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FRNK, the Autonomously Expressed C-Terminal Region of Focal Adhesion Kinase, Is Uniquely Regulated in Vascular Smooth Muscle: Analysis of Expression in Transgenic Mice

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Abstract FRNK, the autonomously expressed carboxyl-terminal region of focal adhesion kinase (FAK), is expressed in tissues that are rich in vascular smooth muscle cells (VSMCs). Here we report the generation of transgenic mice harboring the putative FRNK promoter fused to LacZ and examine the promoter activity in situ via expression of β-galactosidase. The transgenic mice exhibited expression of β-galactosidase predominantly in arterial VSMCs in large and small blood vessels of major organs. Upregulation of β-galactosidase activity was observed in tunica media following carotid injury, indicating that the FRNK promoter is activated in VSMCs in response to injury. Robust expression of β-galactosidase in blood vessels was also detected in the developing embryo. However, expression was also observed in the midline, the nose and skin epidermis, indicating distinct transcriptional regulation of the FRNK promoter in embryogenesis. To analyze FRNK expression in vitro, we identified a 116 bp sequence in the FRNK promoter that was sufficient to function as an enhancer when fused to the minimal actin promoter and expressed in cultured smooth muscle cells. Mutation of AP-1 and NF-E2 binding consensus sequences within this element abrogated enhancer activity, supporting the involvement of this promoter element in VSMC expression of FRNK. J. Cell. Biochem. 95: 1248–1263, 2005.

Key words: focal adhesion kinase; smooth muscle cells; LacZ expression

The regulation of growth and differentiation of vascular smooth muscle cells (VSMCs) is a critical event during development of the vascular system and plays a central role in the cardiovascular diseases such as atherosclerosis, restenosis, and hypertension [Rivard and Andres, 2000]. VSMCs exhibit two distinct cellular phenotypes, a "contractile" and a "synthetic" phenotype, in response to various different environmental cues. Biochemical studies indicate that this phenotypic modulation is mediated by growth factors and extracellular matrix (ECM) proteins, suggesting involvement of cell–cell and cell–matrix interaction in the process [Thyberg, 1996]. Gene ablation of some subunits of integrins, the most widely known adhesion receptor for ECM proteins, showed defects in vascular development, suggesting critical role of ECM, and integrins interaction in organization of vascular system [Rupp and Little, 2001].

Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase that is important for organization and signaling by focal complexes and adhesions [Parsons, 2003]. The clustering

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of integrins in response to cell adhesion triggers the autophosphorylation of FAK, recruits other signaling molecules to complexes with FAK, and promotes formation of structure termed focal complexes and adhesions, structures crucial for cell growth and migration [Parsons, 2003]. A body of evidence supports a role for FAK in signal transduction by growth factor receptors and or ECM components through integrins in VSMCs [Abedi and Zachary, 1995; Hauck et al., 2000].

FAK-related non-kinase, FRNK, is a 42-kDa protein originally observed in chicken embryonic fibroblasts [Schaller et al., 1993]. Ectopic expression of FRNK inhibits the activity of FAK and delays cell spreading, cell migration, and inhibits growth factor activation of the ERK pathway [Schaller et al., 1993; Richardson and Parsons, 1996; Hauck et al., 2000; Taylor et al., 2001]. The amino acid sequence of FRNK is identical to the carboxy-terminal sequences of FAK [Schaller et al., 1993], which includes two proline-rich domains implicated in recruiting signaling proteins such as Graf, Cas, and ASAP1 [Hildebrand et al., 1993; Harte et al., 1996; Liu et al., 2002] as well as a focal adhesion targeting (FAT) domain that is necessary and sufficient to target proteins to focal adhesions [Hildebrand et al., 1993]. Previously we have shown that in rodent tissues, FRNK is expressed in organs rich in VSMCs and in cultured VSMCs [Taylor et al., 2001]. Since FRNK cDNAs are comprised of a unique 5′ non-coding leader sequence fused to the coding sequence of the C-terminus of FAK, we have proposed that transcription of FRNK mRNA is initiated from an independent promoter located in an intron of FAK [Nolan et al., 1999].

To better understand the factors that regulate expression of FRNK in vivo, we generated transgenic mice expressing the LacZ gene under the control of the FRNK promoter and characterized the pattern of β-galactosidase expression in vivo. This approach is necessary because FRNK is immunohistochemically indistinguishable from FAK; hence conventional in situ immunohistochemical approaches to assess FRNK expression are not useful. In postnatal mice, the expression of β-galactosidase was observed predominantly in VSMCs in large and small blood vessels of major organs whereas little activity was observed in other types of tissues rich in visceral smooth muscle cells. Following carotid wire injury, increased β-galactosidase was observed, indicating that the FRNK promoter was stimulated in the neointima and tunica media. In the embryo, the FRNK promoter was highly active in the midline, nose area, and skin in addition to blood vessels. Using in vitro luciferase reporter assays we assessed the molecular control of FRNK expression, identifying a 116 bp sequence in the FRNK promoter that was sufficient to function as an enhancer when fused to the minimal actin promoter and expressed in cultured smooth muscle cells. Furthermore a mutation of AP-1 and NF-E2 binding consensus sequences within this element abrogated enhancer activity, supporting the involvement of this promoter element in VSMC expression of FRNK.

MATERIALS AND METHODS

Cloning of Murine Genomic Sequences

The mouse genomic library 129SVJ, derived from 8-week male spleen DNA and cloned into the Lambda FIX II vector (Stratagene, La Jolla, CA), was screened with the 753 bp EcoNI fragment from the 3′ end of the murine FAK (nucleotides +2151 to +2094). The phage library was plated onto host strain *Escherichia coli* XL-1-Blue MRA and plaques were screened for hybridization to the [α-^{32}P]dCTP labeled probe by standard procedures. DNA from selected phage clones was subjected to sequence analysis to determine location of intron and exon sequences. Upon identification of the exon corresponding to the 5′ leader exon upstream of the first coding exon of FRNK cDNA, a 311 bp sequence derived from this exon was used as a probe for further library screening to isolate four overlapping phage clones. The four genomic clones were sequenced to determine the genomic organization of region 5′of the FRNK coding region. Computer analysis of the potential transcription factor binding sites was performed by the MatInspector V2.2 (Genomatix) based on TRANSFAC 4.0.

Construction of the Transgenic Vector and Generation of Transgenic Mice

Phage clone (8A1) contained an approximately 16 kilobase insert that includes the first coding exon, the 5′ leader exon and the upstream intron containing the putative promoter region. 8A1 was digested with XbaI to isolate a 6 kilobase fragment spanning nucleotides −5338 to +730 of the FRNK genomic sequence

(+1 is the first nucleotide of the 5′ leader exon) corresponding to the 5′ leader exon and parts of the flanking upstream and downstream introns. The XbaI fragment was subcloned into an XbaI site of pBluescript KS- (pBS) plasmid (pBS-Xba6K). The 6 kilobase fragment was reisolated from the pBS-Xba6K by SalI and NotI digestion and inserted into the multicloning sites of the pUC19-lacZ vector. The subsequent vector was modified whereby the EcoRI site originally present in the pBS plasmid was removed by digestion with the two flanking SmaI sites and religated. The 6 kilobase/LacZ sequence was cut out with NotI and EcoRI digestion and purified from an agarose gel for DNA injection. Four transgenic founder lines were generated using standard methods in the Transgenic Core Facility at The University of Virginia. To determine copy numbers of each founder lines, 10 μg of tail DNA was purified, digested with BamHI, and run on a 1.0% agarose gel. Southern blotting was performed using a LacZ probe. The filter was hybridized with the LacZ probe labeled with [α-^{32}P]-dCTP by random priming. The tail DNA of heterozygous Sno^{ex1} gene-targeted mice carrying one copy of LacZ [Pearson-White and McDuffie, 2003] was used as a single copy standard. The transgene-specific bands were quantitated by densitometry to estimate the number of transgenes integrated for each founder. Line Tg-H contained three copies of the transgene, lines Tg-M1 and Tg-M2 contained four copies, and line Tg-L one copy.

Histochemical Staining of Transgenic Mice Tissue

Postnatal tissues or whole embryos from mice were immersed in fixing solution (0.2% glutaraldehyde, 2% formaldehyde, and PBS) for 30 min, rinsed twice with PBS for 10 min, and placed in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.2% NP-40, 0.1% deoxycholate, 1 mg/ml 5-bromo-4-chloro-3-indolyl b-D-galatopyranoside; X-gal, 20 mM Tris-Cl, and PBS pH 7.3) at room temperature overnight in dark. Embryos older than E12.5 were sectioned coronally to permit fixation and dye penetration. Tissues or embryos stained with X-gal were rinsed twice with PBS; paraffin embedded, sectioned in 5 μm slices, and counter stained with eosin. For whole mount observation, embryos were cleared in a 2:1 mixture of benzyl alcohol and benzyl benzoate. For cryosectioning, tissues were trimmed, fixed with 0.2% paraformaldehyde in 0.1 M PIPES, pH 6.9 at 4°C overnight, cryoprotected in 30% sucrose, 2 mM $MgCl_2$ in PBS at 4°C overnight, placed in an optimal cutting temperature compound (OCT; Miles Inc. Elkhart, IN), and stored at −20°C. Frozen sections were fixed with fixing solution and subjected to X-gal reaction or immunohistochemistry. Immunohistochemistry was performed with a mouse monoclonal anti-α-SMA antibody (diluted 1:250, clone IA4, Sigma, St. Louis, MO) and rat anti-CD31 (diluted 1:10, BD Biosciences). Frozen sections were washed with PBS, incubated in 0.3% H_2O_2 in PBS for 20 min to inhibit endogenous peroxidase activity, and saturated with 10% (vol/vol) whole sheep serum for 20 min to block nonspecific binding. Sections were incubated with or without first antibodies for 20 min, washed with PBS three times, and followed by incubation with 1:100 diluted sheep peroxidase-conjugated species-specific secondary antibodies (Amersham Biosciences). The immune complexes formed were visualized by DAB substrate kit (BD Biosciences) and counterstained with hematoxylin. The in situ hybridization was performed using digoxigenin-labeled probes as described previously [Yan et al., 1999]. FRNK mRNA was detected with a DIG-labeled antisense riboprobe synthesized with T7 RNA polymerase from a linealized 309 base fragment of the 5′ leader exon cDNA in PCR-script vector. As a control, a sense RNA probe was synthesized with T3 RNA polymerase from the same cDNA.

