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The Fps/Fes Protein-Tyrosine Kinase Promotes Angiogenesis in Transgenic Mice

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The *fps/fes* proto-oncogene encodes a cytoplasmic protein-tyrosine kinase known to be highly expressed in hematopoietic cells. To investigate *fps/fes* biological function, an activating mutation was introduced into the human *fps/fes* gene which directs amino-terminal myristylation of the Fps/Fes protein. This mutant, myristylated protein induced transformation of Rat-2 fibroblasts. The mutant *fps/fes* allele was incorporated into the mouse germ line and was found to be appropriately expressed in transgenic mice, in a tissue-specific pattern indistinguishable from that of the endogenous mouse gene. These mice displayed widespread hypervascularity, progressing to multifocal hemangiomas. High levels of both the transgenic human and endogenous murine *fps/fes* transcripts were detected in vascular tumors by using RNase protection, and *fps/fes* transcripts were localized to endothelial cells of both the vascular tumors and normal blood vessels by in situ RNA hybridization. Primary human umbilical vein endothelial cultures were also shown to express *fps/fes* transcripts and the Fps/Fes tyrosine kinase. These results indicate that *fps/fes* expression is intrinsic to cells of the vascular endothelial lineage and suggest a direct role of the Fps/Fes protein-tyrosine kinase in the regulation of angiogenesis.

The *fps/fes* proto-oncogene encodes a 92-kDa cytoplasmic protein-tyrosine kinase (PTK) consisting of an amino-terminal domain (N-Fps), a central Src homology 2 (SH2) domain, and a carboxyl-terminal catalytic domain. Fps/Fes is structurally similar to a 94-kDa tyrosine kinase, Fer (16, 26). However, both Fps/Fes and Fer are quite distinct from cytoplasmic PTKs of the Src family, as they lack a negative regulatory tyrosine phosphorylation site in the carboxyl-terminal region, are not modified by N-terminal myristylation, and do not have an SH3 domain, which is present in all members of the Src family. Whereas Fer is widely expressed, expression of Fps/Fes has previously been reported only in hematopoietic cells, and most abundantly in those of the myeloid lineage (9, 11, 27, 28, 36, 37). Low levels of Fps/Fes were also detected in some B-cell lines (27). More recently, expression of *fps/fes* has been detected in several rapidly proliferating embryonic tissues originating from different germinal layers by RNA in situ hybridization (4). A requirement for *fps/fes* in myelopoiesis has been suggested by gene transfer experiments in which a leukemic cell line acquired the ability to undergo terminal myeloid differentiation after transfection with an *fps/fes* expression construct (50). Involvement of Fps/Fes in signaling through the human heterodimeric interleukin-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) receptors as well as the erythropoietin receptor has been suggested (14, 15). In the former case, Fps/Fes is reported to directly associate with the common β chain of the human interleukin-3 and GM-CSF receptors and to be catalytically activated upon ligand binding (15). A more recent study using the same myeloid cell line shows that the Jak2 tyrosine kinase, but not Fps/Fes, is associated with and activated by the GM-CSF receptor (33).

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Oncogenic *fps/fes* alleles have been frequently isolated as avian and feline retroviral transforming genes (19). The viral oncoproteins include amino terminal Gag sequences fused to the Fps/Fes polypeptide. Addition of the viral Gag domain results in constitutive activation of the Fps/Fes tyrosine kinase (10, 38) by a mechanism which appears to involve association with the plasma membrane or the cytoskeleton. If the cellular Fps/Fes kinase does functionally interact with some component of a membrane-spanning receptor complex, then structural alterations which direct its association with the plasma membrane might induce its constitutive activation in the absence of appropriate extracellular signals.

In an attempt to elucidate the biological function of the Fps/Fes kinase, we have generated transgenic mice expressing either the wild-type human *fps/fes* gene or a gain-of-function mutant allele designed to encode a membrane-associated kinase. Transgenic mice which express approximately 10-fold-higher levels of the wild-type Fps/Fes kinase, compared with the endogenous protein, develop normally and display no apparent phenotype (11). We report here that tissue-specific expression of an activated mutant *fps/fes* allele in transgenic mice results in widespread hypervascularity progressing to multifocal hemangiomas. This phenotype correlates with an apparently normal and previously unrecognized pattern of *fps/fes* expression in cells of the vascular endothelial lineage. These observations suggest that the Fps/Fes tyrosine kinase is involved in angiogenesis.

MATERIALS AND METHODS

Construction of expression plasmids and transgenes. Human *fps/fes* sequences were derived from a 13-kbp genomic *Eco*RI fragment previously shown to contain the complete locus (11–13, 35). Expression of this gene in transfected rodent fibroblasts was facilitated by cloning the 13-kbp *Eco*RI fragment into the simian virus 40 (SV40)-based expression plasmid

pECE (8, 12). Although contained within this human genomic sequence, the human *fps/fes* promoter is not active in fibroblasts and therefore requires the tandem upstream SV40 promoter for efficient transcription in Rat-2 cell transfectants.

To generate the mutant *fps/fes* allele, a 1,310-bp *Bgl*II fragment containing exon 2 was subcloned from the pECE *fps/fes* expression plasmid into pUC119 to allow synthesis of single-stranded DNA to carry out oligonucleotide-directed mutagenesis (24). A 45-nucleotide mutagenic oligonucleotide, with 15 heterologous nucleotides flanked on either side by 15 *fps/fes* nucleotides, was used to introduce five additional codons between the second and third codons of the *fps/fes* open reading frame. The *Bgl*II fragment containing this altered sequence was then substituted back into the expression construct. DNA sequencing across the insertion was carried out to confirm the mutation.

