Fes Tyrosine Kinase Expression in the Tumor Niche Correlates with Enhanced Tumor Growth, Angiogenesis, Circulating Tumor Cells, Metastasis, and Infiltrating Macrophages

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Abstract

Fes is a protein tyrosine kinase with cell autonomous oncogenic activities that are well established in cell culture and animal models, but its involvement in human cancer has been unclear. Abundant expression of Fes in vascular endothelial cells and myeloid cell lineages prompted us to explore roles for Fes in the tumor microenvironment. In an orthotopic mouse model of breast cancer, we found that loss of Fes in the host correlated with reductions in engrafted tumor growth rates, metastasis, and circulating tumor cells. The tumor microenvironment in Fes-deficient mice also showed reduced vascularity and fewer macrophages. In co-culture with tumor cells, Fes-deficient macrophages also poorly promoted tumor cell invasive behavior. Taken together, our observations argue that Fes inhibition might provide therapeutic benefits in breast cancer, in part by attenuating tumor-associated angiogenesis and the metastasis-promoting functions of tumor-associated macrophages. Cancer Res; 71(4); 1465–73. ©2010 AACR.

Introduction

The fes proto-oncogene (also known as fps), encodes a Src homology 2 (SH2) domain-containing cytoplasmic protein-tyrosine kinase (PTK) which has been implicated in signaling downstream of receptors for cytokines, growth factors, immunoglobulin, collagen, and endotoxin (reviewed in refs. 1 and 2). Fes expression is restricted to myeloid, endothelial, and subsets of epithelial and neuronal cells (1, 3, 4). Fes was originally identified as a dominant-acting oncoprotein encoded by transforming retroviruses isolated from avian (fpe; ref. 5) or feline (fes; ref. 6) tumors. When expressed in transgenic mice, viral fps induced tumors in lymphoid and mesenchymal tissues (7). These observations suggested that activating mutations in the human fes proto-oncogene might contribute to cancer. Missense mutations were subsequently identified in human colorectal cancers (8), leading to the speculation that activated Fes kinase contributed to these cancers. However, subsequent biochemical and structural modeling analysis revealed that these mutations attenuated rather than activated Fes kinase (9). This revelation raised the novel possibility that Fes might also function as a tumor suppressor. Genetic evidence to support this hypothesis came from studies of transgenic mice expressing polyoma virus middle T (PymT) antigen in the mammary glands. Tumors developed earlier in mice targeted at the fes locus with either null or kinase-inactivating missense mutations (9). The fes promoter was also found to be silenced by methylation in colorectal cancer cell lines, and this correlated with downregulation of Fes expression (10). These apparently contradicting observations argued that Fes may play both oncogenic and tumor suppressor roles. Furthermore, considering the different cell types which express Fes, the cumulative effect on tumorigenesis may depend on both tumor autonomous cell roles and roles in cells of the tumor niche. For example, tissue-specific expression of an activated fes allele in transgenic mice led to hypervascularity and multifocal hemangiomas, correlating with expression in vascular endothelial cells (11), and this same activated fes allele was able to partially rescue the vasculogenesis defect in vascular endothelial growth factor (VEGF) receptor knockout embryos (12). In other studies using fes knockout mice, we observed hypersensitivity to endotoxin, which correlated with abundant Fes expression in macrophages where it regulates TLR4 endocytosis, NFkB signaling, and TNFα expression (13, 14). These phenotypes in transgenic and knockout mice suggested possible roles for Fes in both vascular endothelial and myeloid cells, which might influence tumor progression. Tumor cell autonomous roles for Fes in breast cancer initiation were also suggested by a recent study showing Fes is highly expressed...
and activated in mouse mammary epithelial cells during lactation, where it is associated with E-cadherin based adhe-
sions junctions (4). However, to our knowledge, Fes expression in
breast tumors or tumor cell lines has not been reported.

