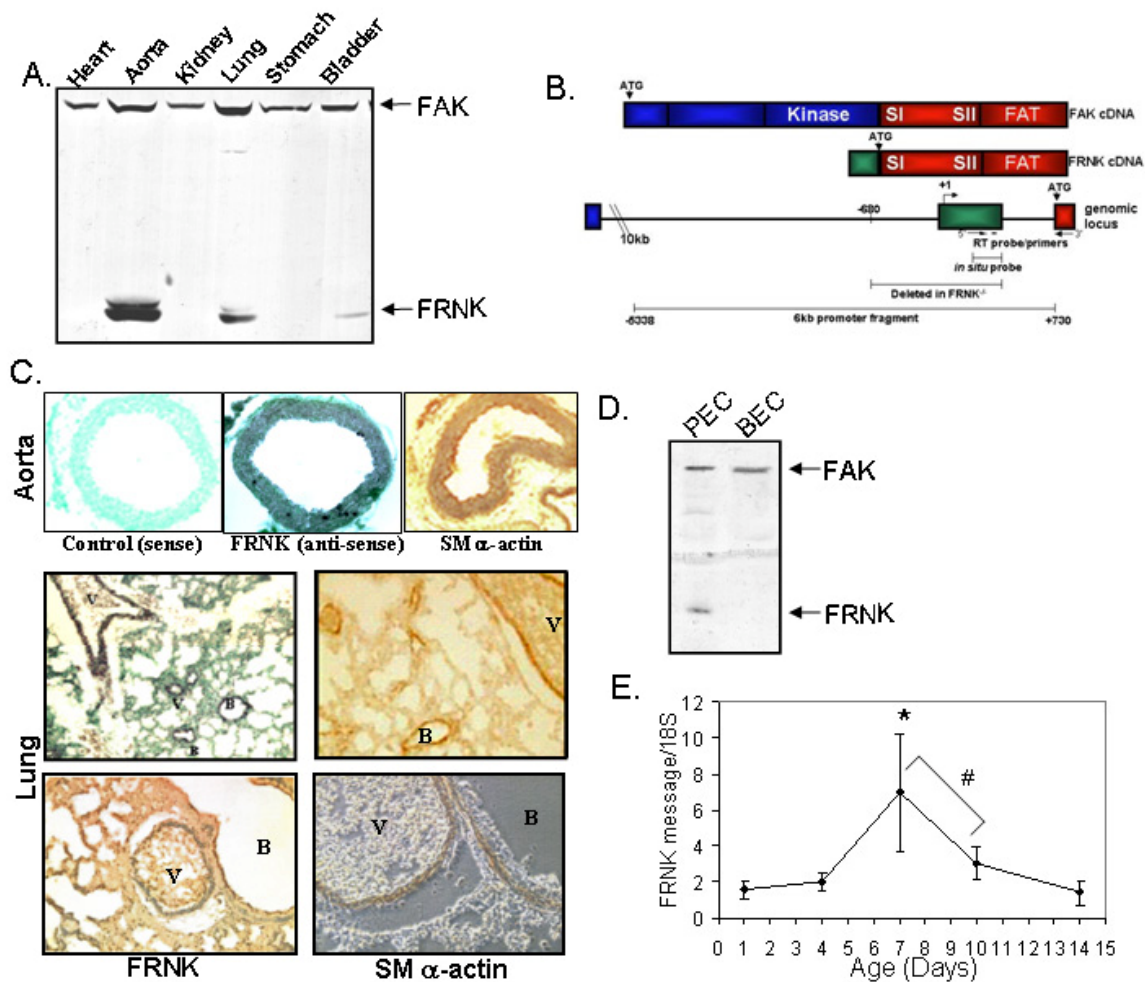
Figure 3.1. FRNK is expressed selectively in smooth muscle containing-tissues. A. Immunoblot showing expression of FAK and FRNK in tissues from 14d postnatal rat. **B.** Schematic of FAK and FRNK cDNA and genomic locus. Shaded areas represent FAK-specific (blue), FRNK-specific (green), or common (red) coding regions. **C.** *In situ* hybridization in postnatal day 7 mouse aorta and lung using sense and anti-sense probes directed against the FRNK non-coding exon. Immunohistochemistry for SMα-actin is shown as a positive control. **D.** Western blot of FRNK and FAK levels in coronary SMC (cSMC), and bovine endothelial cells (BEC). **E.** Quantitative RT-PCR of FRNK message levels in thoracic aorta was dissected from mouse pups 1, 4, 7, 10, and 14d post-natal. Data is normalized to expression of 18S and expressed as the mean +/- SEM (n > 4 for each timepoint, * $p < 0.01$, # $p < 0.05$).
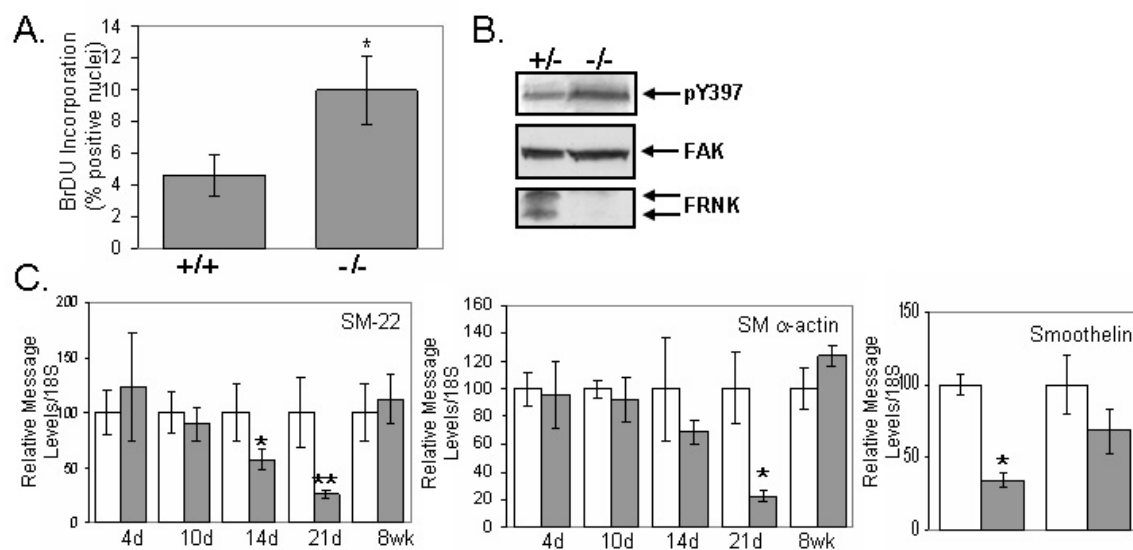
Figure 3.2. FRNK^{-/-} mice exhibit increased SM proliferation and attenuated expression of SM marker genes during development. A. BrdU incorporation in post-natal day 4 thoracic aorta collected from wild type (+/+) or FRNK^{-/-} (-/-) mice. Data is expressed as the mean +/- SEM (n=5, p<0.03). **B.** Western analysis in post-natal day 4 bladders from heterozygous (+/-) or FRNK^{-/-} (-/-) pups. pY397 reveals enhanced active FAK in -/- tissue. **C.** Quantitative RT-PCR for indicated SM differentiation markers in +/+ and -/- thoracic aorta collected from 4 days to 8 weeks post-natal. Data is normalized to expression of 18S and is expressed as the mean percent of wild type expression at each time point +/- SEM (n > 4 for each timepoint, ** p < 0.01, * p < 0.05).
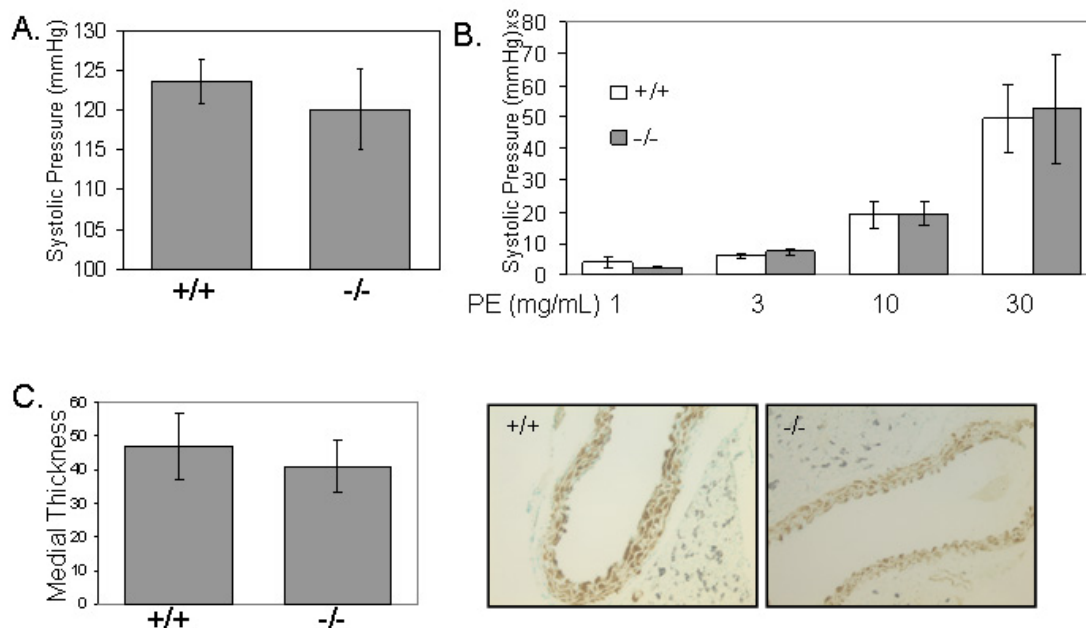
Figure 3.3. FRNK^{-/-} mice do not display abnormal homeostasis. **A.** Wt (+/+) and FRNK^{-/-} (-/-) mice aged 13 weeks were subjected to tail cuff measurement of systolic blood pressure. Data is presented as an average of the calculated mean for each mouse over 6 days, +/- SEM. **B.** Blood pressure was measured in anaesthetized mice aged 14 weeks by aortic catheterization during administration of phenylephrine (PE) into the jugular vein. Data are expressed as the mean +/- SEM. **C.** Aortic medial thickness was measured using ImageJ software in Wt (+/+) and FRNK^{-/-} (-/-) mice aged 13 wks from formalin-fixed, paraffin-embedded sections stained with SM α-actin. Data is expressed as the mean +/- SEM. Sample histology with staining for SM α-actin from mice aged 13 weeks is shown at right.
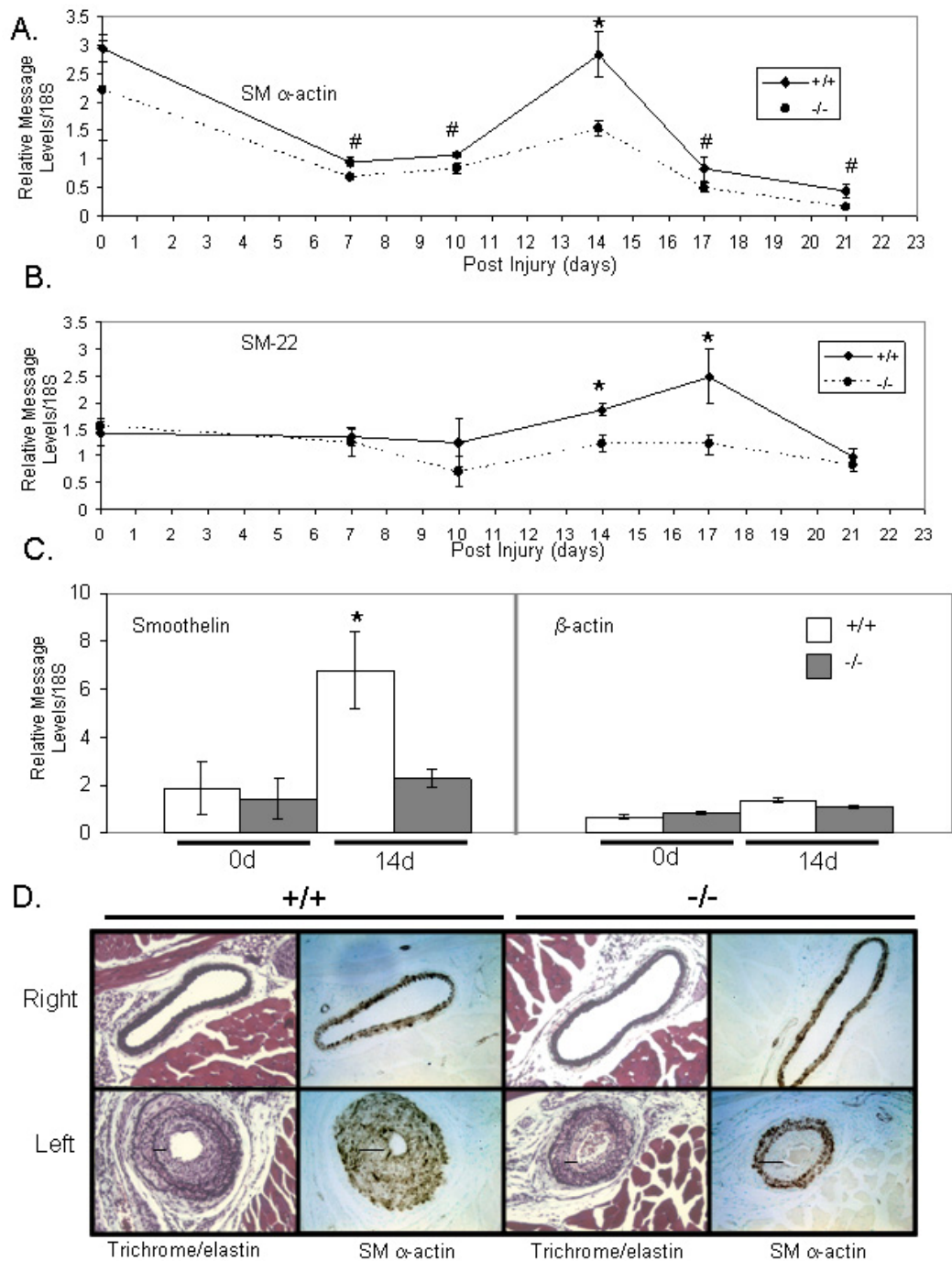
Figure 3.4. FRNK^{-/-} mice show decreased expression of SM marker genes following carotid artery ligation. Wild type (+/+) or FRNK^{-/-} (-/-) mice were subjected to left carotid blood flow cessation as described in the Methods section. **A-C**. Total RNA was extracted from the left and right carotids pre- and post-ligation and processed for real-time quantitative RT-PCR for the indicated SM markers and β-actin (control). Data is presented as the left carotid normalized to expression in the wild type right (control) carotid artery at each timepoint +/- SEM (n >4, * $p<0.01$, # $p<0.05$). **D.** Verhoeff's elastin and immunohistochemistry for SM α-actin staining in the right and left carotid arteries removed from wild type (+/+) or FRNK^{-/-} (**-/-**) mice 14 days following ligation. Black bars indicate thickness of neointima.