Cell culture. Rat-2 fibroblasts (43), human acute myelogenous leukemia (AML) cells, and the murine hemangioendothelioma cell line EOMA were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. The human AML cell line (OCI-2) was obtained from Mark Minden. The EOMA cell line was from Robert Auerbach (31). The murine macrophage cell line BAC.2F5, obtained from Richard Stanley (30), was grown in alpha modified Eagle medium supplemented with 10% fetal bovine serum, 20% L-cell conditioned medium (as a source of colony-stimulating factor 1 [CSF-1]), 292 μg of glutamine per ml, 20 μg of asparagine per ml, and 50 μM 2-mercaptoethanol at 37°C in a 5% CO_2 atmosphere. Bone marrow-derived macrophage cultures were established and grown in BAC.2F5 medium as previously described (11). A Rat-2 transfectant expressing the murine c-*fms* proto-oncogene, which encodes the CSF-1 receptor (CSF-1-R), was provided by Micheal Reedijk (34). Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics Corporation and grown according to the supplier's specifications. Transfection of Rat-2 cells was performed by calcium phosphate coprecipitation as described previously (40). Cotransfection of Rat-2 cells with *fps/fes* expression constructs and pSV_2neo was followed by selection with the antibiotic G418 at 400 μg/ml (39).

In vitro analysis of transfected cell lines. Metabolic labeling of rat cells with [^{3}H]myristic acid (New England Nuclear) was performed as previously described (22). Labeled cells were lysed in radioimmunoprecipitation assay buffer, and Fps/Fes proteins were immunoprecipitated as described previously (12). Immune complex kinase assays were performed on *fps/fes*-expressing rat cells as previously described (12), using crude polyclonal rabbit sera raised against a TrpE-Fps/Fes fusion protein. For Western blotting (immunoblotting) analysis of whole cell lysates, cultured cells were washed and lysed directly in hot sodium dodecyl sulfate (SDS) sample buffer as described previously (21), with the exception that bromophenol blue was excluded from the lysis buffer, and protein concentrations were determined by using a protein assay kit from Sigma (32). Western blotting analysis of mouse tissues and cultured cells has been described previously (11). The Fps/Fes-specific (α-Fps/Fes) antibody was raised in rabbits against a bacterially expressed TrpE-Fps/Fes fusion protein (containing Fps/Fes residues L-401 to Q-405) and affinity purified against a purified glutathione *S*-transferase–Fps/Fes fusion protein (containing Fps/Fes residues Q-381 to E-536). Antibody specific for the c-*fms* gene product was kindly provided by Micheal Reedijk (34).

Generation of transgenic mice. Transgenic mice were generated by DNA microinjection of mouse zygotes as previously described (11). Embryos for microinjection were obtained from CD1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.). The transgene was excised as a 13-kbp *Eco*RI fragment from the mutant *fps/fes* expression plasmid described above and purified away from the vector by low-melting-point agarose gel electrophoresis followed by purification on NACS PREPAC columns (GIBCO BRL).

RNase protection. RNA was purified by the acid guanidinium thiocyanate-phenol-chloroform extraction method (5). Samples of 5 μg of total RNA were subjected to RNase protection analysis as described previously (17). ^{32}P-labeled antisense probes were synthesized with T7 RNA polymerase. The template was a *Hin*dIII-linearized recombinant pGEM3Z (Promega Biotec) plasmid (pGAMH) which contains an insert consisting of nucleotides 740 to 798 of murine β-actin cDNA (42), nucleotides 2565 to 2667 of the murine *fps/fes* cDNA (48), and nucleotides 2546 to 2622 of the human *fps/fes* cDNA (1). These sequences were generated by PCR amplification from cloned DNA (murine β-actin, murine *fps/fes*, and human *fps/fes* cDNAs were kindly provided by Ralph Zirngibl, Andrew Wilks, and Myriam Alcalay, respectively). PCR priming oligonucleotides consisted of 15 nucleotides of homologous sequence, plus 9 additional nonhomologous residues at their 5′ ends, containing restriction endonuclease sites designed to facilitate cloning in the proper orientation (*Xba*I-5′-murine β-actin-3′-*Bam*HI-5′-murine *fps/fes*-3′-*Kpn*I-5′-human *fps/fes*-3′-*Eco*RI-T7 promoter).

Immunohistochemistry. Paraffin sections were dewaxed and stained immunohistochemically for von Willebrand factor with a commercially available probe (Molecular Probes, Inc.) and a Vectastain ABC kit (Vector Laboratories, Inc.). After color development, slides were lightly counterstained with eosin.

In situ hybridization. In situ hybridization was carried out on 8-μm sections of paraffin wax embedded tissues essentially as described elsewhere (47). Digoxigenin-substituted single-stranded RNA probes were synthesized by using digoxigenin-11-UTP (Boehringer Mannheim Biochemicals). The template consisted of a full-length human *fps/fes* cDNA cloned into pGEM-3Z. This was generated by cloning *fps/fes* cDNA as an *Eco*RI fragment from pLF5.13 (1) in the plus orientation into the *Eco*RI site of pECE (8); from this intermediate plasmid (pEF4), the *fps/fes* cDNA insert was directionally cloned as a *Hin*dIII-to-*Xba*I fragment into pGEM-3Z, to produce plasmid pGHF. Antisense RNA transcripts were made from *Hin*dIII-linearized pGHF by using T7 RNA polymerase, while control sense RNA transcripts were generated from *Xba*I-linearized pGHF by using SP6 RNA polymerase.

RESULTS

Construction of an activated mutant *fps/fes* allele. Localization of many membrane-associated polypeptides involves hydrophobic interactions between covalently attached fatty acyl groups and lipid bilayer structures (44). To promote interaction between Fps/Fes and the inner surface of the plasma membrane, we engineered a mutant human *fps/fes* allele which encodes an amino-terminal myristylation target sequence. Oligonucleotide-directed mutagenesis was used to insert an additional 15 nucleotides between the second and third codons of the *fps/fes* open reading frame (Fig. 1A). The resulting amino terminus of the mutant protein contains the sequence GSSKSK (Fig. 1B), which is identical to the amino terminus of the Src PTK and is sufficient to direct amino-terminal myristylation of Src or a recombinant Src-pyruvate kinase fusion protein (3, 22).

