Published in final edited form as:

J Cell Physiol. 2010 November ; 225(2): 562–568. doi:10.1002/jcp.22239.

FRNK Overexpression Limits the Depth and Frequency of Vascular Smooth Muscle Cell Invasion in a Three-Dimensional Fibrin Matrix

LP Brewster^{1,2}, AA Ucuzian^{1,2}, EM Brey^{3,4}, M Liwanag^{3,5}, AM Samarel⁶, and HP Greisler^{1,2,3,7}

¹Department of Surgery; Loyola University Medical Center, Maywood, IL, USA

²Department of Cell Biology, Neurobiology, & Anatomy, Loyola University Medical Center, Maywood, IL, USA

³Edward J. Hines, Jr. VA Hospital, Research Services, Hines, IL, USA

⁴Department of Biomedical Engineering, Illinois Institute of Technology, Chicago, IL, USA

⁵Department of Biology, Millikin University, Decatur, IL, USA

⁶Cardiovascular Institute, Loyola University Medical Center, Maywood, IL, USA

⁷Edward J. Hines, Jr. VA Hospital, Surgical Services, Hines, IL, USA

Abstract

Pathological vascular smooth muscle cell (VSMC) behavior after vascular interventions such as angioplasty or bypass is initiated within the 3-D environment of the vessel media. Here VSMCs proliferate, invade the surrounding matrix, migrate adluminally, and deposit substantial amounts of matrix, leading to myointimal hyperplasia and decreased blood flow to critical organs and tissue. Since focal adhesion kinase (FAK) mediates many of the VSMC responses to these pathologic events, it provides a reasonable pharmacologic target to limit this invasive VSMC behavior and to better understand the cellular pathophysiology of this disease. Here we quantified the effectiveness of disabling FAK in VSMCs with its dominant-negative inhibitor, FAK related nonkinase (FRNK), in a clinically relevant 3-D assay. We found that FRNK overexpression decreased VSMC invasion (both the length and frequency) in this matrix. These effects were demonstrated in the presence and absence of chemical mitotic inhibition, suggesting that FAK's effect on cellular matrix invasion, migration, and proliferation utilize separate and/or redundant signaling cascades. Mechanistically, FAK inhibition decreased its localization to focal adhesions which led to a significant decrease in FAK autophosphorylation and the phosphorylation of the serine/threonine kinase, AKT. Together these findings suggest that disruption of FAK signaling may provide a pharmaceutical tool that limits pathological VSMC cell behavior.

Keywords

Vascular Smooth Muscle Cell (VSMC); Three-Dimensional Modeling; myointimal hyperplasia; focal adhesion kinase (FAK); FAK-related nonkinase (FRNK)

Correspondence to: LP Brewster.

Corresponding author: lukebrewst@aol.com 2160 South First Avenue, Maywood, IL, 60153 Fax: 708-216-6300; Office: 708-216-8541.

Introduction

Narrowing or re-stenosis secondary to myointimal hyperplasia (IH) continues to be a common long-term complication for patients after vascular operations and interventions. IH requires direct intimal injury and the requisite growth factors to be available to cells at the time of injury. However IH does not occur if either the injury or growth factors are not present simultaneously (Allaire and Clowes, 1997; Lindner and Reidy, 1991). Intimal injury disrupts the normally quiescent endothelium and its basement membrane, creating an inflammatory state and the desegregation of endothelial cells (EC) and VSMCs. The VSMC response to this injury is coordinated by both integrin and growth factor signaling (Carragher et al., 1999; Hedin et al., 1999). The VSMCs themselves proliferate in the vessel media, invade the denuded extracellular matrix (ECM), and migrate toward the vessel lumen where they continue to proliferate and deposit exuberant amounts of ECM proteins (Schwartz et al., 1995).

This pathological VSMC behaviour (matrix invasion, migration, and proliferation) can be investigated in single cell systems through traditional two dimensional (2-D) in vitro assays (Hauck et al., 2002; Hauck et al., 2000; Taylor et al., 2001). However 2-D assays may be more applicable to ECs, which exist in vivo as an intimal monolayer, than they are for VSMCs, which exist in the 3-D multilayered vessel media (Cukierman et al., 2001; Stegemann et al., 2005). Since the contribution of VSMCs' to IH is regulated in a dynamic 3-D environment, where the ECs are diminished in number and no longer provide a confluent or quiescent barrier to VSMC invasion, an in vitro 3-D co-culture assay may provide both a more complex and realistic environment to test novel therapeutic approaches and study cellular pathophysiology. Furthermore, 3-D assays allow for control of particular variables such as the effect of a particular matrix (e.g. fibrin) or specific growth factors that can more realistically be mimicked through a combination of variables (Griffith and Swartz, 2006).

