FRNK Expression Promotes Smooth Muscle Cell Maturation During Vascular Development and After Vascular Injury


**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 laboratory previously reported that a specific focal adhesion kinase (FAK) inhibitor termed FRNK (FAK Related Non-Kinase) is selectively expressed in large arterioles when SMCs 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 postnatal vessel growth and after vascular injury. We also show that FRNK expression is regulated by transforming growth factor (TGF)-β and that forced expression of FRNK in cultured cells induces serum- and TGF-β-stimulated SM marker gene expression, whereas 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 after vascular injury. (*Arterioscler Thromb Vasc Biol. 2008;28:000-000*)

**Key Words:** integrins  ■  smooth muscle  ■  differentiation  ■  vascular remodeling

While medial smooth muscle cells (SMCs) 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 progrowth and migratory signals. This unique plasticity is critical for proper vessel development, blood pressure homeostasis, and injury repair processes.

A number of secreted growth factors (ie, platelet derived growth factor [PDGF-BB], sphingosine 1-phosphate [S1], TGF-β [TGF-]) and contractile agonists (angiotensin II, endothelin-1, and thromb) have been shown to regulate SMC phenotype in vitro and in vivo. 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. Indeed, genetic ablation of either fibronectin or the α5 integrin fibronectin receptor results in embryonic lethality associated with impaired SMC investment of both embryonic and extraembryonic vessels. 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.

One of the major proteins involved in the integrin intracellular signaling cascade is the nonreceptor protein tyrosine kinase, focal adhesion kinase (FAK), which is strongly and rapidly activated by various aforementioned growth factors and by ligation of all β, β, or β-containing integrins. 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 integrin−/− embryos. Interestingly, our laboratory recently showed that FAK activity is regulated in a unique fashion in SMCs, whereby a separate protein comprising the carboxyterminus of FAK, termed FRNK (FAK Related Non Kinase), which acts as a dominant-interfering
mutant for FAK, is selectively expressed in SMCs with very high levels found in the large arteries. FRNK transcription results from the use of an alternative start site within the FAK gene, and FRNK expression is independently regulated by a distinct promoter embedded within FAK intronic sequences.\textsuperscript{12,13} 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 after injury, when SMCs 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 SMCs in vivo. Herein we present evidence that FRNK\textsuperscript{−/−} mice exhibit repressed SM marker gene expression during postnatal vessel growth and after vascular injury. These data highlight the possibility that extrinsic signals regulate the SMC gene profile by modulating the dynamic expression of FRNK.

**Methods**

Mice were housed in an AALAC accredited University Animal Care Facility, and all experimental procedures were approved by the University of North Carolina animal care and use committee (IACUC). All quantitative data represent at least 3 separate experiments presented as mean±SEM. Means were compared by 2-tailed Students t test, and P<0.05 was considered statistically significant as indicated by an asterisk. All other data including Western analysis are representative of at least 3 individual experiments. Please see Online Supplemental Data section (available online at http://atvb.ahajournals.org) for a complete description of the animal models, reagents, DNA constructs, and general methods used for these studies.

**Results**

The 41/43 kDa FRNK protein is selectively expressed in the vasculature from approximately E12.5 onward with highest levels observed in large arteries and lung of neonatal rats (postnatal day 4 to 14) (Figure 1B).\textsuperscript{12,14} FRNK protein was also detected in cells derived from human aorta and coronary arteries and coronary SMC cultures derived from explanted proepicardial organs, but not in cultured endothelial cells (supplemental Figure I). As previously reported (and depicted in Figure 1A), FRNK expression is regulated by a promoter embedded within the fak gene and frnk transcription initiates from a noncoding exon located approximately 300 bp upstream of the FRNK translational start site in the mouse gene.\textsuperscript{12,13} Because 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, because the frnk noncoding exon is selectively protected in RNA isolated from SMC and SMC-containing tissues,\textsuperscript{12} 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 postnatal 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 1C, top). Some visceral SMC staining was apparent as well, with high levels observed in the lung (Figure 1C) and bladder (not shown). FRNK expression in the lung was localized to the smooth muscle lining of the bronchi in addition to vessels, similar to the staining pattern observed for SM\textalpha-actin.\textsuperscript{16} Further analysis of FRNK expression in the developing mouse vasculature using quantitative RT-PCR revealed a dynamic upregulation of FRNK mRNA in aorta between 7 and 10 days postnatal, corroborating our previous Western analysis (Figure 1D).