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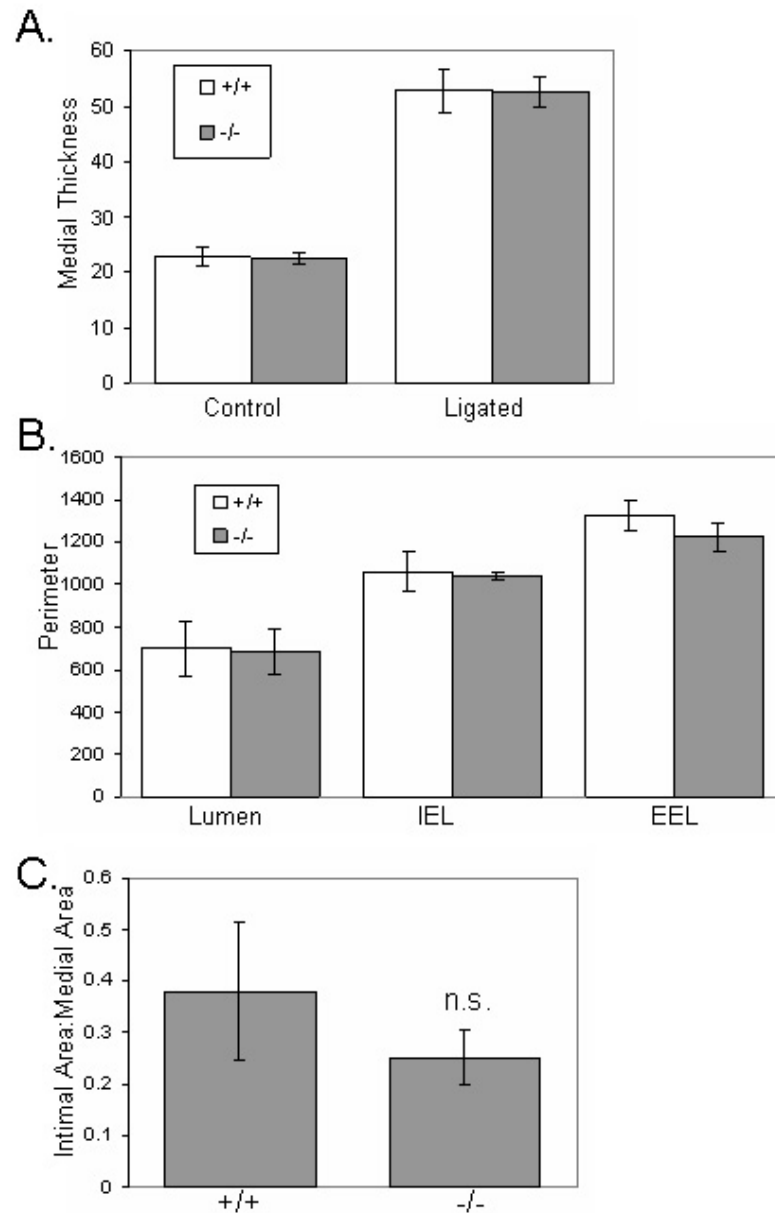
Figure 3.5. FRNK$^{-/-}$ mice show no differences in vessel growth in response to carotid artery ligation. Wild type (+/+) and FRNK$^{-/-}$ (-/-) mice were subjected to ligation of the left carotid artery for 14 days. Measurements were taken from trichrome/elastin stained sections using ImageJ (NIH). **A.** The distance between the IEL and EEL in the right and left carotid was measured at 9 points along the 3mm region of remodeled vessel for each individual animal. Data is presented as the average of the mean for each animal, +/- SEM. **B.** The circumference of the lumen, IEL, and EEL was measured three times from the left carotid of each animal. **C.** Ratio of intimal area:medial area in the left, injured carotid. Data for each panel is presented as the average of the mean for each animal, +/- SEM.
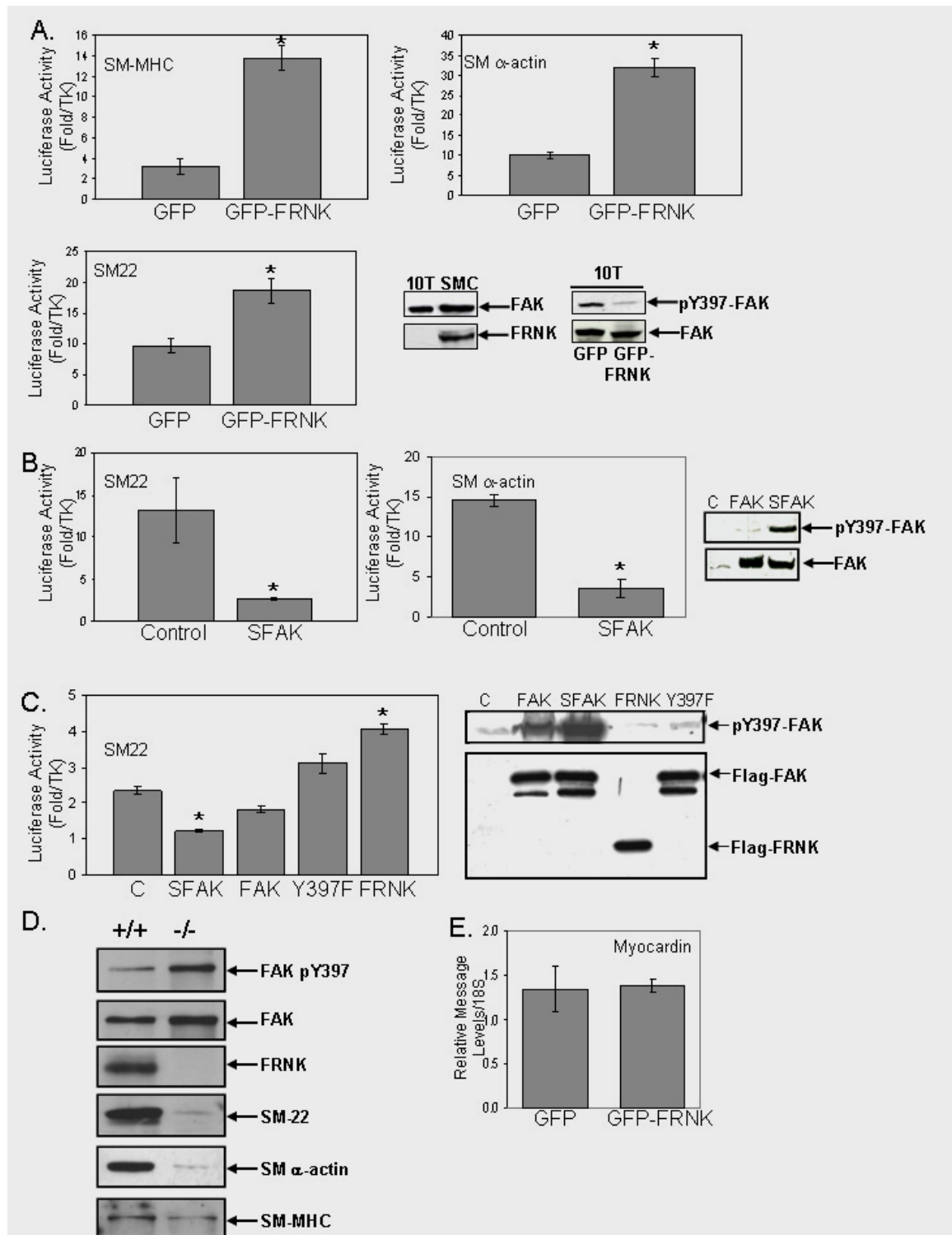
Figure 3.6. SM marker gene expression is induced by FRNK expression. **A.** 10T1/2 cells were transfected with SM-MHC, SM α-actin, or SM22 luciferase reporter plasmids. 24 hours later cells were infected with either GFP or GFP-FRNK adenovirus for an additional 24 hours prior to luciferase detection. Bottom right: Western analysis of 10T1/2, SMC or 10T1/2 infected with GFP or GFP-FRNK adenovirus as described above. **B.** (Left) 10T1/2 cells were co-transfected with SM22 or SM α-actin luciferase and empty vector or SuperFAK and reporter assays were performed as described above. (Right) Western analysis of 10T1/2 cells transfected with Flag vector (C), Flag-FAK, or Flag-SuperFAK (SFAK). **C.** Rat aortic SMC were co-transfected with SM22-luciferase reporter construct and with either empty vector (C), SFAK, FAK, Y397FFAK or FRNK in the presence of serum. (Left) Luciferase activity was measured 48 hrs following transfection. (Right) Western analysis shows activation state of FAK using phospho-specific FAK pY397 antibody and expression of Flag-tagged constructs with Flag antibody. **D.** Western analysis of primary cell isolates generated from pools of thoracic aortas harvested from 6 week post-natal wild type (+/+) or FRNK$^{-/-}$ (-/-) mice. Cultured cell data are representative of three separate isolates of wild type and FRNK$^{-/-}$ collected at identical passage numbers. **E.** Rat Aortic SMC were infected with GFP or GFP-FRNK as described above. Total RNA was collected and RT-PCR was used to measure myocardin expression. Promoter data are expressed as mean +/- SEM for at least 3 separate experiments. Western analyses are representative of at least 3 experiments.
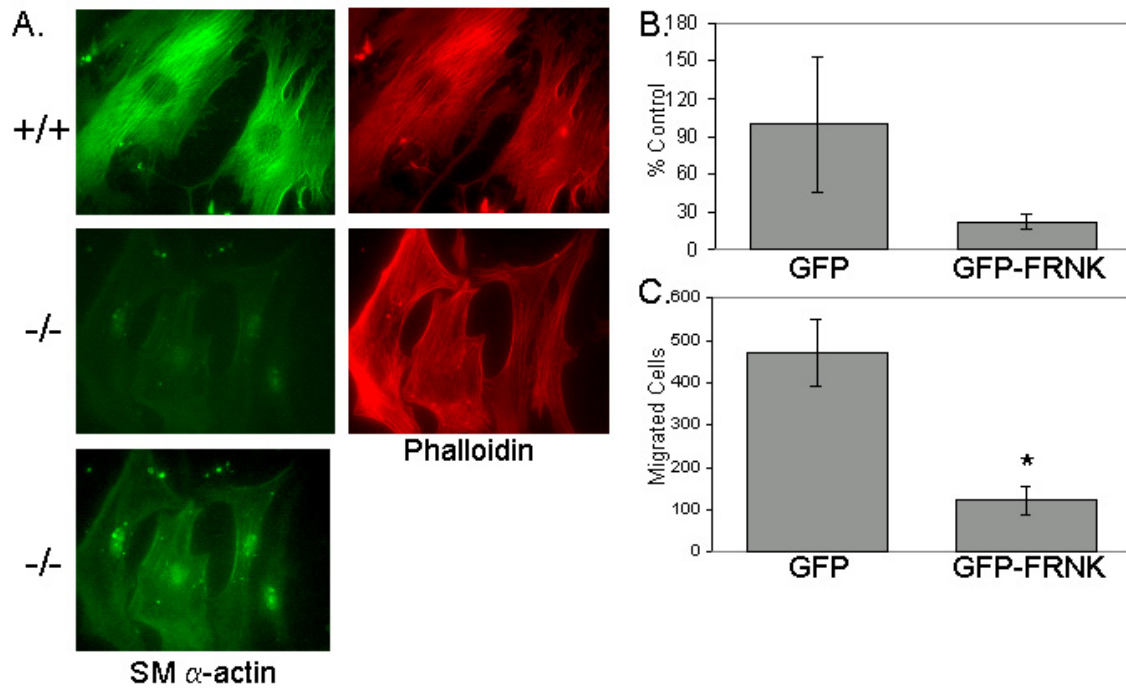
Figure 3.7. Re-expression of FRNK attenuates serum-dependent growth and migration in FRNK^{-/-} cells. A. FRNK^{-/-} (-/-)or WT (+/+) cells were maintained in serum, fixed in 4% paraformaldyhyde, and stained with an antibody for SM α-actin and phalloidin. The top two left pictures were taken at the same level of exposure, and the bottom picture is a longer exposure to differences in SM a-actin expression. **B.** FRNK^{-/-} cells were infected with GFP or GFP-FRNK adenovirus for 24 hours. The next day, cells were treated with BrdU (30 ug/mL) for 4 hours. After fixation, cells were stained with an antibody for BrdU and positive cells were counted. **C.** Cells were infected as described above for 24 hours and subsequently plated onto transwell filters (see materials and methods). After 18 hours, migrated cells were counted.
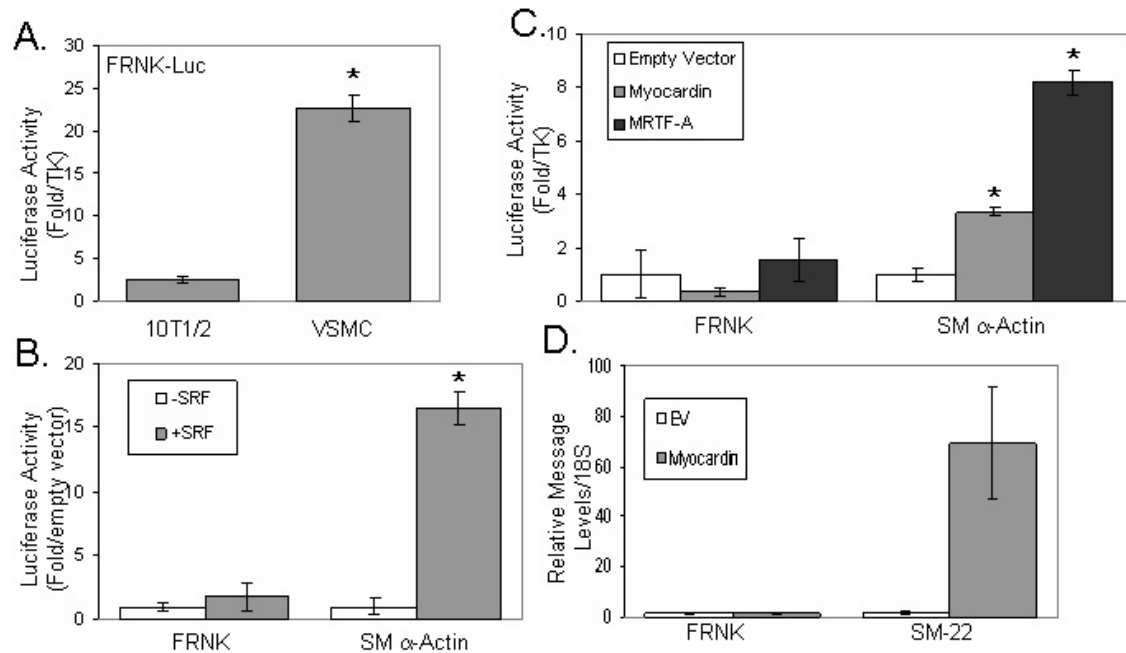
Figure 3.8. Activity of the FRNK promoter is regulated in a SM-specific but CArG-independent fashion. A. 10T1/2 or VSMC were transfected with 6kb FRNK-Luc or TK-Luc and assayed for luciferase activity 48 hours later. **B.** SRF$^{-/-}$ ES cells were co-transfected with either FRNK-Luc or SM α-actin-Luc in the presence of empty vector or SRF and processed for luciferase activity at 24 hr. **C.** Rat aortic SMC were transfected with FRNK-luc or SMα–actin-luc in the presence of empty vector, myocardin, or MRTF-A and were processed for luciferase activity at 48 hr. **D.** 10T1/2 cells were transfected with Flag or Flag-Myocardin. Total RNA was collected and RT-PCR was performed to measure levels of FRNK. Data from reporter assays is normalized to TK promoter activity in the presence of empty vector for each reporter. RT data is normalized to 18S. All data are presented as the mean +/- SEM of 3-5 experiments.
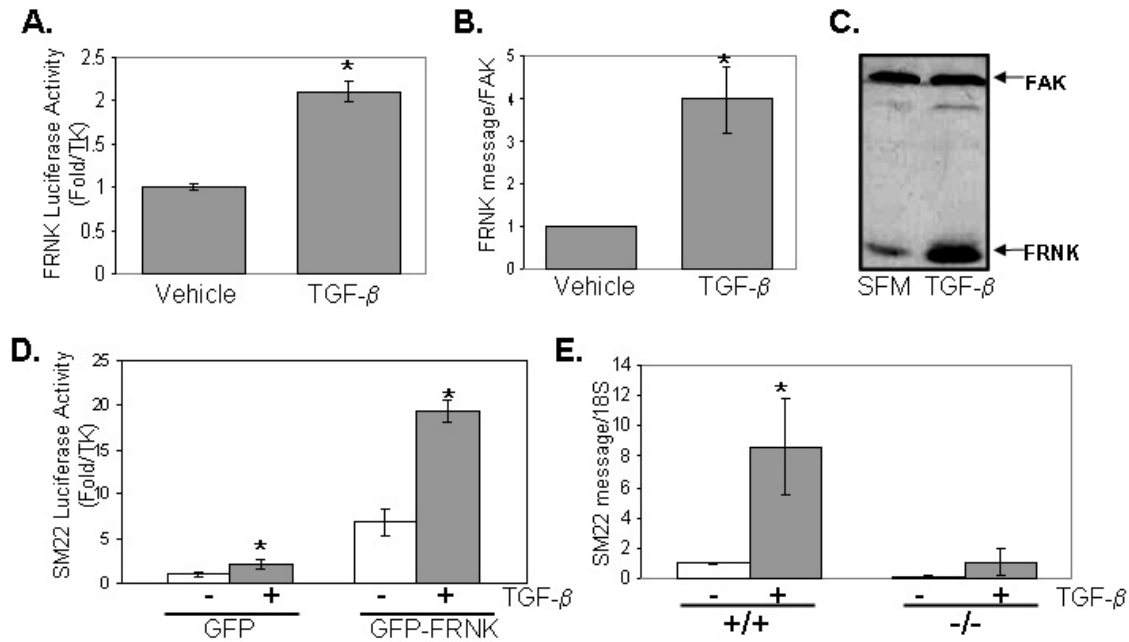
Figure 3.9. TGF-β treatment increases levels of FRNK expression. A. 10T1/2 cells were transfected with FRNK-Luc in serum-containing media. 24 hours later, media was replaced with serum-free media and cells were dosed with vehicle or TGF-β (1 ng/mL) for an additional 24 hr at which time cells were assayed for luciferase activity. The data are expressed as the mean +/- SEM (n=3, p<0.01). **B.** Quantitative RT-PCR for FRNK was performed on RNA isolated from SMC grown in serum-free media and treated with 1 ng/mL TGF-β or vehicle for 18 hr. Data are presented as fold increase in FRNK expression relative to untreated cells and is expressed as the mean +/- SEM (n=3, p<0.01). **C.** Western analysis for FRNK and FAK in serum-starved SMC treated with vehicle or TGF-β (1 ng/mL) for 24 hrs **D.** 10T1/2 cells were transfected with SM22-luc and infected with GFP or GFP-FRNK adenovirus 24 hours later. Cells were serum starved and treated with TGF-β as described above and processed for luciferase activity 24 hr later. **E.** Quantitative RT-PCR for SM-22 message in wild type (+/+) or FRNK^{-/-} (-/-) cells serum starved and treated with TGF-β as described above for 24 hr. Data is normalized to presence of 18S and is expressed as the mean +/- SEM (n=3, *p<0.05).