Since integrins and growth factors share molecular pathways and signaling proteins, the targeted disruption of these pathways may be beneficial to limiting IH. FAK is a critically important, nonreceptor protein tyrosine kinase that coordinates both integrin and growth factor signaling cascades (Sieg et al., 2000). Upon activation, FAK localizes to focal adhesion complexes with its carboxy-terminal focal adhesion targeting domain. After integrin engagement, FAK is activated by autophosphorylation of its Tyr-397 residue (pFAK-Y397). FAK then binds various docking proteins including Src and PI(3)K, initiating their signaling cascades. Together these events are critical to the coordination of integrin-mediated cell survival, migration, and proliferation (Schlaepfer, 1999). Since FAK participates in the regulation of many of the cellular activities that lead to IH (Avraham et al., 2003; Mitra et al., 2005; Walker et al., 2003), it is a potential therapeutic target for limiting IH.

Here we utilize FAK's dominant-negative inhibitor, termed FAK-Related Non-Kinase (FRNK). FRNK is under the control of its own intronic promoter and shares FAK's carboxy-terminal region including its FAT domain. However FRNK lacks FAK's autophosphorylation site as well as its kinase domain (Nolan et al., 1999). FRNK can function by disrupting FAK signaling through its competitive localization to focal adhesions. This displaces FAK, abrogates its signaling, and interferes with actin filament assembly (Heidkamp et al., 2002).

By creating a realistic 3-D model for investigating VSMC matrix invasion, migration, and proliferation, this study quantifies the effect of FAK disruption on pathological VSMC behaviour and identifies likely intracellular mechanisms for these findings.

Materials and Methods

Animal care complied with The Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996) and the “Principles of Laboratory Animal Care” (National Institutes of Health publication no. 85-23, revised 1985). Canine jugular vein ECs (passages 2-5) and common carotid artery VSMCs (primary cells) were harvested and identified as published previously (Brewster et al., 2004; Zarge et al., 1997).

Adenoviral Infection

VSMCs were plated onto collagen coated polystyrene plates. After 24 hours of culture, VSMCs were infected with Adv-GFP-FRNK (100 MOI $\times$ 24 hours) or Adv-GFP (100 MOI $\times$ 24 hours). Infection efficiency was confirmed by counting % green fluorescent cells per high power fields. VSMCs demonstrating $> 90\%$ transfection efficiency were used.

3-D Invasion Assay

The delineation of individual cell types (VSMCs versus ECs) involved in matrix invasion was established by labeling either VSMCs or, in alternate groups, ECs with the cell membrane stain PKH-26 (Sigma, Saint Louis, MO) as has been described previously (Fox et al., 1999). The labeled cells were then suspended together (15,000 VSMC:5000 EC) in medium to create an EC deficient environment (Xue and Greisler, 2002). In parallel experiments to study VSMCs in the absence of ECs, additional groups were used in which the ECs were eliminated from the assay, but otherwise the assay was the same.

In all groups, the cells were then incubated in suspension culture for 48 hours to form a multicellular aggregate. These cell aggregates were embedded in 200 μ L fibrin hydrogel anchored with a nylon ring, and placed in 24 well plates with growth media as described previously (Xue and Greisler, 2002). The invading cells were then analyzed over time by fluorescence microscopy to determine EC and VSMC invasion of the matrix concomitantly using replicate groups that were differently labeled. Each group had 6 replicates, and the experiment was performed twice with consistent results. The viability of cells in all groups during the time course of the experiment was indicated by the absence of typical morphological characteristics seen in apoptotic sprouts such as cellular debris and discontinuous appearing sprouts.

To test whether FRNK overexpression can limit VSMC invasion in this assay, FAK signaling was disrupted by infecting VSMCs and ECs (100 moi, 24h) Adv-GFP-FRNK prior to cell suspension; Adv-GFP served as a control for the nonspecific effects of Adv infection. The viruses were generated as described previously (Heidkamp et al., 2002). Digital images captured the average length of invasion at selected time points, and the length of invasion was then quantified in microns (μ) as mean length of invasion $\pm$ standard deviation (Xue and Greisler, 2002). Measurement sites with no cellular invasion were excluded from mean average length analysis but were quantified separately as the frequency of invasion. The invading cells were digitally photographed at selected time points using a Zeiss Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc, Thornwood, NY) and Axiovision software (version 5.1; Germany). The digital photographs were aligned with the grid evenly divided into 36 intervals using Adobe Photoshop (version 5.0; Adobe Systems Inc, San Jose, CA). For each assay, the length of invasion was measured using Axiovision software at the furthest point at all 36 intervals, and the average length of invasion was calculated for each image by subtracting the average length from the radius of the pellet. The frequency of invasion was calculated and used as a density index of the invading cellular network defined as the percentage of grid lines with crossing sprouts per 36 grid lines.

