Angiogenesis by Fibroblast Growth Factor 4 Is Mediated through an Autocrine Up-Regulation of Vascular Endothelial Growth Factor Expression

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Angiogenesis by Fibroblast Growth Factor 4 Is Mediated through an Autocrine Up-Regulation of Vascular Endothelial Growth Factor Expression[1]

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ABSTRACT

The infection of normal mouse mammary EF43 cells by a retroviral vector carrying either *Fgf-3* (EF43.*Fgf-3*) or *Fgf-4* (EF43.*Fgf-4*) cDNA resulted in the transformation of cells displaying different tumorigenic potentials in nude mice (A. Hajitou and C-M. Calberg-Bacq, Int. J. Cancer, *63:* 702–709, 1995). EF43.*Fgf-4* produced rapidly developing tumors at all sites of inoculation, whereas EF43.*Fgf-3* produced slowly growing tumors only in the mammary fat pad. Cells infected with the vector carrying the selection gene alone (EF43.C) were not tumorigenic. The angiogenic properties of these cells were tested in an *in vitro* angiogenesis model using human umbilical vein endothelial cells (HUVECs) cultured at the surface of a type I collagen gel and their capacity to form tube-like structures on invasion of the gel. Only the conditioned medium (CM) of EF43.*Fgf-4* induced an angiogenic morphotype in HUVECs. In parallel, the mRNA expression of matrix metalloproteinase 1 and c-ETS-1 was increased in the HUVECs displaying a differentiated phenotype, whereas the tissue inhibitor of matrix metalloproteinase 1 mRNA level was decreased. Recombinant human fibroblast growth factor 4 (FGF-4) did not induce an angiogenic phenotype in HUVECs by itself. By Western blot analysis, a high expression of vascular endothelial growth factor (VEGF) was detected in the EF43.*Fgf-4* CM. This result was confirmed by Northern blot analysis of total RNA extracted from the three cell types; the steady-state level of VEGF mRNA was low and equivalent in EF43.C and EF43.*Fgf-3,* whereas it was strongly increased in EF43.*Fgf-4.* Culturing EF43 cells carrying only the selection gene with increasing concentrations of recombinant human FGF-4 resulted in a dose-dependent stimulation of VEGF. The induction of the angiogenic morphotype and the parallel modulations of the biosynthetic phenotype in HUVECs were completely suppressed by adding a neutralizing antibody directed against VEGF to EF43.*Fgf-4* CM. Furthermore, inhibition of protein kinase C by bisindoylmaleimide suppressed the angiogenic phenotype induced by the CM of EF43.*Fgf-4.* Our results point to an indirect angiogenic activity of FGF-4 through the autocrine induction of VEGF secretion by EF43.*Fgf-4* cells, an original signaling pathway that might be significant in tumor progression and metastasis.

INTRODUCTION

FGFs[3] form a family of structurally related heparin-binding polypeptides encoded by at least 14 different genes (1–3) among which products FGF-1 to FGF-9 have been characterized (4–6). Unlike FGF-1, FGF-2, and FGF-9, which lack a signal peptide, the other FGFs are secreted. The FGFs and their transmembrane receptors are essential during development (reviewed in Ref. 6), and the expression of each member of the family and its receptors is spatiotemporally and tightly regulated. Except for FGF-1 and FGF-2, which are ubiquitous, and FGF-7, which is present in various epithelia, the other FGFs are not produced or very weakly produced in normal adult tissues. When ectopically expressed, they may be oncogenic. *Fgf-4, Fgf-5,* and *Fgf-6* were originally isolated as genes able to transform NIH3T3 cells in culture (7). *Fgf-3* has been identified as a main target of mouse mammary tumor virus and is involved in mouse mammary tumorigenesis (8). *Fgf-4* is also activated by proviral insertion in mouse mammary tumors (9), although much less frequently than *Fgf-3* (10). *Fgf-4* expression has been associated with progression toward a metastatic phenotype in mouse mammary carcinogenesis (11). There is a strong selection for the activation of these two genes in cooperation with *Wnt-1* oncogene expression in the induction of mouse mammary tumors (12).

In a previous work, we infected normal mouse mammary EF43 cells (13) recently identified as myoepithelial cells[4] with retroviral vectors carrying either *Fgf-3* (EF43.*Fgf-3*) or *Fgf-4* (EF43.*Fgf-4*) or the selection gene alone as control (EF43.C; Ref. 14). The parental EF43 as well as the EF43.C cells were not tumorigenic when inoculated in nude mice. By contrast, the EF43.*Fgf-4* cells induced rapidly developing and aggressive tumors, whatever the site of injection. The EF43.*Fgf-3* cells produced slowly developing tumors after a lag period of quiescence, but only after injection in the mammary fat pad.

In vivo, the switch from a quiescent tumor to an invasive neoplasm is accompanied by neovascularization, or angiogenesis, a process essential for tumor expansion (15, 16). The rapid development of the EF43.*Fgf-4*-induced tumors was evocative of a sustained growth, probably through a neovascularization process. In this work, we analyze the angiogenic capacities of EF43 cells producing either FGF-3 or FGF-4 by testing the medium conditioned by these cells in culture. The *in vitro* angiogenesis model used HUVECs and their capacity to invade a type I collagen gel and to form pseudocapillary structures on angiogenic stimulation (17, 18). Our results demonstrate an indirect angiogenic activity through the autocrine induction of VEGF in EF43.*Fgf-4* but not in EF43.*Fgf-3* cells.

MATERIALS AND METHODS

Cell Cultures and CM. HUVECs were isolated according to Jaffe *et al.* (19), as described previously (20). The cells were cultured on gelatin-coated tissue culture dishes in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 5% HDS, 5% FCS (Life Technologies, Inc.), 20 μg/ml endothelial cell growth factor (Boehringer Mannheim, Mannheim Germany), 50 μg/ml heparin, 10 mM HEPES, penicillin (100 units/ml), and streptomycin (100 μg/ml) and used at passage 2. The endothelial origin of the cells was assessed as described previously (20). Endothelial cell growth factor and heparin supplementation was suppressed when performing the experiments.

Mouse mammary cells were derived from the EF43 cell line (13) by infection with retroviral vectors carrying *Fgf-3* (EF43.*Fgf-3*), *Fgf-4* (EF43.*Fgf-4*), or the empty vector as control (EF43.C), as described previously (14). The cells were cultured in DMEM (Life Technologies, Inc.) containing 10% FCS, 5 ng/ml mouse EGF (Sigma, Deisenhofen, Germany), 1 μg/ml bovine insulin (Boehringer Mannheim), penicillin (100 units/ml), and streptomycin (100 μg/ml).

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[3] The abbreviations used are: FGF, fibroblast growth factor; BIM, bisindoylmaleimide; CM, conditioned medium; EGF, epidermal growth factor; FCS, decomplemented FCS; HDS, heat-decomplemented pooled human serum; HUVEC, human umbilical vein endothelial cell; MMP, matrix metalloproteinase; PKC, protein kinase C; TIMP, tissue inhibitor of matrix metalloproteinase; VEGF, vascular endothelial growth factor; rh, recombinant human.

[4] A. Hajitou and C-M. Calberg-Bacq. FGF-3 and FGF-4 elicit distinct oncogenic properties in mouse mammary myoepithelial cells, submitted for publication.

For preparing CM, cultures of the three infected cell types were seeded at specific cell densities to take into account the growth rate of each cell type to obtain a similar number of cells in the dish at the time of CM collection. The cultures were washed three times with serum-free RPMI 1640 with a 2-h incubation for the last wash and then incubated in the same medium (6 ml; 5×10^6 cells). The CM was collected after 24 h, centrifuged to remove cell debris, passed through a 0.22-μm filter, and stored at 4°C.