We reasoned that upregulation of FRNK in the neonatal vessels may serve to buffer FAK-dependent signals in this environment that is particularly rich in growth factors and matrices known to be upstream activators of FAK. Thus we sought to identify some of the mechanisms involved in the transient and dynamic regulation of FRNK expression in postnatal vessels. To determine the relative stability of FRNK protein, we treated SMCs with cyclohexamide for various times and analyzed FRNK and FAK levels. In support of the idea that FRNK expression can be tightly regulated, we found that FRNK protein turnover is relatively rapid, with an apparent half-life of approximately 4.5 hour (supplemental Figure IIA). The level of FAK protein was not significantly changed during the 8-hour time-course examined, consistent with previous reports that FAK protein is extremely stable with a half-life exceeding 20 hours.\textsuperscript{20} We next sought to identify factors that can modulate dynamic FRNK expression during vascular morphogenesis. We first used a FRNK promoter reporter construct to define factors that regulate FRNK transcription. A fragment comprising approximately 6 kb 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.\textsuperscript{21} Importantly, we found that the corresponding FRNK promoter attached to a luciferase reporter (FRNK-Luc) exhibited high activity in cultured SMCs in comparison to 10T1/2 cells (consistent with the levels of FRNK expressed in these two cell types; supplemental Figure IIB). 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, coexpression of SRF and FRNK-Luc in SRF−/− ES cells did not alter basal FRNK-Luc promoter activity in these cells, whereas reconstitution of SRF induced expression of a SM\textalpha-actin-Luc construct approximately 15-fold (Figure 2A). Moreover, when expressed in SMCs, the FRNK-Luc construct was unresponsive to overexpression of the myocardin family of SRF cofactors, which stimulated strong activation of the SM\textalpha-actin-Luc reporter (supplemental Figure IIC). Furthermore, ectopic expression of myocardin did not alter FRNK message levels but induced a striking 60-fold increase in SM22b message (Figure 2B). Collectively, these data provide strong support for FRNK being expressed in a non-SRF/CARG dependent fashion as previously suggested.\textsuperscript{22}

We next screened a number of cytokines/growth factors known to be released after vessel injury for their ability to increase FRNK promoter and protein levels in cultured rat aortic SMCs. We found that TGF-\textbeta 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 2C through 2E), whereas other...
agonists including the potent SMC mitogens PDGF-BB, S1P, angiotensin II, thrombin, and basic-fibroblast growth factor had no effect (data not shown). The ability of SB 431542 (a selective inhibitor of the TGF-β1 activin receptor-like kinase; ALK-5) to dramatically reduce expression of FRNK in cultured SMCs under serum-starved conditions or after TGF-β treatment strongly supports the idea that TGF-β is a major regulator of FRNK expression (Figure 2E).

Because TGF-β induces FRNK expression and SM differentiation, we theorized that FRNK might promote SMC phenotypic switching from a synthetic to contractile state. 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 upregulate SMC-specific gene expression on stimulation with serum, S1P, or TGF-β.19,20 Whereas FRNK protein is not expressed in detectible levels in 10T1/2, FRNK message is detectible by quantitative RT-PQR (data not shown) and forced expression of FRNK in these cells inhibited FAK activity and resulted in a 2- to 4-fold increase in SM22, SMα-actin, and SM-MHC promoter activity, without effecting myocardin levels (Figure 3A; supplemental Figure IIIA and IIIB). 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 SuperFAK21) that leads to enhanced FAK activity as assessed by phosphorylated Y397FAK levels. As expected, SuperFAK expression in 10T1/2 cells significantly reduced SM22 (Figure 3B) and