Mitomycin C (MMC) Treatment

MMC limits cellular proliferation, and this experiment was performed to identify whether FRNK's effect on limiting VSMC invasion was solely linked to proliferation, or whether there was an independent and direct effect on cell invasion and migration. Here, 500,000 VSMCs were plated in 60mm Petri dishes and allowed to incubate overnight. Cells were treated with 10ug/mL of MMC for 1 hour prior to aggregation (described below). Control VSMCs were plated in 60mm petri dishes, incubated overnight, but were not treated with MMC prior to aggregation.

Western Blot Analysis

The ratio of overexpressed GFP-FRNK to endogenous FRNK protein in transfected cells was determined using anti-total FAK antibodies which cross-react with the homologous C-terminus of FRNK. Total and phosphorylated levels of Akt (Ser 473) were determined in VSMCs overexpressing either GFP or GFP-FRNK. The cellular expression levels of FAK, pFAK-Y397, and alpha-smooth muscle actin using standard methods (Heidkamp et al., 2002). Densitometry was performed using UN-SCAN-IT™ software (Silk Scientific, Inc., Orem, UT). The assays were replicated and analyzed together.

Immunohistochemical Localization of FAK and FRNK

Immunohistochemical localization of FAK and FRNK in VSMCs were employed as described previously (Eble et al., 1999; Heidkamp et al., 2002). This experiment was performed in duplicate with similar results.

Statistics

Student's unpaired t-test was used to compare the length of invasion and protein expression levels between test groups for planned comparisons at determined time points; these values were expressed as mean $\pm$ standard deviation. The frequency of cellular invasion was determined as the presence or absence of measures per 10° intervals along the radius of a superimposed 360° overlay and was analyzed using the Chi Square test.

For the VSMC alone invasion assay, repeated measures mixed ANOVA with post-hoc Tukey tests were performed to statistically analyze overall differences in the invasion curves for the VSMC-alone assay between treatment and control groups, and to statistically analyze differences in trends among subjects over time.

An alpha level of .05 was used to determine significance between two groups, and this level was corrected for multiple measures (alpha=.02) when comparisons were made between three groups (uninfected, GFP-infected, and GFP-infected cells). All statistical tests were analyzed using Microsoft Excel software (Redmond, WA).

RESULTS

In Co-culture VSMCs are the predominant cell type invading the matrix

When cellular invasion of VSMC and EC aggregates were examined with epifluorescence, VSMCs but not ECs migrated from the co-culture cell pellet into the 3-D matrix. In simultaneous experiments with either VSMCs or ECs stained with PKH, there was robust VSMC invasion at day 14. This is in contrast to the minimal EC invasion at the same time point (Figure 1A). VSMC invasion was prominent by day 3 but minimal in ECs out to stagnation of invasion (20d). Thus, the 3-D co-culture assay assessed VSMC invasion, evaluating both the frequency and length of matrix invasion in an EC-deficient 3-dimensional environment.

FRNK limits the length and frequency of VSMC invasion in co- culture

Since the clinical delivery of FRNK after angioplasty or stenting would involve exposure predominantly to VSMCs but also to some ECs, both VSMCs and ECs were infected with either Adv-GFP or Adv- GFP-FRNK. Figure 1B depicts a typical experiment in which cellular invasion of GFP- or GFP-FRNK infected co-culture aggregates that were imaged by light and epifluorescent microscopy. Adv-GFP infected cellular invasion of the matrix was robust and visualized under both light and epifluorescent microscopy; uninfected cells behaved similarly to GFP-infected cells. In contrast, there was no invasion seen in epifluorescent microscopy of GFP-FRNK infected cells at any time point using standard imaging.

To statistically analyze our results, the length of cellular invasion was quantified using measurements from light microscopy and not epifluorescence. Here the length of invasion was significantly reduced in the GFP-FRNK infected group as compared to the GFP-infected and uninfected groups at all time points (Figure 1C). The length of cellular invasion increased over time within each group out to stagnation (day 20), but it was consistently decreased in the GFP-FRNK infected group when compared to GFP and uninfected control groups [Day 3: $85\mu\pm66\mu$ vs. $234\mu\pm74\mu$ and $224\mu\pm131\mu$ respectively; $P<.02$. Day 20: $577\mu\pm151\mu$ compared to $809\mu\pm238\mu$ and $848\mu\pm310\mu$; respectively; $P<.02$]. This suggests that GFP-FRNK infection has a prolonged inhibitory effect in these cells over time. GFP-FRNK infected cells also demonstrated a significant decrease in the frequency of cellular invasion at each time point up to 20 days (Table 1), consistent with a prolonged inhibition of VSMC matrix invasion by FRNK overexpression and not simply a delay in this behavior. Thus FRNK overexpression decreased both the frequency and length of VSMC invasion in this 3-D co-culture assay. Single cell culture of VSMCs using the same 3-D assay was utilized to determine the effect of proliferation on these findings.