CHAPTER IV

CARDIAC NEURAL CREST-SPECIFIC INACTIVATION OF FOCAL ADHESION KINASE LEADS TO
OUTFLOW TRACT DEFECTS ASSOCIATED WITH ENHANCED SMOOTH MUSCLE CELL
MATURATION AND IMPAIRED MOTILITY[2,3]

ABSTRACT

Focal Adhesion Kinase (FAK) is strongly activated by integrin/matrix interactions and growth factor receptor engagement, and the observation that *fak*^{-/-} mice die between E8.5-10 with notable defects in vessel integrity suggests that FAK may play an important role in vascular maturation. In the current study we demonstrated that conditional deletion of FAK in *wnt-1*- or *nkx2-5*-expressing cells led to persistent truncus arteriosus that was incompatible with post-natal life. Given that aorticopulmonary septation involves the precise spatial and temporal coordination of several smooth muscle cell (SMC) processes including migration, proliferation, differentiation, and apoptosis, these studies suggest that FAK plays a critical role in SMC development. Indeed, our studies in FAK null aortic SMC cultures revealed a dual function for FAK in regulating SMC phenotype. FAK inactivation did not influence cell growth or survival, but markedly attenuated serum- and PDGF-BB-dependent chemotaxis. In addition, FAK inactivation also promoted serum- and TGF-β-stimulated SMC differentiation marker gene expression, which was mediated at least in part, by p38 Map Kinase. To our knowledge these studies are the first to define a cell autonomous role for FAK in the developing outflow tract and our results provide interesting new insights into the mechanisms underlying some of the most common congenital defects in humans including Tetralogy of Fallot and the DiGeorge Syndrome.

INTRODUCTION

The investment of newly formed endothelial cell tubes with differentiated smooth muscle cells (SMC) is a very important process during vessel formation and requires intricate regulation of SMC motility, growth, and differentiation. Mature medial SMC express high levels of contractile genes (i.e. myosin heavy chain (SM-MHC), SM α-actin, SM22α among others) and their presence is essential for maintaining vessel tone and for providing dynamic control of blood pressure (see (47) for review). Although there is no master regulator of SMC specification, serum response factor (SRF) binding to conserved CArG (CC(A/T)$_6$GG) promoter elements is required for the expression of most SMC differentiation marker genes and it is well known that SRF activity is regulated through interactions with additional cell-type-selective and ubiquitous transcription factors. The potent SRF co-factors of the myocardin family (myocardin and myocardin-related transcription factors, MRTF-A and B) are particularly strong activators of SMC-specific gene expression. The importance for myocardin in SMC differentiation is underscored by the finding that germline deletion of myocardin leads to embryonic lethality by E9.5 accompanied by a near complete lack of SMC lining major vessels (140). Extensive evidence indicates that extracellular matrix signaling is also an important regulator of SMC growth and differentiation as deletion of either fibronectin (FN), the α_5 integrin FN receptor, or focal adhesion kinase (FAK) (the kinase that mediates $\alpha5$-dependent signaling) each results in extraembryonic and embryonic vessel defects leading to lethality in the mouse from E8.5 to E10 (115, 116, 141).

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

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signaling by supporting the formation of multi-protein signaling complexes that lead to downstream activation of a number of signaling molecules that have been previously implicated in the regulation of SMC phenotype including ERK, JNK, and the small GTPases, Rac and Rho (142). Importantly, our lab 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 (122). These results suggest that FAK activity is tightly controlled in SMC especially during the transition from the synthetic to contractile phenotype.

It is clear that SMC play an important part in the intricate and complex series of events that must occur during the remodeling of the pharyngeal arches into an asymmetric aortic arch with appropriate division of the great vessels, but their precise functions are not yet known. The cardiac outflow tract and pharyngeal arches contain SMC derived from two distinct embryological origins, the cardiac neural crest and the secondary heart field. Elegant cell tracing studies in the chick and mouse have revealed that the tunica media at the base of the aorta and pulmonary trunk is invested by vascular smooth muscle derived from *nkx2-5*-expressing cells from the secondary heart field (143). Additional studies revealed that the vascular smooth muscle layers in the aorta from the aortic root (just distal to the *nkx2-5*-derived field) to the point of insertion of the ductus arteriosis, as well as the SMC within the internal carotid and proximal subclavian arteries are derived from *wnt-1*-expressing cardiac neural crest (CNC) cells (12, 144,

145). Interestingly, both *wnt-1-* and *nkx2-5-*expressing cells contribute to development of conotruncal septal SMC that divide the outflow tract into the aorta and pulmonary artery, allowing for dual systemic and pulmonary circulation (12, 145).

It is becoming clear that mutations that regulate cardiac neural crest differentiation into SMC lead to outflow tract defects. Indeed, germline deletion of MRTF-B leads to embryonic lethality associated with impaired SMC differentiation and aberrant outflow tract (OFT) development, as does *wnt-1* targeted deletion of myocardin (16, 17, 146). In addition, TGF-β, which promotes SMC differentiation (147-149), and PDGF-BB, which promotes SMC growth and motility, are important extrinsic regulators of SMC phenotype and genetic ablation of receptors for these genes also resulted in defective OFT morphogenesis (150, 151). The identification of the signaling pathways linking these extrinsic factors to SRF activation will be very important for our understanding of the control of SMC phenotype during development and disease.

The aim of the present study was to determine whether FAK activation plays a direct role in regulating SMC function during vascular development. We present evidence that FAK deletion (by homologous recombination) in either *wnt-1* or *nkx2-5* derived cells results in OFT development defects including persistent truncus arteriosis (PTA). However these phenotypes were not accompanied by reduced SMC differentiation, instead our studies revealed that ablation of FAK enhanced TGF-β stimulated SMC differentiation but attenuated PDGF-mediated SMC motility. These studies are the first to define a cell autonomous role for FAK in SM development and they provide interesting new insights into mechanisms underlying some of the most common congenital defects in humans.

MATERIALS AND METHODS

Generation of FAK-deficient mice

FAK^nk mice: Mice homozygous for insertion of loxP sites flanking the kinase domain of FAK ($fak^{flox/flox}$) (generated by Louis Reichardt and Hilary Beggs) were bred with $nkx2\text{-}5^{Cre}$ knock-in mice from Robert Schwartz (Institute of Biotechnology, Houston, TX) as previously described (152, 153). Genetic controls are $fak^{flox/flox}$ $nkx2\text{-}5^{wt/wt}$ and FAK^nk mice are $fak^{flox/flox}$ $nkx2\text{-}5^{wt/Cre}$.

FAK^wnt mice: To generate $fak^{+/-}$ mice, $fak^{flox/flox}$ mice were bred with $E2A^{Cre}$ mice to mediate germline Cre-mediated recombination of one floxed allele. $wnt\text{-}1^{Cre}$ mice were obtained from Andrew McMahon (12) and crossed with $fak^{flox/flox}$ to obtain $fak^{flox/+}wnt\text{-}1^{Cre}$ progeny. After backcrossing to C57/Bl6 mice, resulting $fak^{+/-}$ mice were bred with $fak^{flox/+}wnt\text{-}1^{Cre}$ mice to obtain $fak^{flox/-}wnt1^{Cre}$ (FAK^wnt) or $fak^{flox/-}wnt1$ (genetic control) mice. Genotypes were determined for all mice by obtaining DNA from tail snips and performing PCR. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Antibodies and Reagents

The Pyk2, phospho-specific ERK1/2, phospho-specific p38, and phospho-specific Smad 3 antibodies were purchased from Cell Signaling. Anti-vinculin, SMα-actin, and α-tubulin antibodies were purchased from Sigma. The N-term specific anti-FAK, C-term specific anti-FAK, phospho-specific Smad2, and anti-ERK2 antibodies were purchased from Upstate. Paxillin antibody was purchased from BD Transduction Laboratories and anti-Cyclin D1 was purchased from Santa Cruz. The anti-phospho Y397FAK antibody

was purchased from BioSource and the Texas-Red phalloidin was purchased from Molecular Probes. SM-22 antibody was a generous gift from Mario Gimona and SM-MHC antibody was obtained from U. Groeschel-Stewart. PDGF-BB, TGF-β, and SB-203580 were purchased from Calbiochem. 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.

Constructs

The promoter reporter constructs: SM α-actin (from -2560 to +2784), SM22 (from -450 to +88) and SM-MHC (from -4200 to +11600) luciferase constructs used have been previously described (128). 6xSBE-luciferase was a gift from Li Li (Wayne State University) (119). Flag-FAK was a generous gift from Dr. Tom Parsons (University of Virginia) and was previously described (154).

Cell Culture and Agonist Treatment

Aortic smooth muscle cells were isolated from either Wistar Rats or $fak^{flox/flox}$ 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 (125). Each of our preparations is routinely tested for expression of smooth muscle-specific markers (by immunohistochemistry) and the ability to drive SM-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.

Cell Growth Assay

$fak^{flox/flox}$ SMC (pre-treated with CRE or Lac-Z adenovirus for 72 hr) were trypsinized and plated onto a 96-well microplate (5 x 10^3 cells/well). Cells were serum starved for 24 hr and treated with PDGF-BB (20ng/ml) or EGF (100 ng/ml) for 48 hr. Cells were incubated with the formazen dye, WST-1 (10 μl; Roche) for 4 hr and the absorbance was read at 450 nM as per manufacturers instructions. Caspase activity was determined using the Caspase-Glo kit (Promega). To measure proliferating cells, BrdU (Sigma, 30 μg/mL) was administered to SMC grown on chamber slides. After 4 hrs, cells were fixed in 4% paraformaldehyde and stained using a BrdU detection kit (Invitrogen).

Transwell Assay

SMC were trypsinized and resuspended in DMEM:F12 containing 1% penicillin/streptomycin and 0.1% bovine serum albumin. Approximately 20,000 cells were plated on transwell filters (8 μm pore size) precoated with FN. After 7 hours, the cells were fixed in 4% paraformaldyhyde and the remaining cells in the upper chamber

were removed with a cotton swab. Migrated cells were stained with crystal violet and counted.