To elucidate the involvement of Fes in breast cancer, we
have employed a tumor cell orthotopic mouse mammary
gland engraftment model designed to separately examine
tumor cell autonomous and niche roles of Fes. Manipulation
of Fes expression in the engrafted breast carcinoma cells had
no effect on growth at the orthotopic injection site or metast-
tasis. However, when Fes expression was eliminated in the
niche, significant reductions in tumor growth rates and
metastasis were observed. These defects correlated with
reductions in tumor-associated vascularity, macrophages,
and circulating tumor cells. Bone marrow–derived fes-null
macrophages were less proficient at promoting the in vitro
invasive properties of cocultured tumor cells, or of being
induced to invade by tumor cells. These observations are
consistent with tumor progression roles of Fes acting at
the level of the vascular endothelial cells and macrophages.
This study provides novel genetic evidence that the Fes
protein-tyrosine kinase represents a potential therapeutic
target in breast cancer, where Fes inhibition in macrophages
and vascular endothelial cells would attenuate their tumor
promoting roles.

Materials and Methods

Cell culture

The highly metastatic AC2M2 mouse mammary carcinoma
cell line (15) was routinely cultured in Dulbecco’s Modified
Eagle Medium (Invitrogen) supplemented with 10% fetal
bovine serum (FBS, Sigma), 2 mM l-glutamine and antibi-
tics/antimycotics (Invitrogen), and maintained at 37°C with
5% CO2 in a humidified incubator. These cells were trans-
duced with lentivirus expressing green fluorescence protein
(GFP). For some experiments, these GFP-expressing AC2M2
cells were transduced with pMSCVpuro (Clontech) retro-
viruses encoding C-terminally Myc-epitope tagged wild type,
kine-deduced fes-null Fes (K888R; ref. 16), or kinase-activated (V-terminally
myristoylated) Fes (11).

Mice

The previously established fes-null (fes−/−) strain (13) was
crossed with nude mice (Ncr-Foxn1nu/nu; Taconic) to produce
hybrid wild-type (fes+/-; nu/nu) and fes-null nude (fes−/−; nu/nu) mice. Mice were housed in the Animal Care Facility
and procedures were carried out according to the guidelines
of the Canadian Council on Animal Care, with the approval of
the institutional animal care committee.

Orthotopic mammary gland engraftment model

After being anesthetized with 250 mg/kg avertin, age-
matched wild-type and fes-null female nude mice were injected
with 7,500 GFP-expressing AC2M2 cells into the fourth
mammary gland (15). Tumor growth at the orthotopic site was
measured by ultrasound imaging (VisualSonics Vevo 770) 12,
14, 16, 19, and 22 days after engraftment. On day 23, the tumors
were removed and mice were kept alive to assess metastasis. On
day 35, mice were euthanized, lungs were dissected, and GFP-
expressing metastatic nodules were biophotonically imaged
using a Pan-A-See-Ya Panorama imaging system. Images were
captured using a Hamamatsu R/W ORCA-ER digital camera
with excitation filter at 470 nm/20 nm and an emission filter at
525 nm/20 nm.

In vivo macrophage imaging

Mice were engrafted with AC2M2 cells as described earlier.
On day 13, these tumor-bearing mice were injected with 200
μL of 20 mg/mL tetrathymylrhodamine conjugated dextran
beads (Invitrogen). After 2 hours, mice were perfused with 1%
paraformaldehyde in PBS and mammary glands were dis-
sected and imaged by Leica TCS SP2 confocal microscopy.