Carotid Wire Injury

Mice were injured using the method of Lindner et al. [1993] modified by Manka et al. [2002]. Both injured left and uninjured right carotid arteries were excised 14 days after surgery. Tissues were stained with X-gal as described and serial 5-μm sections were prepared from the paraffin-embedded blocks for histology.

Promoter-Luciferase Reporter Plasmids

PCR was performed to subclone a 2.2 kb fragment spanning from −1934 to +302 into PCR-script vector using the PCR-script cloning kit (Stratagene). The insert was then removed from PCR-script by SacI and SmaI digestion and cloned into the SacI/SmaI sites of the pGL3-

Basic Vector (Promega) to construct pGL3-2.2K. Inserts of the constructs listed in Figure 7A were amplified by PCR using a 5′ primer with KpnI overhang and an appropriate 3′ primer with either internal NsiI site (nucleotide position −677) or XhoI overhang. The PCR fragments were swapped with the KpnI/XhoI or KpnI/NsiI fragment of pGL3-2.2K at either KpnI/XhoI sites in pGL3 or KpnI and a NsiI site at −677. pGL3-5.6K-5′ half and pGL3-2.2K-5′ half was generated by PCR using a primer flanking NsiI site at −677 and swapped with a NsiI/XhoI fragment of pGL3-5.6K or pGL3-2.2K, respectively. For pGL3-5.6K, a 6 kilobase XbaI fragment of pBS-Xba6K was subcloned to the NheI site of the pGL3-Basic Vector. The consequent plasmid was digested with NheI and XhoI and swapped with a NheI/XhoI fragment of pGL3-2.2K to adjust the 3′ end to other constructs. PGL3-5.6K-0.4K and pGL3-3.0K-0.4K were generated by digestion of pGL3-5.6K and pGL3-3.0K with HindIII, respectively, to remove internal HindIII fragment and followed by self ligation. To make point deletions in pGL3-3.0K d#2 and pGL3-3.0K d#3, primers with each deletion were synthesized and used as 5′ PCR primers. The primer pair was designed to amplify the fragment from −1735 to −1325 with two internal HindIII sites. The subsequent PCR product with mutation was digested with HindIII and was swapped with the wild type fragment of pGL3-3.0K. pβLuc24 [Ogino and Yasuda, 1998] was kindly provided by Dr. H. Ogino, Department of Biology, University of Virginia. A 116-bp sequence from −1735 to −1620 was amplified by PCR and inserted in the KpnI/PstI site of pβLuc (pβLuc-E1). The second PCR was performed using a pair of primers with PstI and XhoI sites on both the ends. The sequences of oligonucleotides for PCR were 5′-TTTCTGCAGCTCGAGAGCTTTTCACTGGA-ATGTAT-3′ and 5′-TTTCTCGAGCTGCAGC-TGCTGAGTCAAAG-3′. The PCR product was purified, digested with PstI, and inserted at a PstI site of pβLuc-E1 to generate pβLuc-E2. pβLuc-E3 was constructed by inserting the PCR fragment digested with XhoI to an XhoI site of the first PCR fragment inserted in pβLuc-E2. The mutated fragment of pβLuc-mutE2 was generated by PCR using an oligonucleotide with a mutation underlined (5′-TTTCTCGAGCT-GCAGCTGCTGAATCAAAG-3′) and used for ligation with pβLuc-E1. pβLuc-mutE3 was constructed by inserting the wild type PCR fragment at XhoI site of pβLuc-mutE2. Positive control plasmid, the pGL3-Promoter Vector, was purchased from Promega.

Cell Culture and Luciferase Assays

9E11G and P19 cells were provided by Dr. G. K. Owens in Department of Molecular Physiology and Biological Physics, University of Virginia. Cells were maintained in alpha-minimum essential medium (alpha-MEM, GIBCO) supplemented with 7.5% fetal bovine serum. For firefly luciferase assay, cells were seeded at a density of 1.5×10^4 cells/cm^2 in 6-well plates (Corning Glass; Corning, NY). To correct for differences in transfection efficiency, 0.1 μg of the Renilla Luciferase reference plasmid (pRL-TK Vector, Promega) was added to 2.5 μg of the firefly luciferase reporter plasmids. Transfections were performed using Superfect (Qiagen Inc., Valencia, CA) and cell extracts were prepared with lysis buffer according to the manufacturer's instructions (Promega) 24 h after transfection. Firefly luciferase activity was measured with a luminometer and the Dual-luciferase^R reporter assay system (Promega). The ratio of firefly luciferase activity to renilla luciferase activity in each sample served as measure of normalized luciferase activity. Each sample was examined in triplicate and was repeated at least three times to assure reproducibility.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts from 9E11G and P19 were prepared by BD^{TM} Transfactor Extraction Kit (BD Biosciences Clontech; Palo Alto, CA). Double-stranded oligonucleotides were generated by hybridizing equimolar amounts of complementary single-stranded DNA oligonucleotides and end-labeled using [γ^{32}P] dATP and polynucleotide kinase. Consensus oligonucleotides for AP-1, AP-1 mutant, NF-E2, and NF-E2 mutant were obtained from a commercial source (sc-2501, sc-2514, sc-2527, and sc-2528, Santa Cruz Biotechnology, Inc.). Incubation was performed at room temperature for 20 min using 5 μg of nuclear extract in a binding buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10% glycerol, 0.1 mM DTT and 1 pmol of [^{32}P]ATP-labeled probe with or without ten times molar excess of a cold competitor oligonucleotide. The reaction mixture was loaded onto a 6% acrylamide gel, run in 0.5× Tris borate–

EDTA buffer at 200 V for 2 h. The gel was dried and exposed to X-ray film.

RESULTS

Generation of Transgenic Mice Harboring a 6 Kilobase FRNK Promoter Region

A 16-kilobase genomic DNA fragment was sequenced to determine the boundaries of the first coding exon of FRNK, the 5′ leader exon and the upstream intron (Fig. 1). Sequence comparison of the mouse and chicken FRNK genomic loci revealed that the organization of the FRNK gene is well conserved between the two species. The intronic DNA of each species exhibits a 5′ leader exon (320 and 538 bp for mouse and chicken, respectively) and a short intron that separates the leader exon and the first coding exon (556 bp in mouse, Fig. 1). The intron separating the leader exon and the last coding exon of the catalytic domain of FAK is more than 15 kilobases in mouse, while the corresponding chicken intron is shorter (approximately 6-kilobases). To assess the expression of FRNK in vivo, transgenic mice were generated using a 6-kilobase fragment containing the leader exon and putative promoter region fused to the LacZ gene (Fig. 1). Four independent transgenic founder lines (Tg-H, Tg-M1, Tg-M2, and Tg-L) were obtained and Southern blot analysis indicated that each line contained one (Tg-L), three (Tg-H), and four copies (Tg-M1 and Tg-M2) respectively of the transgene (Materials and Methods). The transgene line, Tg-H, exhibited the highest level of LacZ staining and Tg-L line showed the lowest levels of LacZ expression, however, in each case, the overall pattern of LacZ expression was identical (data not shown).

Expression of LacZ in Multiple Organs

LacZ expression, as determined in Materials and Methods, was most clearly visible in aorta and the pulmonary arteries but not detected in vena cava or coronary arteries (Fig. 2A and B). LacZ expression was also detected in arteries of the brain (Fig. 2E) and kidney (Fig. 2F) as well as thoracic aorta, abdominal aorta, and carotid arteries (not shown). Thus the initial survey of LacZ expression revealed expression in arterial blood vessels in virtually all of the major organs of postnatal transgenic mice. In contrast, LacZ expression was absent in the muscle walls of tissues that are rich in visceral smooth muscles such as stomach and intestine, although the surrounding mesenteric vessels were positive for β-galactosidase activity (Fig. 2C and D). We confirmed the restricted LacZ expression in aorta and arteries by histology. By X-gal staining of cryosections, we observed LacZ expression in the thoracic organs and the kidney (Fig. 3A, arrows), but absent in trachea (not shown), bronchus in the lung (Fig. 3A,B), cardiac muscle in the heart, walls of a bladder, skeletal muscle (Fig. 3A), esophagus, and uterus (not shown).

