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Disruption of FRNK Expression by Gene Targeting of the Intronic Promoter Within the Focal Adhesion Kinase Gene

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Abstract FRNK, a non-catalytic variant of focal adhesion kinase (FAK), is expressed in major blood vessels throughout mouse development and is postulated to play a role in regulating cell adhesion and signaling in vascular smooth muscle cells (VSMCs). The FRNK transcriptional start site lies within an intron of the FAK gene, suggesting that the FRNK gene is a ''gene within a gene''. Here, we identified a 1 kb intronic sequence of the FAK gene that is necessary for endogenous FRNK expression. Deletion of this sequence in gene-targeted mice abolished FRNK expression, showing the direct involvement of the FAK intron in the regulation of FRNK expression. The level of FAK expression was normal in the FRNK-deficient mice, indicating that FAK and FRNK are transcriptionally regulated by distinct promoters. The FRNK-deficient mice were viable, fertile, and displayed no obvious histological abnormalities in any of the major blood vessels. Western blot analysis showed that FRNK–deficient and wild-type (WT) cells had comparable levels of steady-state and adhesion-dependent FAK autophosphorylation. Despite the fact that ectopic expression of FRNK suppresses focal adhesion formation in cultured cells, these results suggest that endogenous FRNK is not essential for development or the formation of the mouse vasculature. J. Cell. Biochem. 102: 947–954, 2007.

Key words: gene expression; cell adhesion; vascular smooth muscle cells

Focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase located in focal adhesions, becomes activated in response to integrin engagement and initiates numerous intracellular signaling pathways that regulate cellular process such as migration, proliferation, and cell survival [Parsons et al., 2000]. FAK function is regulated in part by tyrosine phosphorylation, which creates an SH2 binding site for Src tyrosine kinases, enabling the activated FAK/Src complex to phosphorylate downstream substrates [Calalb et al., 1995; Eide et al., 1995]. FAK activation results in induction of other signaling cascades involving serine/threonine kinases such as extracellular signal-regulated kinases (Erks), lipid kinases, phosphatases, and regulators of GTPases [Schaller et al., 1994; Hildebrand et al., 1996; Schlaepfer and Hunter, 1997; Renshaw et al., 1999]. Another regulatory mechanism of FAK function has been described which depends on the expression of FRNK, a 42-kDa protein whose amino acid sequence is identical to the non-catalytic carboxyl-terminal sequence of FAK [Richardson and Parsons, 1996]. Ectopic expression of FRNK delays cell spreading and inhibits cell migration in various types of cultured cells [Schaller et al., 1993; Richardson and Parsons, 1996; Taylor et al., 2001]. Although the precise mechanism for the inhibitory effects of FRNK is unknown, several mechanisms have been proposed to explain the function of FRNK. First, given that the focal adhesion targeting domain of FRNK [Hildebrand et al., 1993] is shared with FAK

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and indispensable for the inhibitory effects [Sieg et al., 1999; Mortier et al., 2001], it is hypothesized that FRNK competes with FAK for focal adhesion localization. Secondly, FRNK may compete for binding to FAK-associated signaling molecules such as Graf, Cas, and ASAP1 [Harte et al., 1996; Hildebrand et al., 1996; Liu et al., 2002]. Alternatively, given that FRNK overexpression inhibits autophosphorylation of FAK in cultured cells [Richardson and Parsons, 1996; Hauck et al., 2000], FRNK may directly or indirectly facilitate the dephosphorylation of FAK, resulting in termination of the downstream signaling events.

By reporter gene expression analyses, we previously proposed that the FRNK expression is governed by an alternative promoter located within an intron of the FAK gene [Nolan et al., 1999; Hayasaka et al., 2005]. An alternative promoter leads to multiple mRNA transcripts and protein products, which can generate diversity in gene expression and tissue specificity [Ayoubi and Van De Ven, 1996]. These lines of evidence give rise to the intriguing possibility that FAK function is modulated by the expression of FRNK under the control of the alternative promoter. To date, it has been reported that FRNK is predominantly expressed in tissues that are rich in vascular smooth muscle cells (VSMCs) and that the level of FRNK protein increases in response to carotid artery injury [Taylor et al., 2001]. Thus, despite the possible importance of FRNK in the induction and/or maintenance of the smooth muscle phenotype within cells of the vasculature, little is known about the regulation of its gene expression and potential role of FRNK in VSMCs.