Promoter Assays

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

RhoA Pulldown Assay

Activity of RhoA was measured as previously described (128). Briefly, cell lysates were incubated with 40 μg of a GST-rhotekin Rho binding domain fusion protein immobilized to glutathione-Sepharose 4B beads (Amersham Biosciences) for 45 min at 4°C in binding buffer (50 mM Tris, pH 7.6, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 0.5 mM $MgCl_2$). Beads were washed three times (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM $MgCl_2$) and resuspended in 2x Laemmli buffer. Proteins were separated using SDS-PAGE, transferred to 0.2 μm polyvinylidene difluoride, and probed with anti-RhoA (26C4) (Santa Cruz Biotechnology). Loading controls (typically 10%) were taken from each lysate sample prior to pull downs.

Western Blotting

Western blots were performed using the appropriate antibodies at a 1/1000 dilution. Blots were washed in TBS-T (TBS plus 0.05% Triton-X), followed by

incubation with either horseradish peroxidase conjugated rabbit anti-mouse antibody or Protein A (Amersham) at a 1/2000 dilution. Blots were visualized after incubation with chemiluminescence reagents (ECL, Amersham).

Immunocytochemistry

Cells were processed for immunocytochemistry using previously published methods (122). 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) or anti-FAK (N-term 1:250). After washing with PBS, slides were incubated for 1 hour with Texas Red-conjugated donkey anti-mouse antibodies (2 µg/ml) or Texas Red-conjugated phalloidin to detect filamentous actin.

Histology and Staining

Pregnant females or postnatal pups were sacrificed at indicated times. Whole embryos or neonatal heart and lungs were formalin-fixed, paraffin embedded, and sectioned to 8 µm. To examine morphology, sections were stained with hemotoxylin and eosin. To detect expression of SM markers, immunohistochemistry was performed with antibodies to SM α-actin and SM-22. Briefly, sections were deparaffinized and rehydrated in graded ethanols. For detection of SM-22, sections underwent citrate buffer antigen retrieval (Vector). All sections were treated with 0.23% hydrogen peroxide to block endogenous peroxidases and permeabilized in 0.4% TritonX-100. Slides were blocked in 5% goat serum and incubated with primary antibody overnight. For SM α-

actin, protein was detected using HRP linked anti-mouse secondary and diaminobenzioline. For SM-22, biotinylated anti-rabbit secondary was used with the ABC detection kit (Vector).

Statistics

All results are representative of three independent experiments. Data is presented as the mean +/- standard error of the mean (SEM). Significance was determined using Student's *t* test.

RESULTS

To examine the role of FAK in SMC development, we utilized the *wnt-1^{Cre}* line that induces recombination in a substantial portion of the proximal outflow tract SMC. These well characterized mice generated high efficiency recombination c.a. E9.5 in CNC-derived SMCs populating pharyngeal arch arteries 3, 4, and 6 and the truncus arteriosis and by E11.5 they induced recombination within the SMC precursors invading the conotruncal cushions and the aorticopulmonary septum (12). *fak$^{flox/flox}$* mice were first interbred with *wnt-1^{Cre}* transgenics and the *fak$^{+/fl}$/wnt-1^{Cre}* offspring were subsequently crossed with mice heterozygous for the FAK allele (*fak$^{+/-}$*). At P0, we found the appropriate Mendelian distribution of *fak$^{flox/-}$wnt-1$^{Cre/wt}$* (herein referred to as FAK^{wnt}) mice, however the FAK^{wnt} mice died within the first two weeks of life (Table 4.1). Gross evaluation of FAK^{wnt} neonates at P0-P1.5 revealed no significant difference in external appearance from the genetic control littermates. Indeed, FAK^{wnt} and genetic control neonates were similar in size and had normal limb and cranial-facial development (data not shown). However, gross morphology of the cardiac outflow tract of FAK^{wnt} mice revealed aberrant septation and branching of the major outflow vessels (Figure 4.1).

We next performed a histological analysis of FAK^{wnt} and littermate genetic control hearts at selected time points from E12.5-P0 to evaluate outflow tract development. We found that 100% of the FAK^{wnt} embryos exhibited PTA, indicating that FAK expression in CNC cells is necessary for appropriate septation of the truncus arteriosis (Figure 4.1). Examination of other neural crest derived tissues such as cranial bones and thymic lobes revealed no obvious abnormalities, indicating that the initial delamination, migration, and proliferation of neural crest was normal. We next examined

SM marker gene expression in the FAK^{wnt} mice to determine whether FAK was necessary for SMC differentiation. Somewhat suprisingly, we found that the FAK^{wnt} mice exhibited a thick layer of SM α-actin- and SM-22-positive SMC within the walls of the un-septated truncus arteriosis (Figure 4.2) and carotid arteries (data not shown). Indeed, SM-22 staining appeared particularly intense in the OFT of FAK^{wnt} in comparison to similar regions of the genetic controls (arrows, Figure 4.2b). We previously showed that conditional deletion of FAK in *nkx2-5*-expressing cells (FAK^{nk}) resulted in completely penetrant ventricular septal defects accompanied by significant outflow tract abnormalities, including PTA. Since *nkx2-5*-dervied SMC also contribute to formation of the aortic root and septum, we next examined FAK^{nk} mice for defects in smooth muscle maturation. Similar to what we observed in the FAK^{wnt} mice, we found that the single vessel arising from FAK^{nk} embryos that exhibited PTA was fully invested with SMC, as assessed by robust SM α-actin staining (Figure 4.3). Collectively, these studies indicate that FAK is not required for neural crest cell function or the differentiation of *wnt-1* or *nkx2-5* expressing cells to SMC, but is essential for the formation of the aorticopulmonary septum during OFT development.

Aorticopulmonary septation occurs between E11.5 and E12.5 when a wedge of CNC-derived SMC extends from the dorsal region of the aortic sac and ultimately fuses to the ventrally growing conotruncal septum to partition the aortic sac into distinct aortic and pulmonary channels and align the great vessels with the left and right ventricles respectively. While almost nothing is known about the regulation of SMC during this morphogenetic process, several fundamental cellular functions including directional motility, cell growth and cell differentiation are likely involved. In order to evaluate the

necessity for FAK in these cellular processes, we next established an aortic SMC culture from $fak^{flox/flox}$ mice. As shown in Figure 4.4, 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. 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 (Figure 4.4b).

We first evaluated the effect of FAK depletion on SMC growth and survival. We found no significant difference in the rate of BrdU incorporation in continuously growing $fak^{flox/flox}$ cells treated with LacZ or Cre adenovirus (Figure 4.5A). We also found comparable rates of cell growth under serum-free conditions and in cells stimulated with the SMC mitogens PDGF-BB and EGF (Figure 4.5B). Accordingly, mitogen-stimulated ERK activity and cyclin D protein levels were similar in Cre- and LacZ- treated cells (Figures 4.5C and 4.5D), indicating that FAK was not required for activation of the major mitogenic Ras/ERK signaling pathway in SMC. Consistent with our findings that deletion of FAK did not alter cell growth, we observed no significant difference in cell survival as measured by caspase 3/7 activity (Figure 4.5E). Collectively, these data indicate that defective aorticopulmonary septation in the FAK^{wnt} and FAK^{nk} mice is not likely due to inhibition of SMC proliferation or aberrant apoptosis.

We next examined the effect of FAK on SMC motility, another candidate cellular response critical for aorticopulmonary septation. Using a modified Boyden chamber assay, we demonstrated that PDGF-stimulated chemotaxis was dramatically reduced in FAK-null SMC (Figure 4.6A). This inhibition was observed across a wide range of FN

concentrations, indicating that these effects were not due to altered adhesiveness (data not shown). Interestingly, a similar significant reduction in chemotaxis was observed when a modest level of serum (1%) was used as a chemoattractant. However, this effect could be overcome by the strong chemotaxic stimulus induced by higher concentrations of serum (10%) indicating that impaired chemotaxis was not due to a global structural deficiency in the FAK-null SMC (Figure 4.6B). The marked decrease in PDGF-BB-stimulated chemotaxis observed in the FAK-null SMC is likely due to defective recruitment of factors to focal adhesion complexes that are required to maintain stable lamellipodial projections (LEM and JMT manuscript in preparation). Since PDGF is known to be highly expressed in the conal truncus in this critical developmental window, and has been implicated in guiding cells during aorticopulmonary septal formation, it is possible that the cause of the PTA in FAK^{wnt} and FAK^{nk} mice was due to a defective response of SMC to this chemokine (150, 155).

We observed a qualitative increase in SMC-marker gene expression in the OFT of the FAK^{wnt} mice. Since premature SMC differentiation may affect whether a SMC can populate the aortic sac, we next strove to determine whether SMC marker genes might be elevated in FAK-null SMC cultures. We first examined the protein levels of SRF-target genes in LacZ- and Cre-treated SMC maintained in 10% serum. As shown in Figure 4.7A, we observed a striking increase in expression of the canonical SM marker gene, SM-MHC in FAK-null SMC. Consistent with this observation, another SRF-target gene vinculin was more highly expressed in FAK-null SMC (156), while expression of the paxillin (a non-SRF dependent focal adhesion protein) was similar between FAK-null and control SMC (Figure 4.7A). We next asked if TGF-β-induced SM marker gene

expression was altered in FAK-null SMC. As shown in Figure 4.7B, while basal levels of SM α-actin were similar in serum-starved Cre and LacZ-treated SMC, TGF-β induced a more marked up-regulation of SM α-actin protein levels in Cre-treated compared to LacZ treated SMC. To test whether this effect was due to enhanced SMC-specific transcription, we examined the responsiveness of an SM-22 promoter/luciferase reporter in these cells. As shown in Figure 4.7C, SM-22 activity in control SMC was induced 2-fold by TGF-β, but by 10-fold in FAK-null cells (top panel). A similar enhancement in TGF-β induced expression of the SM α-actin and SM-MHC promoters was also observed in FAK-null SMC (Figure 4.7C middle, bottom panels). Importantly, ectopic expression of wild type FAK reversed the enhanced expression of SM α-actin induced by TGF-β (Figure 4.7D). Taken together, our findings provide evidence that FAK depletion attenuates PDGF-stimulated chemotaxis, but promotes TGF-β-stimulated SMC maturation.

We next strove to determine the precise mechanisms by which FAK inactivation enhances SMC differentiation, since this finding could have important implications on the regulation of SMC phenotype during development and disease. The canonical pathway by which TGF-β stimulates SMC gene transcription is through activation of Smad2/3 transcription factors. TGF-β-mediated phosphorylation and subsequent nuclear localization of Smad2 and Smad3 can stimulate SMC-specific transcription (117, 118, 148), and Smad binding elements (SBE) have been identified in a number of SMC-specific promoters. However, we found that FAK deletion did not significantly enhance Smad2/3 activity. Indeed, a consistent lag in Smad2 and Smad3 phosphorylation was apparent in FAK-null SMC, following TGF-β treatment indicating that the initial phase

of SMAD signaling may actually be FAK-dependent (Figure 4.8A). Further, TGF-β stimulated similar activation of a multimerized Smad binding element promoter/reporter construct (6xSBE/Luc) in Cre- and LacZ-treated *fak$^{flox/flox}$* SMC, indicating that FAK likely acts to enhance TGF-β-stimulated SMC gene expression by a Smad-independent mechanism (Figure 4.8B).

It is becoming clear that RhoA activation is an important determinant of SMC differentiation marker gene expression. Indeed the strong RhoA agonist, sphingosine-1-phosphate (S1P), has been shown to induce SMC differentiation through a mechanism involving RhoA dependent translocation of the potent SRF co-factors MRTF-A and B (128, 157). Interestingly, previous studies have shown that FAK-null fibroblasts exhibit enhanced basal RhoA activity, indicating the possibility that this pathway might be involved in promoting SMC maturation in FAK-null SMC (158). However, we found that basal and S1P-stimulated RhoA activity were comparable in LacZ- and Cre-treated SMC (Figure 4.9A) and that TGF-β treatment did not lead to detectible levels of RhoA activation at select time points examined from 2 min-4 hrs in either LacZ- (Figure 4.9B) or Cre-treated cells (data not shown). In agreement with our biochemical assays, we did not observe enhanced nuclear localization of either MRTF-A or MRTF-B in vehicle-, S1P-, or TGF-β-treated FAK-null SMC in comparison to similarly treated FAK containing SMC (data not shown). Collectively, these data argue against enhanced RhoA activation as a major mechanism involved in the modulation of TGF-β induced SM marker gene expression in FAK-null SMC.

Another downstream signaling molecule implicated in TGF-β stimulated gene expression is the MAPK family member p38. p38 is known to be a critical player in

skeletal muscle myotube formation and has been recently implicated in promoting cardiac and smooth muscle differentiation and in the conversion of fibroblasts to SM α-actin expressing myofibroblasts (159-161). We found that the p38α/β subunit inhibitor, SB203580 (10 μM) dramatically reduced TGF-β stimulated SM marker gene promoter-reporter expression in 10T1/2 SM precursor cells (Figure 4.10A). This effect was not due to toxicity, as parallel experiments revealed that SB203580 treatment did not significantly affect PDGF-stimulated SMC chemotaxis (data not shown). We next evaluated the levels of phosphorylated (active) p38 in LacZ- and Cre-treated cells following TGF-β treatment. As shown in Figure 4.10B, the time course of p38 activation was prolonged and the peak p38 activity was more pronounced in FAK-null SMC in comparison to FAK-containing SMC. As expected, treatment with SB203580 reversed the elevated SM gene expression observed in FAK-null SMC (data not shown). Thus, enhanced p38 activity may account, at least in part, for the observed increase in SM marker gene expression observed in FAK null SMC.

DISCUSSION

In the current study we demonstrated that conditional deletion of FAK in *wnt-1-* or *nkx2-5*-expressing cells led to PTA that was incompatible with post-natal life. Given that aorticopulmonary septation involves the precise spatial and temporal coordination of several SMC processes including migration, proliferation, differentiation, and apoptosis, these studies suggest that FAK plays a critical role in SMC development. Indeed, our studies in FAK-null aortic SMC cultures revealed a dual function for FAK in regulating SMC phenotype. FAK inactivation attenuated serum- and PDGF-BB-dependent chemotaxis, and also promoted serum- and TGF-β-stimulated SMC differentiation marker gene expression. To our knowledge these studies are the first to define a cell autonomous role for FAK in the developing OFT and provide interesting new insights into the mechanisms underlying some of the most common congenital defects in humans including Tetralogy of Fallot and the DiGeorge Syndrome.