***In Vitro* Angiogenic Assay.** The *in vitro* angiogenic assay based on the model of Montesano and Orci (17) was essentially performed as described previously (18). Briefly, HUVECs suspended in 5% FCS, 5% HDS, and 90% RPMI 1640 were seeded on a 0.4-mm-thick collagen gel (6×10^4 cells/cm^2) and allowed to attach and spread for 3 h. The gel was made of RPMI 1640 containing 1 mg/ml purified type I collagen and was allowed to polymerize for 10 min at 37°C (20). After spreading of the cells, the medium was replaced by a mixture containing 90% CM, 5% FCS, and 5% HDS and was renewed daily. In some assays, a blocking polyclonal antibody directed against the rh 165-amino acid form of VEGF (rhVEGF$_{165}$; Chemicon, Temecula, CA; AB1442) was added to the CM of EF43.*Fgf-4* at 10 μg/ml and preincubated for 1 h at room temperature before renewal of the medium. Rabbit IgG directed toward an irrelevant antigen at the same concentration was used as control. When needed, rhFGF-2 (Boehringer Mannheim), rhFGF-4 (ICN Pharmaceuticals, Costa Mesa, CA), or rhVEGF$_{165}$ (Sigma) was added to the RPMI 1640.

Histological Analysis. HUVECs cultured on a collagen gel for 8 days in the presence of EF-43.*Fgf-4* CM as described above were fixed with 3% paraformaldehyde in PBS for 2 h, washed overnight in PBS supplemented with 6.8% sucrose, dehydrated with acetone, and embedded in methacrylate, with all steps being carried out at 4°C. Serial sections of 2 μm were stained with propidium blue.

Western Blot Analysis of VEGF. The analysis was performed on 1 ml of CM dialyzed overnight against 100 ml of 200 mM ammonium acetate, lyophilized, and electrophoresed under nonreducing conditions on a 15% SDS-polyacrylamide gel according to the technique of Laemmli (21). Western blotting was performed as described by Towbin *et al.* (22) using a polyclonal antibody antirecombinant human VEGF$_{165}$ (Chemicon; AB1442) and, as secondary antibody, peroxidase-conjugated swine antirabbit IgG (Dako, Copenhagen, Denmark; P0217). Peroxidase was revealed by the enhanced chemiluminescence assay (Amersham Corp.).

Western Blot Analysis of FGF-4. The analysis was performed on 5 ml of CM partially purified on heparin-sepharose as described by Souttou *et al.* (23). Western blotting was performed as described previously using a rabbit anti-FGF-4 generously provided by Dr. C. Basilico (New York University School of Medicine, New York, NY).

Northern Blot Analysis. Total RNA was purified according to Chirgwin *et al.* (24) with slight modifications, as described previously (25). When required, the collagen gel was digested by incubation with 0.1 ml of 1% bacterial collagenase (Sigma, C-9891) in RPMI 1640 for 10 min at 37°C, and the cells were harvested by centrifugation at 550 × *g* for 5 min. Northern blot analysis was performed as described previously (25). Human probes were insert fragments of plasmids Gem 3collK4 for MMP1 (26), pTIMP for TIMP-1 (27), Gem-β-actin for β-actin, pH3a for MMP2 (28), pSS38 for TIMP2 (29), and ETS-1 for c-ETS-1 (30). An end-labeled oligonucleotide specific for human and mouse 28S recombinant RNA (Promega) was used to monitor the amount of loaded RNA. The probe for murine VEGF was a complete VEGF$_{164}$ cDNA generously provided by P. D'Amore (Laboratory for Surgical Research, Children's Hospital, Boston, MA). All probes were labeled using [^{32}P]dATP and [^{32}P]dCTP (ICN Pharmaceuticals Inc.; 3000 Ci/mmol) and a random-primed DNA labeling kit (Boehringer Mannheim). Prehybridization was performed in 50% formamide, 2 × SSC, 5 × Denhardt's solution, 0.1% SDS, and 5% dextran sulfate. Hybridization was performed for 16 h in the same mix at 42°C for all the probes except for MMP1, which required 52°C. The signal intensities were measured by laser scanning densitometry (Ultroscan LKB, Uppsala, Sweden).

RESULTS

Induction of an Angiogenic Phenotype in HUVECs by CM from EF43.*Fgf-4*. HUVECs plated on a collagen gel were cultured in presence of CM from the three types of infected mouse mammary cells to examine whether soluble factors produced by cells presenting different tumorigenic potentials *in vivo* could affect the behavior of endothelial cells in the *in vitro* angiogenic assay.

In control RPMI 1640, HUVECs seeded at the surface of a type I collagen gel attached, spread, and proliferated to form a monolayer presenting, at confluence, a typical cobblestone-like morphology (Fig. 1*a*). In the presence of CM from EF43.C (Fig. 1*b*) or from EF43.*Fgf-3* (Fig. 1*c*), the HUVECs maintained their typical monolayer pattern for up to 14 days. They remained superficial and never invaded the gel. In the presence of CM from EF43.*Fgf-4*, the morphology of the culture was disturbed by a progressive loss of the uniform cobblestone-like pattern and by the acquisition by the cells of a spindle-shape morphology (Fig. 1*d*). After 6–9 days of culture in daily renewed CM of EF43.*Fgf-4*, an appreciable number of HUVECs

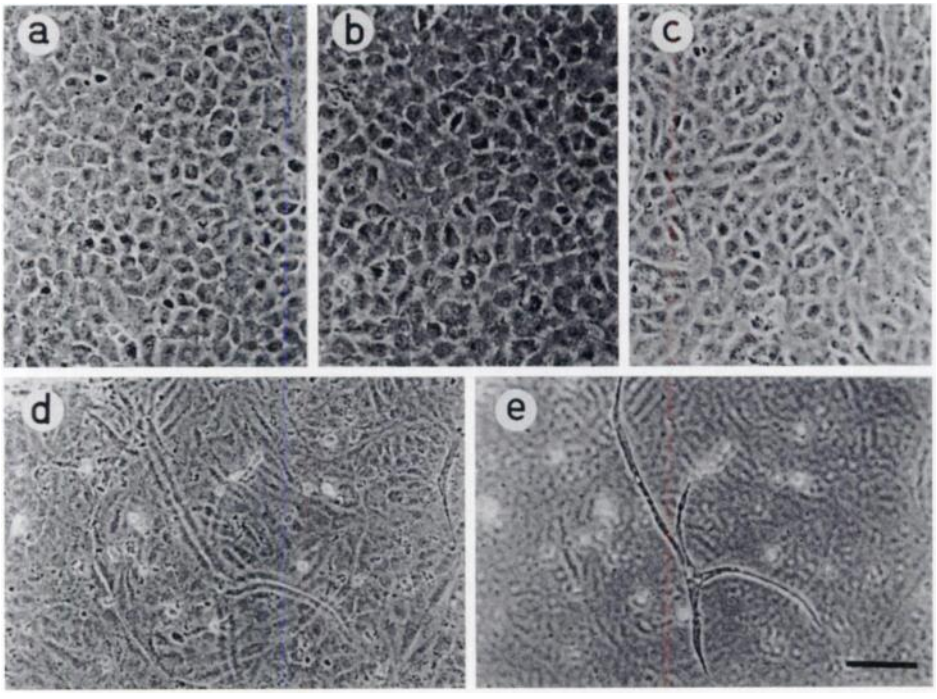

Fig. 1. Phase-contrast micrographs of HUVECs cultured on a collagen gel for 8 days with unconditioned medium (*a*) and with medium conditioned by EF43.C (*b*), by EF43.*Fgf-3* (*c*), and by EF43.*Fgf-4* (*d*). *e*, photograph of the same field as in *d* but focused on a branching tube-like structure 20 μm under the HUVEC monolayer. *Bar*, 25 μm.