Flow cytometry measurement of macrophages and
endothelial cells

Tumor-bearing mammary glands were harvested from wild-
type and fes-null nude mice 7 days after engraftment as
described earlier, and digested in tissue digestion buffer con-
taining 0.1 mg/mL collagenase (Sigma), 0.072 mM CaCl2 and
20% FBS at 37°C for 3 hours. Single-cell suspensions were
obtained by filtering twice through 70 μm cell strainers (BD
Bioscience). Samples were centrifuged at 700 rpm for 5 min-
utes, and resuspended in 2 mL of ice-cold PAB [phosphate-
buffered saline with 1% (w/v) bovine serum albumin]. Aliquots
were incubated with 0.1 μg/mL phycoerythrin (PE)-conjugated
rat antimouse F4/80 antibody (Caltag) for macrophages, PE-
conjugated rat IgG2a (Molecular Probes) for isotype con-
trol, or propidium iodide (Invitrogen) for cell viability.
Samples were then washed with ice-cold PAB, fixed for 15
minutes with 2% formaldehyde (Sigma) at 37°C, resuspended
in 500 μL PAB, and analyzed by flow cytometry. Peritoneal
macrophages (obtained as described previously; ref. 14) were
used as positive controls.

Immunoblotting

Lysates from mammary glands, tumors, or cell cultures
were prepared in RIPA lysis buffer and assessed by immuno-
blotting as described in detail previously (4). Protein levels
were determined by densitometry using Image Pro software.
Antibodies used in immunoblotting included anti-Fes, which
is a rabbit polyclonal serum that cross-reacts with Fes and Fer
(ox-FpsE2; ref. 3), anti-Myc ascites (1–9E10.2 mouse hybri-
doma from American Type Culture Collection), anti-RasGap
rabbit polyclonal (17), anti-tubulin mouse monoclonal
(Sigma), or anti-PECAM-1, which is rabbit polyclonal serum
raised against a GST-fusion protein containing the complete
cytoplasmic domain of mouse PECAM-1 (18).

Immunofluorescence analysis of tumors and stroma

Tumors and associated mammary glands were removed from
age-matched wild-type and fes-null mice 7 days after
engraftment, fixed in 4% paraformaldehyde, and parafin
embedded. Tissue sections (5 μm) were deparaffinized with
toluene and rehydrated through an ethanol gradient. Antigen
was retrieved by heating sections in 10 mM sodium citrate
(pH 6.0) at 95°C in a steam cooker for 20 minutes. After cooling at room temperature for 20 minutes, sections were rinsed 3 times with PBS, and incubated in 0.2% Triton/PBS for 10 minutes, followed by 3 PBS rinses. Sections were then blocked with 3% BSA for 20 minutes. Following 3 washes in PBS, sections were incubated overnight with PECAM-1 rabbit polyclonal antibody (1:50 dilution in blocking buffer) at 4°C. Sections were washed 3 times in PBS before addition of Alexa Fluor 546 goat antirabbit IgG (1:200 dilution in blocking buffer, Invitrogen) for 1 hour at room temperature. After thorough washing with PBS, sections were mounted with Mowiol (Calbiochem) and imaged using Olympus BX51 fluorescence microscopy with excitation filter at 545 nm and emission filter at 576 nm. Quantification of vasculature in tumor stoma was performed using Image Pro software.

**Tail vein injection lung metastasis model**

Five \( \times 10^5 \) GFP-expressing AC2M2 cells were injected into the tail veins of wild-type or fes-null nu/nu mice and 22 days later lungs were dissected and biophotonically imaged as described earlier. Numbers and sizes of metastatic lesions were determined using Image Pro software.

**Detection of circulating tumor cells**

Twenty-two days after engraftment with GFP-expressing AC2M2 cells, peripheral blood was collected by cardiac punctures performed under deep anesthesia with isofluorane. Five hundred microliters of blood samples were subjected to hypotonic shock with 5 mL distilled water, followed by isotonic restoration with 1.5 mL 0.6M KCl. Samples were then washed with 50 mL PBS to remove red blood cell debris, resuspended in 500 µL PAB, and analyzed by flow cytometry.

**Collagen invasion assay**

Primary bone marrow–derived macrophages were obtained as previously described (19). MTLn3-GFP rat breast cancer cells were cocultured for 18 hours with bone marrow–derived macrophages (BMM) from wild-type or fes-null mice in the presence of colony stimulating factor (CSF)-1 and 10% FBS. Cocultures were then overlaid with collagen I gels and invasion into the collagen gel after 24 hours was quantified as previously described (20).