To directly assess the significance of FRNK expression in the biological properties of VSMCs and in the regulation of FAK function, we have examined the consequences of FRNK ablation in gene-targeted mice. In this article, we provide genetic evidence that FRNK expression is in fact regulated by a promoter within an intron of the FAK gene. Although no serious abnormalities were observed in the FRNK knockout mice or VSMCs explanted from the mice, this system provides the ideal model to allow continued studies on the function of endogenous FRNK.

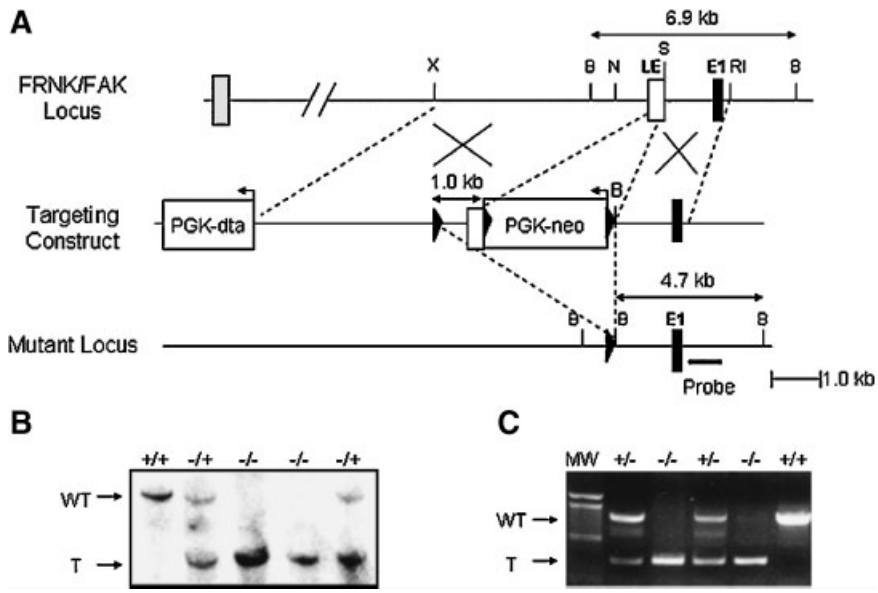

Fig. 1. Targeted disruption of the FRNK promoter. **A**: schematic representation of the FRNK targeting strategy. **Top**: structure and partial restriction map of the FRNK promoter locus. The noncoding LE unique to FRNK is shown as an open box and the coding exon common to FAK and FRNK (E1) as a solid. The shaded box represents an exon located upstream of the FRNK LE that is unique to FAK. B, BamHI; N, NsiI; RI, EcoRI; S, SacII; X, XbaI. **Middle**: the targeting construct after linearization with XhoI. The third LoxP sequence (black triangle) is inserted at the NsiI site in the 5′ arm of the targeting vector so that the 1 kb FRNK promoter locus and entire neo cassette can be deleted at the following step. The diphtheria toxin A fragment gene (dta) and the neomycin resistance gene express under the control of the mouse phosphoglycerate kinase (PGK) promoter (PGK-neo). **Bottom**: the targeted allele after Cre-mediated recombination. The 1 kb FRNK promoter locus and entire neo cassette were deleted. The probe used in Southern blot analyses is shown (probe). **B**: Southern blot analysis of the offspring from a FRNK HET intercross. Tail DNA was digested with BamHI and hybridized to the radiolabel probe. The WT locus is 6.9 kb and the targeted locus (T) is 4.7 kb. **C**: PCR analysis of the offspring from a FRNK HET intercross.