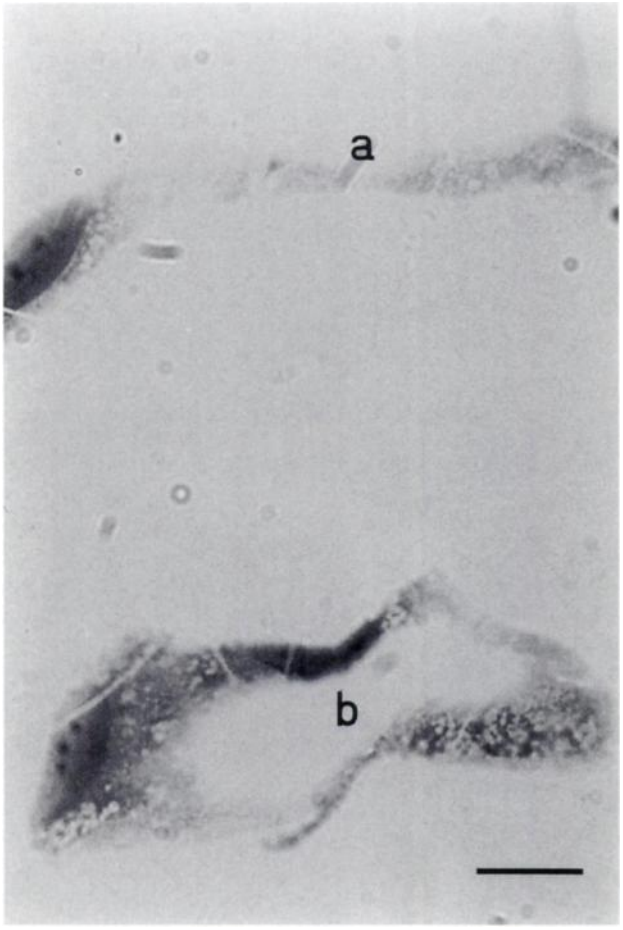

Fig. 2. Transverse section of HUVECs cultured on a collagen gel for 8 days with medium conditioned by EF43.*Fgf-4* shows the monolayer of cells at the surface of the collagen gel (*a*), and cells within the collagen gel associated into tube-like structures and delineated a lumen (*b*). *Bar*, 4 μm.

migrated within the collagen gel and organized into elongated and branched structures as observed by phase-contrast (Fig. 1, *d* and *e*). Histological examination (Fig. 2) revealed a continuous monolayer of HUVECs at the top of the collagen gel, and HUVECs associated into tube-like structures and delineated a lumen inside of the gel.

Modulation of the HUVEC Phenotype by the CM of EF43.*Fgf-4*. The phenotype of the HUVECs was evaluated by measuring the steady-state level of mRNA coding for proteolytic enzymes involved in the extracellular matrix degradation (MMP1 and MMP2), their physiological inhibitors (TIMP1 and TIMP2), and ETS-1, a recognized transcription factor playing a role in *in vitro* and *in vivo* angiogenesis (31, 32). Total RNA was extracted from HUVECs cultured for 8 days in the presence of CM from the three infected cell types. HUVECs maintained in the CM of EF43.C did not differ from the cells in contact with the CM of EF43.*Fgf-3* in the expression of MMP1, TIMP1, or c-ETS-1 (Fig. 3, *Lanes 1* and *2*). The angiogenic phenotype induced by CM of EF43.*Fgf-4* was associated with an increased expression of the mRNA coding for MMP1 (Fig. 3, *Lane 3*) and a decreased expression of the mRNA coding for its physiological inhibitor, TIMP1 (Fig. 3, *Lane 3*), resulting in a MMP1:TIMP1 mRNA ratio increased by a factor of 20. A significant increase in c-ETS-1 mRNA expression was also observed in this condition (Fig. 3, *Lane 3*), whereas the mRNA level for MMP2, TIMP2, and β-actin, which were all constitutively expressed, was not significantly modulated in any of the culture conditions (Fig. 3). This pattern of expression of mRNA represents additional support for the differentiated phenotype of the HUVECs under the activity of CM of EF43.*Fgf-4*.

Induction of VEGF Expression by FGF-4. rhFGF-4 added to HUVECs at a concentration as high as 50 ng/ml did not induce any morphological change in the HUVECs cultured on a collagen gel (data not shown), in agreement with its lack of angiogenic properties as demonstrated *in vivo* and *in vitro* by Jouanneau *et al.* (33). This observation suggested that another factor or factors present in the CM of EF43.*Fgf-4* might be responsible for the angiogenic properties. We tested rhFGF-2, one of the first described angiogenic factors. It was less efficient in the induction of tube-like structures in our experimental model, even at a concentration as high as 20 ng/ml, whereas $rhVEGF_{165}$ proved to be a potent stimulator (Fig. 4).

Western blot analysis revealed the actual presence of VEGF in the CM of EF43.*Fgf-4* (Fig. 5*A*), whereas in the CM of EF43.C and EF43.*Fgf-3*, it was nearly undetectable for a similar amount of proteins loaded in each lane, as shown by Coomassie Blue staining of samples run in parallel (Fig. 5*B*). The VEGF detected in the EF43.*Fgf-4* CM is most likely the 164-amino acid murine form, because it migrates at the same position as $rhVEGF_{165}$. This result was confirmed by measuring the steady-state level of $VEGF_{164}$

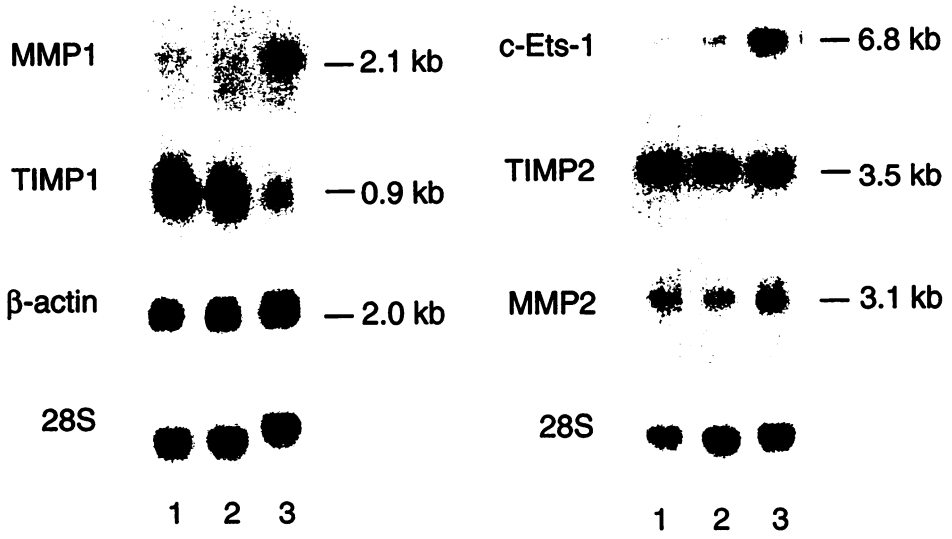

Fig. 3. Northern blot analysis of mRNA steady-state levels. Total RNA was extracted from HUVECs cultured on a collagen gel for 8 days with medium conditioned by EF43.C (*Lanes 1*), EF43.*Fgf-3* (*Lanes 2*), and EF43.*Fgf-4* (*Lanes 3*). The 28S rRNA was hybridized on the same membranes with a specific oligonucleotide, as detailed in "Materials and Methods," to normalize the amount of total RNA loaded in each lane.