**Statistics**

All statistical analysis was done by using GraphPad Prism 5 software. All error bars represent standard error of the mean (SEM). \( P \) values were calculated by Student’s \( t \)-test and 2-way analysis of variance (ANOVA) analysis. Data sets with \( P \leq 0.05 \) were considered statistically significant.

**Results**

**Fes plays a tumor-promoting role in the niche**

Preliminary experiments were performed to explore potential tumor cell autonomous roles for Fes in mammary tumorigenesis. GFP-expressing AC2M2 mouse breast carcinoma cells were transduced with retroviruses encoding wild-type, kinase-dead, or activated Fes (Supplementary Fig. S1A). These cells were engrafted into the mammary glands of nu/nu mice and tumor growth was monitored by ultrasound measurement for 22 days. Primary tumors were then removed to promote metastatic growth and lung metastasis was examined 13 days later. No significant differences in tumor growth rates at the orthotopic injection site were observed (Supplementary Fig. S1B) and there were no significant differences in the frequency of metastasis to the lungs (data not shown). Thus, Fes did not appear to play a tumor cell autonomous role in this AC2M2 tumor engraftment model.

We next asked whether Fes expression in the niche cells, rather than in tumor cells, might influence tumor growth and metastasis. To facilitate these experiments, we crossed nu/nu mice with fes knockout mice (13) and established colonies of wild-type and fes-null nu/nu mice. GFP-expressing AC2M2 cells were orthotopically engrafted into the mammary glands of wild-type and fes-null mice and tumor growth and metastasis was monitored as described earlier. Tumor growth at the orthotopic site was significantly slower in fes-null mice (\( P < 0.0001 \)) relative to wild-type mice (Fig. 1) and the frequency of lung metastasis was also significantly reduced in fes-null mice, with 70% of wild-type mice versus 50% of fes-null mice developing metastasis (Supplementary Fig. S2). Also, among mice displaying lung metastasis wild-type mice displayed significantly more metastatic nodules (\( P = 0.0055 \); Fig. 2A). Although there was a trend toward smaller sized metastatic lung nodules in the fes-null mice, this difference did not achieve statistical significance (\( P = 0.1215 \); Fig. 2B).

**Reduced vascularity in the fes-null tumor niche**

The reduction in tumor engraftment growth rate and metastasis frequency in fes-null mice suggested Fes might be playing a tumor promoting role, which is intrinsic to cells making up the tumor niche. Angiogenesis promotes tumor growth and metastasis, and Fes is expressed in vascular...
endothelial cells and has been implicated in angiogenesis (11, 12, 21–23). We therefore examined the vascularity in wild-type and fes-null tumors and associated tissues. PECAM-1 immunofluorescence staining revealed reduced numbers of blood vessels in fes-null stroma (Supplementary Fig. 3). Quantification of these data showed a significant reduction in vessel density in fes-null tumor stroma (P = 0.0251; Fig. 3A). When the percentages of vessels of different sizes were compared, we found that the reduced vascularity was not associated with any particular size of vessels (Fig. 3B).

To further quantify the vascular content of tumors and associated stroma, we next performed quantitative immunoblotting analysis using PECAM-1 antibody. Tumors were allowed to grow for 7 days after AC2M2 cell injection. Lysates were then prepared from dissected tumors (stroma-free), the tumor-associated stroma (tumor-free), as well as from uninvolved mammary glands of tumor-bearing mice or mammary glands from non–tumor-bearing mice. PECAM-1 immunoreactivity was significantly reduced in tumors (P = 0.0124; Supplementary Fig. S4A and Fig. 4) and tumor-associated stroma (P = 0.0284; Supplementary Fig. S4B and Fig. 4) from fes-null mice relative to wild-type mice. There was also a trend toward lower PECAM-1 levels in fes-null uninvolved mammary glands from tumor-bearing mice (P = 0.0580; Supplementary Fig. S4C and Fig. 4) and mammary glands from non–tumor-bearing control mice (P = 0.1233; Supplementary Fig. S4D and Fig. 4), but these differences were not statistically significant.