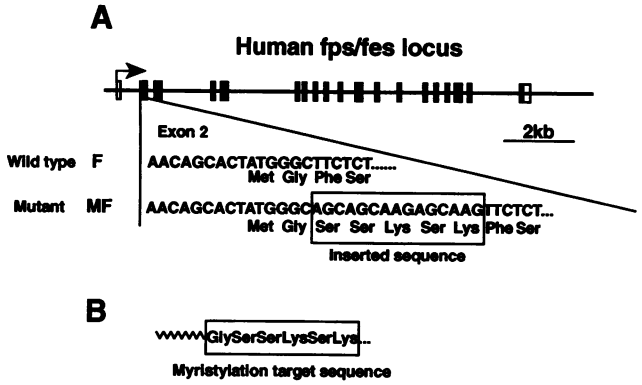

FIG. 1. Schematic diagram of wild-type and mutant human *fps/fes* alleles. The top line in panel A illustrates the 13-kbp *Eco*RI fragment which contains the complete human *fps/fes* locus. The transcriptional start site is indicated by the horizontal arrow, and the coding regions of exons 2 through 19 are shown as filled rectangles. The bottom portion of panel A shows the exon 2 nucleotide and encoded amino acid sequences of the wild-type (F) and mutant (MF) alleles. The boxed sequences are the only differences between the two alleles. Panel B shows the expected structure of the mutant gene product; the wavy line represents a cotranslationally added myristic acid group.

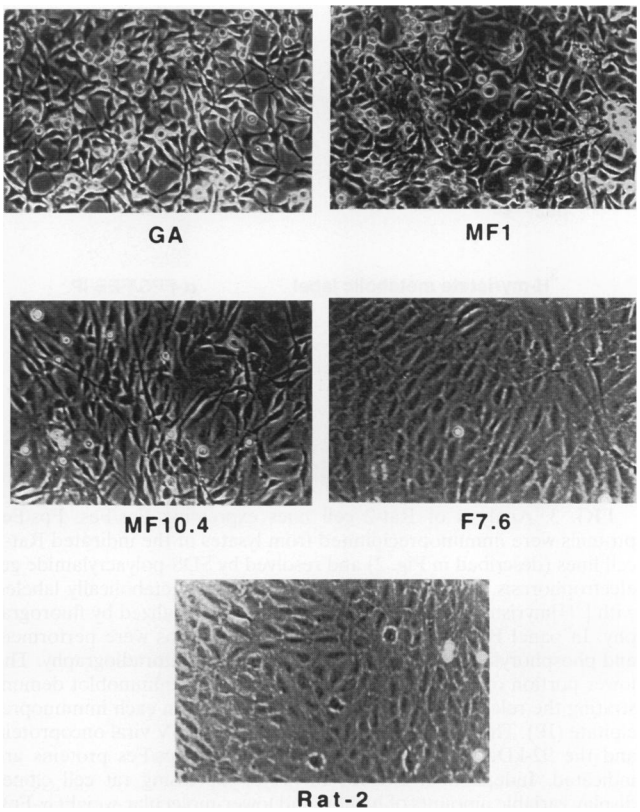

FIG. 2. *fps/fes*-expressing Rat-2 cell lines. GA and MF1 cell lines express the GA FeSV v-*fes* oncogene and the mutant human *fps/fes* alleles, respectively. These lines were derived from foci of transformed Rat-2 cells which were subsequently cloned in soft agar. MF10.4 and F7.6 express the mutant and wild-type human *fps/fes* alleles, respectively. These lines were generated by cotransfection with pSV_2neo and selection in G418. Rat-2 is the parental cell line from which all these clones were derived.

Transformation of rat fibroblasts by the mutant *fps/fes* allele. The mutant *fps/fes* allele (designated MF, for myristylated Fps/Fes) was initially characterized in transfected Rat-2 fibroblasts lines. As the *fps/fes* gene is not transcriptionally active in fibroblasts, a tandem upstream SV40 promoter was added to facilitate these in vitro studies (12). Expression constructs encoding the mutant (MF) or wild-type (F) *fps/fes* alleles were cotransfected into Rat-2 fibroblasts along with pSV_2neo, and Fps/Fes-expressing G418-resistant clones were characterized by Western blotting. While clones expressing wild-type *fps/fes* were morphologically indistinguishable from the parental Rat-2 cells, 3 of 19 MF clones displayed an elongated fusiform morphology (Fig. 2, MF10.4). There was no correlation between level of kinase expression and morphological transformation in these 19 MF clones (data not shown). Focus assays using the F and MF expression constructs were also performed on Rat-2 cells. In several experiments, no foci were observed with the F allele, even after maintenance of the transfected monolayers for up to 2 months. In contrast, the MF allele reproducibly gave rise to a small number of foci (one to two per 10 μg of DNA) which required up to 6 weeks to develop. These cells grew in soft agar and displayed a highly refractile morphology similar to that of Rat-2 cells transformed with a viral *fps/fes* allele encoded by the Gardner-Arnstein feline sarcoma virus (GA FeSV) (Fig. 2, MF1 and GA).

Myristylation of the mutant Fps/Fes polypeptide. Fatty acylation of the MF-encoded Fps/Fes variant was investigated by metabolic labeling with [^{3}H]myristate. Radiolabeled cell lysates were immunoprecipitated with a rabbit polyclonal α-Fps/Fes antiserum. Half of the immunoprecipitates were then analyzed by immunoblotting with affinity-purified α-Fps/Fes antibody (Fig. 3A, bottom), while the other half was analyzed by fluorography (Fig. 3A, top). While similar levels of the MF- or F-encoded 92-kDa polypeptides were present in these cell lines, incorporation of [^{3}H]myristate is seen only in the MF-encoded protein or in the 110-kDa retroviral Gag-Fps/Fes GA FeSV fusion protein. The relative levels of expression and [^{3}H]myristate incorporation were similar for the GA FeSV and MF proteins. This finding demonstrates that the amino terminus of the MF-encoded protein is efficiently recognized by myristate transferase. Wild-type Fps/Fes is not modified by myristylation, as no incorporation of tritium was detected.