Invasion of VSMCs is limited by FRNK in a proliferation-dependent and independent fashion

FRNK overexpression decreased matrix invasion by VSMCs into fibrin hydrogels over the duration of the experiment compared to GFP controls ($p < .001$ for both the length and frequency of invasion) (Figure 2A and B). At Day 3, length of invasion was $55.8 \pm 34.4 \mu\text{m}$ in FRNK overexpressing cells vs. $101.4 \pm 29.2 \mu\text{m}$ in GFP controls ($p = .020$). This persisted at the completion of the experiment (Day 9: $217.0 \pm 36.3 \mu\text{m}$ in FRNK overexpressing cells vs. $361.8 \pm 69.4 \mu\text{m}$ in GFP controls ($p = .010$)). There was also a statistically significant decrease in the frequency of invasion in FRNK overexpressing cells at all time points ($p < .005$ for each time point). Finally there was a plateau in the observed length of invasion in FRNK-overexpressing VSMCs at later time points while GFP controls demonstrated continued invasion over the course of the experiment. This is consistent with a statistically significant difference observed in the trends of each invasion curve over time ($p < .001$).

To delineate the effect of proliferation on VSMC invasion in this assay, VSMCs were pre-treated with mitomycin C (MMC). Here inhibition of matrix invasion by FRNK persisted in MMC treated, non-proliferating SMCs over the duration of the nine day experiment vs. MMC treated GFP controls ($p < .016$ and $p < .005$ for the length and frequency of invasion, respectively) (Figure 2A and B). A statistically significant decrease in the frequency of invasion for FRNK overexpressing cells was observed at the earlier time points ($p < .001$ and $p = .05$, respectively) but not at the conclusion of the experiment. A similar plateau of length of invasion was observed at later time points in non-proliferating FRNK overexpressing cells. This was consistent with an observed significant difference in the trends of each length of invasion curve over time ($p < .001$).

FRNK displaces FAK from focal adhesion complexes

GFP-FRNK infection led to ~3.5 fold increase in cellular FRNK content (Figure 3A). Since FRNK has been demonstrated to displace FAK from focal adhesions in neonatal rat ventricular myocytes (Heidkamp et al., 2002), we utilized immunohistochemistry and confocal microscopy to demonstrate that GFP-FRNK displaced FAK from focal adhesion complexes in VSMCs, while GFP-infected VSMCs retained the normal incorporation of endogenous FAK into focal adhesion complexes (Figure 3B).

FRNK limits FAK autophosphorylation and AKT phosphorylation but does not increase alpha smooth muscle actin

When normalized to percent of GFP infected VSMC lysate, endogenous FAK expression was not significantly decreased and trended upwards after GFP-FRNK infection ($100\% \pm 13\%$ vs. $118\% \pm 7\%$; $P=.09$). However as seen in Figure 4, FAK autophosphorylation (pFAK-Y397) was significantly decreased in GFP-FRNK-infected VSMCs when compared to GFP-infected VSMCs ($100\% \pm 4\%$ vs. $23\% \pm 1\%$; $P=.002$) (Figure 4A and B). Again the uninfected group yielded similar results to the GFP control ($P>.1$) and was similarly greater than the GFP-FRNK-infected VSMCs ($P=.01$).

Total Akt levels were 2.7 fold greater in VSMCs overexpressing FRNK, but this was not statistically significant ($p = .202$). However the phosphorylation of Akt in FRNK overexpressing cells was significantly decreased compared to that of GFP controls when normalized to total Akt levels ($p<.001$) (Figure 4 C and D).

Alpha-smooth muscle actin (α -SMA) levels are maintained by VSMCs when they exist in their quiescent contractile state. However after vessel injury VSMCs switch to a synthetic phenotype and exhibit downregulation of α -SMA (Owens, 1995; Stegemann et al., 2005). To determine whether FRNK overexpression preserved a contractile state in these VSMCs, we determined the α -SMA levels by Western blot in VSMCs that were either uninfected, GFP-infected, or infected with GFP-FRNK. We did not find an absolute or relative increase in α -SMA levels in the GFP-FRNK-infected cells. Rather both the GFP-infected and the GFP-FRNK VSMCs had similar levels of expression to each other ($83\% \pm 8\%$ vs. $81\% \pm 11\%$; $P=.7$) (Figure 5).