Reduced involvement of tumor-associated macrophages in fes-null mice

Another Fes-expressing cell type that has been implicated in regulating tumorigenesis is the macrophage. In particular, alternatively activated M2-like macrophages are implicated in promoting tumor-associated angiogenesis and metastasis (24–26). We therefore examined the involvement

Figure 2. Reduced metastasis in fes-null mice. AC2M2 cells were engrafted into wild-type and fes-null mice and allowed to grow for 22 days. Tumors were then removed to promote tumor growth at metastatic sites and lungs were removed for biophotonic imaging of metastatic lesions 13 days later. A, significantly more metastatic lesions per lung were observed in wild-type relative to fes-null mice lungs (P = 0.0055; n = 19 for each group). B, the size of metastatic lesions (as assessed by pixel numbers per lesion) in fes-null mice appeared somewhat smaller than in wild-type mice, but this difference did not reach statistical significance (P = 0.1215).

Figure 3. Reduced vascularity in fes-null tumor stroma. AC2M2 cells were engrafted into wild-type and fes-null mice. On day 7 postinjection, tumor-bearing mammary glands were dissected, fixed, and paraffin embedded. Five micrometers of tissue sections were deparaffinized and stained with PECAM-1 antibody. Composite images of the stromal tissues surrounding each tumor were assembled and fluorescent pixels in this tumor boundary area were quantified using Image Pro software. The density (A) and size distribution of the blood vessels (B) were determined. Significantly fewer vessels per mm² were present in fes-null tumor stroma (P = 0.0251), but there was no difference in the size distribution of these vessels (<50 pixels, P = 0.3562; 50–100 pixels, P = 0.4400; 100–500 pixels, P = 0.1226; 500–1000 pixels, P = 0.2371; 1000–3000 pixels, P = 0.2222; >3000 pixels, P = 0.0790; n = 4 for each group).
cell migration and invasion

Fes-null promote their invasive potential (20, 31, 32). We therefore associated macrophages are thought to promote angiogenesis in human breast cancer (27). Alternatively activated tumor-relate with increased neovascularization and poor prognosis as associated macrophage content in fes-null mammary glands (Supplementary Fig. S4). Uninvolved glands from tumor-bearing mice as well as mammary glands from nontumor bearing control mice were also assessed (n = 4 for each group). Significantly lower PECAM-1 levels were observed in both tumors (P = 0.0124) and stroma (P = 0.0284) from fes-null mice. Trends toward reduced PECAM-1 levels in fes-null mice were also observed in uninvolved mammary glands from tumor-bearing mice (P = 0.0580) and mammary glands from nontumor bearing control mice (P = 0.1233), but these were not statistically significant.

of macrophages in this engraftment model. Macrophages were initially detected in vivo by taking advantage of their propensity to phagocytose dextran beads. Tetramethylrhodamine-conjugated beads were injected i.v. into tumor-bearing mice 13 days after engraftment of GFP-expressing AC2M2 cells. Tumor-bearing mammary glands were removed 2 hours later and imaged by confocal fluorescence microscopy. In wild-type mice, large numbers of dextran-labeled macrophages were clearly observed at the tumor front and within the tumor (Supplementary Fig. S5). In contrast, few macrophages were seen in fes-null tumors and associated stroma.

Because Fes-deficiency may have compromised macrophage phagocytosis of dextran beads (14), we also used surface antigen labeling and flow cytometry to quantify macrophages in tumors and surrounding stromal tissues. Single-cell suspensions were prepared from intact tumor-bearing mammary glands removed 7 days after tumor cell injection and the suspensions were prepared from intact tumor-bearing mammary glands for each group. There were significantly fewer macrophages in fes-null mice 7 days posttumor cell injection. Cells were stained with PE-conjugated F4/80 antibody to identify macrophages and the relative percentages of stained cells were determined by flow cytometry. There were significantly fewer macrophages in tumor and associated stroma from fes-null relative to wild-type mice (P = 0.0369; n = 6 for both groups).