The mutant Fps/Fes kinase is biochemically activated. To explore the effects of amino-terminal myristylation on Fps/Fes tyrosine kinase activity, wild-type or mutant Fps/Fes proteins were immunoprecipitated from cell lysates and subjected to an immune complex kinase assay. Although approximately the same amount of Fps/Fes protein was precipitated from each of the expressing rat cell lines (Fig. 3B, bottom), the myristylated proteins from either the MF1 or MF10.4 line were more highly autophosphorylated than the wild-type protein from the F7.6 line (Fig. 3B, top). The in vitro autophosphorylation activity of the mutant protein more closely resembled that of the viral Gag-Fps/Fes oncoprotein.

Vascular hyperplasia in transgenic mice. The mutant human *fps/fes* allele was excised from the SV40-based expression construct and used to generate transgenic mice by zygote DNA microinjection. This 13-kbp genomic *Eco*RI fragment was previously shown to direct appropriate tissue-specific expression in transgenic mice, independent of the integration site, suggesting that it has all of the transcriptional elements required for physiological *fps/fes* expression (11). Of five transgenic founder animals obtained, two died as neonates, one was severely runted and died at 4 weeks of age, and two

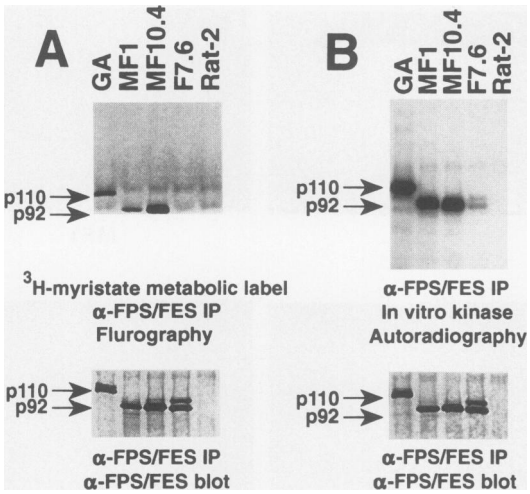

FIG. 3. Analysis of Rat-2 cell lines expressing Fps/Fes. Fps/Fes proteins were immunoprecipitated from lysates of the indicated Rat-2 cell lines (described in Fig. 2) and resolved by SDS-polyacrylamide gel electrophoresis. In panel A, the cells were first metabolically labeled with [^{3}H]myristate, and labeled proteins were visualized by fluorography. In panel B, immune complex kinase reactions were performed, and phosphorylated proteins were visualized by autoradiography. The lower portion of each panel shows an α-Fps/Fes immunoblot demonstrating the relative amounts of Fps/Fes proteins in each immunoprecipitate (IP). The positions of the 110-kDa GA FeSV viral oncoprotein and the 92-kDa wild-type and mutant human Fps/Fes proteins are indicated. Independent isolates of *fps/fes*-expressing rat cell clones display variable amounts of higher- and lower-molecular-weight α-Fps/Fes-reactive species, and the basis of the different mobilities is not known.

survived and were bred to stable lines. One line had only one or two copies of the transgene and showed no apparent phenotype (data not shown). The other line (MF1) has approximately 10 copies of the MF allele, and transgenic neonates were easily identified by their darker color. This phenotype was found to be due to an abnormally high degree of vascularity in the subcutaneous brown adipose tissues. Immunohistochemical analysis with an antibody directed against von Willibrand factor, which is abundantly expressed in vascular endothelial cells (18, 20), showed that the subcutaneous adipose layer of the transgenic neonates contains elevated levels of von Willebrand factor-positive cells relative to normal littermates (Fig. 4B and D, respectively). Histological examination of this adipose layer clearly showed a higher degree of vascularity in transgenic compared to control tissue (Fig. 4A and C, respectively).

This hypervascularity is apparent in all tissues, and as these animals age, they develop widespread vascular hyperplasia, progressing to multifocal hemangiomas most frequently associated with lymph nodes, the gut mesentery, and the uterus. Figure 4E shows a section through one such tumor isolated from the axillary node region of an MF1 transgenic animal. The normal architecture of the node is largely disrupted by invading vascular spaces. Vascular hyperplasia is also seen in the surrounding meschenchymal tissues (Fig. 4F). All MF1 mice became anemic and succumbed to the systemic effects of vascular hyperplasia such as internal hemorrhaging. The median life span of MF1 transgenic animals was approximately 7 months.

Transgene expression in tumors and normal tissues. RNA transcripts derived from both the human and murine *fps/fes* alleles were detected in MF1 transgenic tissues and at very high levels in hemangiomas (Fig. 5). The RNase protection probe used in this analysis consists of antisense sequences corresponding to 3′ noncoding portions of the murine (102 nucleotides) and human (77 nucleotides) *fps/fes* mRNAs, together with a short stretch of the murine β-actin (59 nucleotides) antisense sequence, which serves as an internal control for sample recovery during the procedure. Low similarity between human and murine *fps/fes* sequences in the 3′ noncoding region allows this probe to distinguish between the human and murine transcripts. In control CD1 bone marrow and in a murine macrophage cell line (BAC.2F5), only the murine transcript was seen. In contrast, in the human *fps/fes*-expressing Rat-2 line (F7.6) and the human AML line, only the human transcript was detected. In tissues of MF1 mice, the same coincidental patterns of human and murine *fps/fes* transcripts were seen as previously described for transgenic mice expressing the wild-type human *fps/fes* allele (11).