MATERIALS AND METHODS

Construction of the Targeting Vector

A 5.7 kb XbaI/SacII genomic fragment of the murine FRNK locus was subcloned into the pBluescript KS- (pBS) plasmid to generate pBS-LA (Fig. 1A). pBS-LA was digested at the internal NsiI site to insert a synthetic LoxP fragment (5′-ataacttcgtataatgtatgctatacgaagttatcccgggccctccctga-3′) 0.7 kb upstream of the FRNK leader exon (LE). The 5.7 kb SalI/NotI fragment including the LoxP sequence was cloned into the same sites of the pGKNeolox2dta + kmcs targeting vector (provided by Gene targeting and Transgenic facility, University of Virginia) immediately upstream from a neomycin resistance gene flanked by LoxP sites. A 2.1 kb SacII/EcoRI genomic fragment downstream of the LE of FRNK was subcloned into pBS and modified to generate MluI and SacII sites at the end of the insert The 2.1 kb MluI/SacII fragment was ligated into the same sites of the targeting vector downstream from the Lox-Neo-Lox cassette.

Generation of FRNK-Deficient Mice

The FRNK targeting vector was linearized with KpnI and electroporated into IB10 ES cells. Cells were selected in the presence of G418, and resistant clones were analyzed by Southern blotting using the protocol and probes for genotyping to distinguish homologous recombinants. One of four homologous recombinants was transfected with a Cre-expression plasmid, pIC-Cre [Gu et al., 1993], to initiate Cre-mediated recombination. Colonies were selected and screened by Southern blotting to identify a clone with a recombination at the LoxP sites in the FRNK sequence and the end of the Neo cassette. This clone was injected into C57BL/6 E3.5 blastocysts and chimeric mice were obtained. The resulting male chimeras were mated with C57BL/6 females and agouti offspring (F1) were genotyped. Homozygous FRNK-deficient offspring from a cross of the F1 agouti FRNK heterozygous (HET) mice were genotyped by Southern blotting and used for experiments.

Genotyping by Southern Hybridization and PCR

Genotyping was performed by PCR and Southern blotting using tail DNA purified by DNeasy Tissue Kit (Qiagen, Inc., Valencia, CA). PCR was performed using a primer set, 5′aaggaagtcacccgccagct 3′ and 5′gcctgctctaaac tggaa 3′, which corresponds to the sequences outside of the LoxP sites. The product size from the WT and targeted allele are 1 kb and 310 bp, respectively. For Southern blotting, 10 μg of tail DNA was digested with BamHI and run on a 1.0% agarose gel. The DNA was transferred by the alkaline transfer method onto a GeneScreen Plus membrane (PerkinElmer Life and Analytical Sciences, Waltham, MA). A 1.7 kb genomic fragment corresponding to the endogenous sequence downstream of the exon 1 was labeled with [α-^{32}P] dCTP by random priming and used as a probe.

Isolation and Culture of Vascular Smooth Muscle Cells

The cells from a single aorta were explanted as previously described [Ray et al., 2001]. Briefly, the thoracic aortas from 6-week-old mice were excised and the adventitia were removed using microdissecting scissors under a dissecting microscope. A smooth tube of aorta was cut into pieces, digested in type II collagenase for 4 h, and resuspended in the culture medium (DMEM, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum. Cells were used for experiments between passages 4 and 10.

Northern Blot Analysis

Poly A RNA was isolated from cultured aortic VSMCs using the mRNA Isolation Kit (Roche Applied Science, Indianapolis, IN). PolyA RNA (0.5 μg) was loaded on a 1% agarose gel and run in a buffer containing 2.2 M formaldehyde. The gel was soaked for 10 min in 50 mM NaOH and subjected to alkaline transfer onto a Hybond-N + membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Hybridization was carried with 25 ng of ^{32}P-labeled cDNA probe encoding the FAK carboxyl terminal domain common to FAK and FRNK (nucleotides 2,296–3,213) or a GAPDH cDNA control fragment at 42°C in 50% formamide, 5 × SSPE, 5 × Denharts, 1% SDS, and 100 μg/ml ssDNA. Washes were carried out 2 × SSC and 0.1% SDS twice for 20 min at room temperature. The hybridized membrane was autoradiographed for 3 days at −70°C with an intensifying screen.