Fes-null macrophages are deficient in promoting tumor cell migration and invasion

Tumor-associated macrophages have been shown to correlate with increased neovascularization and poor prognosis in human breast cancer (27). Alternatively activated tumor-associated macrophages are thought to promote angiogenesis and metastasis, in part through paracrine interactions with vascular endothelial cells (28–30) and with tumor cells which promote their invasive potential (20, 31, 32). We therefore asked if fes-null macrophages were deficient in these properties. To examine this we cultured primary BMMs from wild-type or fes-null mice in isolation or in cocultures with MTLn3 rat mammary adenocarcinoma cells in a collagen matrix-based invasion assay model (20). BMMs from each genotype or MTLn3 cells invaded to the same degree when cultured in isolation. In cocultures, BMMs from both genotypes promoted the invasion of MTLn3 cells, but fes-null BMMs were significantly less effective in doing so (P = 0.005; Fig. 6). MTLn3 cells also promoted the invasion of BMMs in cocultures, but the fes-null BMMs were significantly less responsive (P = 0.002; Fig. 6).

Fes-deficiency in the lung niche does not compromise tumor cell invasion and initial growth rates at the metastatic site

Angiogenesis is believed to play a rate-limiting effect on tumor growth. Reduced tumor growth rates at the primary site in fes-null mice might therefore be due in part to roles for Fes in promoting vascular endothelial cell migration, proliferation, and establishment of new vessel structures. However, metastasis also involves interactions between tumor and endothelial cells, both at the level of escape from the primary site and extravasation at the metastatic site. To explore this latter event we injected GFP-expressing AC2M2 cells into the tail veins of wild-type and fes-null nu/nu mice and quantified the number and sizes of lung metastatic lesions that formed after 22 days using biophotonic imaging of resected lungs (Supplementary Fig. S6). No significant differences were seen, suggesting that Fes-deficiency in the lung microvascular bed did not influence the process of tumor cell adhesion and invasion through the endothelium, or the initial metastatic growth rate.

Figure 4. Reduced vascularity in tumors and associated stroma in fes-null mice. Wild-type and fes-null mice were engrafted with AC2M2 cells and tumors were allowed to grow for 7 days. Tumors were dissected away from associated stroma and separate lysates for each were analyzed by immunoblotting for vascular content using PECAM-1 as a marker for endothelial cells and RasGAP as a protein normalization control (Supplementary Fig. S4). Uninvolved glands from tumor-bearing mice as well as mammary glands from nontumor bearing control mice were also assessed (n = 4 for each group). Significantly lower PECAM-1 levels were observed in both tumors (P = 0.0124) and stroma (P = 0.0284) from fes-null mice. Trends toward reduced PECAM-1 levels in fes-null mice were also observed in uninvolved mammary glands from tumor-bearing mice (P = 0.0580) and mammary glands from nontumor bearing control mice (P = 0.1233), but these were not statistically significant.

Figure 5. Reduced macrophages in fes-null tumor-bearing mammary glands. Single-cell suspensions were prepared from tumor-bearing mammary glands of wild-type and fes-null mice 7 days posttumor cell injection. Cells were stained with PE-conjugated F4/80 antibody to identify macrophages and the relative percentages of stained cells were determined by flow cytometry. There were significantly fewer macrophages in tumor and associated stroma from fes-null relative to wild-type mice (P = 0.0369; n = 6 for both groups).
Fes deficiency in the niche compromises the escape of tumor cells from the primary tumor site