This analysis confirmed that the MF human transgene is expressed in the same pattern as the endogenous murine gene and argue against ectopic expression of the mutant *fps/fes* allele. The high degree of *fps/fes* expression seen in bone marrow is thought to originate from myeloid cells, which are enriched in this tissue. As expression of *fps/fes* was previously thought to be largely confined to the myeloid lineage, the levels of RNA seen in the hemangiomas suggested either a substantial myeloid infiltration or expression in some other component of the tumor. Consistent with the latter possibility, we detected *fps/fes* transcripts in the murine hemangioendothelioma cell line EOMA and in primary HUVEC. This observation demonstrates transcription of the *fps/fes* proto-oncogene in cells of the vascular endothelial lineage.

Western blotting analysis also showed that MF1 mice had elevated levels of tissue-specific Fps/Fes protein expression relative to normal CD1 mice (data not shown). High levels of Fps/Fes were detected in the spleen, lung, lymphatics, and gut mesentery of MF1 transgenic mice, and lower levels were detected in thymus, brain, heart, kidney, testes, small intestine, and testicular fat pads. In control CD1 mice, Fps/Fes was also detectable in those tissues which showed highest levels of protein in the MF1 mice. The tissue-specific pattern of protein expression was consistent with the RNase protection analysis.

Expression of Fps/Fes in normal endothelial cells. Expression of *fps/fes* in primary endothelial cells has not previously been documented. The vascular hyperplasia seen in the MF1 transgenic mice suggests that Fps/Fes may normally be involved in regulating proliferation of endothelial cells. To confirm that *fps/fes* is normally expressed in this lineage, we looked for Fps/Fes protein in primary HUVEC and the murine hemangioendothelioma cell line EOMA. The antibody used in this analysis was raised and affinity purified against a bacterially expressed fusion protein containing human Fps/Fes residues L-401 to Q-445. This sequence is highly conserved between human and murine Fps/Fes, and these antibodies detect human and murine Fps/Fes proteins equally well. Western blotting revealed that HUVEC express levels of the Fps/Fes protein which are comparable to those seen in human and murine myeloid cell lines (AML and BAC.2F5) or primary bone marrow-derived macrophage cultures from control CD1 mice (Fig. 6A). We also detected lower levels of Fps/Fes in the EOMA cell line. Overexpression of Fps/Fes protein is seen in primary bone marrow macrophage cultures from MF1 transgenic mice or transgenic lines expressing wild-type human *fps/fes* (F2 and F3). An identical Western blot was also probed with antibody to CSF-1-R receptor (α-CSF-1-R), which is abundantly expressed in macrophages (Fig. 6B). Other con-

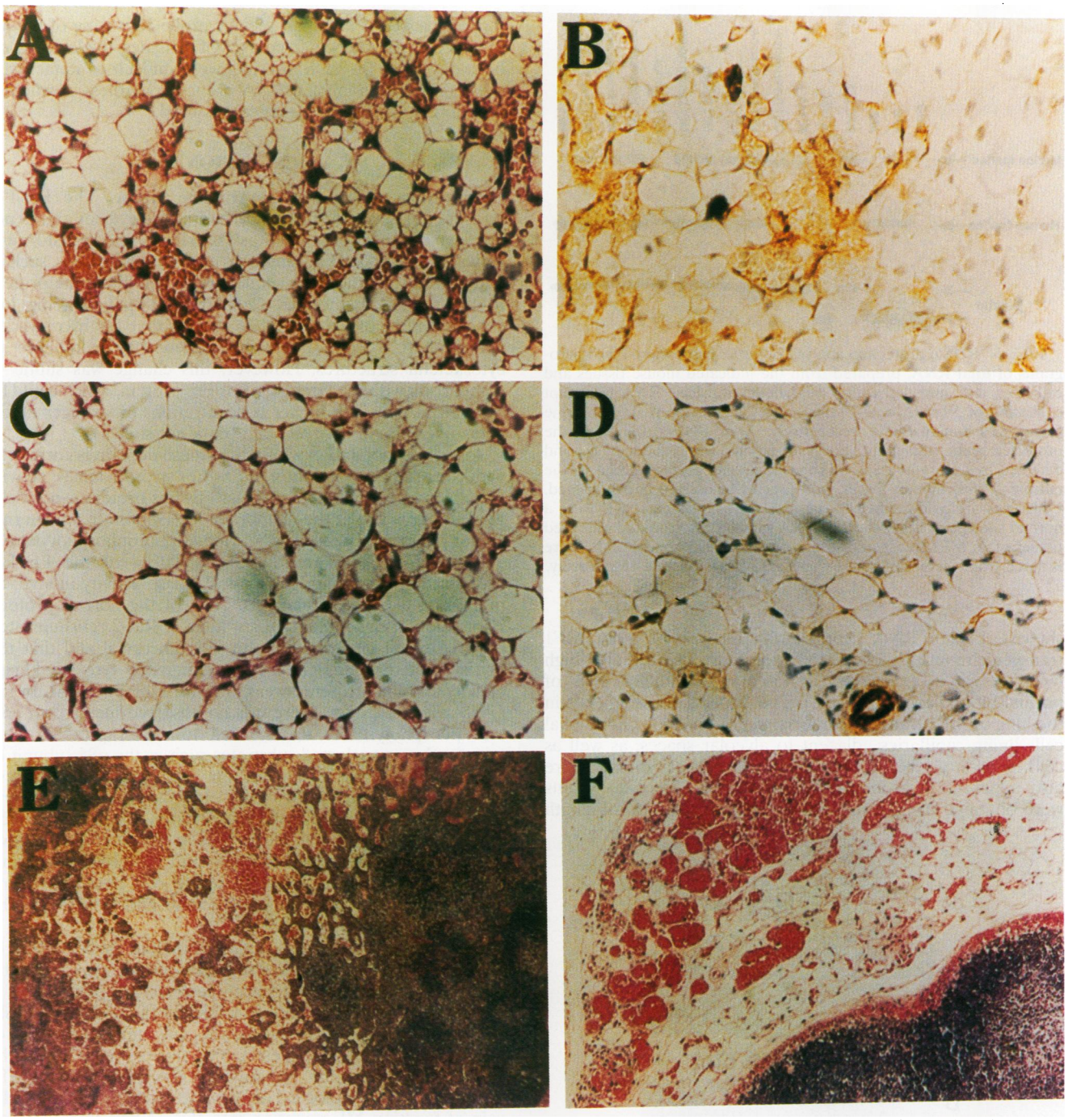

FIG. 4. Histological and immunocytochemical analysis of subcutaneous brown adipose tissue and histological analysis of an endothelial tumor. Cross sections through the trunk region were prepared from paraffin-embedded tissues of neonatal mice (A through D) or a tumor excised from the axillary lymphatic region of and adult transgenic mouse (E and F). Sections in panels A, C, E, and F were stained with eosin and hematoxylin; sections in panels B and D were stained with antibody against an endothelial cell marker, von Willebrand factor. Panels A and B are from a transgenic animal; panels C and D are from a control littermate.