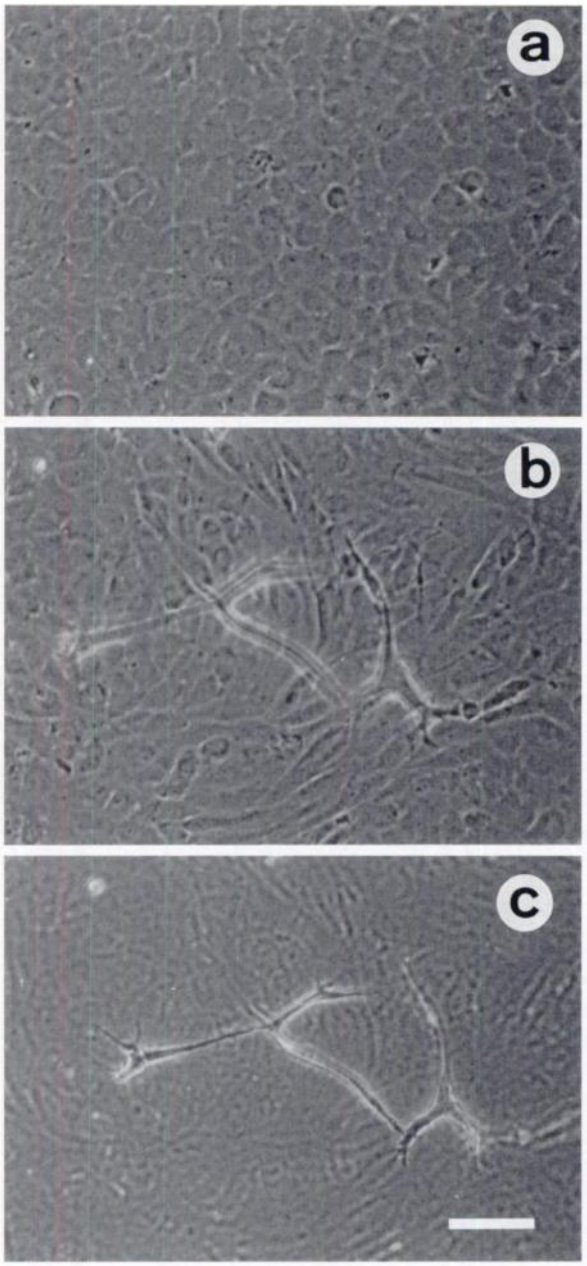

Fig. 4. Phase-contrast micrographs of HUVECs cultured on a collagen gel for 6 days with control medium (*a*) or with 20 ng/ml rhVEGF$_{165}$ (*b*). *c*, photograph of the same field as in *b* but focused on a branching tube-like structure 15 μm under the HUVEC monolayer. *Bar*, 25 μm.

mRNA in the three cell types by Northern blot analysis. Its level was low in EF43.C and in EF43.*Fgf-3* and was strongly enhanced in EF43.*Fgf-4* (Fig. 6). Moreover, culturing EF43.C in the presence of increasing concentrations of rhFGF-4 for 48 h and then conditioning the medium for 24 h as described in "Materials and Methods" resulted in a dose-dependent induction of VEGF$_{164}$ secretion already detectable by Western blot analysis in CM supplemented by 10 ng/ml rhFGF-4 (Fig. 7).

The Angiogenic Phenotype of HUVECs Induced by EF-43.*Fgf-4* Is Mediated by VEGF. Preincubation of the CM of EF-43.*Fgf-4* with a VEGF neutralizing antibody for 1 h abolished the induction of the angiogenic morphotype in the HUVECs. Under these conditions, cells multiplied until confluence but did not invade the collagen gel and kept the cobblestone-like morphology throughout the dish (Fig. 8*b*). A similar treatment performed with an irrelevant antibody at the same IgG concentration did not affect the induction of tube-like structures by the CM of EF-43.*Fgf-4* (Fig. 8*a*).

The phenotypic changes induced by the CM of EF43.*Fgf-4*, *i.e.*, an increased level of MMP1 and c-ETS-1 mRNAs and the reduced expression of TIMP1 mRNA, were suppressed by the neutralizing anti-VEGF antibody, whereas the irrelevant antibody had no effect (Fig. 9).

The EF43.*Fgf-4*-induced Angiogenic Morphotype Is Mediated through a PKC-dependent Pathway. BIM added to the CM of EF43.*Fgf-4* completely suppressed the formation of tube-like structures. In the presence of this PKC inhibitor, the monolayer pattern was maintained for up to 14 days, and no cell was seen invading the collagen gel (Fig. 10*A*). In spite of the abolition of the angiogenic morphotype by BIM, the accompanying increased mRNA steady-state level of both MMP1 and c-ETS-1 was not suppressed, whereas the reduced expression of TIMP1 reverted to a high level (Fig. 10*B*). This suggested that induction of the angiogenic morphotype and up-regulation of MMP1 and c-ETS-1 are mediated through distinct signaling pathways. By contrast, down-regulation of TIMP1 introduced by the CM of EF43.*Fgf-4* is PKC dependent.

DISCUSSION

The induction and maintenance of tumor blood vessel supply are essential requirements for the growth of solid cancers and their dissemination. Tumoral angiogenesis has been related to the produc-

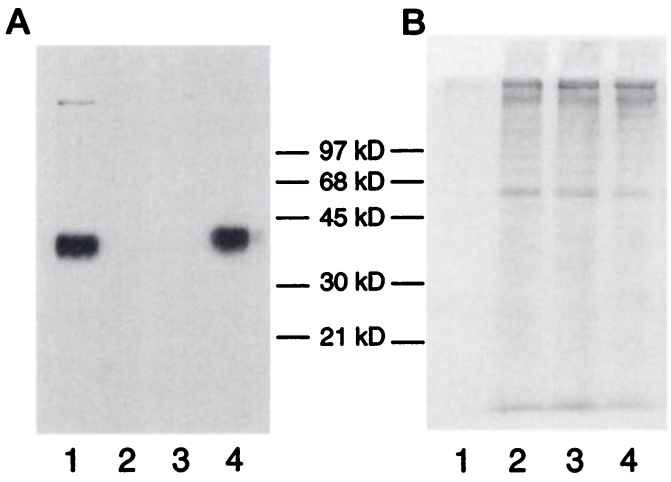

Fig. 5. *A*, Western blot analysis of medium conditioned by EF43.C (*Lanes 2*), EF43.*Fgf-3* (*Lanes 3*), and EF43.*Fgf-4* (*Lanes 4*). As a positive control, 20 ng of rhVEGF$_{165}$ were loaded in *Lanes 1*. *B*, Coomassie Blue staining of a SDS-PAGE run in parallel and loaded with the same samples as in *A*.

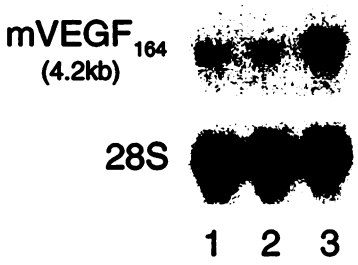

Fig. 6. Northern blot analysis of mRNA steady-state levels for mouse VEGF$_{164}$. Total RNA was extracted from EF43.C (*Lane 1*), EF43.*Fgf-3* (*Lane 2*), and EF43.*Fgf-4* (*Lane 3*). The 28S rRNA was hybridized on the same membrane with a specific oligonucleotide as detailed in "Materials and Methods" and was used to normalize the amount of total RNA loaded in each lane.