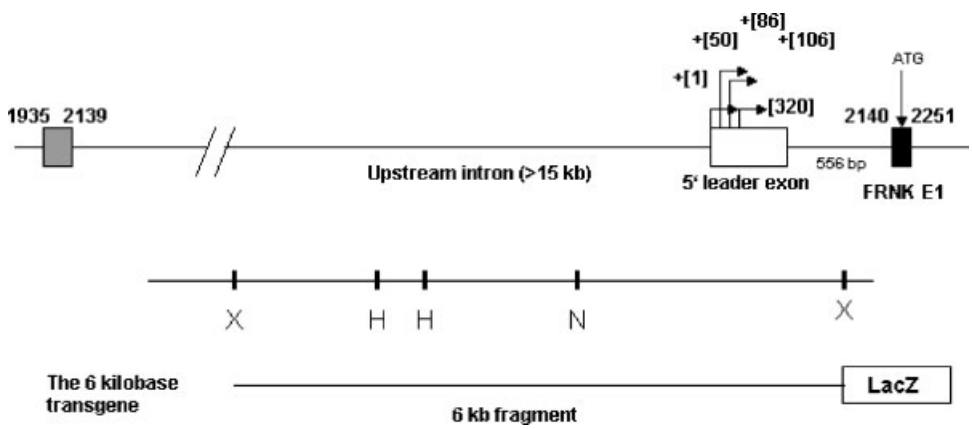

Fig. 1. Genomic sequence and structure of the FRNK promoter. Restriction map and exon/intron structure of the murine FRNK gene. The boundaries of the 6 kilobase XbaI fragment used to generate FRNK transgenic mice are indicated with an arrow. The shaded box denotes the last coding exon of the catalytic domain of FAK. The open box denotes the position of the 5′ leader exon of FRNK and the closed box denotes the first coding exon of FRNK (FRNK E1) that is shared with FAK. Locations of intron/exon boundaries are denoted by numbers which correspond to the previously published nucleotide sequences of mouse FAK cDNA [Hanks et al., 1992]. Transcriptional start sites are indicated by angled arrowheads. Abbreviations of restriction sites: X, XbaI; N, NsiI; H, HindIII.

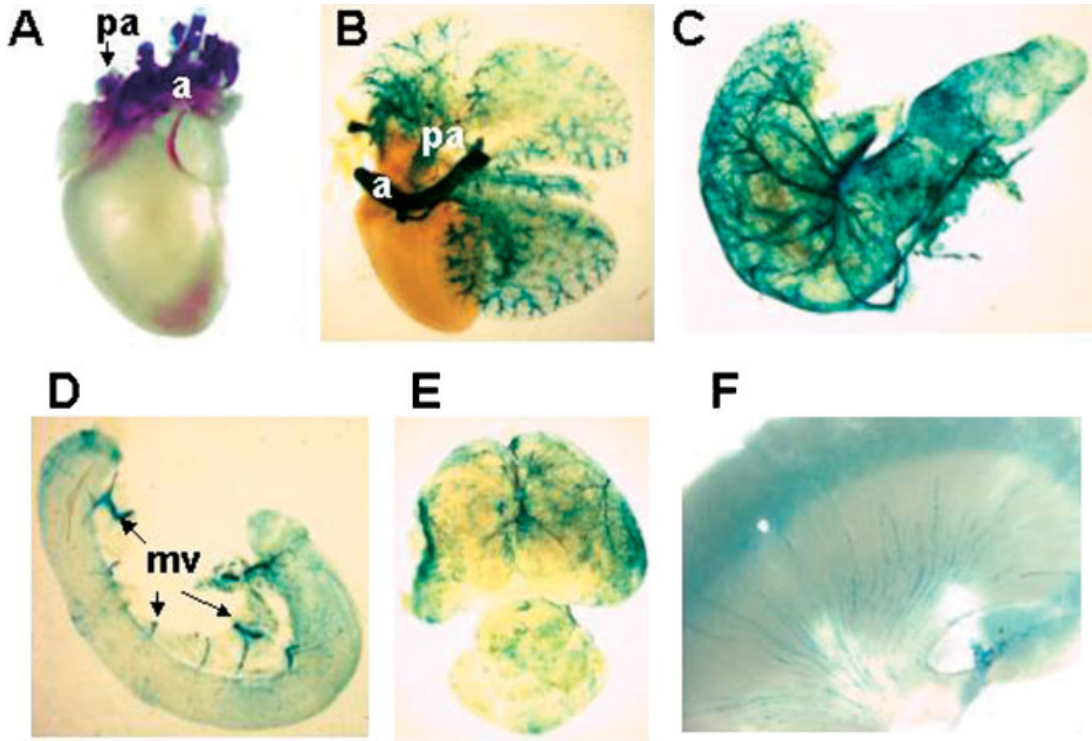

Fig. 2. LacZ expression in major organs in postnatal mice. **A**: Anterior view of the heart from a postnatal (3 weeks old) transgenic mouse; pa, pulmonary arteries; a, aorta. **B**: View of lung lobes with a heart showing intense staining of arteries. **C**: Staining of stomach showing that LacZ expression is restricted to surrounding blood vessels. **D**: Small intestine with attached mesenteric vessels (mv) showing LacZ activity. **E**: Posterior view of a brain whole mount with X-gal staining of arteries. **F**: Coronal section of the kidney showing LacZ expression of the renal vasculature.

To identify the type of cells that express LacZ in blood vessels, immunohistochemical staining was carried out with antibodies to alpha-smooth muscle actin and an endothelial cell specific marker, CD31, using thoracic aorta from transgenic mice. As shown in Figure 3B, LacZ expression overlapped the expression of alpha-smooth muscle actin (left and middle panels), but appeared to not significantly co-localize with CD31 (right panel), indicating that LacZ is preferentially expressed in VSMCs.

Transgenic LacZ Expression is Regulated by the Injury Response

We previously showed that FRNK expression is increased within 14 days after carotid balloon injury in the rat [Taylor et al., 2001]. To determine if the FRNK promoter of the transgene is regulated similarly to that of the endogenous promoter in smooth muscle cells upon injury, we performed carotid wire injury in the transgenic mice. To reduce the background expression of LacZ in the smooth muscle cells and to facilitate observation of the induction of LacZ expression level, we used the lowest LacZ expressing line (Tg-L). At 14 days after injury, LacZ expression was enhanced in injured carotid (Fig. 4, right panel), whereas the control non-injured carotid showed little staining of LacZ (left panel), indicating that the 6 kilobase FRNK promoter of the transgene mimics endogenous tissue-specific responses in postnatal transgenic mice. Interestingly, LacZ expression was enhanced predominantly in the neointima area that is composed of newly synthesized VSMCs (shown by an arrowhead). These observations indicate that the 6 kilobase DNA fragment contains sufficient sequence information to respond to the signals induced upon vascular injury and indicates that the FRNK promoter is likely up-regulated in the neointima upon injury.

LacZ Expression in the Embryo

To date, little is known about the expression profile of FRNK during embryogenesis; in part due to the difficulties of detection and lack of knowledge as to what types of cells express FRNK. Having shown that transgenic LacZ expression occurs in VSMCs and changes in

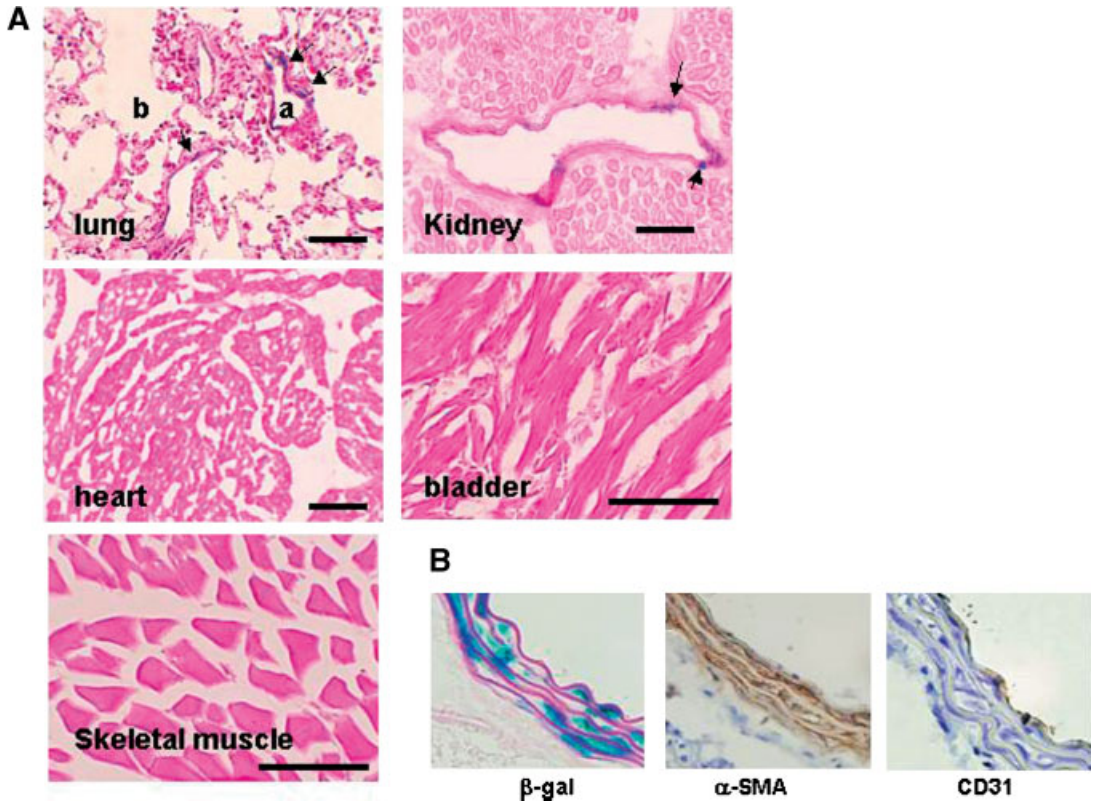

Fig. 3. Analysis of tissues and smooth muscle cells from transgenic mice. **A**: X-gal staining of lung, kidney, heart, bladder, and skeletal muscle. Whole organs were fixed with 0.2% paraformaldehyde and cryosectioned prior to X-gal staining. LacZ expression is shown in arteries in the lung and kidney (indicated as arrows), and absence of staining in the cardiac, skeletal, and visceral tissues. Bar; 100 μm; a, aorta; b, bronchus. **B**: Immunohistochemical staining with anti-alpha smooth muscle actin α-SMA antibody, anti-CD31 antibody, and LacZ expression in the thoracic aorta. Frozen sections were incubated with detection antibodies and peroxidase-conjugated species-specific secondary antibodies, visualized using DAB substrate kit, and counter stained with hematoxylin as described in Materials and Methods.