trols include rat fibroblasts (Rat-2) and Rat-2 transfectants expressing CSF-1-R (FMS) or the wild-type (F7.6) or mutant (MF10.4) *fps/fes* allele.

In situ analysis of *fps/fes* expression. To directly identify *fps/fes*-expressing cells, an RNA in situ hybridization analysis was performed on transgenic mouse tissues. The highest levels of *fps/fes* transcripts were seen in the endothelial component of the hemangiomas (Fig. 7A and C). We also detected *fps/fes* transcripts in cells lining blood vessels in the mesenchyme surrounding the tumors (Fig. 7C). The morphology and location of the *fps/fes*-expressing cells are consistent with their being vascular endothelial cells. Immunocytochemical analysis of these tumors with antibody directed against von Willibrand factor showed an identical pattern of staining (data not

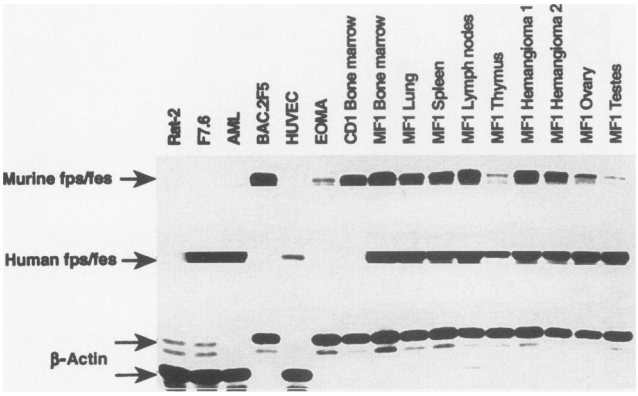

FIG. 5. RNase protection analysis of *fps/fes* expression. Five micrograms of total RNA, prepared from the indicated mouse tissues and cell lines, was annealed with a ^{32}P-labeled antisense RNA transcript consisting of 59, 102, and 77 nucleotides of murine β-actin, murine *fps/fes*, and human *fps/fes*, respectively. After RNase treatment, the protected probe fragments were resolved on a 8% sequencing gel and detected by autoradiography. Probe fragments corresponding to the murine *fps/fes*, human *fps/fes*, and β-actin transcripts are indicated. Cells lines are as described in the text; CD1 bone marrow is from a normal control mouse. All other RNA samples are from the indicated tissues of an MF1 transgenic mouse. MF1 hemangiomas 1 and 2 were isolated from the gut mesentery and axillary lymphatics, respectively.

shown). In situ RNA hybridization analysis of other MF1 transgenic mouse tissues, such as the lung, also revealed high levels of *fps/fes* transcripts in vascular endothelial cells of normal blood vessels (Fig. 7E). The *fps/fes*-expressing cells in the lung of MF1 transgenic mice appear to include endothelial cells lining capillaries adjacent to alveolar spaces, as well as cells lining larger blood vessels. Lower levels of expression are also apparent in bronchial epithelial cells. Identical results were obtained using a full-length human probe, a full-length murine probe, or a human-specific probe consisting of unique 3′ noncoding sequence. In control CD1 tissues, the lower levels of endogenous *fps/fes* transcripts were difficult to detect with these digoxigenin-labeled RNA probes, regardless of whether the human or murine sequence was used.

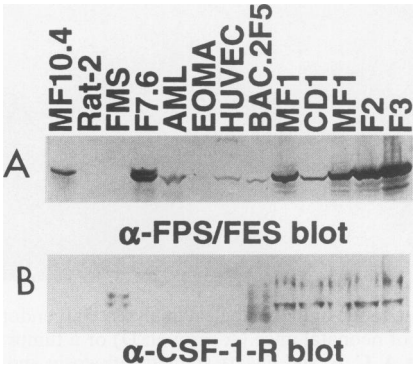

FIG. 6. Western blotting analysis Fps/Fes and CSF-1-R in myeloid and endothelial cells. Whole cell lysates were prepared from the indicated cell lines and primary cultures. Fifty micrograms of protein was resolved by SDS-polyacrylamide gel electrophoresis and blotted to nitrocellulose. Identical blots were probed with affinity-purified α-Fps/Fes polyclonal rabbit antibody or a crude rabbit polyclonal α-CSF-1-R antibody. Detection was accomplished by using a secondary goat anti-rabbit alkaline phosphatase conjugate followed by a color reaction. Cells lines are described in the text. MF1, CD1, F2, and F3 are primary bone marrow-derived macrophage cultures from transgenic mice expressing the mutant human *fps/fes* gene (MF1), the normal control mice (CD1), or two independent transgenic lines expressing the wild-type human *fps/fes* gene (F2 and F3), respectively.

DISCUSSION

Activation of the Fps/Fes tyrosine kinase. The Fps/Fes tyrosine kinase is normally localized in the cytosol and is not oncogenic. However, both Fps/Fes kinase and transforming activities are induced by amino-terminal modifications, such as addition of retroviral Gag sequences, which direct the protein to the plasma membrane. Cellular fractionation using low concentrations of Triton X-100 showed that the myristylated MF protein is more tightly associated with membrane or cytoskeletal structures. However, this change was subtle in comparison with the viral Gag-Fps/Fes protein, which could be solubilized only with higher concentrations of stronger detergents like sodium deoxycholate (data not shown).

