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Modulation of Tumor Angiogenesis by Conditional Expression of Fibroblast Growth Factor-2 Affects Early but not Established Tumors[1]

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ABSTRACT

Fibroblast growth factor-2 (FGF2) is a pleiotropic heparin-binding growth factor endowed with a potent angiogenic activity *in vitro* and *in vivo*. To investigate the impact of the modulation of FGF2 expression on the neovascularization at different stages of tumor growth, we generated stable transfectants (Tet-FGF2) from the human endometrial adenocarcinoma HEC-1-B cell line in which FGF2 expression is under the control of the tetracycline-responsive promoter (Tet-off system).

After transfection, independent clones were obtained in which FGF2 mRNA and protein were up-regulated compared with parental cells. Also, the conditioned medium of Tet-FGF2 transfectants caused proliferation, urokinase-type plasminogen activator up-regulation, migration, and sprouting of cultured endothelial cells. A 3-day treatment of Tet-FGF2 cell cultures with tetracycline abolished FGF2 overexpression and the biological activity of the conditioned medium without affecting their proliferative capacity.

Tet-FGF2 cells formed tumors when nude mice received s.c. injections. The administration of 2.0 mg/ml tetracycline in the drinking water before cell transplantation, continued throughout the whole experiment, inhibited FGF2 expression in Tet-FGF2 tumor lesions. This was paralleled by a significant decrease in the rate of tumor growth and vascularization to values similar to those observed in lesions generated by parental HEC-1-B cells. Tetracycline administration 20 days after tumor cell implant, although equally effective in reducing FGF2 expression and inhibiting tumor vascularity, only minimally impaired the growth of established Tet-FGF2 tumors.

The results indicate that FGF2 expression deeply affects the initial tumor growth and neovascularization of HEC-1-B human endometrial adenocarcinoma in nude mice. On the contrary, the growth of established tumors appears to be independent of the inhibition of FGF2 expression and decreased vascular density. The possibility that a significant reduction of angiogenesis may not affect the progression of large tumors points to the use of antiangiogenic therapy in early tumor stage.

INTRODUCTION

New blood vessel formation represents an important step in tumor growth (1). Tumor angiogenesis is controlled by positive and negative modulators produced by neoplastic, stromal, and infiltrating cells (2). Among them, FGF2[3] was one of the first identified angiogenic growth factors (3, 4). FGF2 is a heparin-binding protein that induces cell proliferation or differentiation in a variety of cell types of mesodermal and neuroectodermal origin (5–7). It induces cell proliferation, che-

motaxis, and protease production in cultured endothelial cells (4, 8). *In vivo*, FGF2 shows angiogenic activity in different experimental models (9), and it is thought to play a role in the growth and neovascularization of solid tumors. Various tumor cell lines express FGF2 *in vitro* (8, 10–14). *In situ* hybridization and immunolocalization experiments have shown the presence of FGF2 mRNA and/or protein in neoplastic cells, endothelial cells, and infiltrating cells within human tumors of different origin (15–19). Antisense cDNAs for FGF2 and FGFR-1 inhibit neovascularization and growth of human melanomas in nude mice (20). Also, a significant correlation between the presence of FGF2 in cancer cells and advanced tumor stage has been reported (21).

In the last few years, various angiogenic factors other than FGF2 have been identified. Among them, VEGF appears to play a major role in tumor neovascularization (22). Indeed, VEGF antagonists, which include neutralizing antibodies (23), antisense-VEGF cDNA (24), and dominant-negative VEGF receptor mutant (25), can inhibit tumor growth in different experimental models. Also, VEGF levels in tumor biopsies correlate with blood vessel density of the neoplastic tissue and may be of prognostic significance (26, 27).

At variance with VEGF, FGF2 is released by producing cells via an alternative secretion pathway (28, 29), and it accumulates in the ECM, where it is mobilized by ECM-degrading enzymes (30, 31). Accordingly, FGF2 is detectable in the urine of patients with a wide spectrum of cancers (32, 33) and in the cerebrospinal fluid of children with brain tumors (34). Interestingly, the appearance of an angiogenic phenotype correlates with the export of FGF2 during the development of fibrosarcoma in a transgenic mouse model (35). These data suggest that FGF2 production and release may occur *in vivo* and may influence solid tumor growth and neovascularization by autocrine and paracrine modes of action. Accordingly, neutralizing anti-FGF2 antibodies affect tumor growth under defined experimental conditions (36–38). Relevant to this point is the recent observation that a secreted FGF-binding protein that mobilizes stored extracellular FGF2 can serve as an angiogenic switch for different tumor cell lines, including squamous cell carcinoma and colon cancer cells (39). Interestingly, targeting of FGF-binding protein with specific ribozymes reduces significantly the growth and vascularization of xenografted tumors in mice (39) despite the high levels of VEGF produced by these cells (40). These data suggest that modulation of FGF2 expression may allow a fine-tuning of the angiogenesis process even in the presence of significant levels of VEGF.