Western Blotting and Antibodies

Lung extracts were prepared from 3-week-old mice by homogenization in modified radioimmunoprecipitation assay buffer (RIPA) [Kanner et al., 1989] containing 1 mM sodium orthovanadate and Complete EDTA-free protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). For preparation of whole cell lysates of VSMCs, subconfluent cultures of passages 2 or 4 were washed twice in CMF-PBS (calcium, magnesium free phosphate buffered saline, 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM disodium phosphate, 1.4 mM potassium phosphate, pH 7.2) and lysed by scraping in RIPA buffer. Protein concentration was measured by the BCA assay kit (Pierce Biotechnology, Inc., Rockford, IL). To obtain lysates from cells spreading on fibronectin, VSMCs were starved 60 h in culture medium containing 0.1% serum prior to seeding on fibronectin-coated dishes. VSMCs were detached by trypsin and EDTA, treated with 0.5 mg/ml trypsin inhibitor, and 2×10^5 cells were seeded in each well of 6-well dishes in culture medium containing 1% serum. Dishes were washed twice with CMF-PBS, and RIPA lysates were harvested at the indicated times. 100 μg of lung lysate or 50 μg of VSMCs lysate were boiled in sample buffer, electrophoresed on 10% SDS polyacrylamide gels, and transferred to nitrocellulose. Filters were blocked overnight at 4°C with Tris-buffered saline (50 mM Tris/HCl, pH 7.6, 150 mM NaCl) containing 0.05% Tween-20, 2% gelatin, 0.1% NaN_3, and 5% skim milk. Polyclonal anti-myosin heavy chain antibody was kindly provided by Dr. G. Owens (University of Virginia, Charlottesville, VA). For a loading control, monoclonal anti-cortactin antibody (4F11) was used. FAK and FRNK were detected by a rabbit polyclonal antibody generated against a part of C-terminal sequence of FAK (cat# 06–543, Upstate Biotechnology, Lake Placid, NY). FAK autophosphorylation was detected using polyclonal rabbit anti-pY397 FAK (BioSource International, Camarillo, CA). Binding of HRP-conjugated secondary antibodies was detected by ECL (GE Healthcare Bio-Sciences Corp.).

Measurement of Growth Rate and Cell Adhesion to Fibronectin

For measurement of the growth rate, cultured VSMC at passage 4 were harvested, and 5×10^4 cells were plated in each well of a 6-well dishes. After 16 h incubation in the presence of 10% serum, cells were starved in medium without serum for 24 h. Then, cells were stimulated by 10% serum by replacement of culture medium, and the number of cells in each well was counted at 24, 48, and 72 h after stimulation. To analyze cell spreading on fibronectin, 5×10^4 VSMCs were starved 60 h in culture medium containing 0.1% serum prior to seeding on fibronectin-coated dishes. VSMCs were detached by trypsin and EDTA, treated with 0.5 mg/ml trypsin inhibitor, and seeded in each well of 6-well dishes in culture medium containing 1% serum. Dishes were washed twice with CMF-PBS 40 min after seeding and the number of adherent cells was counted. Cell spreading was microscopically observed at 30 min, 1, 2, and 5 h after seeding.

Immunoflorescence cell staining

VSMCs were seeded at a density of 5×10^4 cells on fibronectin-coated coverslips in the presence of 1% serum overnight, and then the cells were starved in 0.1% serum for 60 h. Coverslips were washed twice with CMF-PBS, fixed with 4% paraformaldehyde in PBS for 20 min at RT, and permealized with 0.5% Triton X-100/PBS for 2 min. Cells were stained with antibodies to paxillin or FAK (Transduction Laboratories, Franklin Lakes, NJ), vinculin (Sigma-Aldrich, St. Louis, MO), pY397 phosphoFAK or pS722 phosphoFAK (BioSource International, Camerillo, CA). FITC and Texas Red secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Actin stress fibers were stained with Texas Red-X phalloidin (Molecular Probes, Eugene, OR). Images were captured using a 60× objective on a Nikon E600 microscope equipped with a Hamamatsu Orca Camera and the Openlab image acquisition software (Improvision Inc., Lexington, MA). In order to compare the levels of staining between samples, images were captured using the same exposure times, and the levels were adjusted equally using Adobe Photoshop.