Autophosphorylation within the kinase domain is correlated with constitutive stimulation of Fps/Fes tyrosine kinase activity (46). Hence, it is possible that the Fps/Fes protein is normally recruited to a site at the membrane in response to an external stimulus and is subsequently activated by autophosphorylation. We therefore speculate that myristylation of the Fps/Fes protein might stimulate its tyrosine kinase activity in vivo, by mimicking a transient activated state of the normal protein. Expression of this mutant *fps/fes* allele in Rat-2 cells indicated that the novel myristylation site was functional and that the myristylated protein had increased in vitro tyrosine kinase activity and had acquired weak transforming potential. In the G418-selected clones, there was no observed increase in protein phosphotyrosine levels when total cell proteins were examined or on selected substrates such as the p85 subunit of phosphatidylinositol 3′-kinase, phospholipase Cγ, or Ras–GTPase-activating protein (data not shown). Furthermore, neither the wild-type nor the myristylated kinase was detectably tyrosine phosphorylated in vivo in these cell lines. However, in the focus-derived MF line and the GA FeSV-transformed line, elevated levels of whole cell phosphotyrosine correlated with readily detectable phosphotyrosine on the activated kinases (data not shown).

Specific expression of a gain-of-function *fps/fes* allele in transgenic mice. We have previously shown that a 13-kbp human genomic fragment encompassing the *fps/fes* gene is expressed in transgenic mice in a fashion that is indistinguishable from that of the normal mouse gene and independent of the site of integration, suggesting that this fragment has all the elements required for physiological *fps/fes* expression. To investigate *fps/fes* biological activity, we have incorporated the gain-of-function MF mutation into this 13-kbp fragment and obtained a line of transgenic mice that express the mutant protein in appropriate tissues. Unlike the wild-type *fps/fes* transgene, which has no obvious phenotypic effect, the mutant MF transgene appeared to be toxic, as judged by the low number of transgenic MF founder animals obtained and the difficulty in establishing a stable line of transgenic mice. As the cellular *fps/fes* gene is abundantly expressed in hematopoietic cells of the myeloid lineage, the MF1 transgenic mice might have been expected to display a phenotype reflecting disruption of myelopoiesis. Although we have detected signs of extramedullary hematopoiesis, in the form of myeloid progenitor cells in hemangiomas, and other indications of increased in vitro proliferative potential of myeloid progenitors (unpublished data), there was no apparent hematopoietic disease. In