Interestingly, PTA has been associated with a block in SMC differentiation within the truncus of mice harboring null mutations in the BMP receptor, Alk-2 and MRTF-B as well as those with CNC-targeted expression of a dominant negative Notch variant (15-17, 162). However, similar to our studies, CNC-targeted ablation of TGFβR2, PDGFRα/β, and Gata 6 each lead to fully penetrant PTA without a noticeable reduction in SMC number or SMC maturation (150, 151, 163). In fact, Choudhary, *et al.* noted that at a stage prior to aorticopulmonary septal formation, the cells at the distal end of the aortic sac (where aorticopulmonary wedge formation is initiated) prematurely expresses very high levels of SM marker genes in *wnt-1^Cre^/tgfβr2* mutants (151). Taken together, these finding may suggest that precise regulation of SMC phenotype within the

walls of the aortic sac is necessary for subsequent formation of the aorticopulmonary septum. Since PDGF (164), GATA6 (165), and now FAK have each been implicated in the maintaining SMC in an immature, synthetic state, it is tempting to speculate that precocious SMC maturation may occur when these molecules are inactivated. However, it is also clear from our studies and others that both PDGF and FAK are also necessary for directional motility, indicating that lack of these molecules could impede motility of either neural crest or secondary heart-field derived mesencymal cells into the conotruncal region. Thus, although we do not yet know the precise causative factor(s) for PTA, it is possible that both precocious SMC maturation and defective motility lead to the aberrant morphogenetic patterning of the cardiac OFT observed in the FAK[nk] and FAK[wnt] mice.

Our lab recently showed that FAK activity is regulated in a unique fashion in SMC by the selective expression of a separate protein comprising the carboxyterminus of FAK, termed FRNK (FAK Related Non Kinase) that acts as a dominant-interfering mutant for FAK (122). FRNK expression is very high in large arterioles and is greatly increased after birth and from two to three weeks following vessel injury, times when SMC are transitioning from a synthetic to contractile phenotype (122). Although FRNK null mice exhibited no overt phenotype (166), FAK activity was enhanced in FRNK^{-/-} SMC tissues while SMC differentiation marker gene expression was reduced during post-natal vessel growth and following vascular injury (unpublished observations, RLS and JMT). Interestingly, FAK-null mouse embryonic fibroblasts exhibit a myofibroblast appearance with increased SM α-actin expression further supporting a role for FAK activation in the regulation of SMC phenotype (133).

In addition, recent studies also suggest that FAK promotes 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 (134). Stable expression of FRNK in embryonic stem (ES) cells was shown to induce cardiac α-myosin heavy chain and sarcomeric myosin expression (135). 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 (167), 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 it is possible that limiting FAK activity might enhance SRF-dependent muscle cell differentiation at the expense of cartilaginous cell differentiation(168). 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.

The well spread morphology of our FAK-null SMC differs from previously described embryonic FAK-null fibroblasts which were more rounded, possibly due to enhanced RhoA activation (169, 170). It is important to emphasize, however, that FAK-null fibroblasts expressed high levels of Pyk2 and that compensatory pathways are frequently up-regulated in cells isolated from conventional knock-out mice. Importantly,

our results are in excellent agreement with previous work from Tilghman, *et al.* who demonstrated that repression of FAK expression in *fak*flox/flox fibroblasts by adenoviral mediated CRE delivery or siRNA did not result in significant morphological changes (121). We did however observe a consistent increase in vinculin protein levels in the absence of FAK, supporting our hypothesis that SRF-dependent gene transcription is elevated in FAK-null cells (171).

Of the several potential mechanisms by which FAK activity could modulate TGF-β dependent SMC differentiation, our data support a role for p38. Although FAK and TGF-β have been shown to affect RhoA activity (160, 169, 170), neither of these signals altered this pathway in our SMC culture model. FAK activation is also required for full ERK activity in some models and it has been previously shown that ERK-dependent activation of the SRF co-factor, Elk-1, negatively regulates SMC marker gene expression by competitively interfering with the myocardin binding to SRF (172-175). While FAK deletion in SMC attenuated PDGF-stimulated migration, we did not observe any significant difference in either the kinetics or amplitude of PDGF- or EGF-stimulated ERK activation in FAK-null SMC. In contrast, we did observe a significant enhancement of p38 activity in FAK-null SMC which was required for enhanced SMC differentiation marker gene expression. Importantly, p38 activity has been shown to promote smooth, cardiac, and skeletal muscle differentiation as well as SM α-actin expression in fibroblasts (159-161). p38 activity is not required for skeletal muscle specification, but inhibition of p38 activity markedly attenuated expression of a subset of MyoD target genes classified as late muscle structural genes (159). This effect may be due to impaired p38-dependent recruitment of the SWI-SNF chromatin remodeling complex to the

promoters of these target genes (176). In addition, p38 has been shown to directly phosphorylate the SRF-cofactors, MEF2A, C, D and GATA-4 at residues located within the transactivation domain of each of these proteins and this phosphorylation is necessary for their full transcriptional activities (159). Interestingly, SRF is itself a target for phosphorylation by MK2 (a downstream effector of p38) and activation of MK2 is necessary for TGF-β stimulated myofibroblast differentiation (177, 178). Whether p38 functions in a similar fashion to regulate SRF or additional SRF-cofactors involved in SMC differentiation is not yet known.

FAK is a multifunctional protein that associates with a number of adapter molecules through well defined protein interaction sites, and it is also possible that specific FAK binding partners could be involved in modulating the SMC phenotype. Interestingly, we recently reported that the FAK-interacting protein leupaxin shuttles from focal adhesions to the nucleus, where it acts as an SRF co-factor to enhance SM marker gene expression (179). Furthermore, we showed that expression of a constitutively active FAK variant leads to sequestration of leupaxin within focal adhesions and reduces leupaxin-dependent gene transcription (179). Conversely (although a suitable leupaxin antibody is not available to detect endogenous leupaxin in our primary mouse SMC cultures) we have noted that ectopically expressed GFP-leupaxin exhibits a more nuclear restricted localization in FAK-null SMC in comparison to FAK containing SMC (unpublished observations). Studies to determine the relative contributions of (or relationship between) leupaxin and p38 in regulating SMC phenotypes in FAK null SMC are ongoing in our laboratory.

In conclusion, our studies indicate that FAK plays a direct role in regulating the formation of the aorticopulmonary septum during outflow tract development. We postulate that direct modulation of FAK activity (or the intrinsic shifting of FAK/FRNK expression) likely mediates a balance between SMC migratory and contractile capacities necessary for proper morphogenesis of this structure. With respect to adult onset disease, is clear that dynamic SMC phenotypic switching is a key factor in the development and progression of restenotic lesions. Since the data from our current and previous studies indicate that FAK activity promotes SMC migration and down regulation of differentiation marker gene expression, it will be of future interest to determine whether SMC-restricted FAK inactivation (or FRNK expression) in large vessels at later timepoints might control neo-intimal formation by preventing SMC phenotypic modulation.

Table 4.1. Variable offspring from $fak^{+/-}$ x $fak^{+/fl}$ $wnt\text{-}1^{+/Cre}$ intercross.

	P0	P14	
	Percentage	Percentage	*fak* Copy Number
$fak^{+/+}$ $wnt\text{-}1^{+/+}$	19	15	2
$fak^{+/+}$ $wnt\text{-}1^{+/Cre}$	9	8	2
$fak^{fl/+}$ $wnt\text{-}1^{+/+}$	23	8	2
$fak^{fl/+}$ $wnt\text{-}1^{+/Cre}$	16	15	1
$fak^{+/-}$ $wnt\text{-}1^{+/+}$	2	31	1
$fak^{+/-}$ $wnt\text{-}1^{+/Cre}$	5	15	1
$fak^{fl/-}$ $wnt\text{-}1^{+/+}$	7	8	1
$fak^{fl/-}$ $wnt\text{-}1^{+/Cre}$	19	0	0