RESULTS

Generation of FRNK-Deficient Mice

We previously showed that FRNK transcription initiates from a non-coding exon (the LE) that is located within an intron of FAK [Nolan

et al., 1999], which suggests that the LE and the sequences upstream from it contain the regulatory elements for FRNK transcription. We, therefore, hypothesized that a deletion in the FRNK promoter would abrogate FRNK expression without affecting the expression of FAK. A series of deletion mutants were tested using luciferase reporter assays which showed a DNA fragment lacking 1 kb of FRNK genomic sequence corresponding to the 0.3 kb LE and the 0.7 kb upstream sequences completely lacked promoter activity (H. Hayasaka, unpublished data). Based on this finding, FRNK knockout mice were generated by targeting this 1 kb region of the genome for deletion (Fig. 1A). The resulting offspring were genotyped by Southern blotting (Fig. 1B) and PCR (Fig. 1C) to identify the mice that were homozygous for deletion of the FRNK promoter sequence. To confirm that FRNK expression was lost with this targeting strategy, Northern blotting was performed with a probe that reacts to the common carboxyl terminus of FAK and FRNK. The 2.2 kb FRNK mRNA was undetectable in FRNK-deficient animals, whereas the 4.2 kb FAK mRNA and a control (GAPDH) mRNA were detected at levels comparable to the wild-type (WT) littermates (Fig. 2A). Western blotting analysis with lung lysates showed that the intensity of the 41 and 43 kD FRNK bands was reduced by half in HET FRNK mice compared with that in the WT and undetectable in the FRNK-deficient littermates (Fig. 2B). The level of FAK protein was not affected by the FRNK knockout. These lines of evidence indicate that the 1 kb sequence including the LE is crucial for transcription of FRNK but is dispensable for expression of FAK.

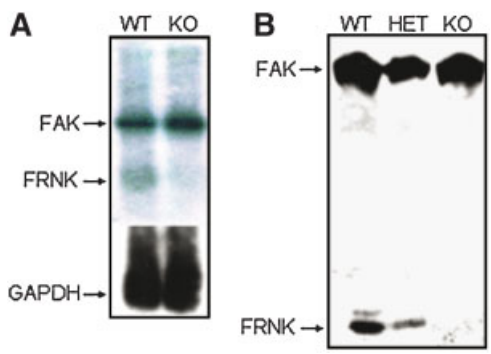

Fig. 2. **A**: Northern blot analysis of explanted VSMC from WT and FRNK-deficient (KO) littermates. PolyA RNA was blotted with a carboxyl terminal probe common to FAK and FRNK. FRNK mRNA was undetectable in the FRNK knockout mouse. GAPDH expression is shown as a loading control. **B**: Western blot analysis of lung lysates derived from FRNK-deficient and WT littermates. The total FAK expression level was similar irrespective of genotypes. FRNK expression in HET was decreased to half of that in WT and was undetectable in homozygous FRNK-deficient (KO) mouse. The total FAK expression level was similar irrespective of genotypes.