expression are induced in response to injury, we examined LacZ expression in embryos from E8.5 to E13.5. LacZ expression was initially detected at E9.5 in subclavian arteries, dorsal aortas, and the front nasal mass along with the midline (Fig. 5A). The expression of LacZ in the vasculature was more visible and marked in the basilar artery and umbilical artery at E11.5

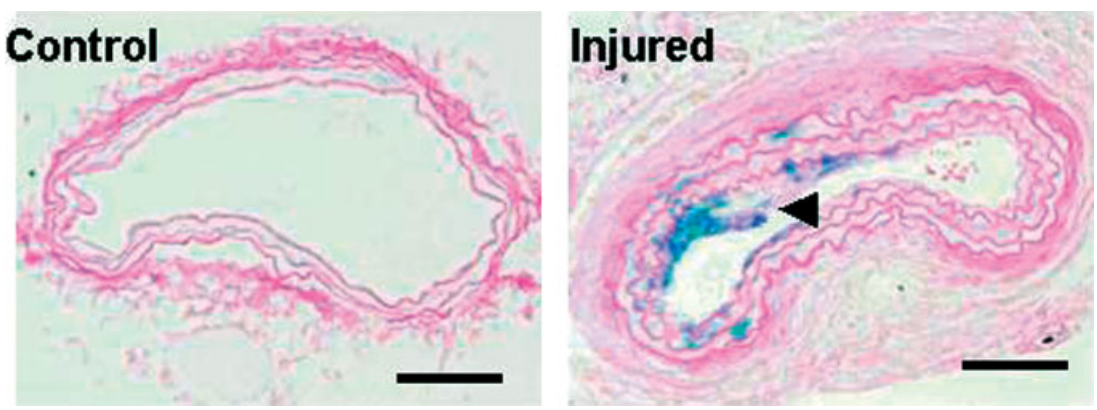

Fig. 4. Induction of LacZ expression upon carotid wire injury. Images represent vessel cross-sections from a control right common carotid and an injured left common carotid artery at 14 days post injury. Whole carotids were stained with X-gal and sectioned. Images reveal elevated LacZ expression in a part of tunica media and neointima. Bar, 100 μm. Neointima formed by injury is indicated by arrowhead.

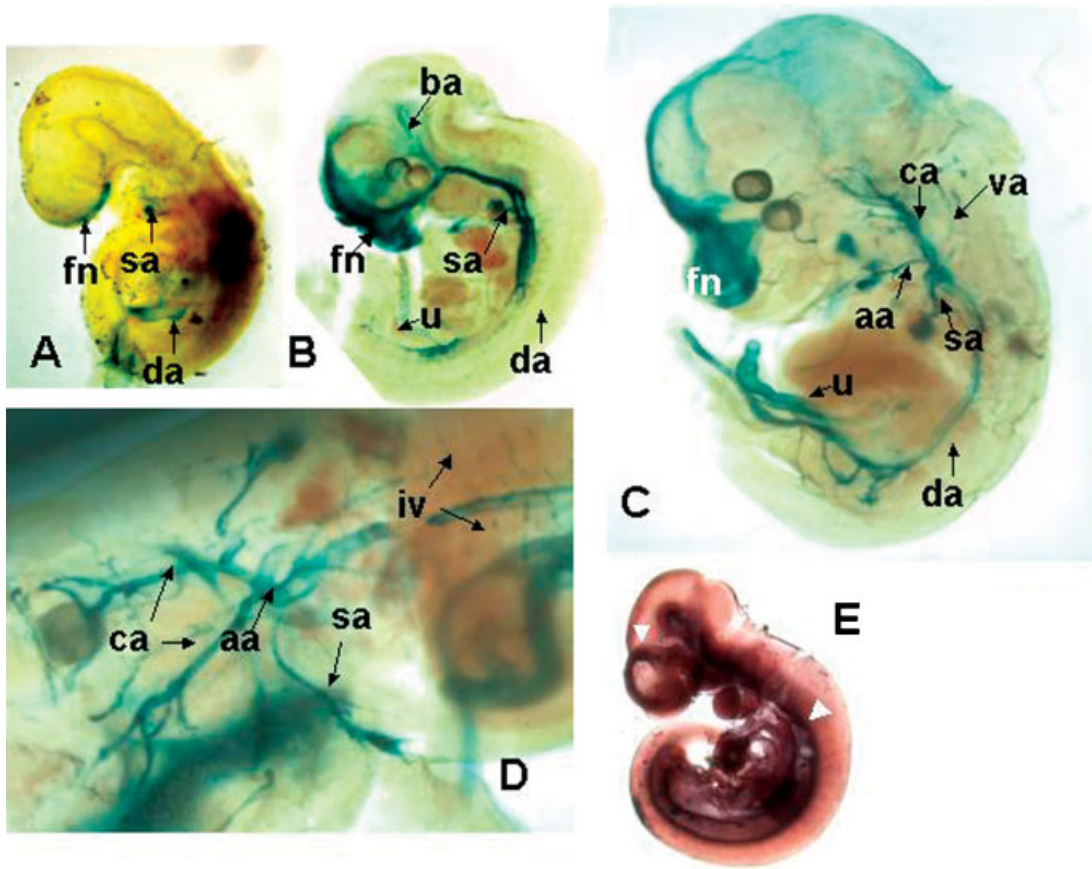

Fig. 5. LacZ expression in developing embryos. Whole-mount images of transgenic embryos stained with X-gal. **A**: LacZ expression is shown in the front nasal mass (sn), subclavian arteries (sa), and dorsal aorta (da) at E9.5. **B**: At E11.5, LacZ expression was detected in basilar arteries (ba), subclavian arteries (sa), and umbilical artery (u). **C**: At E13.5, LacZ expression was observed in major vessels including vertebral artery (va), carotid arteries (ca), and aortic arches (aa). **D**: High magnification of posterior view at E13.5 shows intercostal vessels (iv) staining in addition to other arteries. **E**: FRNK expression in a E10.5 embryo defined by whole mount in situ hybridization with a FRNK riboprobe.

(Fig. 5B) and increased continuously through E13.5 (Fig. 5C). LacZ expression was detectable in major arterial blood vessels including carotid arteries, vertebral arteries, aortic arches, and intercostal vessel at E12.5 (not shown) and E13.5 (Fig. 5C and D). In coronal sections, LacZ expression was detected in the aortic arch (Fig. 6A), in dorsal arteries and truncus arteriosus but absent in trachea, esophagus, and atriums at E12.5 (Fig. 6B). Thus, the 6 kilobase FRNK promoter fragment directs gene expression preferentially in arterial blood vessels in the embryo. LacZ expression was unexpectedly seen in the front nasal mass from the early embryonic stage (Fig. 5A). In the front nasal mass, LacZ expression appeared to increase through E9.5 and E11.5 (Fig. 5A and B) and was expanded to nasal septum by E13.5 (Fig. 6C). LacZ expression was also detectable in the head at E12.5 (not shown) and E13.5 (Fig. 5C). The LacZ expression in the head was localized to epidermis (Fig. 6D) and the staining in this area persisted at the late embryonic stages (not shown). A similar pattern of LacZ expression in embryos was observed in all four independent founder lines. The expression of endogenous FRNK in the LacZ-positive regions was confirmed by whole mount in-situ hybridization using an E10.5 embryo using a FRNK-specific probe corresponding to the 5′ leader exon (Fig. 5E, arrowheads). The pattern of LacZ expression indicates that the 6 kilobase DNA fragment promotes gene expression in the vasculature, nose, and epidermis of the head during embryogenesis.