Paracrine signaling between tumor cells and macrophages at the primary tumor site may play an important role in metastasis by promoting the ability of tumor cells to gain access to the vasculature (31–33). Reduced metastasis in fes-null mice (Fig. 2) coupled with observations suggesting that Fes may potentiate paracrine signaling between macrophages and tumor cells contributing to mutually enhanced invasive potential (Fig. 6) led us to examine the number of circulating tumor cells (CTC) in this engraftment model. GFP-expressing AC2M2 cells were injected into the mammary glands of wild-type and fes-null nu/nu mice and tumors were allowed to grow for 22 days. Peripheral blood was then assessed for the presence of CTCs by flow cytometry (Fig. 7). A significantly higher percentage of GFP⁺ nucleated cells was detected in the blood of tumor-bearing wild-type relative to fes-null mice (P = 0.047; Fig. 7A). Although tumor sizes were smaller in all but one of the fes-null mice at the time of analysis, there was no apparent correlation between levels of CTCs and tumor sizes in the wild-type or fes-null mice (Fig. 7B).

Discussion

Mammalian Fes and orthologous avian Fps were initially discovered as viral oncogenic PTKs encoded by tumor-causing retroviruses (5, 6). Subsequent studies demonstrated the transforming and tumorigenic potential of those viral Fes/Fps oncoproteins (7). Collectively, those observations argued that cellular Fes, when activated by mutation or inappropriately overexpressed, could promote tumorigenesis through dominant acting tumor cell autonomous functions. We were therefore surprised to find that ectopic overexpression of wild-type, kinase-activated, or kinase-dead cellular Fes had no apparent effect on the in vivo tumorigenic potential of the tumor cell line used in these engraftment studies. It should also be noted that we were unable to detect endogenous Fes expression in AC2M2 cells, and Fes does not appear to be expressed in any of the breast cancer cell lines we have examined (data not shown); nor has its expression in breast cancer been reported in the literature, to our knowledge.

Considering that Fes is also expressed in vascular endothelial and myeloid lineages, we next explored the possibility that it might influence tumorigenesis through effects on cells in the tumor niche. We had previously shown that transgenic expression of an activated fes allele correlated with hyper-vascularity, and this led to the original observation of Fes...
expression in vascular endothelial cells (11). Subsequent studies have linked Fes activation to signaling in endothelial cells downstream of angiogenic growth factors including VEGF, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF; refs. 12, 21–23). Using PECAM-1 expression as a marker of endothelial cells, we observed a significant reduction in the vascularity of tumors and associated stroma in fes-null mice. There was also a trend toward reduced vascularity in unaffected mammary glands in tumor-bearing mice as well as in glands of control mice; consequently, reduced angiogenesis in fes-null mice may not be strictly tumor-associated. However, these latter reductions were not statistically significant, suggesting that tumor-associated angiogenic signaling may be more sensitive to Fes deficiency. Because angiogenesis is linked to tumor growth and metastasis, these observations were consistent with the observed differences in growth rate and lung metastasis. Thus, reduced tumorigenesis in fes-null mice may be, at least in part, due to loss of proangiogenic Fes signaling functions intrinsic to vascular endothelial cells, and could reflect differential responsiveness to tumor-produced paracrine acting factors including VEGF, PDGF, and FGF.

Other cells of the niche can interact in a paracrine fashion with the endothelium and with tumor cells to promote tumor growth and metastasis. Fes is also expressed in hematopoietic cell types, including platelets (34), mast cells, (35) granulocytic cell types, (36) and macrophages (16). Although we cannot rule out any particular cell type, we focused our attention on macrophages because of the high level of Fes expression in these cells and accumulating evidence linking them to tumorigenesis (24, 28–30, 33, 37–39). Given our observation of decreased numbers of CTCs in fes-null mice, we were particularly intrigued with the recent report correlating tumor cell interactions with macrophages and endothelial cells with clinical metastasis in breast cancer (39). In vitro co-culture experiments revealed a significant defect in the ability of fes-null bone marrow derived macrophages to promote tumor cell invasion into collagen I gels. Furthermore, the tumor cell-induced invasive properties of the macrophages were compromised in Fes-deficient cells. These observations strongly argue that Fes plays roles in metastasis by promoting paracrine interactions between macrophages and tumor cells. However, it should be considered that BMMs in these coculture collagen gel invasion studies, so their precise phenotype may not be expected to precisely recapitulate that of tumor-associated macrophages in vivo. Furthermore, the in vivo niche contains many other cytokines, growth factors, and extracellular matrix components, as well as many other cell types, including fibroblasts, lymphocytes, and vascular and lymphatic endothelial cells. Further studies are required to elucidate the roles of Fes in differentiation of monocyte progenitors into specialized macrophage phenotypes and the ability of these cells to interact with the in vivo tumor niche.