Discussion

In this study we found a marked inhibition of the frequency and length of 3-D invasion of VSMCs treated with GFP-FRNK. Both effects persisted out to stagnation of cellular invasion. It appears that this effect is initiated through the displacement of FAK from focal adhesion complexes by FRNK with the subsequent prevention of FAK autophosphorylation (pFAK-Y397) and AKT phosphorylation, rather than phenotypic modulation of VSMCs (i.e. preserving the contractile phenotype through increased α -SMA levels). Thus this approach may be effective in limiting VSMC pathology even if treatment is initiated after the VSMCs have switched to a synthetic phenotype, which would be desirable clinically.

FAK autophosphorylation is critical to integrin signalling, but its importance may not rely solely on propagating its kinase signalling cascade through autophosphorylation as activation of its kinase domain was not required for epidermal growth factor and platelet derived growth factor chemotactic signalling (Sieg et al., 2000). FAK also is important to the normal Src signalling as the association of Src kinase with FAK is dependent on pFAK-Y397 autophosphorylation. Further downstream this association leads to the activation of numerous critical intracellular signalling cascades, including phosphorylation of FAK's kinase domain (Schlaepfer et al., 2004); The importance of FAK to Src was also supported in *Fak* null keratinocytes that demonstrated impaired Src signalling (Schober et al., 2007).

Thus the prevention of FAK autophosphorylation may disrupt both the promulgation of FAK kinase activity and the signalling pathways of other intracellular proteins (e.g. Src) that dock to FAK after its autophosphorylation.

The antagonistic relationship between FAK and FRNK demonstrated in this study has been supported in single cell type assays. For example, ECs demonstrate increased levels of FAK expression in angiogenic blood vessels, and FAK can promote capillary tube formation (Haskell et al., 2003). Further, FAK overexpression has also been reported to increase EC migration, proliferation, and retinal neovascularization, while FRNK decreased these effects (Kornberg et al., 2004). The overexpression of FAK and FAK tyrosine phosphorylation has been correlated with an invasive phenotype and increased cellular migration in a variety of cells, but these behaviors are not necessarily mediated by the same signaling mechanisms (Hsia et al., 2003; Schlaepfer et al., 2004). In contrast an invasion deficiency was seen in *fak* null fibroblasts (Sieg et al., 1999). Still the in vivo activities of FAK and FRNK may not be as straight forward as they appear, i.e. that FAK stimulates matrix invasion, cell migration, and proliferation, while FRNK inhibits these activities (Nagoshi et al., 2006). FRNK can also down-regulate expression of various MMPs, such as MMP-2 (Hauck et al., 2002), which may help to explain the decreased frequency and/or length of matrix invasion in the MMC treated GFP-FRNK infected group. Here we found that GFP-FRNK limited the invasion of MMC-treated VSMCs significantly compared to MMC-treated GFP control cells (proliferation independent effect) as well as to the GFP-FRNK VSMCs not pretreated with MMC (proliferation dependent effect).

Thus the robust inhibition of the frequency and length of VSMC invasion in this novel 3-D co-culture assay may be attributed to the displacement of FAK from focal adhesions, preventing FAK autophosphorylation and subsequent FAK kinase activity as well as limiting the availability of FAK to be a scaffolding protein for other important intracellular proteins (e.g. src). This desired inhibition of various signalling cascades, many of which promote pathologic VSMC behaviour, make FAK a simple pharmacologic target and explain the proliferative dependent and independent effects of FRNK in MMC-treated VSMCs.

Overall these findings suggest that FRNK plays a critical role limiting VSMC matrix invasion and migration. Future studies using selected mutants of the kinase domain to diminish intrinsic kinase activity will help to better define the role of FAK signalling as well as the structural importance of FAK as a scaffolding molecule for other proteins (e.g. Grb2).

This study does have recognized limitations, namely that the contributions of ECs to the co-culture assay are not well characterized; this was intentional in that we chose to infect both cell types prior to embedding them into fibrin hydrogels in order to make the model more realistic to in vivo gene infection of injured vessels. However the similar findings in the VSMC alone assay, suggests that the mechanistic studies are not dependent on the addition of ECs to this assay. Since we did not visualize GFP-FRNK infected cell invasion under epifluorescence microscopy, we quantified cellular invasion under light microscopy, which does not discriminate between GFP infected and uninfected cells. For this reason we included uninfected cells for comparison to these groups and found them to behave like their GFP control counterparts. Finally the effect of FRNK on cytoskeleton assembly and stability has been published previously (Heidkamp et al., 2002), but this effect was relatively later after infection with FRNK and secondary to FAK dislocation, so we consider this effect to be a consequence of FAK disruption and not the cause of these effects.