Expected percentage: 12.5% for each genotype; P0, n = 43; P14, n = 13.
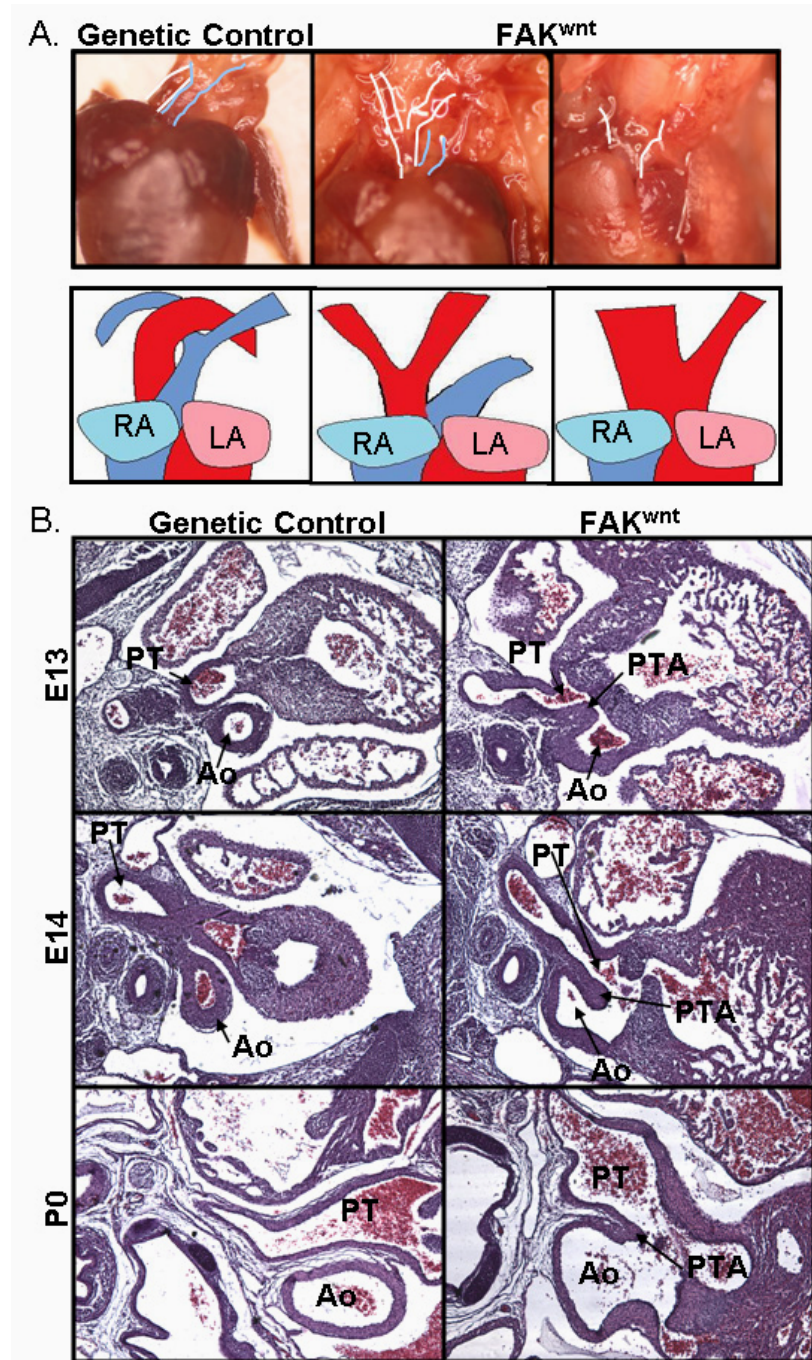
Figure 4.1. Deletion of *fak* from the cardiac neural crest causes septation defects in the truncus arteriosus. **A.** Gross morphology of the cardiac outflow tract in P0 genetic control (left) and two examples of outflow defects in FAK^{wnt} mice (right). Lines are drawn to indicate morphology of the great vessels (white, aorta; blue, pulmonary artery). Schematics below illustrate the nature of the observed defects. RA-right atrium, LA-left atrium. **B.** Transverse sections through the cardiac outflow tract of embryonic day (E) 13, 14 or P0 genetic control (left) or FAK^{wnt} (right) mice stained with H&E. Data are representative of at least 5 mice. PT-pulmonary trunk, Ao-Aorta, PTA-persistent truncus arteriosis.
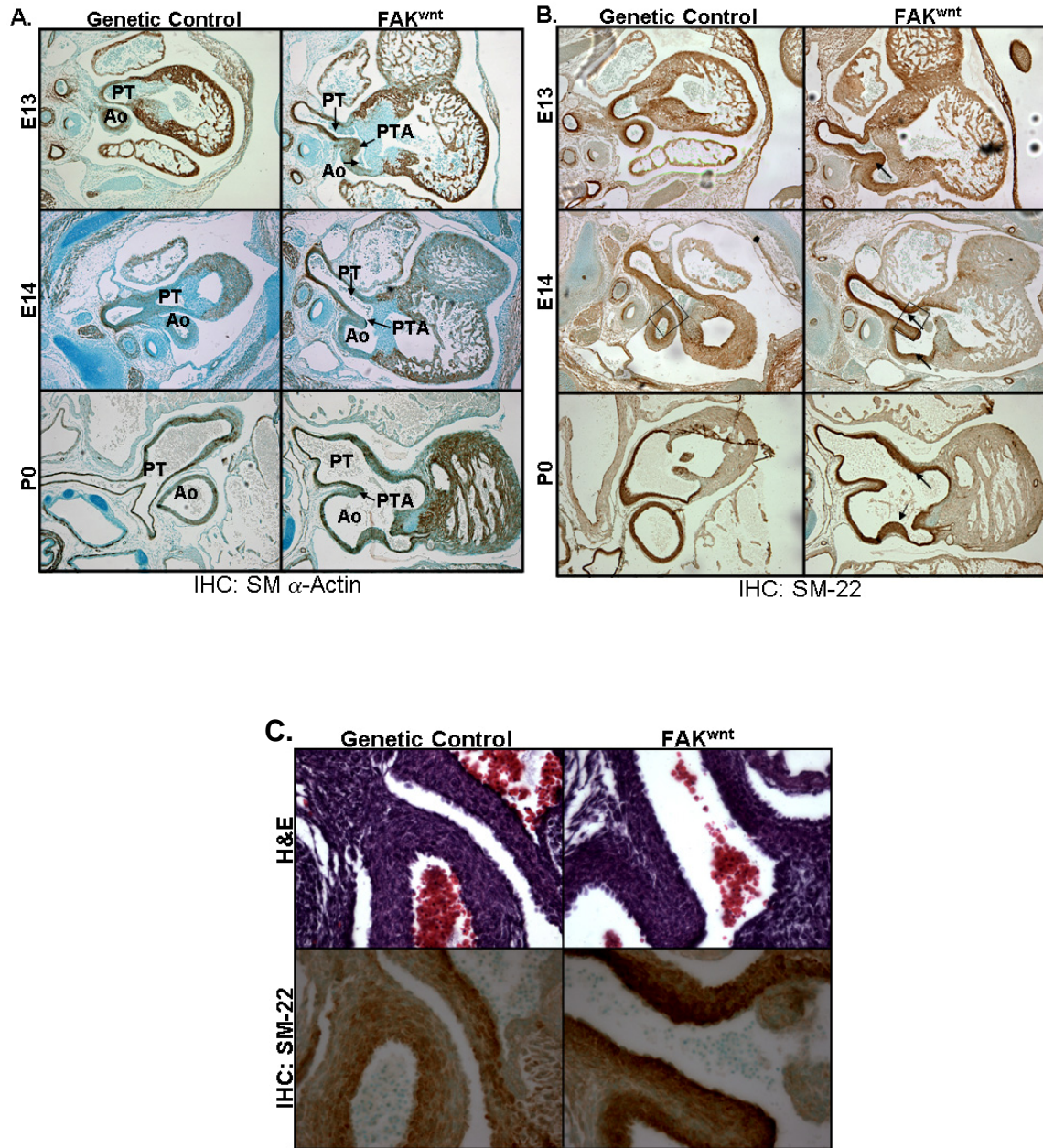
Figure 4.2. PTA is not a result of impaired SMC investment in OFT of FAK^{wnt} embryos.
Immunohistochemistry for SM α-actin (**A**) or SM-22 (**B**) reveals thick layer of differentiated SMC in both genetic control (left) and FAK^{wnt} (right) mice from E13 to P0. Boxes in Panel **B** are shown at higher magnification in Panel **C**. **C.** High power magnification showing cross section through vascular wall of the aorta and pulmonary artery of genetic control embryo and the truncus arteriosis of FAK^{wnt} embryo at E14. SM2-22 immunohistochemistry demonstrates a marked increase in neural creast-derived SMC in FAK^{wnt} OFT. Data are representative of at least 5 mice. PT-pulmonary trunk, Ao-Aorta, PTA-persistent truncus arteriosis.
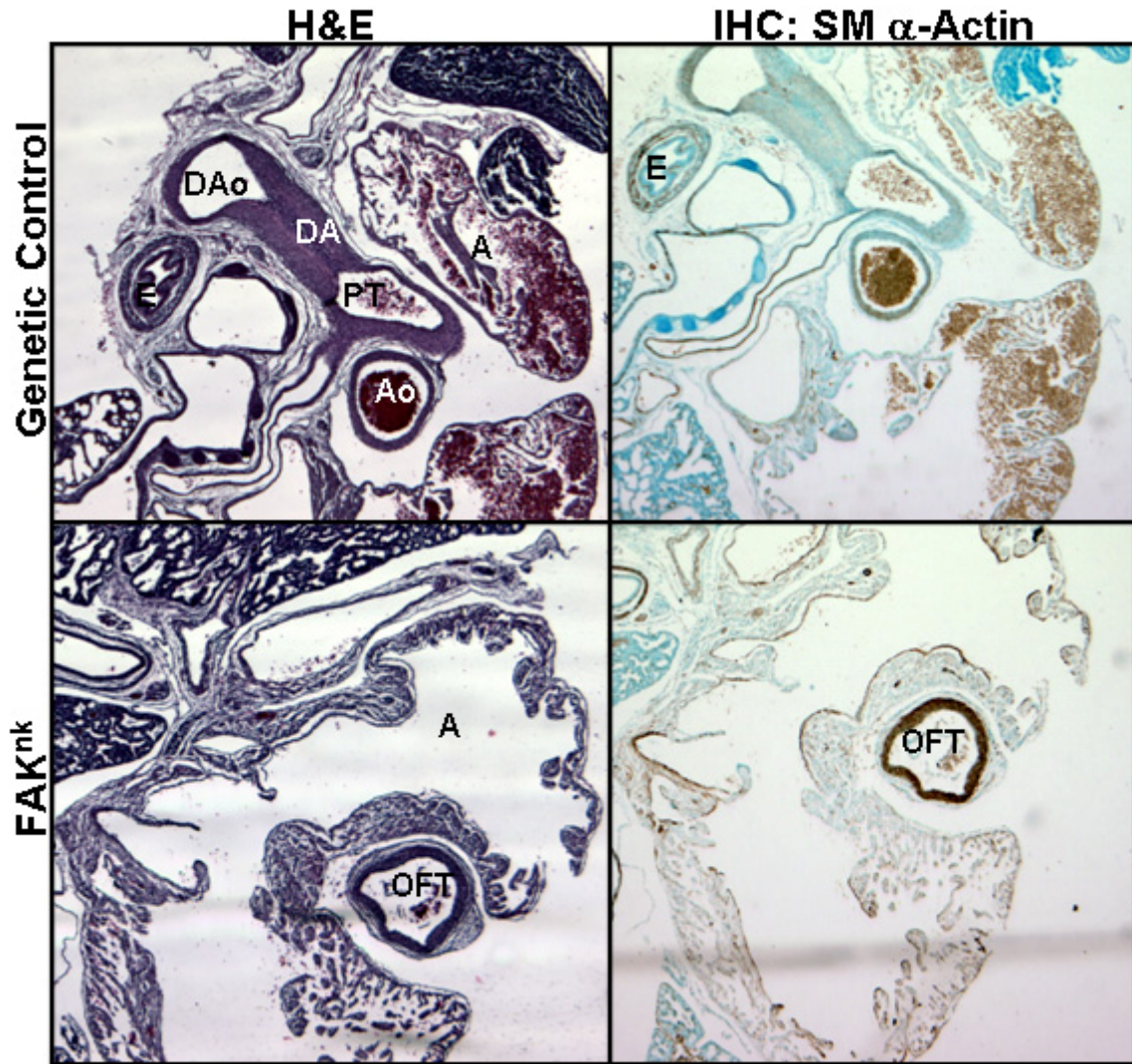
Figure 4.3. Conditional deletion of *fak* in *Nkx2-5*-expressing cells leads to PTA accompanied by pronounced SMC investment within the OFT. Transverse sections through the cardiac outflow tract of P0 genetic control (top) or FAK[nk] mice (bottom). Sections were stained with H&E (left) or processed for immunohistochemistry using an anti-SM α-actin antibody (right). Approximately 15% of FAK[nk] mice presented with PTA. Data are representative of at least 5 mice. DAo-dorsal aorta, PT-pulmonary trunk, OFT-outflow tract, DA-ductus arteriosus, Ao-aorta, A-atrium, E-esophogus.
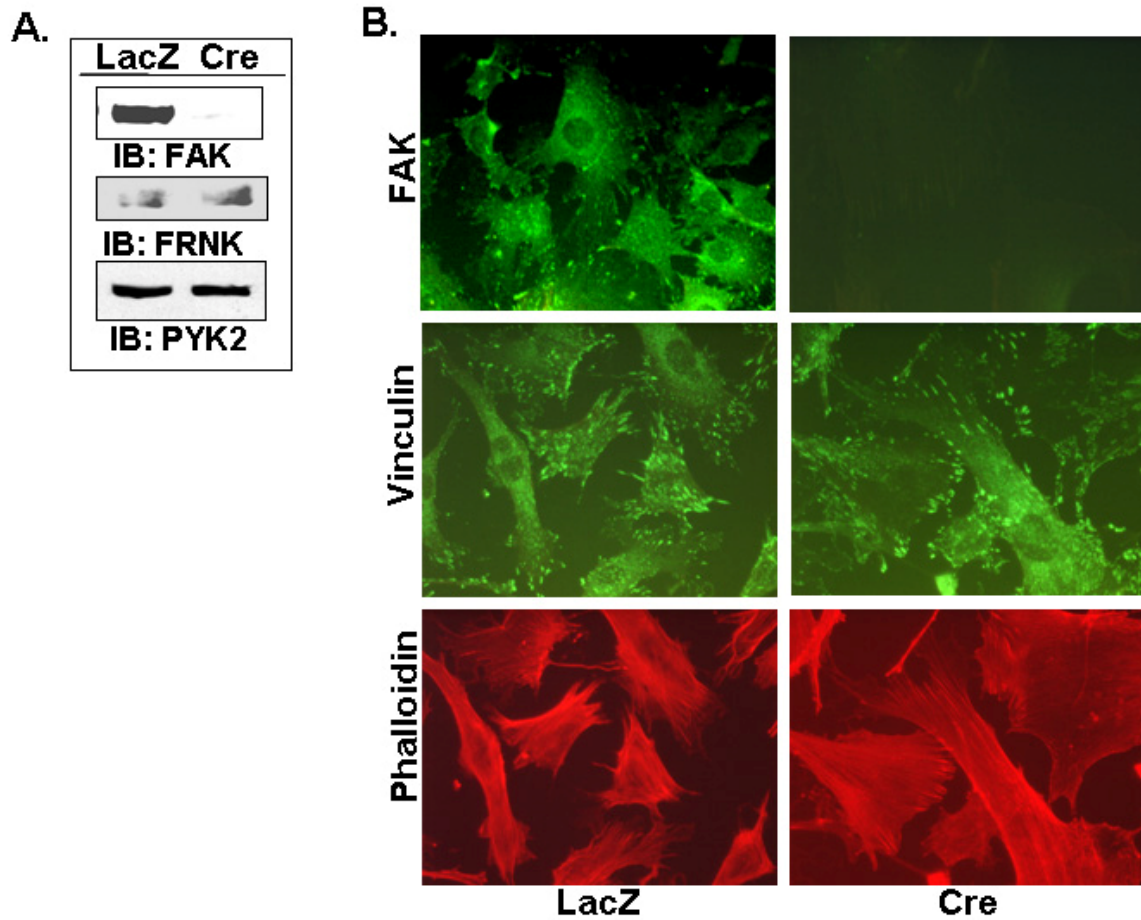
Figure 4.4. Characterization of FAK-null SMCs. **A.** *fak*flox/flox SMC maintained in 10% serum were infected with either LacZ or Cre adenovirus for 72 hrs, cells were lysed, and Western blotting was performed using either anti-C-terminal FAK (that recognizes both FAK and FRNK), or anti-Pyk2 specific antibodies. **B**. *fak*flox/flox SMC maintained in 10% serum 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 Material and Methods section. Data are representative of at least three experiments.
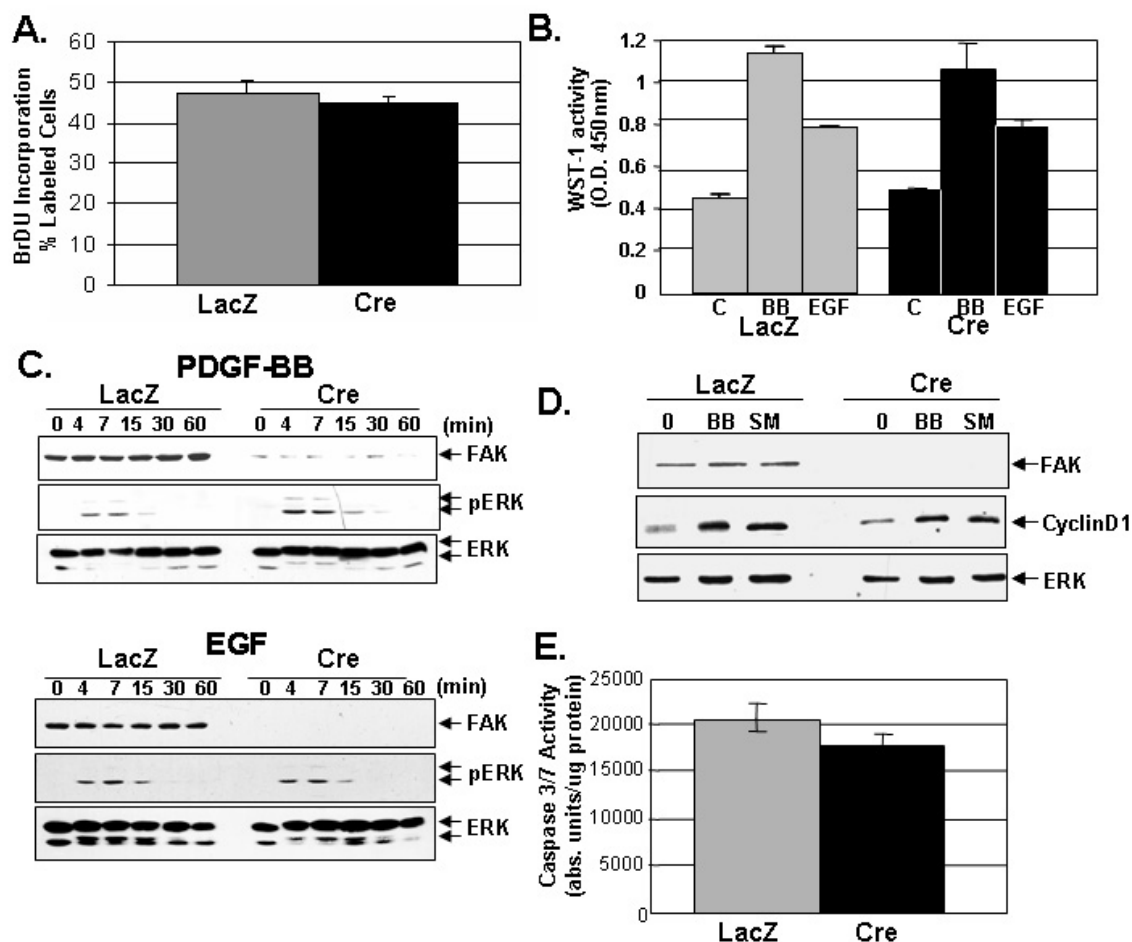
Figure 4.5. FAK depletion does not affect SMC growth or growth factor-stimulated ERK activation. $fak^{flox/flox}$ SMC were infected with either Cre or LacZ adenovirus for 72 hrs. **A.** Cells maintained in serum were assessed for BrdU incorporation as described in Materials and Methods **B.** Cells were serum starved for 24 hrs prior to treatment with either PDGF-BB (20 ng/ml) or EGF (100 ng/ml) for 48 hrs prior to determining WST-1 activity as described in the Materials and Methods section. **C**. Cre or LacZ infected $fak^{flox/flox}$ SMC were serum starved for 24 hrs prior to treatment with either PDGF-BB (20 ng/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. **D**. $fak^{flox/flox}$ SMC were infected with either Cre or LacZ adenovirus for 72 hrs. Cells were then serum starved for 4 hrs prior to treatment with either 10% serum (SM) or PDGF-BB (20 ng/ml) for 24 hrs. Cells were lysed, electrophoresed, and Western blotting was performed with anti-FAK, anti-Cyclin D1 and anti-ERK antibodies. **E**. 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.
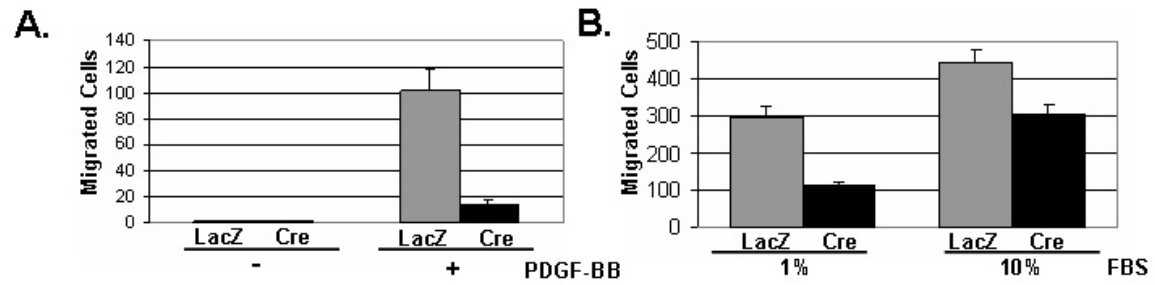
Figure 4.6. FAK depletion attenuates serum- and PDGF-stimulated chemotaxis. Cre- or LacZ-infected *fak*^flox/flox^ SMC (treated as described above) were plated on fibronectin-coated inserts (3 µg/ml; Bio-Coat) in serum-free media using either PDGF-BB (20 ng/ml; panel **A**) or serum (1 or 10%, panel **B**) as the chemoattractant which was applied to the bottom chamber as described in the Methods section. After a 7 hr incubation cells were stained and counted in four fields. Data represent mean +/- SE for 4 separate experiments.
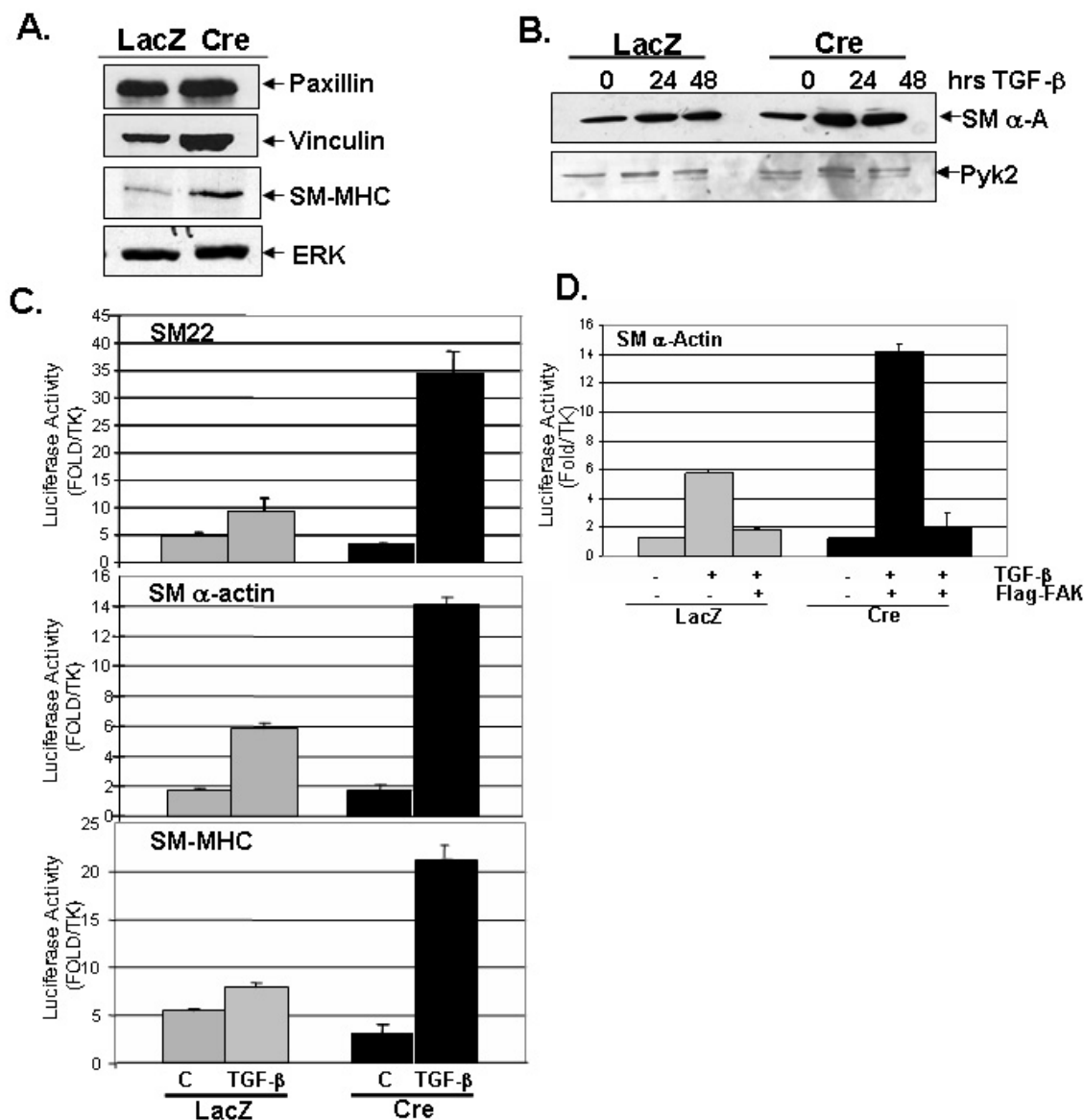
Figure 4.7. FAK depletion enhances SM gene expression. A. *fak*[flox/flox] SMC were infected with either Cre or LacZ adenovirus for 72 hours. Cells maintained in 10% serum were lysed and Western blotting was performed using either anti-paxillin, anti-vinculin, anti-SM-MHC, or anti-ERK specific antibodies. **B.** *fak*[flox/flox] SMC were infected as mentioned above. Cells were serum starved for 24 hrs prior to TGF-β treatment for times indicated. Western blotting was performed with anti-SM α-actin and anti-Pyk2 (loading control) antibodies. **C.** *fak*[flox/flox] SMC were transfected with SM-22, 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 co-transfected with the SM α-actin-luciferase promoter and either empty Flag vector or Flag-FAK. 6 hrs following transfection cells were infected with either Cre or LacZ adenovirus. 24 hrs following infection, cells were rinsed and serum starved for 24 hrs and then treated with 1ng/ml TGF-β overnight. Cells were lysed and luciferase activity was measured. Data is representative of 3 individual experiments.