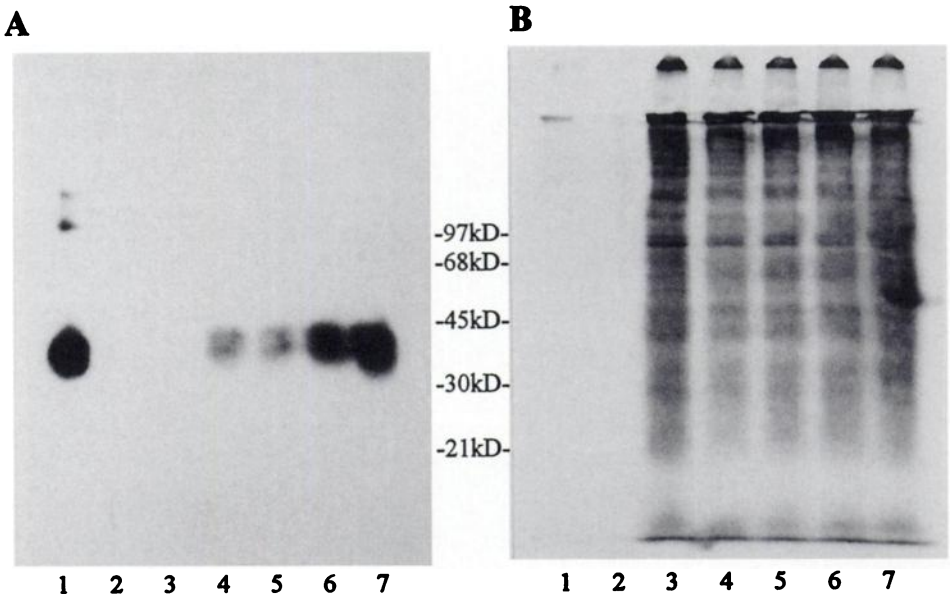

Fig. 7. *A*, Western blot analysis of medium conditioned by EF43.C cultured without (*Lanes 3*) or with 10 (*Lanes 4*), 20 (*Lanes 5*), 50 (*Lanes 6*) or 100 (*Lanes 7*) ng/ml rhFGF-4. $rhVEGF_{165}$ (20 ng/ml) was loaded in *Lanes 1*, and 100 ng of rhFGF-4 were loaded in *Lanes 2*. *B*, Coomassie Blue staining of a SDS-PAGE loaded with the same samples as in *A*.

tion of angiogenesis-promoting growth factors by tumor cells (15). In this work, the angiogenic potential of three mouse mammary cell types that express variable tumorigenic phenotypes was investigated. The cells (EF43) were derived from a clonal outgrowth in a mammary gland culture from a γ-irradiated BALB/c mouse (34) and were recently shown to be of myoepithelial origin.[4] As demonstrated in a previous work (14), the control EF43 cells (EF43.C) infected by the retroviral vector carrying only the selection gene are not tumorigenic. The EF43 cells carrying *Fgf-3* (EF43.*Fgf-3*) produce tumors developing with a significant delay and only when implanted in the mammary fat pad, whereas the EF43 cells carrying *Fgf-4* (EF43.*Fgf-4*) give rise to rapidly growing tumors independently of the inoculation site.

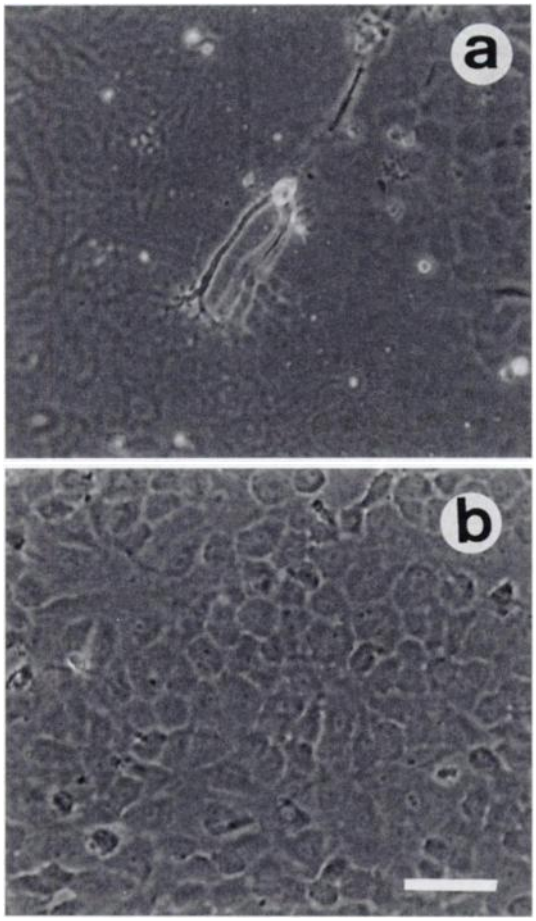

Fig. 8. Phase-contrast micrographs of HUVECs cultured on a collagen gel for 8 days with medium conditioned by EF43.*Fgf-4* in the presence of a control antibody (*a*). In *b*, HUVECs were cultured in the same conditioned medium but in the presence of a neutralizing anti-VEGF antibody. *Bar*, 25 μm.

The overexpression of growth factors is known to confer a tumorigenic potential to normal cells (7, 35, 36). The introduction of *Fgf-4* in various cell types of murine or human origin results in a high production of this growth factor, which clearly modifies their *in vitro* and *in vivo* phenotype and induces several significant hallmarks of transformation including morphological changes, proliferation in low serum conditions, growth in soft agar, and formation of solid tumors (23, 33, 35, 37). Such modifications of the *in vitro* and *in vivo* phenotype are expressed by EF43 cells carrying *Fgf-4*, indicating that they possess functional receptors allowing an autocrine regulation pathway and comply with the observation of Talarico and Basilico (38) that secretion is a prerequisite for *Fgf-4*-induced transformation. Our initial observation that the CM of the FGF-4-producing cells induced an angiogenic phenotype in HUVECs was consistent with the potent oncogenic activity of *Fgf-4* (7, 23, 39, 40). However, the addition of rhFGF-4 to HUVECs up to 50 ng/ml failed to induce an angiogenic phenotype in the HUVECs, a result in agreement with Jouanneau *et al.* (33), who showed that biologically active FGF-4 secreted by rat bladder carcinoma cells or recombinant FGF-4 did not induce tubulogenesis by bovine microvascular endothelial cells using an *in vitro* angiogenesis model similar to ours. rhFGF-4 has been shown to induce neovascularization *in vivo* in the chick chorioallantoic membrane and in rat cornea assays (39). In these *in vivo* assays, however, it is not known if the growth factor is acting directly or indirectly by inducing the expression of angiogenic factors. The *in vitro* mitogenic effect of FGF-4 on endothelial cells reported by Miyagawa *et al.* (41) and Delli Bovi *et al.* (42) addresses only a part of the complex angiogenesis process. The test used in the present study does not involve endothelial cell proliferation (17, 18). It mimicks selected events of the angiogenic process, *i.e.*, migration of endothelial cells into the perivascular stroma, cellular alignment, and