Analysis of Conserved Elements Within the 6 Kilobase Promoter Region

Because the 6 kilobase region of DNA in the transgene showed preferential expression to

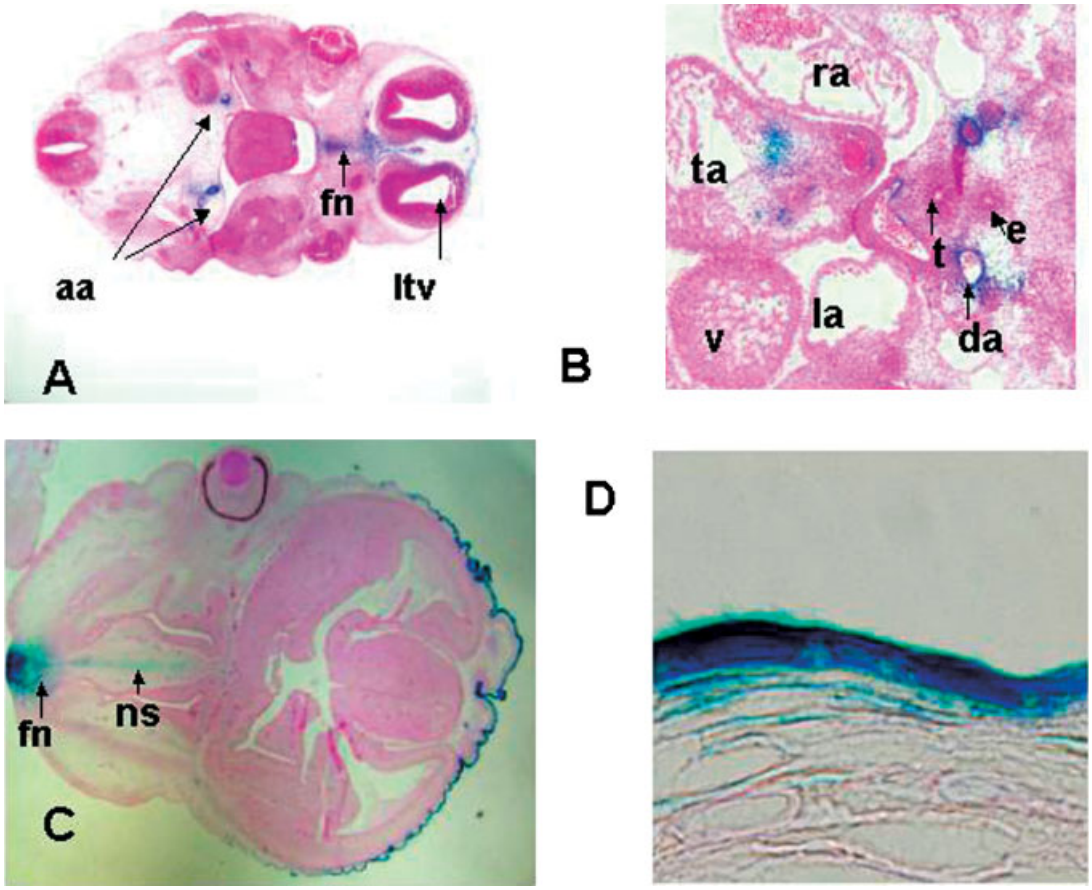

Fig. 6. Detection of LacZ expression in sections of transgenic embryos. Images are from coronal sections (**A**) and transverse sections of the heart region (**B**) at E12.5 and frontal section at E13.5 (**C**). At E12.5, LacZ is highly expressed in arteries, truncus arteriosus and the front nasal mass. At E13.5, nasal septum, front nasal mass and skin of the head showed strong LacZ activity. **D**: High magnification of skin of the head at E13.5, showing elevated LacZ activity in epidermis. Abbreviations: aa, aortic arch; la, left atrium; ra, right atrium; e, esophagus; t, trachea; da, dorsal aortic root; v, ventricle; ta; truncus arteriosus; ltv, lateral telencephalic vesicle; fn, front nasal mass; ns, nasal septum.

VSMCs, we sought to define *cis* regulatory elements in the 6 kilobase region that couple to cell-type specificity of FRNK expression. Segments derived from the 6 kilobase fragment were cloned upstream of the firefly luciferase gene and used in transient expression experiments to measure the relative efficiency of luciferase expression in cultured cells. We confirmed that the 5.6 kilobase fragment (5.6K), which corresponds to the same 5′ upstream sequence of the 6 kilobase transgene, showed prominent luciferase activity in rat primary cultured VSMCs (data not shown). 9E11G cells exhibit properties of smooth muscle cells [Blank et al., 1995] and express the endogenous FRNK, in contrast to the parental undifferentiated mouse embryonic carcinoma cell line, P19 [Taylor et al., 2001]. Upon transfection, the 5.6-kilobase fragment generated higher luciferase activity in 9E11G cells than in P19 cells (Fig. 7A). The 3.0K fragment and 2.2K fragment showed significant luciferase activity in 9E11G cells, approximately 70% of the activity was compared to the 5.6K fragment. Truncation of the 5′ end of this fragment to −1735 (2.0K) resulted in a modest reduction in luciferase activity, whereas truncation to −1355 (1.7K) drastically reduced luciferase activity in 9E11G cells (Fig. 7A). Each of the fragments exhibited only low levels of luciferase activity in the P19 cells, consistent with the putative promoter fragments being more active in the context of smooth muscle cells. In addition, constructs lacking the 0.4 kb sequence from −1735 to −1325 (5.6K–0.4K and 3.0K–0.4K,) were significantly less active than the full length constructs pGL3-5.6K and pGL3-3.0K, respectively (Fig. 7B, left panel), suggesting that the 0.4 kb fragment contains a regulatory sequence that enhances FRNK promoter activity.

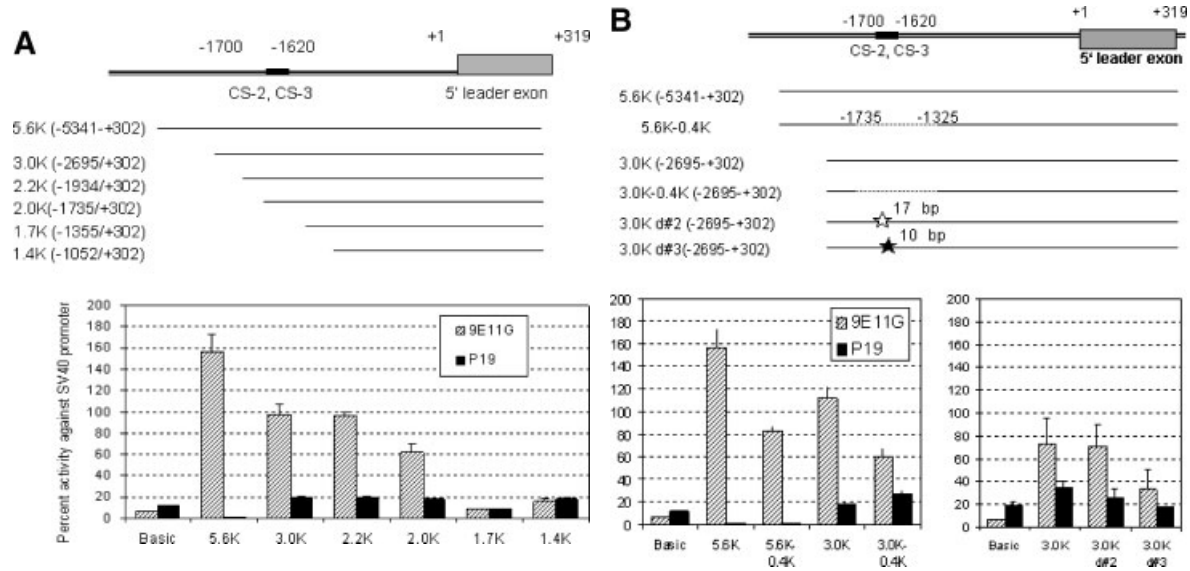

Fig. 7. Analysis of the FRNK promoter in a differentiated smooth muscle cell line (9E11G) and the parental embryonic carcinoma cell line (P19). **A**: Luciferase reporter constructs with 5′ deletions are diagrammatically shown and aligned with a map of the DNA sequences present in the mouse genome (top). Two regions of sequence homology with the chicken locus are denoted by a black box (CS-2 and CS-3). The 5′ leader exon is denoted by a shaded box. Each construct was transfected into cells and luciferase activity determined as described in Materials and Methods. The firefly luciferase activity is shown as percent activity relative to that of SV40 promoter (pGL3-Promoter Vector). **B**: Cells were transfected with the plasmids denoted at the top and luciferase activity determined as described in Materials and Methods. Sequences deleted from pGL3-5.6K or pGL3-3.0K are shown by dotted lines. Point mutations within a 17 bp region containing CS-2 or a 10 bp sequence containing CS-3 are indicated by open and closed stars, respectively. **C**: Sequences of the murine FRNK promoter from −1735 to −1325 and the 5′ leader exon. Potential cis-acting sequences are underlined. The three homologous sequences between murine and chicken (CS-1, CS-2, and CS-3) are denoted in the darken boxes. The nucleotide sequences of the three homologous regions are 100% identical between the two species. The 5′ leader exon is shown by a gray shaded box and the transcription start sites are marked by an angled arrow. The 116 bp sequence used to generate luciferase reporter plasmids with the β-actin basic promoter (panel **D**) is indicated as an open box. D: Individual constructs are diagramed; open boxes denote the β-actin basal promoters and closed boxes denote the luciferase genes. The 116-bp fragment including CS-2 and CS-3 was cloned to pβLuc vector to generate E1, E2, and E3, containing two and three tandem copies of the fragment, respectively. Stars show the point mutation located in the NF-E2/AP-1 consensus of mutE2 and mutE3. Luciferase activity is shown relative to the SV40 promoter control.