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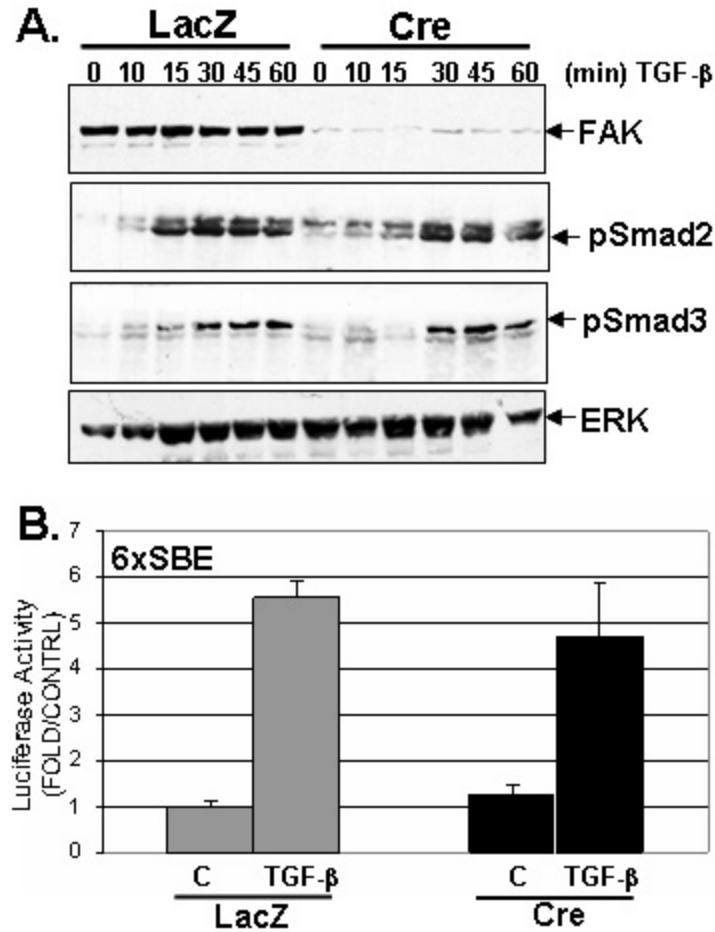
Figure 4.8. FAK depletion does not enhance TGF-β stimulated Smad activation or transactivating capacity. A. *fak*^{flox/flox} SMC were infected with LacZ or Cre adenovirus as mentioned above. Cells were serum starved for 24 hrs prior to TGF-β treatment for times indicated. Western blotting was performed with anti-phospho-Smad2, anti-phospho-Smad3, anti-FAK and anti-ERK antibodies. **B.** *fak*^{flox/flox} SMC were infected with LacZ or Cre adenovirus as mentioned above. Two days following infection, cells were transfected with 6x-SBE-luciferase. After 24 hrs, cells were rinsed and serum starved 8 hours prior to treatment with TGF-β (1ng/mL). 24 hrs later, cells were lysed and luciferase activity was measured. All data represent at least 3 separate experiments.

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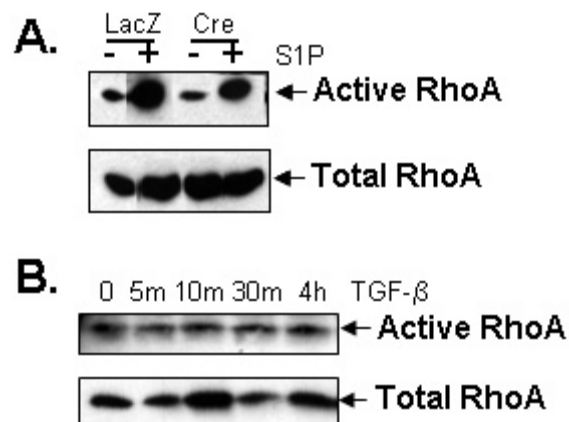
Figure 4.9. FAK depletion does not affect RhoA activity. A. $fak^{\text{flox/flox}}$ SMC were infected with LacZ or Cre adenovirus as mentioned above. Cells were serum starved for 24 hrs prior to S1P (panel A) or TGF-β (panel B) treatment for times indicated. Cell lysates were incubated with GST-rhotekin beads as described in the Materials and Methods section. Active RhoA (present in the precipitations) and total RhoA were detected by Western analysis. Densitometric analysis of three separate experiments revealed no significant difference in basal or S1P-stimulated RhoA activation in Cre- compared to LacZ-infected cells. All data represent at least 3 separate experiments.
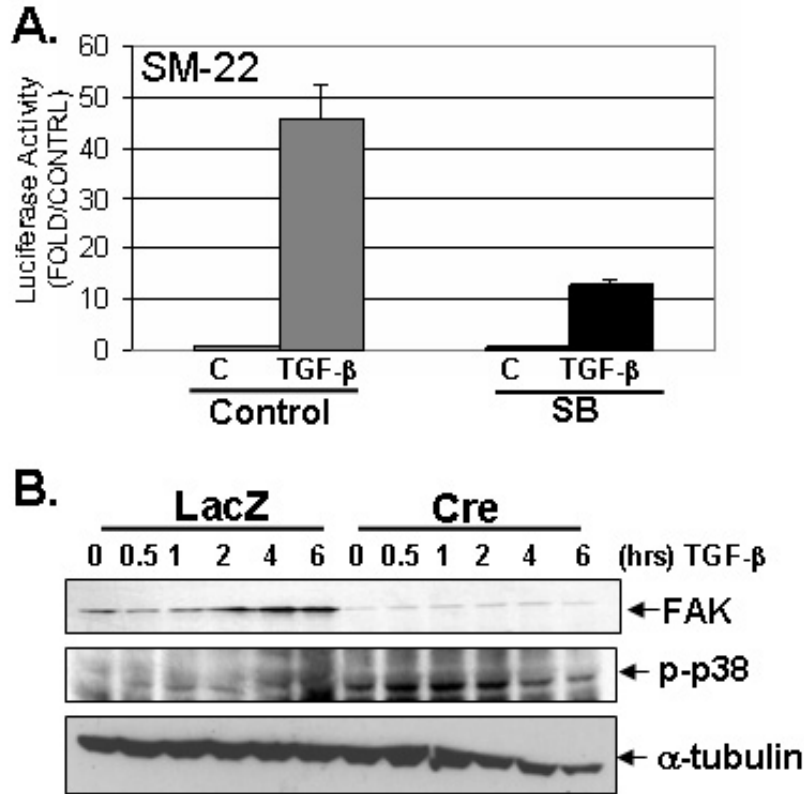
Figure 4.10. p38 activity is necessary for TGF-β enhanced SM gene transcription and is enhanced in the absence of FAK. **A.** 10T1/2 cells were transfected with SM-22 luciferase. 24 hrs later, cells were starved in DMEM containing 0.2% FBS. The next day, cells were pretreated with SB compound (10 µM) for 1 hour, and then treated with TGF-β for 24 hours. Cells were subsequently lysed and assayed for luciferase activity. **B.** *fak*^{flox/flox} SMC were infected with LacZ or Cre adenovirus as mentioned above. Cells were serum starved for 24 hrs prior and again for 2 hrs prior to TGF-β treatment for times indicated. Western blotting was performed with anti-FAK, anti-phospho-p38, and anti-α-tubulin antibodies.

CHAPTER V

DISCUSSION

Within this dissertation, I have presented multiple findings that further delineate a role for focal adhesion kinase and its family member, FRNK, in smooth muscle cell growth and differentiation. Prior to these studies, little was known about how FRNK expression was regulated in smooth muscle cells, or what function FRNK had *in vivo*. Focal adhesion kinase, however, is a well studied protein and its function in a variety of cell types has been previously determined. Since germline deletion of FAK results in embryonic lethality due to general mesodermal defects, it has been thought that FAK plays a significant role in formation of the vasculature and in guidance of smooth muscle cells. Here, by using a variety of integrative *in vitro* and *in vivo* approaches, we have shown that both FAK and FRNK are critical for proper smooth muscle cell function.

FAK: A NEW ROLE IN SMOOTH MUSCLE CELLS

As described previously, others have demonstrated that FAK is essential for proper development during embryogenesis. FAK has also been shown to upregulate proliferation by mediating outside-in signaling from integrins, G-protein coupled receptors, and growth factors. Here we have shown that FAK is required in cardiac neural crest-derived cells for proper septation of the cardiac outflow tract and provided additional evidence that supports an emerging role for FAK as a regulator of differentiation. As described in Chapter IV, FAK has recently been implicated as a regulator of differentiation in skeletal and cardiac muscle cells (134, 135).

In addition to playing an important role in formation of the cardiac outflow tract during embryogenesis, we also expect that FAK is an important regulator of proliferation and differentiation in smooth muscle cells derived from other origins. Moreover, we

hypothesize that FAK activity is important for smooth muscle cell growth and neointima formation following vascular injury. However, it has been difficult to conditionally delete FAK in smooth muscle derived from distinct origins because of the lack of effective Cre-expressing mouse lines. Future studies include looking at the role of FAK in maturation of other vessels at early postnatal timepoints when smooth muscle cells are maturing and thickening the vascular wall. It is also a goal of our laboratory to determine a role for FAK following vascular injury by carotid artery ligation. These studies will provide further insight into FAK's role as a modulator of smooth muscle cell phenotype.