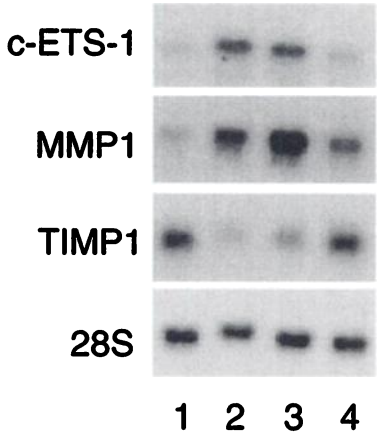

Fig. 9. Northern blot analysis of mRNA steady-state levels. Total RNA was extracted from HUVECs cultured on a collagen gel for 8 days with medium conditioned by EF43.*Fgf-3* (*Lane 1*) or EF43.*Fgf-4* (*Lanes 2–4*) in the presence of a control antibody (*Lane 3*) or a neutralizing anti-VEGF antibody (*Lane 4*). The 28S rRNA was hybridized on the same membrane with a specific oligonucleotide as detailed in "Materials and Methods" and was used to normalize the amount of total RNA loaded in each lane.

the generation of a patent lumen, a morphogenic process that is not induced directly by FGF-4. These results suggested that another factor present in the CM of EF43.*Fgf-4* was responsible for its angiogenic activity. The weak morphogenetic effect of FGF-2 in our *in vitro* angiogenesis assay as compared with recombinant VEGF and the extensive vascularization of FGF-4-induced tumors suggested that VEGF might be a good candidate. A number of growth factors and cytokines as EGF, FGF-7, platelet-derived growth factor-BB, tumor necrosis factor α, transforming growth factor β, or interleukin 1β have been reported to induce the expression of VEGF in a variety of cultured cells (43). Moreover, it was recently reported that the activation of oncogenic H-ras also stimulated the expression of VEGF mRNA (44). In addition, the correlation between the up-regulation of VEGF and malignant progression in a mouse skin carcinogenesis model (45), the association of VEGF expression with high microvessel density in primary breast carcinomas, and poor prognosis due to early relapse (46) are further elements supporting the relationship between VEGF in EF43.*Fgf-4* cells and their aggressive phenotype. Western blot analysis of CM collected from the three cell types demonstrated that $VEGF_{164}$ was only secreted by EF43.*Fgf-4* cells, whereas Northern blot analysis revealed that this overexpression was at least partially controlled at a pretranslational level through an increased steady-state level of its mRNA. These results suggested that $VEGF_{164}$ expression was induced in response to FGF-4 production through an autocrine loop in EF-43.*Fgf-4* cells. The up-regulated expression of $VEGF_{164}$, by adding recombinant FGF-4 to EF-43.C, the control cells carrying only the selection gene, is a direct argument supporting this mechanism. Cooperation between VEGF and another growth factor like FGF-2 (47) cannot be ruled out. However, the inhibition of tube-like structures formation and the suppression of associated phenotypic changes by a VEGF-neutralizing antibody clearly demonstrate that VEGF is the main factor responsible for the angiogenic events triggered by the EF43.*Fgf-4* cells. In contrast, FGF-3 overexpression in EF-43.*Fgf-3* cells reported by Hajitou and Calberg-Bacq (14) did not up-regulate VEGF, suggesting that this characteristic is not a common feature of all members of the FGF family. This could be related to the retardation of FGF-3 in the Golgi, even if the production of this protein was sufficient to confer EF43.*Fgf-3* cells with a tumorigenic phenotype (14). The lack of induction of an angiogenic phenotype in HUVECs by EF-43.*Fgf-3* cells could be the reason for the delayed development of tumors.

In parallel to the morphogenetic events resulting in the formation of tube-like structures, the EF43.*Fgf-4* CM induced in HUVECs an increased expression of the mRNA coding for MMP1 and c-ETS-1 and a decreased expression of the mRNA coding for TIMP1. An up-regulation of the MMP1 and c-ETS-1 gene expression in HUVECs

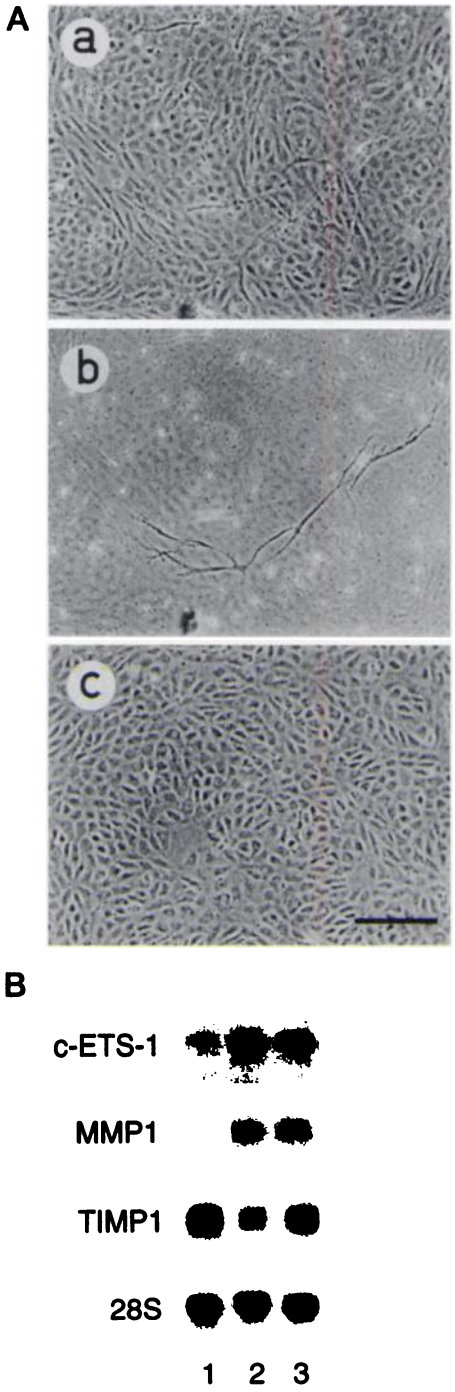

Fig. 10. *A*, phase-contrast micrographs of HUVECs cultured on a collagen gel for 8 days with medium conditioned by EF43.*Fgf-4*. In *a* and *c*, the microscope is focused on the gel. *b*, photograph of the same field as in *a* but focused on a branching tube-like structure 20 μm under the HUVEC monolayer. In *c*, HUVECs were cultured in presence of 2.5 μg/ml BIM. *Bar*, 50 μm. *B*, Northern blot analysis of total RNA extracted from HUVECs cultured in the presence of CM of EF43.C (*Lane 1*) or in the presence of CM of EF43.*Fgf-4* (*Lanes 2* and *3*) in the absence (*Lane 2*) or presence (*Lane 3*) of 2.5 μg/ml BIM. The 28S rRNA was hybridized on the same membrane with a specific oligonucleotide as detailed in "Materials and Methods" and was used to normalize the amount of total RNA loaded in each lane.

by VEGF has been described previously (32, 48). Degradation of type I collagen was indeed considered necessary for angiogenesis *in vitro* (49), and c-ETS-1, a known transactivating factor of MMP1, could participate in the up-regulation of this enzyme in HUVECs. EGF and FGF-2 were also reported to induce protease expression in endothelial cells through an overexpression of c-ETS-1 (32). This overexpression, mediated through a paracrine activity in our most tumorigenic cells, agrees with the observed correlation between peritumoral (fibroblasts and endothelial cells) expression of c-ETS-1 and tumor invasiveness (50). It has to be noted that the pathways leading to tubulogenesis and overexpression of the MMP1 and c-ETS-1 genes are at least partly divergent, because only the morphogenic events are inhibited by a PKC inhibitor. VEGF has indeed been reported to activate PKC-dependent phospholipase D, which could regulate cell migration involved in angiogenesis (51). A regulation of TIMP1 in HUVECs by VEGF has not been reported. The CM of EF-43.C and EF43.*Fgf-3* induced an increase of TIMP1 mRNA in HUVECs as compared to unconditioned medium (data not shown). This stimulation was not observed with the CM of EF43.*Fgf-4*, suggesting that VEGF is able to suppress this overexpression to the level of constitutive expression. The lack of modulation of MMP2 and TIMP2 mRNA steady-state levels in HUVECs agrees with similar observations of the effect of VEGF by Unemori *et al.* (48).