In conclusion, this study supports the importance of FAK signaling for 3-D VSMC invasion into fibrin hydrogels, and it provides proof of concept that GFP-FRNK overexpression can

disrupt FAK signaling and limit VSMC invasion under these conditions. Here FAK signaling was disrupted by FRNK displacing FAK from focal adhesions, preventing the autophosphorylation of FAK, and subsequent phosphorylation of AKT. This disruption of FAK signaling is a novel potential therapeutic target for the prevention of the VSMC component of intimal hyperplasia because it limits matrix invasion, cell proliferation, and migration in a clinically desirable manner.

Acknowledgments

These studies were supported by the NIH (HPG) HL41272, (AMS) HL34328, (LPB) HL078151, (EMB) HL074594; the Department of Veteran's Affairs (HPG, EMB); and the American Heart Association (AAU) AHA 0815674G

Contract Sponsor: National Institutes of Health

Contract Grant #: R01 HL41272, R01 HL34328, F32 HL078151, F32 HL074594

Contract Sponsor: Department of Veterans Affairs

References

- Allaire E, Clowes AW. Endothelial cell injury in cardiovascular surgery: the intimal hyperplastic response. *Ann Thorac Surg.* 1997; 63(2):582–591. [PubMed: 9033355]
- Avraham HK, Lee TH, Koh Y, Kim TA, Jiang S, Sussman M, Samarel AM, Avraham S. Vascular endothelial growth factor regulates focal adhesion assembly in human brain microvascular endothelial cells through activation of the focal adhesion kinase and related adhesion focal tyrosine kinase. *J Biol Chem.* 2003; 278(38):36661–36668. [PubMed: 12844492]
- Brewster LP, Brey EM, Tassiopoulos AK, Xue L, Maddox E, Armistead D, Burgess WH, Greisler HP. Heparin-independent mitogenicity in an endothelial and smooth muscle cell chimeric growth factor (S130K-HBGM). *Am J Surg.* 2004; 188(5):575–579. [PubMed: 15546573]
- Carragher NO, Levkau B, Ross R, Raines EW. Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. *J Cell Biol.* 1999; 147(3):619–630. [PubMed: 10545505]
- Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. *Science.* 2001; 294(5547):1708–1712. [PubMed: 11721053]
- Eble DM, Spragia M, Ferguson A, Samarel AM. Sarcomeric myosin heavy chain is degraded by the proteasome. *Cell Tissue Res.* 1999; 296:541–548. [PubMed: 10370141]
- Fox D, Kouris GJ, Blumofe KA, Heilizer TJ, Husak V, Greisler HP. Optimizing fluorescent labeling of endothelial cells for tracking during long-term studies of autologous transplantation. *J Surg Res.* 1999; 86(1):9–16. [PubMed: 10452862]
- Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. *Nat Rev Mol Cell Biol.* 2006; 7(3):211–224. [PubMed: 16496023]
- Haskell H, Natarajan M, Hecker TP, Ding Q, Stewart J Jr, Grammer JR, Gladson CL. Focal adhesion kinase is expressed in the angiogenic blood vessels of malignant astrocytic tumors in vivo and promotes capillary tube formation of brain microvascular endothelial cells. *Clin Cancer Res.* 2003; 9(6):2157–2165. [PubMed: 12796381]
- Hauck CR, Hsia DA, Puente XS, Cheres DA, Schlaepfer DD. FRNK blocks v-Src-stimulated invasion and experimental metastases without effects on cell motility or growth. *Embo J.* 2002; 21(23):6289–6302. [PubMed: 12456636]
- Hauck CR, Hsia DA, Schlaepfer DD. Focal adhesion kinase facilitates platelet-derived growth factor-BB-stimulated ERK2 activation required for chemotaxis migration of vascular smooth muscle cells. *J Biol Chem.* 2000; 275(52):41092–41099. [PubMed: 10998418]
- Hayasaka H, Simon K, Hershey ED, Masumoto KH, Parsons JT. FRNK, the autonomously expressed C-terminal region of focal adhesion kinase, is uniquely regulated in vascular smooth muscle: analysis of expression in transgenic mice. *J Cell Biochem.* 2005; 95(6):1248–1263. [PubMed: 15962314]