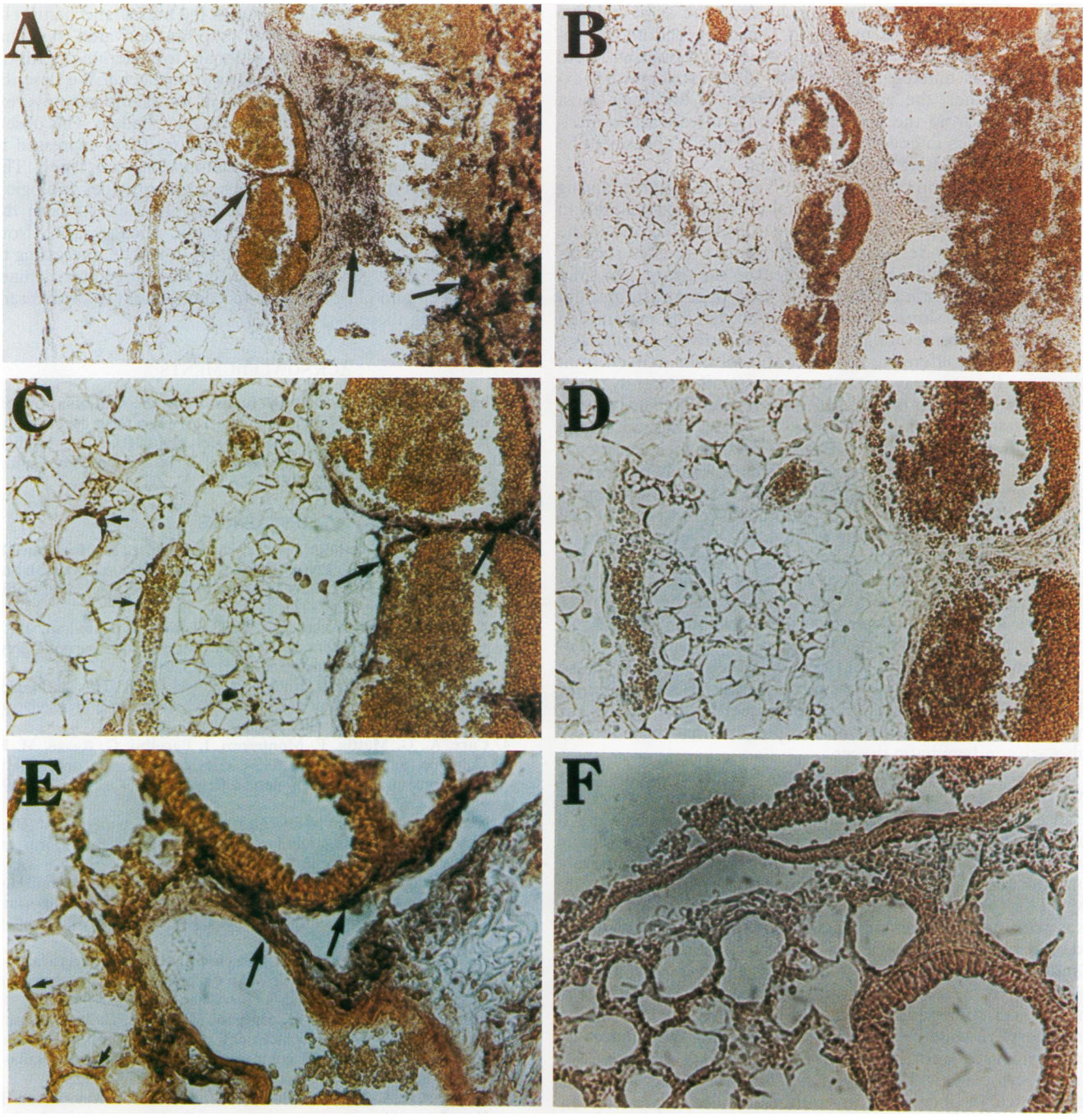

FIG. 7. In situ RNA analysis of *fps/fes* expression in an endothelial tumor. Sections from an endothelial tumor (A through D) or lung (E and F) of an MF1 transgenic mouse were hybridized with antisense (A, C, and E) or sense control (B, D, and F) digoxigenin-substituted human *fps/fes* RNA transcripts. Hybridizing RNA probes were subsequently detected by using an alkaline phosphatase-conjugated antidigoxigenin antibody followed by a color reaction. Panels C and D are higher magnifications of selected fields from panels A and B. Large arrows in panels A and C indicate the vascular endothelial component of the tumor. Smaller arrows in panel C indicate normal endothelial cells lining blood vessels in the mesentery adjacent to the tumor. Large arrows in panel E indicate endothelial cells lining larger vessels adjacent to bronchial spaces, while the smaller arrows indicate the positions of capillaries beside alveolar spaces.

contrast, there was a marked effect on vascular development, ultimately manifested by the development of hemangiomas.

***fps/fes* is expressed in endothelial cells.** In situ analysis specifically localized *fps/fes* transcripts to endothelial cells of the tumors and normal blood vessels of transgenic mice. Independent evidence for intrinsic expression of *fps/fes* in endothelial cells comes from RNase protection analysis and Western blotting of primary HUVEC, in which levels of *fps/fes* expression were comparable to those detected in myeloid cells. The expression of an activated *fps/fes* variant in endothelial cells may explain the hypervascular phenotype of MF mice. Since RNase protection analysis clearly showed that the hu-

man and murine transcripts appeared in the same relative quantities in all tissues examined, including endothelial tumors, in which very high levels of both transcripts were detected, it is unlikely that the MF allele is ectopically expressed. Rather, the vascular phenotype of MF mice most likely results from the presence of constitutively active myristylated Fps/Fes in endothelial cells, in which it appears to be involved in regulating cell proliferation.

Upon completion of vasculogenesis and angiogenesis during development, cells of the endothelial lineage remain largely in a quiescent state. Rapid proliferation of endothelial cells in the adult is generally restricted to angiogenic processes such as wound healing, follicular development in the ovary, and solid tumor growth or other disease processes. In contrast, myeloid cells have much shorter lives and are continually replenished from rapidly proliferating bone marrow-derived progenitors. The distinct proliferative characteristics of cells derived from these two lineages may cause them to respond differently to the activation of a gene involved in their regulation. Thus, the in vivo effects on myeloid cells may be largely masked by the inherently higher proliferative status of this lineage. In contrast, the normally quiescent endothelial cells may be intrinsically more sensitive to an activated Fps/Fes protein which may delay or reduce the tendency of endothelial cells to exit the cell cycle upon completion of the normal angiogenic program.

As the MF1 transgenic animals age, endothelial cell proliferation continues, ultimately progressing to wide spread vascular hyperplasia and eventually to multifocal hemangiomas. The prevalence of hypervascularity in lymphatic tissues, in which large cavernous hemangiomas are nearly always found in older animals, suggests that the endothelial cells of these tissues are particularly susceptible to the effects of *fps/fes* activation. Hemangiomas are also frequently seen in the gut mesentery and the uterus, and it is possible that these tumors also arise in lymphatic tissues.

The vascular hyperplasia and multifocal hemangiomas in MF1 mice are reminiscent of a similar phenotype seen in transgenic or chimeric mice expressing polyomavirus middle T antigen (mT) (2, 49). mT is thought to contribute to cellular transformation by associating with and biochemically activating endogenous cellular Src or other members of the Src family of PTKs (6). Although tyrosine phosphorylated mT protein was seen in those hemangiomas, an associated PTK was not identified. Thus, it is unclear which, if any, member of the Src family is activated in these tumors. As there is no reason to assume that mT expression was restricted to endothelial cells in the mT transgenic mice, and Src or other Src family members are widely expressed, it is intriguing that tumors of other lineages were not seen. Given that mT activates Src family cytoplasmic PTKs, and we have observed a similar hypervascular phenotype in transgenic mice expressing an activated Fps/Fes kinase, it is possible that the endothelial lineage is particularly sensitive to the activation of tyrosine kinases in general. In this regard, it is interesting that vanadate, a potent inhibitor of tyrosine phosphatases, has angiogenic properties and that several known angiogenic factors are ligands for receptor PTKs (7, 23, 29, 41). Several of these receptor tyrosine kinases are structurally related to the platelet-derived growth factor receptor, which has been shown to associate with and activate cytoplasmic PTKs of the Src family (6, 25).

The molecular mechanisms by which the Fps/Fes kinase participates in mitogenic signaling are not yet known. However, if the upstream regulators, or downstream targets, of the normal Fps/Fes kinase are membrane-associated molecules, then mutations which promote association of Fps/Fes with the plasma membrane might circumvent the normal mechanism by which this kinase is recruited into an activated signaling pathway. We have used this rationale to generate a gain-of-function mutation in the *fps/fes* proto-oncogene. We describe here a prominent hypervascularity in transgenic mice overexpressing this activated mutant human *fps/fes* allele. This observation has led to the observation that *fps/fes* is expressed in endothelial cells as well as hematopoietic cell types. The vascular hyperplasia seen in these mice provides biological evidence for a role of Fps/Fes in angiogenesis. As both the vascular endothelial and hematopoietic lineages arise from common mesodermal progenitors (45), it will be interesting to see if the *fps/fes* gene is expressed in this progenitor hemangioblast cell and plays a role in its commitment to or differentiation along these two lineages.

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