FRNK AS A REGULATOR OF SMOOTH MUSCLE CELL DIFFERENTIATION

This work provides evidence that FRNK regulates differentiation of smooth muscle cells. FRNK is frequently used as a tool by investigators to attenuate FAK activity and FAK-dependent downstream mitogenic and migratory pathways. Likewise, our laboratory has also demonstrated that FRNK can inhibit PDGF-stimulated growth and migration in vascular smooth muscle cells (93). We originally hypothesized that in the absence of FRNK, smooth muscle cells would lack a mechanism to negatively regulate FAK activity, and therefore would be hyperplastic. Although proliferation did increase in smooth muscle organs in developing FRNK$^{-/-}$ mice, we did not see any effect on vessel wall diameter in adult animals. Instead, our *in vitro* and *in vivo* evidence have shown a more significant role for FRNK in regulating smooth muscle cell differentiation. In particular, we were able to show that overexpression of FRNK results in higher activity of smooth muscle gene promoters. Using our *in vivo* model, we also made the interesting discovery that in the absence of FRNK, there are lower levels of smooth

muscle gene expression both during development and following vascular injury. This evidence suggests that methods to enhance FRNK expression might provide means to control unwanted proliferation.

Herein, we show that TGF-β upregulates FRNK expression and that expression of FRNK can enhance the response of smooth muscle cells to treatment with TGF-β. At this point it unclear how FRNK is able to enhance this response, but there are potential mechanisms currently under investigation. Recently, our laboratory identified leupaxin as a mediator of smooth muscle cell differentiation that binds FAK (179). It is possible that by forming inhibitory heterodimers with FAK, FRNK is able to allow increased localization of leupaxin to the nucleus to stimulate smooth muscle gene expression. It is also possible that FRNK could mediate p38 MAPK signaling, which can act downstream of TGF-β and promote differentiation.

REGULATION OF FRNK EXPRESSION

Previous studies have indicated that FRNK is specifically expressed in smooth muscle-containing tissues (93, 96). Hayasaka *et al.* recently generated a mouse model in which LacZ expression is under control of the 6kb FRNK promoter (96). This region directed vascular specific expression of LacZ in arteries and arterioles. However, this clearly does not contain all of the elements that control FRNK expression. Notably, this promoter does not direct expression in visceral smooth muscle, while our in situ analysis reveals that FRNK is highly expressed not only in the lung vasculature, but in airway smooth muscle and bladder. In addition, our work here indicates that there is a low level of FRNK expression in the developing coronary vasculature, but the LacZ promoter-

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reporter mouse demonstrates no expression seven days following birth in these vessels. Interestingly, we have recently been able to detect transient expression of FRNK days after birth in the mouse. These findings are important to consider when determining what factors regulate FRNK expression. Future studies with transgenic animal models are needed to elucidate the exact expression pattern of FRNK. Present studies of FRNK expression have only included a portion of the entire non-coding sequence that directs FRNK expression. It is likely that regions exist outside of the well-studied 6kb region that regulates expression of FRNK in coronary vascular or lung airway smooth muscle. Therefore, mouse models that utilize these regions may better illustrate the tissues where FRNK is expressed. It would be possible to generate transgenic lines using portions of the FRNK promoter cloned from BACs to examine promoter regions out of the 6kb construct. A caveat of the current promoter-reporter model is that LacZ expression can only be observed in those tissues that activate the promoter at the examined developmental time. An alternate model would use a region of the FRNK promoter to drive expression of Cre recombinase. By crossing this mouse with a *Rosa26* reporter mouse, any cell that even transiently expressed FRNK would be marked by LacZ throughout development.

As discussed previously, the majority of smooth muscle-specific gene expression is regulated by serum response factor binding to CArG-elements within the promoters of these genes. This work demonstrates that FRNK is not regulated by SRF, or by its potent co-activators, myocardin and MRTF-A. However, as discussed in the Introduction, several other smooth muscle specific genes are also regulated in a CArG-independent fashion. In the study of HRCBP, the authors identified highly conserved elements within

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the promoter that were necessary for HRCBP expression and under the control of the transcriptional enhancer Mef2C (103). In this study, we chose to undertake a similar approach and assume that the most important regions of the FRNK promoter are likely those regions highly conserved across species. Indeed, two regions over 75% conserved across rat, mouse, and human were identified and shown to be necessary and sufficient for smooth muscle specific expression in cultured cells. Although additional studies are required to determine what factors regulate these conserved regions, we have made much progress using novel screening methods to identify enhancers of the FRNK promoter.

At this point, it is interesting to point out the innovative methods now employed to identify functional regions of non-coding gene elements. It is most important to determine what elements regulate gene expression *in vivo*. Investigators are currently moving towards initially screening for important elements by generating sets of promoter-reporter mice that each contains a portion of a non-coding region of interest. Creemers, *et al.* were able to identify a short region of the myocardin promoter that directed expression of LacZ during embryogenesis (180). After locating this region, they were able to identify specific activators of the myocardin promoter using the screen described in Chapter II and by Chang, *et al.* (109, 180). As the generation of transient transgenic animals becomes more cost effective for investigators, this will be a direct way to find significant non-coding regions responsible for regulation of gene expression.

CLINICAL IMPLICATIONS

Although the majority of this work utilized murine models, the goal of each study is always to contribute to medical knowledge that will eventually be used in a clinical

setting. Our studies are relevant to two human disorders, congenital heart defects associated with aberrant outflow tract development and restenosis post-stent implantation.

Proper development of the heart and outflow tract is critical for successful human development. DiGeorge syndrome is an example of a congenital heart defect generally exhibiting persistent truncus arteriosis (PTA), as seen here in FAK-deficient mice. In addition to PTA, DiGeorge syndrome is also characterized by an interrupted aortic arch, malformation of the thymus and/or parathyroid glands, craniofacial dysmorphology, and psychological disorders (181). This overall defect in neural crest development has been linked to a microdeletion on chromosome 22q11. Studies have indicated that the microdeletion is the gene Tbx1, a known regulator of cardiac development. However, not all DiGeorge patients have a deletion of Tbx1, nor do they all share the same severity of symptoms (181). It is likely that the variety of DiGeorge symptoms is modified by other genes. Since this work implicates FAK deletion in contributing to PTA, it is possible that FAK may be a candidate modifier gene in this congenital defect.

The incidence of hypertension and atherosclerosis in the United States population is severe. Smooth muscle cell growth contributes to the development of these diseases that can each lead to heart attack and stroke. Since this work has made clear the contribution of FAK and FRNK to smooth muscle cell proliferation, this could be manipulated to attenuate medial thickening during hypertension and invasion and growth of atherosclerotic plaques. In addition, modulation of TGF-β has been linked with development of hypertension (182, 183).   When TGF-β is allowed to accumulate, its presence causes premature differentiation and a constricted vessel lumen. In this case, it

may possible that TGF-β stimulates the release of FRNK and in turn is better able to activate smooth muscle specific gene expression.

As described in Chapter I, restenosis following surgery to clear blocked atherosclerotic arteries is significant medical condition. Since FAK is known to be activated following vascular injury, it is important to understand the downstream pathways that lead to smooth muscle cell growth and migration. Because FRNK is known to negatively regulate FAK activity, it is possible that FRNK could be potentially used as a therapeutic in vascular stents. Current stent technology is utilizing other agents that are able to reduce the amount of cellular proliferation within the vessel following injury. If transcriptional activators of the FRNK promoter are identified, it is possible that a treatment could be formulated to increase their activity to induce FRNK expression in the vessel.

A MODEL FOR FAK AND FRNK IN SMOOTH MUSCLE CELLS

The studies presented here in this dissertation have elucidated new roles for FAK and its family member FRNK in vascular smooth muscle cells. Previously, FAK was known as an integrator of proliferative signals from a variety of outside sources. FRNK also acts as a dominant negative and can attenuate PDGF-stimulated proliferation and migration in smooth muscle cells (93).

FAK has a well-described role as a mediator of signaling from integrins, growth factor receptors, and G-protein coupled receptors. Agonists such as fibronectin, PDGF, and angiotnsin II all activate FAK by phosphorylation of Y397. This signaling is particularly important during development, when smooth muscle cells or pericytes must

migrate towards sites of vessel formation and then proliferate to form a medial layer around the vessel. As the vessel matures and smooth muscle begins to differentiate into contractile cells, the complement of growth factors an matrix around the vessel begins to change. The microenvironment becomes rich with TGF-β and herein we demonstrate that this factor upregulates FRNK expression *in vitro*. FRNK expression is also increased *in vivo* during development at times that correspond to expression of pro-differention markers. It is likely that FRNK is expressed at these times to attenuate FAK-depdendent proliferation and migration and to enhance expression of smooth muscle genes.

The expression of FRNK following vascular injury also lends support to this model. As discussed previously, smooth muscle cells retain the ability to switch between contractile and proliferative phenotypes. Immediately following vascular injury, pro-proliferative agonists such as PDGF are released, resulting in smooth muscle cell growth. In response, TGF-β is released within the vessel and is likely a major contributor to the increase of FRNK expression. As smooth muscle cells exhibit pathological growth, FRNK is then expressed to negatively regulate FAK activity and promote the re-expression of smooth muscle genes (see Figure 5.1).
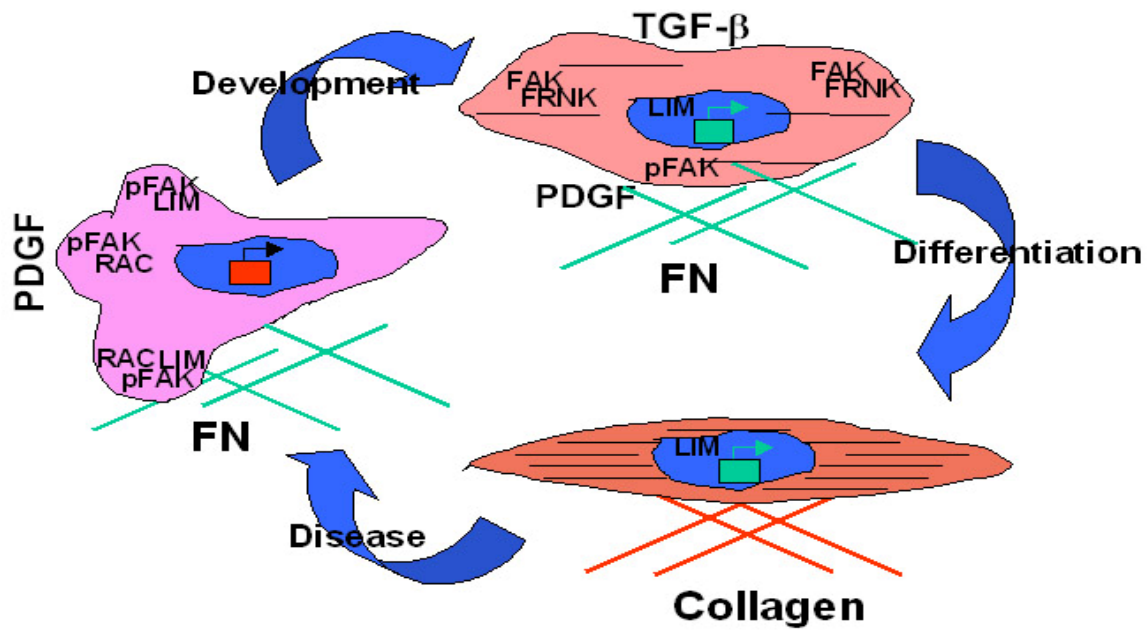
Figure 5.1. Model for FAK/FRNK signaling in smooth muscle cells. In the developing vasculature, SMC are embedded in an environment rich in fibronectin and PDGF. These cells are predicted to have high levels of FAK and Rac activity, and thus be highly motile. As the vessels mature TGF-b is induced and FRNK expression is increased. This leads to FAK inhibition and translocation of LIM factors such as leupaxin into the nucleus where they can promote differentiation. In the adult vasculature high levels of collagen are produced and the cells are quiescent. However upon injury, fibronectin is re-expressed and PDGF secretion is increased. FAK is reactivated and LIM proteins are suggested and the cells revert to a de-differentiated motile phenotype.
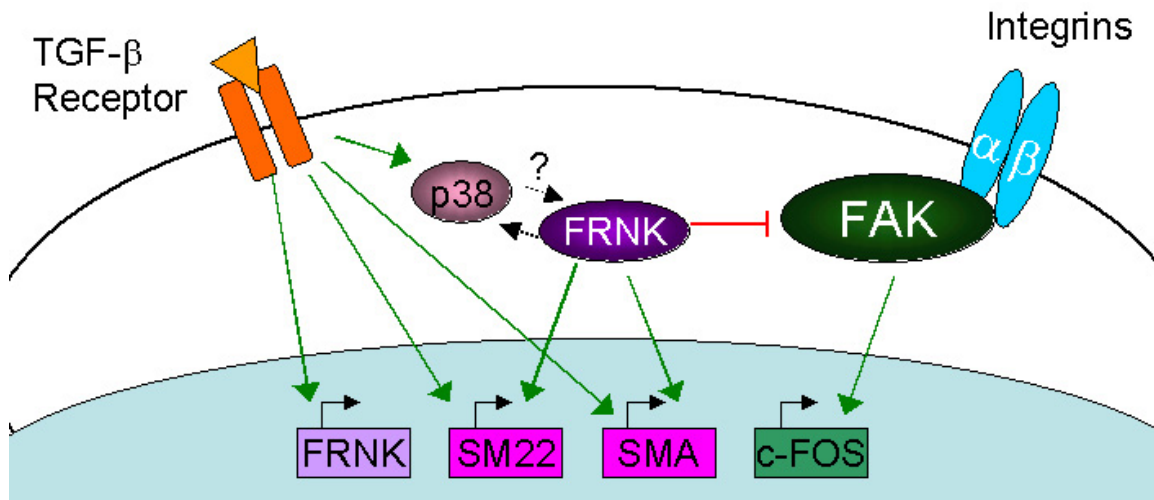
Figure 5.2. Model for TGF-β signaling with FAK and FRNK. TGF-β signaling through Smad proteins and other element can stimulate stranscription of SM genes, such as SM22 and smooth muscle α-actin (SMA). However, our work indicates that TGF-β upregulates FRNK expression in a Smad-independent fashion. FRNK is also able to upregulate expression of SM genes and concurrently block FAK-dependent integrin signaling that increases expression of proliferative genes. This model suggests that p38, an alternative downstream signaling pathway for TGF-β, may be more highly stimulated in the presence of FRNK.

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