- Hedin U, Roy J, Tran P, Lundmark K, Rahman A. Control of Smooth Muscle Cell Proliferation-The role of the basement membrane. *Thrombosis & Haemostasis*. 1999; 82:23–26. (S). [PubMed: 10695481]
- Heidkamp MC, Bayer AL, Kalina JA, Eble DM, Samarel AM. GFP-FRNK disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes. *Circ Res*. 2002; 90(12):1282–1289. [PubMed: 12089066]
- Hsia DA, Mitra SK, Hauck CR, Streblow DN, Nelson JA, Ilic D, Huang S, Li E, Nemerow GR, Leng J, Spencer KS, Cheresch DA, Schlaepfer DD. Differential regulation of cell motility and invasion by FAK. *J Cell Biol*. 2003; 160(5):753–767. [PubMed: 12615911]
- Kinsella M, Tran P, Weiser-Evans M, Reidy M, Majack R, Wight T. Changes in Perlecan Expression during vascular injury: role in the inhibition of smooth muscle cell proliferation in the late lesion. *Arterioscler Thromb Vasc Biol*. 2003; 23:608–614. [PubMed: 12615671]
- Kornberg LJ, Shaw LC, Spoerri PE, Caballero S, Grant MB. Focal adhesion kinase overexpression induces enhanced pathological retinal angiogenesis. *Invest Ophthalmol Vis Sci*. 2004; 45(12):4463–4469. [PubMed: 15557455]
- Lindner V, Reidy MA. Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. *Proc Natl Acad Sci U S A*. 1991; 88(9):3739–3743. [PubMed: 2023924]
- Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol*. 2005; 6(1):56–68. [PubMed: 15688067]
- Nagoshi Y, Yamamoto G, Irie T, Tachikawa T. Expression of FAK-related non-kinase (FRNK) coincides with morphological change in the early stage of cell adhesion. *Med Mol Morphol*. 2006; 39(3):154–160. [PubMed: 16998626]
- Nolan K, Lacoste J, Parsons JT. Regulated expression of focal adhesion kinase-related nonkinase, the autonomously expressed C-terminal domain of focal adhesion kinase. *Mol Cell Biol*. 1999; 19(9):6120–6129. [PubMed: 10454559]
- Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev*. 1995; 75(3):487–517. [PubMed: 7624392]
- Schlaepfer DD. Signaling through focal adhesion kinase. *Prog Biophys Mol Biol*. 1999; 71:435–478. [PubMed: 10354709]
- Schlaepfer DD, Mitra SK, Ilic D. Control of motile and invasive cell phenotypes by focal adhesion kinase. *Biochimica et Biophysica Acta*. 2004; 1692:77–102. [PubMed: 15246681]
- Schober M, Raghavan S, Nikolova M, Polak L, Pasolli HA, Beggs HE, Reichardt LF, Fuchs E. Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. *J Cell Biol*. 2007; 176(5):667–680. [PubMed: 17325207]
- Schwartz SM, deBlois D, O'Brien ER. The intima. Soil for atherosclerosis and restenosis. *Circ Res*. 1995; 77(3):445–465. [PubMed: 7641318]
- Segev A, Nili N, Strauss B. The role of perlecan in arterial injury and angiogenesis. *Cardiovascular Research*. 2004; 63:603–610. [PubMed: 15306215]
- Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, Schlaepfer DD. FAK integrates growth-factor and integrin signals to promote cell migration. *Nat Cell Biol*. 2000; 2(5):249–256. [PubMed: 10806474]
- Sieg DJ, Hauck CR, Schlaepfer DD. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J Cell Sci*. 1999; 112(Pt 16):2677–2691. [PubMed: 10413676]
- Stegemann J, Hong H, Nerem R. Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype. *J Appl Physiol*. 2005; 98:2321–2337. [PubMed: 15894540]
- Taylor JM, Mack CP, Nolan K, Regan CP, Owens GK, Parsons JT. Selective expression of an endogenous inhibitor of FAK regulates proliferation and migration of vascular smooth muscle cells. *Mol Cell Biol*. 2001; 21(5):1565–1572. [PubMed: 11238893]
- Tran PK, Tran-Lundmark K, Soininen R, Tryggvason K, Thyberg J, Hedin U. Increased intimal hyperplasia and smooth muscle cell proliferation in transgenic mice with heparan sulfate-deficient perlecan. *Circ Res*. 2004; 94:550–558. [PubMed: 14739157]

- Walker HA, Whitelock JM, Garl PJ, Nemenoff RA, Stenmark KR, Weiser-Evans MC. Perlecan up-regulation of FRNK suppresses smooth muscle cell proliferation via inhibition of FAK signaling. *Mol Biol Cell*. 2003; 14(5):1941–1952. [PubMed: 12802066]
- Xue L, Greisler H. Angiogenic effect of fibroblast growth factor-1 and vascular endothelial growth factor and their synergism in a novel in vitro quantitative fibrin-based 3-dimensional angiogenesis system. *Surgery*. 2002; 132:259–267. [PubMed: 12219021]
- Zarge JI, Huang P, Husak V, Kim DU, Haudenschild CC, Nord RM, Greisler HP. Fibrin glue containing fibroblast growth factor type 1 and heparin with autologous endothelial cells reduces intimal hyperplasia in a canine carotid artery balloon injury model. *J Vasc Surg*. 1997; 25(5):840–848. discussion 848-849. [PubMed: 9152311]