Classically, activated inflammatory M1-like macrophages are induced by IFN-γ as well as other factors including lipopolysaccharide (LPS), TNFα, and granulocyte macrophage colony stimulating factor (GM-CSF); and they mediate resistance to microbial infections as well as antitumorigenic properties (reviewed in ref. 40). Alternatively, activated M2-like macrophages are induced by interleukin (IL)-4 and IL-13; and in response to other cytokines, including TGF-β, IL-10, and CSF-1, they acquire tumor-promoting properties, and also suppress inhibitory M1 macrophage functions (reviewed in ref. 41). We observed fewer phagocytic F4/80+ macrophages in fes-null tumor-associated stroma, and we speculate that tumor promoting M2-like macrophages may be reduced in fes-null tumor stroma.

Fes-deficient mice had slightly reduced numbers of circulating myeloid cells and slightly increased numbers of GM-CSF induced colony-forming unit granulocyte-macrophage colonies in methylcellulose assays (13, 16). These and other studies have implicated Fes in hematopoietic differentiation along the granulocyte–monocyte lineage. However, there have been no studies that specifically addressed the effects of Fes deficiency on macrophage polarization into M1- versus M2-like phenotypes. However, in vitro studies have shown that activated Fes can promote differentiation of bi-potential U937 cells into macrophages at the expense of the alternative granulocytic fate (42) and can also promote survival and granulocytic differentiation of 32D cells upon IL-3 removal (43). These observations indicate subtle roles for Fes in regulating myeloid differentiation that merit further analysis. Perhaps of more relevance to this study, it will be important to determine if Fes modulates the response of monocytes to IFN-γ or IL-4/IL-13, which drive M1- or M2-like polarization, respectively (25, 44). Based on our observations, we speculate that Fes-deficient macrophages will be relatively refractory to IL-4– or IL-13–induced M2-like polarization. Previous studies reported that Fes interacts with the IL-4Rα chain in B cells, and potentiates recruitment of PBK to IRS2 (45, 46) However, we are unaware of reports linking Fes to IL-4/IL-13 signaling in macrophages. We also speculate that Fes-deficient macrophages will be hypersensitive to M1-polarization. We have previously shown that fes-null mice display hyperinflammatory responses to LPS (13), which were further characterized in vivo by increased leukocyte recruitment to locally inflamed tissues and increased levels of systemic TNFα (47). Cultured fes-null macrophages displayed prolonged LPS-induced activation of NFκB, increased TNFα production, and reduced internalization of the TLR4 receptor complex (14). It will be important to determine if Fes-deficiency promotes an M1-like polarization at the expense of M2 macrophages. In that case, Fes inhibition might not only interfere with tumor-promoting functions of M2 polarized macrophages, but might also promote the M1-based antitumor functions. In our current working model, Fes-deficiency may skew macrophages toward a proinflammatory M1 polarity that could contribute to tumor initiation. This might have played a role in the earlier tumor onset seen in the Fes-deficient MMTV-PymT transgenic mouse model of mammary tumorigenesis (9). At later stages of tumorigenesis where tumor associated macrophages tend to skew more towards a protumorigenic M2 polarized state, Fes-deficiency might attenuate that process, leading to reduced tumor progression, as observed in the orthotopic engraftment model used in this study.
In summary, our observations suggest that systemic inhibition of Fes might provide antitumorigenic benefits in cancer by inhibiting endothelial cell intrinsic angiogenic signaling as well as inhibiting the tumor promoting properties of tumor-associated macrophages.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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