FGF-4 has been reported to induce the expression of 21 genes in NIH3T3 cells (52). Even if most of the induced genes are proliferation specific, the authors did not find an induction of VEGF, suggesting that the autocrine loop observed in our study could be cell-type specific. This hypothesis is also supported by the divergent results reported in the literature. *Fgf-4*-transfected human breast MCF7 cells produce tumors described as abundantly vascularized (35), as does the high producer clone HH9 isolated from *Fgf-4*-transfected HBL 100 (23). The tumors produced by our EF43.*Fgf-4* cells are also largely vascularized (data not shown). On the other hand, the rat bladder carcinoma cell line NBT-II, transfected with *Fgf-1* coupled to a signal peptide forms rapidly growing carcinomas that are highly vascularized, whereas the same cells transfected by *Fgf-4* and producing a biologically active form of FGF-4 induce slowly growing tumors as untransfected cells (33). The failure of some *Fgf-4*-transfected cells to display increased angiogenesis might be related to the panel of receptors expressed at the cell surface (53). It could also depend on the developmental origin of the cells, the mammary gland for EF43, MCF7, and HBL 100 and the urinary bladder for the nonresponsive NBT-II. Additional investigations are needed to define these determinants.

In a recent review, D'Amore and Shima (16) stated that tumor angiogenesis could be an example of a physiological process initiated by tumor growth-induced hypoxia. Our observations demonstrating that FGF-4 up-regulates VEGF expression in well-oxygenated *in vitro* conditions does not favor that proposition and would support the alternate hypothesis of the same authors stating that an event written in the genetic program of the tumor cells is the leading cause of the process. The autocrine loop of FGF-4 and the resulting induction of VEGF might represent that signaling pathway operating in tumor progression and metastasis.

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REFERENCES

1. Yamasaki, M., Miyake, A., Tagashari, S., and Itoh, N. Structure and expression of the rat mRNA encoding a novel member of the fibroblast growth factor family. J. Biol. Chem., *271:* 15918–15921, 1996.
2. Smallwood, P. M., Munoz-Sanjuan, I., Tong, P., Macke, J. P., Hendry, S. H., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Nathans, J. Fibroblast growth factor (FGF) homologous factors: new members of the FGF family implicated in nervous system development. Proc. Natl. Acad. Sci. USA, *93:* 9850–9857, 1996.
3. Coulier, F., Pontarotti, P., Roubin, R., Hartung, H., Goldfarb, M., and Birnbaum, D. Of worms and men: an evolutionary perspective on the fibroblast growth factor (FGF) and FGF receptor families. J. Mol. Evol., *44:* 43–56, 1997.
4. Mason, I. J. The ins and outs of fibroblast growth factors. Cell, *78:* 547–552, 1994.
5. Friesel, R. E., and Maciag, T. Molecular mechanisms of angiogenesis: fibroblast growth factor signal transduction. FASEB J., *9:* 919–925, 1995.
6. Goldfarb, M. Functions of fibroblast growth factors in vertebrate development. Cytokine Growth Factor Rev., *7:* 311–325, 1996.
7. Basilico, C., and Moscatelli, D. The FGF family of growth factors and oncogenes. Adv. Cancer Res., *59:* 115–165, 1992.
8. Dickson, C., Smith, R., Brookes, S., and Peters, G. Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region int-2. Cell, *37:* 529–536, 1984.
9. Peters, G., Brookes, S., Smith, R., Placzeck, M., and Dickson, C. The mouse homolog of the hst/k-FGF gene is adjacent to *int-2* and is activated by proviral insertion in some virally induced mammary tumors. Proc. Natl. Acad. Sci. USA, *86:* 5678–5682, 1989.
10. Clausse, N., Smith, R., Calberg-Bacq, C. M., Peters, G., and Dickson, C. Mouse mammary tumor virus activates *Fgf-3/int-2* less frequently in tumors from virgin than from parous mice. Int. J. Cancer, *55:* 157–163, 1993.
11. Murakami, A., Tanaka, H., and Matsuzama, A. Association of hst gene expression with metastatic phenotype in mouse mammary tumors. Cell Growth Differ., *1:* 225–231, 1990.
12. Shackleford, G., Macarthur, C., Kwan, H., and Varmus, H. Mouse mammary tumor virus infection accelerates mammary carcinogenesis in *Wnt-1* transgenic mice by insertional activation of *int-2/Fgf-3* and *hst/Fgf-4*. Proc. Natl. Acad. Sci. USA, *90:* 740–744, 1993.
13. Günzburg, W. H., Salmons, B., Schalaeffli, A., Moritz-Legrand, S., Jones, W., Sarkar, N. H., and Ullrich, R. Expression of the oncogenes *mil* and *ras* abolishes the *in vivo* differentiation of the mammary epithelial cells. Carcinogenesis (Lond.), *9:* 1849–1856, 1988.
14. Hajitou, A., and Calberg-Bacq, C-M. Fibroblast growth factor 3 is tumorigenic for mouse mammary cells orthotopically implanted in nude mice. Int. J. Cancer, *63:* 702–709, 1995.
15. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, *86:* 353–364, 1996.
16. D'Amore, P. A., and Shima, D. T. Tumor angiogenesis: a physiological process or genetically determined? Cancer Metastasis Rev., *15:* 205–212, 1996.
17. Montesano, R., and Orci, L. Tumor-promoting phorbol esters induce angiogenesis *in vitro*. Cell, *42:* 469–477, 1985.
18. Antoine, N., Greimers, R., Deroanne, C., Kusaka, M., Heinen, E., Simar, L. J., and Castronovo, V. AGM-1470, a potent angiogenesis inhibitor, prevents the entry of normal but not transformed endothelial cells into the G_1 phase of the cell cycle. Cancer Res., *54:* 2073–2076, 1994.
19. Jaffe, E. A., Nachman, R. L., Becker, C. G., and Minick, C. R. Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. J. Clin. Invest., *52:* 2745–2756, 1973.
20. Deroanne, C. F., Colige, A. C., Nusgens, B. V., and Lapière, C. M. Modulation of expression and assembly of vinculin during *in vitro* fibrillar collagen-induced angiogenesis and its reversal. Exp. Cell Res., *224:* 215–223, 1996.
21. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.
22. Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA, *76:* 4350–4354, 1979.
23. Souttou, B., Gamby, C., Crepin, M., and Hamelin, R. Tumoral progression of human breast epithelial cells secreting FGF2 and FGF4. Int. J. Cancer, *68:* 675–681, 1996.
24. Chirgwin, J. M., Przybyla, A. E., Mac Donald, R. J., and Rutter, W. J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry, *18:* 5294–5299, 1979.
25. Lambert, C. A., Soudant, P. E., Nusgens, B. V., and Lapière, C. M. Pretranslational regulation of extracellular matrix macromolecules and collagenase expression in fibroblasts by mechanical forces. Lab. Invest., *66:* 444–451, 1992.
26. Angel, P., Poting, A., Mallik, U., Rahmsdorf, H. J., Schorpp, M., and Herrlich, P. Induction of metallothionein and other mRNA species by carcinogens and tumor promoters in primary human skin fibroblasts. Mol. Cell. Biol., *6:* 1760–1767, 1986.
27. Docherty, A. J. P., Lyons, L., Smith, B. J., Wright, E. M., Stephens, P. E., and Harris, T. J. R. Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid-potentiating activity. Nature (Lond.), *318:* 66–69, 1985.
28. Levy, A. T., Cioce, V., Sobel, M. E., Garbisa, S., Grigioni, W. F., Liotta, L. A., and Stetler-Stevenson, W. G. Increased expression of the M_r 72,000 type IV collagenase in human colonic adenocarcinoma. Cancer Res., *51:* 439–444, 1991.
29. Stetler-Stevenson, W. G., Krutsch, H. C., and Liotta, L. Tissue inhibitor of metalloproteinase (TIMP-2). A new member of the metalloproteinase inhibitor family. J. Biol. Chem., *264:* 17374–17378, 1989.
30. Wernert, N., Raes, M. B., Lassalle, P., Dehouck, M-P., Gosselin, B., and Stehelin, D.