Figure 1. FRNK is expressed selectively in smooth muscle containing-tissues. A, Schematic of FAK and FRNK cDNA and genomic locus. Shaded areas represent FAK-specific (blue), FRNK-specific (green), or common (red) coding regions. B, Western analysis of 14 days postnatal rat tissues. C, In situ hybridization for FRNK and SMα-actin in postnatal day 7 mouse aorta and lung. D, Quantitative RT-PCR for FRNK in thoracic aorta. (Mean±SEM n=4, *P<0.01, #P<0.05).
SMα-actin reporter gene expression (supplemental Figure IIIC). Similarly, ectopic expression of FRNK and FAK variants in primary rat aortic SMC revealed an inverse relationship between FAK activity and SM gene expression (ie, low FAK activity correlates with high levels of SM markers, Figure 3C; supplemental Figure IIID). Taken together, our findings provide compelling evidence that FAK activity limits SMC differentiation, and upregulation of FRNK promotes differentiation by attenuating FAK activity.

We next examined TGF-β–stimulated SM gene expression in our cell culture models. We found that TGF-β and FRNK acted in concert to induce SM22 reporter activity in 10T1/2 cells (Figure 3D). Importantly, similar results were observed when we analyzed endogenous SM gene expression by Western analysis. SM22 protein was undetectible in 10T1/2 cells under basal conditions, but either expression of FRNK or treatment of cells with TGF-β for 48 hours induced a modest increase in SM-22, and when combined, these agents markedly increased SM22 expression (Figure 3E). FRNK and TGF-β each individually led to a similar marked induction of SMα-actin protein levels in 10T1/2 cells, however the combined treatment had a more modest effect, most likely because of the already high levels of SMα-actin induced by each factor alone. 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.

We next strove to evaluate FRNK’s function to regulate SMC phenotype in postnatal vessels. FRNK−/− mice were recently generated by a strategy that resulted in deletion of a 1-kb fragment that included the noncoding exon and 700 bp of upstream fak intronic sequence (see schematic in Figure 1A). 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.14 Because 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 postnatal vessels from wild-type or FRNK−/− mice. As shown in Figure 4A, medial SMC tissue from postnatal FRNK−/− vessels exhibit a significantly higher index of BrdU incorporation in comparison to littermate control vessels.
FRNK−/− pups reveal lack of FRNK protein and higher levels of active pY397FAK, in comparison to wild-type littermate controls, whereas total FAK protein levels are unaltered (Figure 4B).

We next analyzed the dynamic expression of the contractile genes, SMα-actin, SM-22, and smoothelin B in the aorta of postnatal control and FRNK−/− mice by quantitative RT-PCR. Expression of these contractile genes is enhanced approximately 6- to 10-fold from 4 day postnatal to adult vessels in wild-type mice (data not shown). Interestingly, we found that FRNK−/− vessels contain significantly fewer transcripts of these genes in the 2- to 3-week period of vessel development when compared to age and littermate controls, when FRNK protein is most highly abundant in wild-type vessels (Figure 4C).12 However, no significant differences in SM marker gene expression were observed in adult aorta (see data for 8-week animals in Figure 4C) or carotid vessels isolated from wild-type or FRNK−/− mice (Figure 5A), indicating that the lag in SM differentiation observed in the neonatal vessels was normalized during vessel maturation. In spite of these transient significant differences in SMC phenotype, we did not detect any significant difference in medial thickness of FRNK−/− adult arterioles (aorta or carotids) in comparison to littermate control vessels analyzed from 7 days postnatal to adult (supplemental Figure IV). Also, we found that adult FRNK−/− mice did not exhibit significant changes in mean systolic blood pressure (as assessed by tail cuff measurements) or pressor responses (as assessed by intracardiac catheterization) after phenylephrine treatment in comparison to wild-type littermate controls (supplemental Figure IV), indicating that FRNK expression does not function to regulate vascular homeostasis in adult mice (see Supplemental Data section for further discussion).