FAK Phosphorylation in FRNK-Deficient Mice

Homozygous FRNK-deficient mice were born in a Mendelian distribution and showed no obvious defects in their development, survival, or fertility. Therefore, we looked more specifically at the major aortic vessels in which FRNK is normally expressed. We found that the cross-sections of FRNK-deficient aortic vessels from 8-week-old mice looked normal (Fig. 3A), and no differences were noted up to 6 months of age (data not shown). Considering that phosphorylation at FAK Y397 is down regulated upon FRNK over expression in cultured cells [Hauck et al., 2000; Taylor et al., 2001], we assessed the possibility that FRNK directly regulates the phosphorylation level of FAK in VSMCs. To this end, we established primary VSMC cultures from WT or FRNK-deficient mice. The VSMC cultures were characterized by Western blotting which showed a substantial level of smooth muscle myosin heavy chain, a marker for smooth muscle cells (Fig. 3B). The cells were also examined on a single cell basis by immunostaining for another maker of VSMCs, smooth muscle α-actin. Although the percentage of cells that were positive for smooth muscle α-actin was comparable between the two cell lines, there was considerable heterogeneity in the cultures (58% α-actin positive for WT and 53% for FRNK-deficient cells). In these cultures, the level of FAK Y397 phosphorylation was indistinguishable between WT and FRNK-deficient VSMC (Fig. 3B). The phosphorylation of other sites in FAK (S722, Y861, and S911) was also similar between the two types of cells (data not shown). These lines of evidence indicate that loss of FRNK does not increase the basal level of FAK Y397 phosphorylation in cultured VSMCs.

Next, we examined whether the kinetics of FAK phosphorylation during cell attachment to fibronectin-coated plates is affected by loss of FRNK expression. The fibronectin-stimulated FRNK-deficient cells showed an increased level of FAK Y397 phosphorylation within 15 min after fibronectin-stimulation and the phosphorylation persisted up to 60 min (Fig. 3C). The kinetics of FAK Y397 phosphorylation was

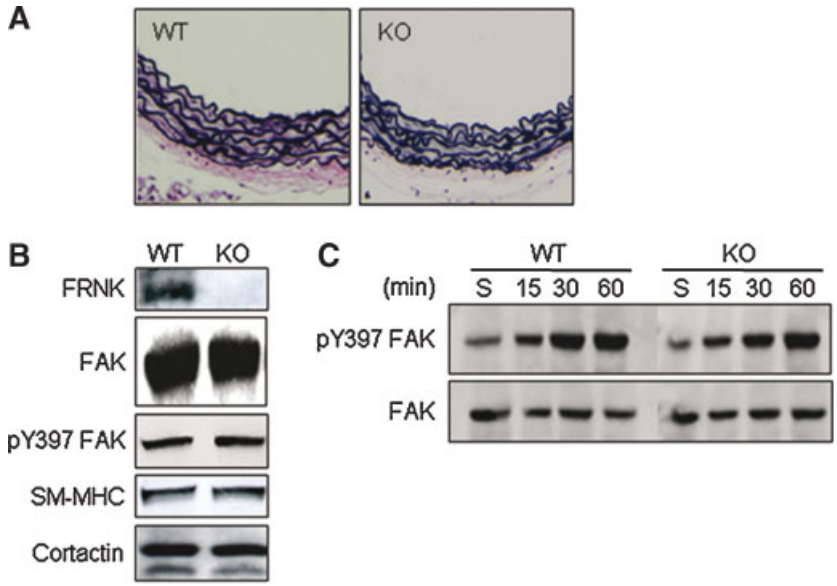

Fig. 3. Histology of aortic blood vessels and the levels of phosphotyrosine 397 of FAK (pY397 FAK) in FRNK-deficient VSMCs. **A**: cross-sections of thoracic aortas stained with Van Gieson's solution (8-week-old). No difference was noted between FRNK-deficient and WT littermates. **B**: whole cell extracts of cultured VSMCs (passage 4) from FRNK-deficient and WT littermates were subjected to Western blotting using anti-pY397 FAK antibody. The levels of FAK, smooth muscle myosin heavy chain and cortactin are shown as loading controls. **C**: levels of pY397 FAK in FRNK-deficient VSMC upon attachment to fibronectin. Serum-starved VSMCs were plated on fibronectin-coated dishes and total lysates were harvested in suspension (S), and at 15, 30, and 60 min. Total FAK is shown as a loading control.

similar between FRNK-deficient and WT cells, indicating that FAK phosphorylation is regulated independently of FRNK expression.