C-ets1 proto-oncogene is a transcription factor expressed in endothelial cells during tumor vascularization and other forms of angiogenesis in humans. Am. J. Pathol., *140:* 119–127, 1992.
31. Vandenbunder, B., Pardanaud, L., Jaffredo, T., Mirabel, M. A., and Stehelin, D. Complementary patterns of expression of c-ets1, c-myb, and c-myc in the blood forming system of the chick embryo. Development (Camb.), *107:* 265–274, 1989.
32. Iwasaka, C., Tanaka, K., Abe, M., and Sato, Y. Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metalloproteinase-1 and the migration of vascular endothelial cells. J. Cell. Physiol., *169:* 522–531, 1996.
33. Jouanneau, J., Moens, G., Montesano, R., and Thiery J-P. Fgf-1 but not Fgf-4 secreted by carcinoma cells promotes *in vitro* and *in vivo* angiogenesis and rapid tumor proliferation. Growth Factors, *12:* 37–47, 1995.
34. Adams, L. M., Ethier, S. P., and Ullrich, R. L. Enhanced *in vitro* proliferation and *in vivo* tumorigenic potential of mammary epithelium from BALB/c mice exposed *in vivo* to γ-radiation and/or 7,12-dimethylbenz(*a*)anthracene. Cancer Res., *47:* 4425–4431, 1987.
35. Mc Leskey, S. W., Kurebayashi, J., Honig, S. F., Zwiebel, J., Lippman, M. E., Dickson, R. B., and Kern, F. G. Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. Cancer Res., *53:* 2168–2177, 1993.
36. Rak, J., Filmus, J., Finkenzeller, G., Grugel, S., Marmé, D., and Kerbel, R. S. Oncogenes as inducers of tumor angiogenesis. Cancer Metastasis Rev., *14:* 263–277, 1995.
37. Delli Bovi, P., and Basilico, C. Isolation of a rearranged human transforming gene following transfection of Kaposi sarcoma DNA. Proc. Natl. Acad. Sci. USA, *84:* 5660–5664, 1987.
38. Talarico, D., and Basilico, C. The kFGF/hst oncogene induces transformation through an autocrine mechanism that requires extracellular stimulation of the mitogenic pathway. Mol. Cell. Biol., *11:* 1138–1145, 1991.
39. Yoshida, T., Ishimura, K., Sakamoto, H., Yokota, J., Hirohashi, S., Igarashi, K., Sudo, K., and Terada, M. Angiogenic activity of the recombinant hst-1 protein. Cancer Lett., *83:* 261–268, 1994.
40. Kurebayashi, J., McLeskey, S. W., Johnson, D. J., Lippman, M. E., Dickson, R. B., and Kern, F. G. Quantitative demonstration of spontaneous metastasis by MCF-7 human breast cancer cells cotransfected with fibroblast growth factor 4 and *LacZ*. Cancer Res., *53:* 2178–2187, 1993.
41. Miyagawa, K., Sakamoto, H., Yoshida, T., Yamashita, Y., Mitsui, Y., Furusawa, M., Maeda, S., Takaku, F., Sugimura, T., and Terada, M. hst-1 transforming protein: expression in silkworm cells and characterization as a novel heparin-binding growth factor. Oncogene, *3:* 383–389, 1988.
42. Delli Bovi, P., Curatola, A. M., Newman, K. N., Sato, Y., Moscatelli, D., Hewick, R. M., Rifkin, D., and Basilico, C. Processing, secretion and biological properties of a novel growth factor of the fibroblast growth factor family with oncogenic potentialities. Mol. Cell. Biol., *8:* 2933–2941, 1988.
43. Thomas, K. A. Vascular endothelial growth factor, a potent and selective angiogenic agent. J. Biol. Chem., *271:* 603–606, 1996.
44. Arbiser, J. L., Moses, M. A., Fernandez, C. A., Ghiso, N., Cao, Y., Klauber, N., Frank, D., Brownlee, M., Flynn, E., Parangi, S., Byers, H. R., and Folkman, J. Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. Proc. Natl. Acad. Sci. USA, *94:* 861–866, 1997.
45. Larcher, F., Robles, A. I., Duran, H., Murillas, R., Quintanilla, M., Cano, A., Conti, C. J., and Jorcano, J. L. Up-regulation of vascular endothelial growth factor/vascular permeability factor in mouse skin carcinogenesis correlates with malignant progression state and activated H-ras expression levels. Cancer Res., *56:* 5391–5396, 1996.
46. Kolch, W., Martiny-Baron, G., Kieser, A., and Marme, D. Regulation of the expression of the VEGF/VPF and its receptors: role in tumor angiogenesis. Breast. Cancer Res. Treat., *36:* 139–155, 1995.
47. Goto, F., Goto, K., Weindel, K., and Folkman, J. Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gel. Lab. Invest., *69:* 491–493, 1993.
48. Unemori, E. N., Ferrara, N., Bauer, E. A., and Amento, E. P. Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. J. Cell. Physiol., *153:* 557–562, 1992.
49. Fisher, C., Gilbertson-Beadling, S., Powers, E. A., Petzold, G., Poorman, R., and Mitchell M. A. Interstitial collagenase is required for angiogenesis *in vitro*. Dev. Biol., *162:* 499–510, 1994.
50. Wernert, N., Gilles, F., Fafeur, V., Bouali, F., Raes, M. B., Pyke, C., Dupressoir, T., Seitz, G., Vandenbunder, B., and Stehelin, D. Stromal expression of c-Ets1 transcription factor correlates with tumor invasion. Cancer Res., *54:* 5683–5688, 1994.
51. Seymour, L. W., Shoaibi, M. A., Martin, A., Ahmed, A., Elvin, P., Kerr, D. J., and Wakelam, M. J. Vascular endothelial growth factor stimulates protein kinase C-dependent phospholipase D activity in endothelial cells. Lab. Invest., *75:* 427–437, 1996.
52. Guthridge, M. A., Seldin, M., and Basilico, C. Induction of expression of growth-related genes by FGF-4 in mouse fibroblasts. Oncogene, *12:* 1267–1278, 1996.
53. Ornitz, D. M., Xu, J., Colvin, J. S., McEwen, D. G., MacArthur, C. A., Coulier, F., Gao, G., and Goldfarb, M. Receptor specificity of the fibroblast growth factor family. J. Biol. Chem., *271:* 15292–15297, 1996.