Figure 1. VSMC invasion of this 3-D fibrin matrix

Panel A depicts cellular invasion of either the ECs (1) or VSMCs (2) that were differentially labeled with PKH-26. There is little EC but robust VSMC invasion of this matrix by PKH-26 labeled cells. Panel B has representative fluorescent and light microscope quantification of GFP and GFP-FRNK infected cellular invasion in co-culture assay (1: LM demonstrating inhibition of the length and frequency of VSMC invasion in the GFP-FRNK infected group in comparison to GFP infected control group (section 3); Fluorescent imaging of these same cells is demonstrated in sections 2 (GFP-FRNK group) and 4 (GFP). The distance scale represents 500 μ m, and images were taken at 14 days. Panel C quantifies the effect of GFP-FRNK on VSMC invasion using light microscopy. GFP-FRNK overexpression significantly decreased the length of VSMC invasion compared to GFP-infected and uninfected control groups at all time points out to stagnation of invasion (day 20); $n=12$; $P<.02$.

Figure 2. Matrix Invasion into fibrin hydrogels by VSMCs, effect of Mitomycin C on cellular invasion

FRNK overexpression decreased the length (A) and frequency (B) of matrix invasion into fibrin hydrogels over the duration of the experiment compared to GFP control ($p < .001$). Inhibition of matrix invasion by FRNK persisted in MMC treated VSMCs over the duration of the experiment vs. MMC treated GFP controls ($p < .016$ and $p < .005$ for length and frequency of invasion, respectively). Also Figure 2 demonstrates that MMC treatment significantly decreased length and frequency of invasion compared to the cells not treated with MMC.

Figure 3. GFP-FRNK infection increases cellular FRNK content and displaces FAK from focal adhesions in VSMCs

GFP-FRNK infection increases cellular FRNK content 3.5 fold (A) without a discernable difference in total FAK levels in GFP vs. GFP-FRNK treated cells.

(B) Immunohistochemical staining of FAK VSMCs using a Rhodamine-tagged secondary antibody that recognizes a FAK epitope not present on FRNK (first column). FAK's normal localization to focal adhesions is demonstrated in the GFP-infected VSMCs (Top row), but it is markedly decreased and less well localized in GFP-FRNK infected VSMCs (Bottom row). In the GFP-channel (middle column), GFP expression is noted diffusely throughout the GFP-infected VSMCs, while GFP-tagged FRNK localizes to focal adhesions (E) in a similar fashion as FAK localized to focal adhesion complexes in the 1st column. Uninfected cells are demonstrating normal FAK localization. The last column (online supplement in color) demonstrates that co-localization (yellow) of endogenous FAK and GFP-FRNK is a rare event.

Figure 4. GFP-FRNK decreases FAK autophosphorylation and AKT phosphorylation

Representative Western Blot analysis of FAK and AKT levels in GFP and GFP-FRNK infected cells.

Bar graph (A) and Western blot (B) illustrating the significant decrease in FAK autophosphorylation in VSMCs after GFP-FRNK overexpression ($P < .002$) that was not dependent on changes in the level of FAK expression between groups ($P > .1$). Phosphorylated AKT in FRNK overexpressing cells was decreased to 6.4% of GFP controls when normalized to total AKT levels ($p < .001$) even though total AKT levels in VSMCs overexpressing FRNK were 2.7 fold greater, this was not significant ($P = .2$). Error bars represent standard deviations from the results of two independent experiments.

Figure 5. α -SMA is not increased in GFP-FRNK infected VSMCs

α -SMA levels were investigated to determine whether GFP-FRNK increased these levels as would be expected if its mechanism was through maintaining or switching VSMCs into the contractile phenotype. The relative α -SMA expression as normalized to percent uninfected VSMCs are shown in part A. Part B shows a representative Western blot comparing uninfected VSMCs with GFP and GFP-FRNK infected VSMCs.

Table I

FRNK overexpression decreases the frequency of VSMC invasion

Group	Day 3	Day 6	Day 13	Day 20
Uninfected*	97%	99%	99%	100%
GFP-infected†	97%	98%	99%	100%
GFP-FRNK-infected	89%	89%	93%	98%
P values	.00001*†	.00001*†	.0001*†	.03*/.003†

The frequency of cellular invasion was significantly less in the GFP-FRNK infected group compared to either the uninfected* or GFPinfected† control groups out to 20 days in culture.