An alignment of chicken and mouse genomic sequences revealed three completely conserved sequences in the FRNK promoter and the 5′ leader exon (CS-1, CS-2, and CS-3, Fig. 7C). Since the 17 bp CS-2 and 10 bp CS-3 are located in the 0.4 kb sequence, we tested whether these conserved regions are important for promoter activity. We generated the constructs, 3.0K d#2 and 3.0K d#3, which contain a deletion of regions CS-2 and CS-3 respectively. As shown in Figure 7B (right panel), luciferase activity was reduced by removal of CS-3, but activity was not significantly altered by deletion of the CS-2 sequence, suggesting that CS-3 is required for promoter activity. Next, we isolated the 116-bp sequence containing both CS-2 and CS-3 (Fig. 7C) and inserted it upstream of the chicken beta-actin promoter-luciferase vector (Fig. 7D) to examine if the CS-2 and CS-3 sequences activate a basal promoter. In contrast to the construct bearing one copy of the element (E1), two or three copies of the 116-bp sequence (E2 and E3, respectively) stimulated beta-actin promoter activity in 9E11G cells, and this effect was proportional to copy number (Fig. 7D). Expression of a construct bearing a point mutation from C to T proximal to CS-3 (mutE2 and mutE3), (serendipitously introduced during PCR amplification of the 116-bp sequence) failed to stimulate luciferase activity above control levels (Fig. 7D). These results indicate that sequences proximal to CS-3 may function as an enhancer with a basal promoter.

Nuclear Factor Binds to the Proximal CS-3 Element

To confirm the existence of a nuclear factor that interacts with CS-3 in 9E11G cells, we performed electrophoretic mobility shift assay (EMSA) using a 27 bp oligonucleotide fragment

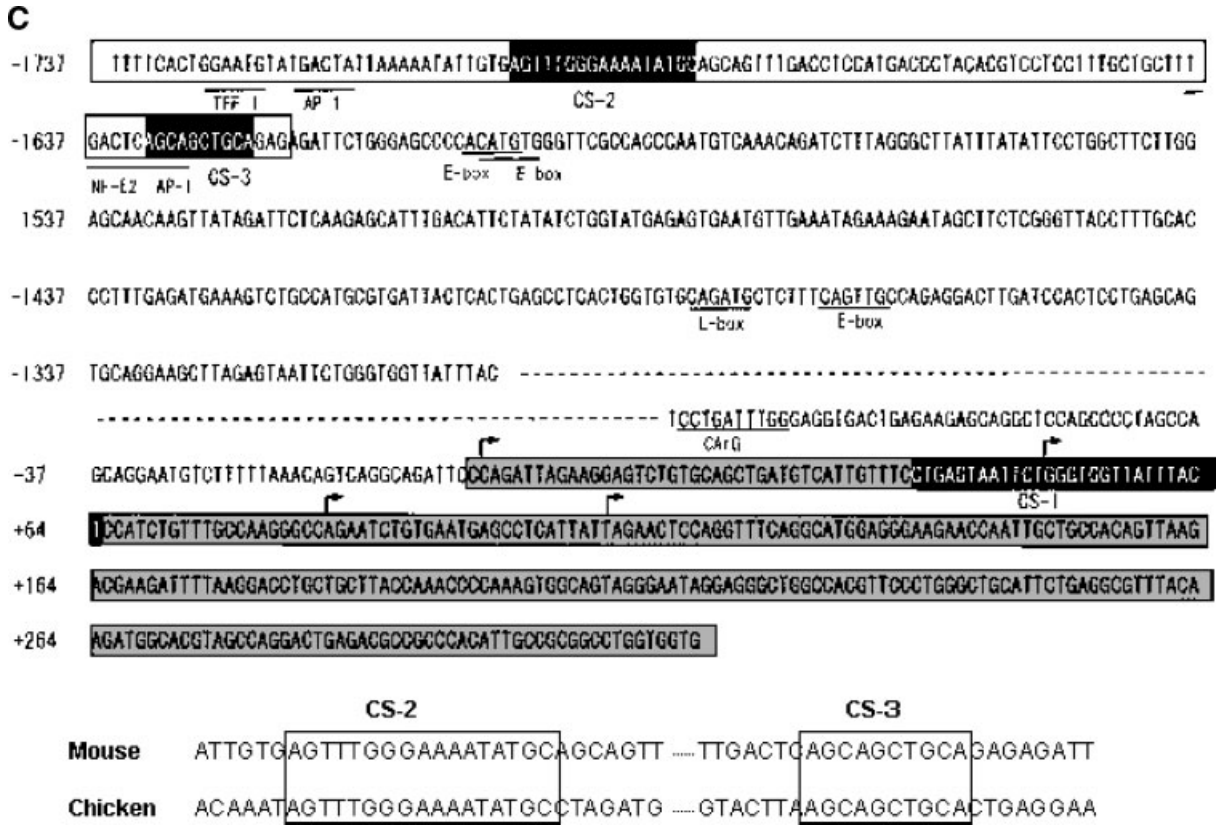

Fig. 7. (*Continued*)

containing CS-3 and the flanking sequence. Two prominent bands were present in EMSA, whereas addition of a ten fold molar excess of a cold competitor reduced the interaction (Fig. 8), suggesting presence in the extract of a nuclear factor that binds to the 27 bp sequence. Because this region also contains AP-1 and NF-E2 consensus binding sequences, we examined whether oligonucleotides containing the AP-1 binding consensus sequence [TGA(C/G)TCA] and/or NF-E2 [TGCTGA(C/G)TCA(T/C)] would compete for binding. As shown in Figure 8A, oligonucleotides containing the AP-1 and NF-E2 binding sequences competed efficiently for binding to the 27 bp probe, whereas those fragments with mutations abrogating AP-1 or NF-E2 binding, competed less efficiently. These data indicate that the extracts contain factors that interact with the CS-3 element as well as the AP-1 and NF-E2 consensus binding sequences. Interestingly, the signal of 27 bp probe–nuclear factor complex was somewhat stronger in 9E11G nuclear extract than in P19 (Fig. 8B), consistent with the possibility that the nuclear factors are more abundant and/or bind more efficiently to CS-3 element in 9E11G cells than in P19 cells.

DISCUSSION

This study presents for the first time insights into the global regulation of FRNK in murine tissues and embryonic development. Whereas previous studies indicated that FRNK transcription is regulated by an alternative promoter located in an intron of FAK 3′ to the sequences encoding the kinase domain and 5′ to the beginning of the exon encoding FRNK, there was little evidence for tissue-specific expression of this promoter. Here we utilize transgenic mice harboring a 6-kilobase segment of DNA containing the mouse FRNK promoter fused to LacZ to investigate the expression of FRNK in tissues of embryonic and postnatal mice.

The pattern of LacZ expression in the adult transgenic mice parallels the expression of the endogenous FRNK observed in tissue and cultured cells [Taylor et al., 2001]. Based on this similar expression pattern and the observation that LacZ expression is enhanced in response to

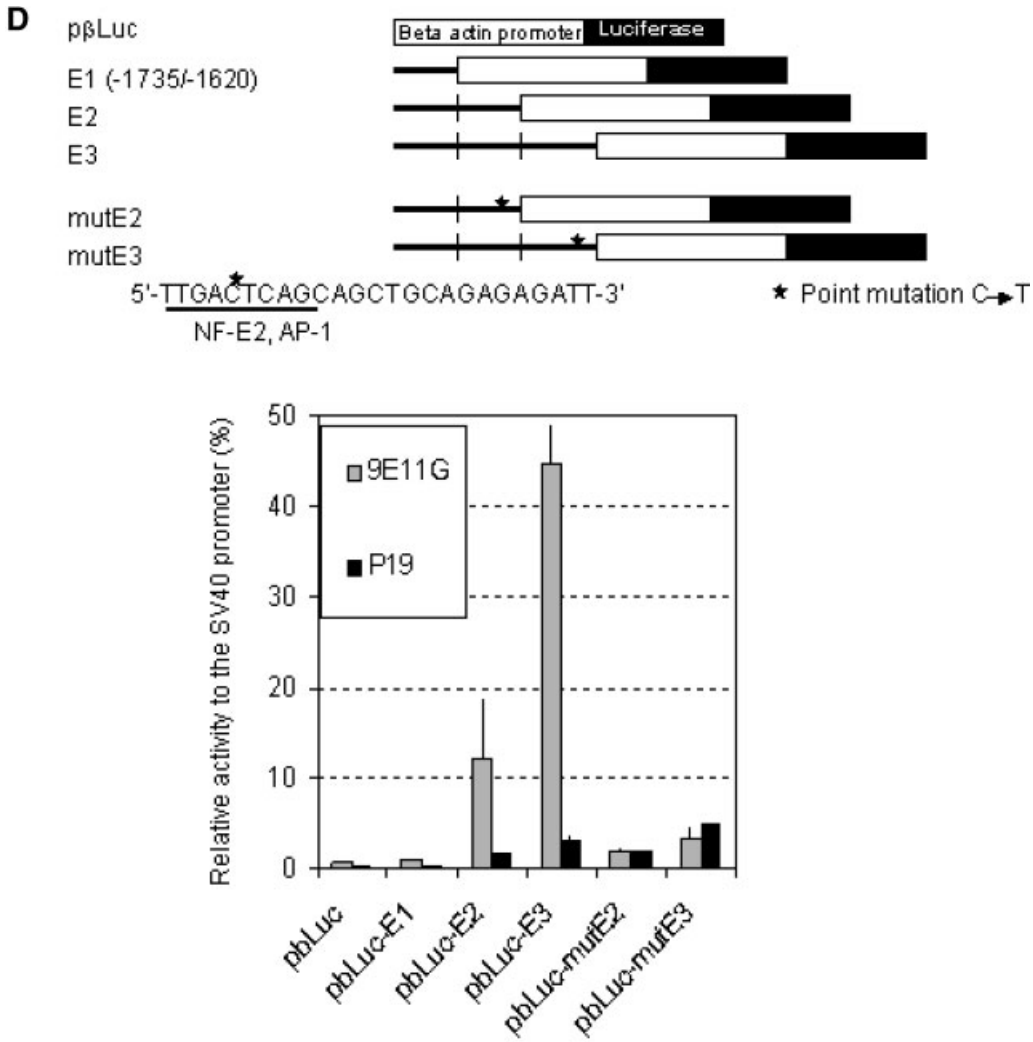

Fig. 7. (*Continued*)