Because we previously reported that FRNK expression is relatively low in adult vessels, but is dramatically upregulated by 14 days after 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 after vascular injury induced by either vessel ligation or surgical procedures.
endothelial denudation. Typically, reduced SMC marker gene expression is apparent within 3 to 7 days after injury followed by a burst of reexpression after approximately 2 to 3 weeks.17,18 To examine a possible involvement of FRNK in the regulation of SMC redifferentiation after vascular injury, we performed quantitative RT-PCR for SM marker genes in wild-type and FRNK−/− carotid arteries 7 to 21 days after carotid ligation. As shown in Figure 5, significant decreases in reexpression of SMα-actin, SM-22, and smoothelin B (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 unligated 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. Although robust SMα-actin staining was observed throughout the media and neointima of wild-type vessels, SMα-actin expression in the neointima of FRNK−/− vessels was markedly reduced (Figure 5D). Although 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 because of the fact that the ligation induces a very strong proliferative response in the wild-type mice (supplemental Figure V). 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 upregulation of FRNK expression after vessel injury is required for appropriate SMC maturation that occurs subsequent to injury repair.

Finally, we used cultures derived from FRNK−/− mice to confirm our previous in vitro and in vivo findings. We found that aortic SMC isolates from FRNK−/− mice consistently exhibited enhanced pY397/FAK, and lower levels of the contractile proteins SM22, SMα-actin, and SM-MHC than SMC isolates from aged matched (and passage-matched) control mice (Figure 6A). As these cells maintained similar morphology (supplemental Figure VI). The low levels of SMC proteins in FRNK−/− SMCs may reflect the inability of these cells to maintain a differentiated phenotype in these progrowth culture conditions (see online Supplemental Data section for further discussion). In support of the notion that FRNK acts to enhance TGF-β dependent gene expression, we found that TGF-β-induced expression of endogenous SM22 was significantly decreased in FRNK−/− SMCs in comparison to matched wild-type SMC isolates (Figure 6B). We also noticed that the FRNK−/− cells exhibited high growth rates and were more motile than control SMC isolates (data not shown). To directly test that these differences were attributable to lack of FRNK expression, we next reconstituted FRNK in FRNK−/− cells. As shown in Figure 6C through 6E, reexpression of FRNK in these cultures significantly increased SMα-actin expression, but significantly decreased both BrdU incorporation and PDGF-stimulated motility.

Discussion

FRNK, a dominant interfering mutant that attenuates FAK activity, exhibits selective and dynamic expression in SMCs. We previously reported that FRNK attenuates SMC proliferation and migration by regulating FAK/Rac1-dependent signaling.23 Herein we have found an additional function for FAK/FRNK signaling in SMCs. We present evidence that FRNK deletion (by homologous recombination) represses SM gene expression during postnatal 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 SMC 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.24 Phosphorylation of Y397 results in subsequent recruitment of the tyrosine kinase Src (or Fyn), which phosphorylates and further activates FAK (and phosphorylates certain FAK binding partners).11 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, whereas expression of a phospho-deficient Y397 FAK cannot. Our findings indicate that FAK activity is enhanced in tissues and cells derived from FRNK−/− mice strongly supports 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 hours, whereas we found that FRNK protein turnover is rapid. We speculate that transient FRNK expression is particularly important in the vasculature, where direct apposition of SMCs with extracellular matrix could lead to high levels of FAK activation and uncontrolled 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. Interestingly, we recently found that FAK-deficient SMC (like FRNK overexpressing SMC) exhibit enhanced TGF-β-stimulated SM marker gene expression (unpublished observations; see Supplemental Data section for further discussion). 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. 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. Because 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 or cofactor recruitment.

Because 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 unresponsive to overexpression of the myocardin family of potent SRF cofactors. Thus, FRNK belongs to a subclass 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) (see Supplemental Data for further discussion). Interestingly, TGF-β, a strong activator of SMC differentiation, led to significant upregulation 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. Direct evidence for the role of FAK in modulating fibroblast-dependent motility was previously shown using FAK−/− fibroblasts, endothelial cells, neurons, and keratinocytes. We previously showed that FRNK expression in SMCs attenuated FAK-dependent PDGF-stimulated chemotaxis, and our recent studies reveal that FAK−/− SMCs exhibit a similar defect (unpublished observations). Because 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.

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Disclosures

None.