Focal Adhesion Formation in FRNK-Deficient VSMCs

Previous experiments have shown that FRNK over expression inhibits adhesion to extracellular matrix and the rate of cell spreading [Richardson and Parsons, 1996]. Based on those findings, we first investigated the efficiency of VSMC attachment to fibronectin-coated dishes. At 20 min after plating, approximately 20% of both the WT and FRNK-deficient VSMCs had attached. The rate of spreading on fibronectin and the rate of proliferation were similarly unaffected by the loss of FRNK (data not shown). Because attachment and cell spreading both depend on the formation of focal adhesions, the VSMCs were examined by immunostaining for markers of focal adhesions. In both WT and FRNK-deficient cells, the actin was organized into prominent stress fibers that ended at paxillin-rich focal adhesions (Fig. 4A, top). The size, number, and distribution of focal adhesions were similar for both cell lines. The relative level of FAK Y397 phosphorylation compared to vinculin staining was not significantly altered in FRNK-deficient cells (Fig. 4, middle). Cellular localization of FAK and the levels of FAK S722 phosphorylation were also unchanged (Fig. 4, bottom). These results suggest that the loss of FRNK expression does not prevent the formation of focal adhesion in cultured VSMCs.

DISCUSSION

In this article, we provide direct genetic evidence that FRNK expression is regulated by an alternative promoter embedded within the gene encoding FAK. Since FRNK cDNA had a unique 5′ leader sequence, it has been postulated that the leader and the upstream sequence mediate the transcriptional regulation of FRNK. The LE and the upstream sequences were previously shown to contain smooth muscle cell-specific transcription regulatory elements in vitro [Nolan et al., 1999; Hayasaka et al., 2005]. In this article, we describe the generation of FRNK-knockout mice. The targeted deletion of regulatory sequences in the FAK intron abolishes FRNK expression in

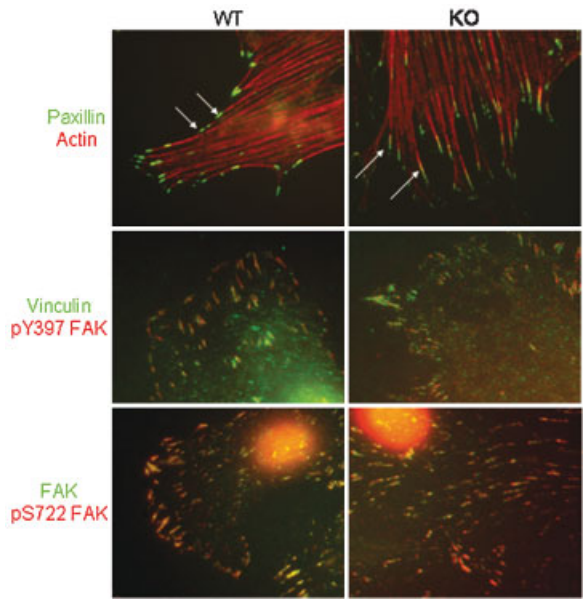

Fig. 4. Mature focal adhesions were observed in FRNK-deficient cells. Cultured VSMCs from WT and FRNK-deficient (KO) mice were stained for markers of focal adhesion (paxillin, FAK, and vinculin) and the actin cytoskeleton. In addition, cells were also stained for phosphorylated FAK. The intensity is comparable between WT and KO cells. Arrows indicate examples of focal adhesions.

lung and VSMCs, providing strong evidence that this region is essential for FRNK expression in vivo. In addition, we showed that FAK expression is normal in the FRNK knockout mice, indicating that FRNK is a "gene within a gene" that its expression is regulated independently from the transcriptional regulation of FAK

This is among a very few reports describing the ablation of "a gene within a gene" and the analysis of its physiological significance in vivo [Gallagher and Herring, 1991; Sobieszek et al., 1997]. Although no basal phenotype has yet been observed in the FRNK-deficient mice, consequences of eliminating FRNK may emerge if these animals are challenged by physiological stress. It will be of special interest to examine recovery from vascular injury in the FRNK-deficient mice, since FRNK expression is upregulated during vascular development and wound healing [Taylor et al., 2001]. Further investigation will be needed to elucidate the significance of FRNK expression under physiological conditions.

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