carotid injury, we propose that the 6-kilobase fragments contains the regulatory information necessary to promote cell type specific expression of endogenous FRNK in the adult. We cannot, of course, rule out that other regulatory elements outside the 6-kilobase fragment contribute to FRNK expression in other tissues. The observation that LacZ expression is up-regulated during VSMC growth response after artificial vascular injury clearly indicates that the 6 kilobase fragment present in the transgene contains regulatory elements capable of the cellular cues induced by vascular injury.

To date, several transgenic mice with the promoters of SMC differentiation marker genes fused to LacZ have been described. In the adult mice, expression of LacZ under the control of smooth muscle α-actin and smooth muscle myosin heavy chain promoters is prominent in both arterial and visceral smooth muscle tissues including the muscular walls of the stomach, intestines, and bladder [Madsen et al., 1998; Mack and Owens, 1999]. We observed that LacZ expression in the adult FRNK promoter-LacZ transgenic mice virtually undetectable in the venous and visceral SMCs, indicating selective expression of FRNK in arterial VSMCs. The selectivity of expression in VSMCs is also reported for the SM22 [Moessler et al., 1996], Crp2/SmLim [Yet et al., 1998], and ACLP promoter-transgenic mice [Layne et al., 2002], where expression of LacZ is restricted to the vascular system but absent in the muscular walls of the visceral organs. The expression pattern of FRNK promoter-LacZ is similar to that observed for the SM22 promoter-LacZ; the

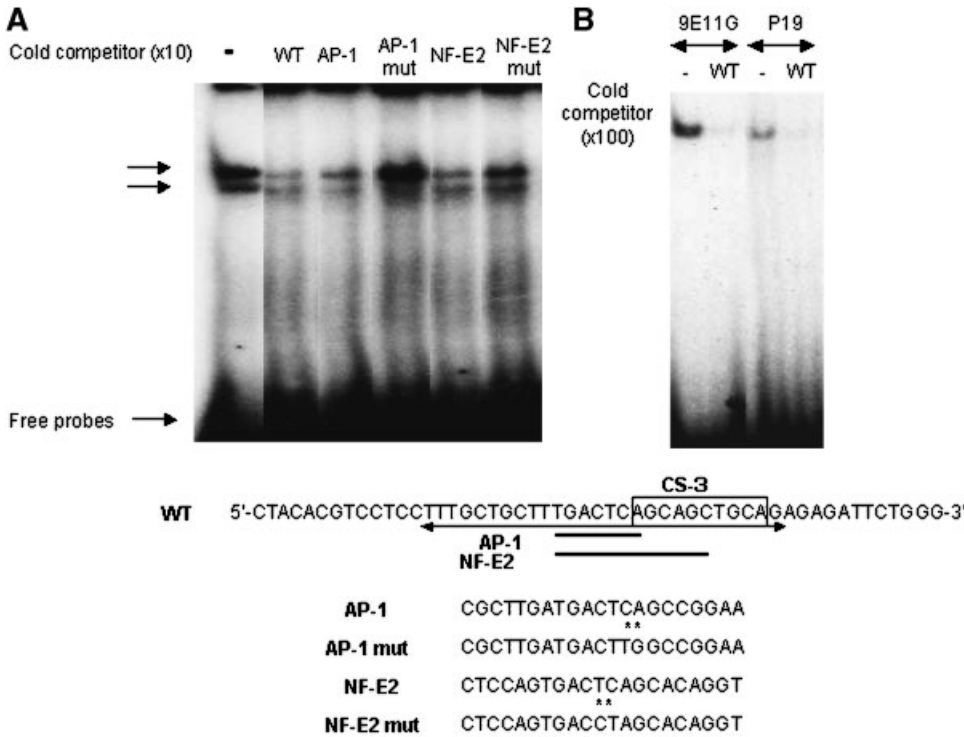

Fig. 8. **A**: Nuclear factor binding to a 27 bp oligonucleotide of the FRNK regulatory region. EMSA was carried out with a nuclear extract from 9E11G cells and the ^{32}P-labeled 27 bp oligonucleotide probe, designated wt and analyzed as described in Materials and Methods. The position of the labeled 27 bp oligonucleotide is indicated by the two arrows. AP-1 and NF-E2 consensus binding sites and CS-3 are denoted as bars and an open box, respectively. Asterisks indicate location of the nucleotide substitution in each probe. For competition experiments, indicated oligonucleotides were added at 10-fold molar excess prior to addition of the radiolabeled probe. **B**: EMSA was carried out with a 5 μg of nuclear extract from 9E11G or P19 cells using the ^{32}P-labeled 27 bp oligonucleotide probe.

first appearance of transgene expression occurring in the dorsal aorta at E9.5 in development [Li et al., 1996; Moessler et al., 1996]. The SM22 gene however appears to be regulated by distinct mechanism from the *FRNK* gene, as evidenced by the downregulation of this gene after vascular injury in the adult and prominent transgene expression in the heart and the rostral somites in the embryo [Li et al., 1996; Moessler et al., 1996; Regan et al., 2000]. Interestingly, LacZ expression was undetectable in the coronary arteries in both FRNK promoter-LacZ and SM 22 promoter-LacZ mice [Li et al., 1996], raising the possibility that the origin of VSMCs of the coronary arteries is distinguished from that of other arterial VSMCs in developmental process. In fact, it is proposed that the coronary VSMCs arise from the epicardial lining to form a vascular plexus that connects to the aorta in later stages, whereas other VSMCs are either from mesoderm, endothelial cells or neural crest [Munoz-Chapuli et al., 2002]. These lines of evidence further support the presence of different subdivisions of VSMCs in which expression of SMC marker genes is dependent on distinct transcriptional regulation [Li et al., 1996; Moessler et al., 1996; Kim et al., 1997].

In FRNK promoter-LacZ embryos, transgene expression was not only restricted to arteries but also observed in the nasal frontal mass in the early embryo and the epidermis in late embryonic stages. The expression of endogenous FRNK in these regions was confirmed by whole mount in-situ hybridization using FRNK-specific probe corresponding to the 5′ leader exon. We speculate that at some point of differentiation process, the cells in the front nasal mass and the epidermis of the head share the same lineage with VSMCs. Studies have proposed that the arterial VSMCs within large and small blood vessels arise from a heterogeneous population of cells including cells of mesoderm and neural crest origin [Gittenberger-de Groot et al., 1999]. In the thorax, head, and neck arteries, the mesectoderm of the neural crest is

the major origin of SMCs [Bergwerff et al., 1998]. Likewise, epidermal differentiation originates from the embryonic ectoderm, followed by invasion of neural crest-derived cells, and the front nasal mass is derived from neural crest-derived mesenchyme [Le Lievre and Le Douarin, 1975; Bergwerff et al., 1998]. Thus, ectopic expression of LacZ in the transgenic embryo may be explained in part by a common lineage of cells outside of blood vessels and the neural crest may contribute to the production of several different types of differentiated progeny.

The present study demonstrated that a 2.2 kilobase fragment (−1934 to +302) is the shortest DNA fragment that will efficiently drive luciferase reporter gene expression in 9E11G cells and that a region between −1735 and −1355 is important for optimal promoter activity. We propose that an enhancer of FRNK may be located in this region. Since deletion of the sequence between −1735 and −1355 does not affect basal FRNK promoter activity in the parental P19 cells, the enhancer appears to be cell-type specific. Virtually all SMC-specific promoter/enhancer elements have a conserved DNA recognition element known as a CArG box, which is the binding site for the serum response factor (SRF) [Kumar and Owens, 2003]. In the FRNK promoter region, a CArG box is located at −84, however, it is absent in the sequence from −1735 and −1355 of FRNK promoter, suggesting that the enhancer in the region is unlikely to be dependent on CArG box—SRF interactions. The absence of a recognizable CArG box in SMC-specific gene promoters has been reported for the mouse aortic carboxypeptidase-like protein (ACLP) and Crp2/SmLim promoters [Yet et al., 1998; Layne et al., 2002]. A positive regulatory element within the ACLP promoter has been mapped to a GC rich sequence that binds Sp1 and Sp3 transcription factors, suggesting that the GC rich elements can promote SMC specificity [Layne et al., 2002]. Furthermore, it has been shown that the Crp2/SmLim promoter is rich in G and C residues and has numerous binding sites for Sp1 [Yet et al., 1998]. In contrast, FRNK promoter does not have GC rich sequence for Sp1 binding in the sequence between −1735 and −1355. This raises the possibility that FRNK expression in VSMC could be regulated by unique factors, although the possibility that a nuclear protein that binds to the enhancer region recruit SRF and/or Sp1 to organize multiprotein complex cannot be excluded.