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DATA SUPPLEMENT

Supplemental Methods

*Antibodies, Constructs, and Reagents:* The SB 431542 compound, cyclohexamide, and anti-SMα-actin, and Flag antibodies were purchased from Sigma. The C-term specific anti-FAK and anti-ERK1/2 antibodies were purchased from Upstate. 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. TGF-β was purchased from Calbiochem. The GFP and GFP-FRNK adenoviruses were generated and expanded as described previously. 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. 6xSBE-luciferase was a gift from Li Li (Wayne State University). Flag-FAK was a generous gift from Dr. Tom Parsons (University of Virginia) and was previously described.

*Cell Culture, Transfections, and Reporter Assays*

Aortic vascular smooth muscle cells (SMC) were cultured from C57bl6 mice or Wistar rats using enzymatic digestion as previously described. 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 Dulbucco’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. For reporter assays, cells were transfected
with appropriate constructs using either Superfect (Qiagen) or Trans-IT (Mirus) transfection reagents according to manufacturer’s protocol. Following transfections, cells were lysed in Glo Lysis Buffer and assayed using Steady Glo Luciferase Assay system (Promega) as directed by the manufacturer. In some experiments, cells were transduced with GFP or GFP-FRNK adenoviurs (10 m.o.i) prior to experimentation.

**Immunocytochemistry and detection of BrdU incorporation in cultured cells**

Cells were processed for immunocytochemistry using previously published methods. 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).

**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. 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 according 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: GCTGCATTCTGAGCGTTTA, Reverse: CAGGATTGTGCACCACCAG, Probe: AGCCAGGACTGAGACGCCGCC), FAK (Forward: GAAAGCAGTAGTGAGCCAACC, Reverse: GAGACTGTCCACTATCTTCTG, Probe: CTCCATGCCTGATAATCTGGCCAG), SM-22 (Forward: TGCAGTGTGGCCCTGATGT, Reverse: TGCTCAGAATCAGCCATTCT, Probe: AGATCGTGGGCGCCTGGGCT), and SM α-actin (Forward: CGCTGTCAAGAACCCTTGAGA, Reverse: CGAAGCCGGCCCTTGAGA, Probe: CAGCACAGCCCTGGTGTGCGAC). 18S primers and probes were a generous gift from Hyung-Suk Kim. Primers and probes for smoothelin were used as described previously.9

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 in serum-free media on transwell filters (8 µm pore size) precoated with FN, using 10% serum-containing medium as the chemoattractant. After 7 hours, the cells were fixed in 4% paraformaldehyde and the remaining cells in the upper chamber were removed with a cotton swab. Migrated cells were counted by indirect fluorescence.

Animal Procedures
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. Genotypes were obtained from tail snip DNA using PCR analysis for primers specific to FRNK.

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. 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 Ab (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<sup>-/-</sup> (-/-) 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.
Supplemental Discussion

While we did not observe significant hemodynamic differences in adult FRNK^-/- mice in comparison to littermate controls, we cannot currently rule out the possibility that significant changes might be apparent at earlier time points. Indeed, we found that SM marker gene expression was significantly reduced in FRNK^-/- vessels from 2-3 weeks following birth but was normalized by 8 weeks (a time point prior to our hemodynamic assessment). However, our data obtained from adult mice does rule out a function for FRNK as a signaling molecule in the regulation of α-adrenergic-dependent vasoconstriction. In addition, these data provide evidence that baseline hemodynamics are not altered in FRNK^-/- mice prior to our surgical manipulation, lending credence to a specific function for FRNK in the re-conversion of SMC from a synthetic to contractile phenotype. Nonetheless, it will be of future interest to evaluate vascular reactivity and blood pressure in younger FRNK^-/- mice.

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) 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. ACLP is also SRF-independent; rather, its promoter is activated by transcription factors Sp1 and Sp3. Interestingly, much like FRNK, CRP2 seems to be most robust in arterial smooth muscle, and its expression is also regulated by TGF-β. CRP2 expression is dependent upon an 800 bp region of its promoter that is independent of Sp1 and Sp3 but dependent on TGF-β mediated activation of ATF2. Although
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.\textsuperscript{16} Expression of HRCBP is dependent on a highly conserved myocyte enhancer factor 2 (MEF2) site within the promoter.\textsuperscript{16} Studies are currently underway in our laboratory to define to what extent FRNK is regulated by some of these known CArG-independent mechanisms that drive SM-selective transcription.

We recently found that like FRNK expression, deletion of FAK from SMC (by homologous recombination) resulted in enhanced TGF-\(\beta\) dependent SM marker gene expression (un-published observations; RLS, LSS, CPM, and JMT). Thus, 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 vascular development and injury repair. There are several putative mechanisms whereby FAK activity might limit TGF-\(\beta\) dependent responses. FAK is a multifunctional protein that associates with a number of adapter molecules through well-defined protein interaction sites, and regulates downstream activation of MapKinases and small GTPases. FAK activation is 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 \textsuperscript{17-20}. RhoA activation has also been shown to be an important determinant of SMC differentiation marker gene expression and previous studies have shown that FAK-null fibroblasts exhibit enhanced basal RhoA activity, indicating the possibility that this pathway might also be involved in promoting SMC maturation in FRNK expressing or FAK-null SMC \textsuperscript{21}. 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 \(^{22}\). 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 \(^{22}\). Studies to determine the relative contributions of (or relationship between) leupaxin, Map Kinases, or Rho A in regulating SMC phenotypes in FRNK expressing and/or FAK-null SMC are currently ongoing in our laboratory.
Supplemental Figure Legends

Online Figure I. FRNK is expressed in coronary SMC. Western blot of FRNK and FAK levels in proepicardial cells (PEC), bovine endothelial cells (BEC), human coronary SMC (HuCSMC), and human aortic SMC (HuAoSMC). Blots were probed with an antibody that detects FAK and FRNK.

Online Figure II. FRNK expression is tightly regulated but myocardin family members do not affect FRNK promoter activity. A. Western blot of Rat SMC treated with cyclohexamide (CHX, 100µg/mL) for indicated times. Densitometry is shown below. B. 10T1/2 or VSMC were transfected with 6kb FRNK-Luc or TK-Luc and assayed for luciferase activity 48 hours later. 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.

Online Figure III. Ectopic expression of FRNK regulates SM marker gene expression but does not alter myocardin levels. A, C. SM-MHC and SMα–actin luciferase assay in 10T1/2 cells expressing GFP, GFP-FRNK, or SuperFAK (SFAK) performed in 10% serum-containing media (Mean +/- SEM; n>=3) B. Rat aortic SMC were infected with GFP or GFP-FRNK and qRT-PCR was used to measure myocardin expression. Data is normalized to the presence of 18S and is presented as the mean +/- SEM (n =3). D. Rat aortic SMC were co-transfected with SM22-luciferase reporter construct and with either empty vector (EV), SFAK, FAK, Y397FFAK. Western blotting was performed 48 hrs following transfection.
**Online Figure IV. FRNK<sup>-/-</sup> mice do not display abnormal homeostasis.** A. Wt (+/+), and FRNK<sup>-/-</sup> (-/-) 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<sup>-/-</sup> (-/-) 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.

**Online Figure V. FRNK<sup>-/-</sup> mice show no differences in vessel growth in response to carotid artery ligation.** Wild type (+/+), and FRNK<sup>-/-</sup> (-/-) 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.
Online Figure VI. FRNK-/- cells are morphologically similar to wild type SMC, but have lower levels of SM α-actin staining. FRNK-/- (-/-) or WT (+/+ ) cells were maintained in serum, fixed in 4% paraformaldehyde, and stained with an antibody for SM α-actin and phalloidin.
Supplemental References


4. Richardson A, Malik RK, Hildebrand JD, Parsons JT. Inhibition of cell spreading by expression of the C-terminal domain of focal adhesion kinase (FAK) is rescued by coexpression of Src or catalytically inactive FAK: a role for paxillin tyrosine phosphorylation. *Mol Cell Biol.* 1997;17:6906-6914.


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On Line Fig I
A. 

Relative Levels of FAK and FRNK over time with CHX treatment.

B. 

Luciferase Activity of FRNK-Luc in 10T1/2 and VSMC cells.

C. 

Luciferase Activity of FRNK and SMα-Actin with different vectors.

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Online Figure II
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