Results from mutational analysis of CS-3 and the 116 bp DNA fragment including CS-3 fused to the basal β-actin promoter with a point mutation in the AP-1/NF-E2 binding consensus raises the possibility that AP-1 and/or NF-E2 family transcription factor may be involved in the regulation of FRNK promoter. Using EMSA, we demonstrated that a nuclear factor(s) in 9E11G cells binds to the CS-3 element and factor binding is influenced by the integrity of the AP-1 and NF-E2 sequences. Nrf1 and Nrf2 of the p45 family are predominantly expressed in mouse VSMCs [Holderman et al., 2002]. Preliminary evidence indicates that antibodies to Nrf1 blocked the formation of nuclear factor–DNA complexes (H.H., unpublished observations) providing evidence for a role of this factor in regulation of FRNK expression. Defining the sequences proximal to CS-3 that function as a VSMC-restricted enhancer of FRNK in vivo is a question that remains to be answered.

To understand a role of VSMCs in maintaining vascular tone and pathological responses in the vessel wall, it is important to understand the factors that regulate expression of SMC specific genes. The evidence that FRNK is expressed in tissues with vascular smooth muscle components supports the idea that FRNK plays an important role in the regulation of migration and proliferation of VSMCs in vivo. Considering that over-expression of FRNK inhibits proliferation of VSMCs in vitro [Hauck et al., 2000; Taylor et al., 2001], it is of interest to know if up-regulation of FRNK promoter results in prevention of restenosis after arterial injury. Since little is known about SMC-specific promoters other than cytoskeletal and contractile proteins, which are generally down-regulated in response to injury, further characterization of FRNK promoter will allow us to understand the molecular mechanisms of up-regulation of SMC-specific promoters in remodeling of blood vessels.

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REFERENCES

Abedi H, Zachary I 1995. Signalling mechanisms in the regulation of vascular cell migration. Cardiovasc Res 30: 544–556.

Bergwerff M, Verberne ME, DeRuiter MC, Poelmann RE, Gittenberger-de Groot AC 1998. Neural crest cell contribution to the developing circulatory system: Implications for vascular morphology? Circ Res 82:221–231.

Blank RS, Swartz EA, Thompson MM, Olson EN, Owens GK. 1995. A retinoic acid-induced clonal cell line derived from multipotential P19 embryonal carcinoma cells expresses smooth muscle characteristics. Circ Res 76:742–749.

Gittenberger-de Groot AC, DeRuiter MC, Bergwerff M, Poelmann RE. 1999. Smooth muscle cell origin and its relation to heterogeneity in development and disease. Arterioscler Thromb Vasc Biol 19:1589–1594.

Hanks SK, Calalb MB, Harper MC, Patel SK. 1992. Focal adhesion protein-tyrosine kinase phosphorylated in response to cell attachment to fibronectin. Proc Natl Acad Sci USA 89:8487–8491.

Harte MT, Hildebrand JD, Burnham MR, Bouton AH, Parsons JT. 1996. p130Cas, a substrate associated with v-Src and v-Crk, localizes to focal adhesions and binds to focal adhesion kinase. J Biol Chem 271:13649–13655.

Hauck CR, Hsia DA, Schlaepfer DD. 2000. Focal adhesion kinase facilitates platelet-derived growth factor-BB-stimulated ERK2 activation required for chemotaxis migration of vascular smooth muscle cells. J Biol Chem 275:41092–41099.

Hildebrand JD, Schaller MD, Parsons JT. 1993. Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. J Cell Biol 123:993–1005.

Holderman MT, Miller KP, Dangott LJ, Ramos KS. 2002. Identification of albumin precursor protein, Phi AP3, and alpha-smooth muscle actin as novel components of redox sensing machinery in vascular smooth muscle cells. Mol Pharmacol 61:1174–1183.

Kim S, Ip HS, Lu MM, Clendenin C, Parmacek MS. 1997. A serum response factor-dependent transcriptional regulatory program identifies distinct smooth muscle cell sublineages. Mol Cell Biol 17:2266–2278.

Kumar MS, Owens GK. 2003. Combinatorial control of smooth muscle-specific gene expression. Arterioscler Thromb Vasc Biol 23:737–747.

Layne MD, Yet SF, Maemura K, Hsieh CM, Liu X, Ith B, Lee ME, Perrella MA. 2002. Characterization of the mouse aortic carboxypeptidase-like protein promoter reveals activity in differentiated and dedifferentiated vascular smooth muscle cells. Circ Res 90:728–736.

Le Lievre CS, Le Douarin NM. 1975. Mesenchymal derivatives of the neural crest: Analysis of chimaeric quail and chick embryos. J Embryol Exp Morphol 34:125–154.

Li L, Miano JM, Mercer B, Olson EN. 1996. Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. J Cell Biol 132:849–859.

Lindner V, Fingerle J, Reidy MA. 1993. Mouse model of arterial injury. Circ Res 73:792–796.

Liu Y, Loijens JC, Martin KH, Karginov AV, Parsons JT. 2002. The association of ASAP1, an ADP ribosylation factor-GTPase activating protein, with focal adhesion kinase contributes to the process of focal adhesion assembly. Mol Biol Cell 13:2147–2156.

Mack CP, Owens GK. 1999. Regulation of smooth muscle alpha-actin expression in vivo is dependent on CArG elements within the 5′ and first intron promoter regions. Circ Res 84:852–861.

Madsen CS, Regan CP, Hungerford JE, White SL, Manabe I, Owens GK. 1998. Smooth muscle-specific expression of the smooth muscle myosin heavy chain gene in transgenic mice requires 5′-flanking and first intronic DNA sequence. Circ Res 82:908–917.

Manka D, von Hundelshausen P, Huo Y, Hanrath P, Sarembock IJ, Ley K, Weber C. 2002. Absence of p-selectin, but not intercellular adhesion molecule-1, attenuates neointimal growth after arterial injury in apolipoprotein e-deficient mice. Circulation 106:1523–1529.

Moessler H, Mericskay M, Li Z, Nagl S, Paulin D, Small JV. 1996. The SM 22 promoter directs tissue-specific expression in arterial but not in venous or visceral smooth muscle cells in transgenic mice. Development 122:2415–2425.

Munoz-Chapuli R, Gonzalez-Iriarte M, Carmona R, Atencia G, Macias D, Perez-Pomares JM. 2002. Cellular precursors of the coronary arteries. Tex Heart Inst J 29:243–249.

Nolan K, Lacoste J, Parsons JT. 1999. Regulated expression of focal adhesion kinase-related nonkinase, the autonomously expressed C-terminal domain of focal adhesion kinase. Mol Cell Biol 19:6120–6129.

Ogino H, Yasuda K. 1998. Induction of lens differentiation by activation of a bZIP transcription factor, L-Maf. Science 280:115–118.

Parsons JT. 2003. Focal adhesion kinase: The first ten years. J Cell Sci 116:1409–1416.

Pearson-White S, McDuffie M. 2003. Defective T-cell activation is associated with augmented transforming growth factor Beta sensitivity in mice with mutations in the *Sno* gene. Mol Cell Biol 23:5446–5459.

Regan CP, Adam PJ, Madsen CS, Owens GK. 2000. Molecular mechanisms of decreased smooth muscle differentiation marker expression after vascular injury. J Clin Invest 106:1139–1147.

Richardson A, Parsons T. 1996. A mechanism for regulation of the adhesion-associated proteintyrosine kinase pp125FAK. [erratum appears in Nature 1996 Jun 27;381(6585):810]. Nature 380:538–540.

Rivard A, Andres V. 2000. Vascular smooth muscle cell proliferation in the pathogenesis of atherosclerotic cardiovascular diseases. Histol Histopathol 15:557–571.

Rupp PA, Little CD. 2001. Integrins in vascular development. Circ Res 89:566–572.

Schaller MD, Borgman CA, Parsons JT. 1993. Autonomous expression of a noncatalytic domain of the focal adhesion-associated protein tyrosine kinase pp125FAK. Mol Cell Biol 13:785–791.

Taylor JM, Mack CP, Nolan K, Regan CP, Owens GK, Parsons JT. 2001. Selective expression of an endogenous inhibitor of FAK regulates proliferation and migration of vascular smooth muscle cells. Mol Cell Biol 21:1565–1572.

Thyberg J. 1996. Differentiated properties and proliferation of arterial smooth muscle cells in culture. Int Rev Cytol 169:183–265.

Yan L, Takekida S, Shigeyoshi Y, Okamura H. 1999. *Per1* and *Per2* gene expression in the rat suprachiasmatic nucleus: Circadian profile and the compartment-specific response to light. Neuroscience 94:141–150.

Yet SF, Folta SC, Jain MK, Hsieh CM, Maemura K, Layne MD, Zhang D, Marria PB, Yoshizumi M, Chin MT, Perrella MA, Lee ME. 1998. Molecular cloning, characterization, and promoter analysis of the mouse Crp2/SmLim gene. Preferential expression of its promoter in the vascular smooth muscle cells of transgenic mice. J Biol Chem 273:10530–10537.