The tetracycline-regulated system (Tet-off system) allows the overexpression of the gene under study in the absence of tetracycline and the decrease in the basal level expression of the gene in the presence of the antibiotic (41). The Tet-off system, therefore, may represent a unique tool to investigate the role of the expression of angiogenic growth factors on tumor growth and neovascularization. Indeed, the Tet-off system has been used to study the effect of the conditional switching of VEGF and FGF-binding protein expression on the growth of human tumor cells injected in nude mice (42, 43).

Recently, we have shown that constitutive FGF2 overexpression

causes a significant increase in the angiogenic activity and tumorigenic capacity of the human endometrial adenocarcinoma HEC-1B cell line (44). In the present study, to investigate the impact of the modulation of FGF2 expression on neovascularization during tumor growth, HEC-1B cells have been transfected with the single expression vector version of the Tet-off system (45) that harbors the human FGF2 cDNA. Stable transfectants were evaluated for their angiogenic and tumorigenic capacity in the absence or in the presence of tetracycline. The results indicate that the growth and vascularization of human endometrial adenocarcinoma HEC-1-B FGF2-transfected tumors strongly depend on FGF2 expression during the initial stages of tumor formation in nude mice. At variance, expansion of established tumors appears to be independent of FGF2 expression and related vascular density.

MATERIALS AND METHODS

Cell Cultures and Transfection. HEC-1-B human endometrial cells were obtained from American Type Culture Collection (Rockville, MD). FGF2-B9 cells, originated in our laboratory by transfection of HEC-1-B cells with the human FGF2 cDNA under the control of human β-actin promoter, constitutively produce and release significant amounts of FGF2 (44).

To generate tetracycline-responsive FGF2-transfectants, a 1108 bp human FGF2 cDNA that encodes for low and high molecular weight FGF2 isoforms (46) was cloned *Eco*RI into SIN-RetroTet vector (45) to give the expression vector pTet-FGF2. Next, HEC-1-B cells plated at 8.0×10^5 cells/100-mm plate were transduced with pTet-FGF2. Because the SIN-RetroTet vector does not harbor any antibiotic resistance gene (45), cells were cotransfected with a calcium phosphate precipitate that contained 20 μg of the expression vector pTet-FGF2, a limiting amount (2 μg) of the pN06 plasmid that harbors the hygromycin resistance gene (kindly provided by G. Persico, I.I.G.B., Naples, Italy), and 40 μg of salmon sperm DNA as a carrier. Under these experimental conditions, pN06-transduced, hygromycin-resistant clones are anticipated to have incorporated also the pTet-FGF2 plasmid. On this basis, 70 μg/ml hygromycin were added to the culture medium 20 h after transfection. After 3 weeks of selective pressure, the hygromycin-resistant clones (Tet-FGF2 cells) were isolated, expanded, and tested for FGF2 expression and tetracycline responsiveness.

HEC-1-B, FGF2-B9, and Tet-FGF2 cells were grown in MEM supplemented with 1% nonessential amino acids, 1% sodium pyruvate, and 10% FBS. Transformed fetal bovine aortic endothelial GM 7373 cells were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ). They correspond to the BFA-1c multilayered transformed clone described previously (47). GM 7373 cells were grown in Eagle's MEM containing 10% FBS, vitamins, and essential and nonessential amino acids. BALB/c mouse aortic endothelial 22106 cells (48) were grown in DMEM added with 10% FBS.

Reagents. Human recombinant M_r 18,000 FGF2 was expressed in *Escherichia coli* and purified by heparin-Sepharose affinity chromatography as described previously (49). Rabbit polyclonal anti-FGF2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The rat monoclonal antimouse CD31 antibody MEC 13.3 was kindly provided by A. Vecchi (Istituto Mario Negri, Milan, Italy). Tetracycline was from Sigma Chemical Co.

FGF2 Protein Production by Tet-FGF2 Transfectants. Transfectants were grown to confluence. Then, cells were incubated in fresh medium for 3 days in the absence or in the presence of 10 ng/ml tetracycline. Conditioned media were collected, and monolayers were washed with 2.0 M NaCl in PBS to elute ECM-bound FGF2 (50). Then, cells were scraped with a rubber policeman and sonicated on ice at 50 W for 20 s in PBS. Quantification of intracellular FGF2 present in the cell lysate and extracellular FGF2, obtained by pooling together the ECM-bound and the free growth factor, was performed using the Quantikine FGF basic Immunoassay (R&D Systems Inc., Minneapolis, MN). Data were normalized for the protein content of the cell extracts.

For immunoblot analysis, 500-μg aliquots of the cell extracts were loaded onto 0.1 ml heparin-Sepharose columns. After a 0.5 M NaCl wash, resin beads were boiled, samples were run on SDS-15% PAGE, and proteins were electrophoretically transferred to nitrocellulose membranes in 20% methanol, 190

mM glycine, and 25 mM Tris-HCl. After transfer, membranes were saturated with PBS containing 3% BSA and 10% FBS and probed for 2 h at room temperature with anti-FGF2 antibody diluted 1:50 in PBS/0.5% BSA. Immunocomplexes were visualized by chemiluminescence Western blotting using the enhanced chemiluminescence Western blotting kit (Amersham Life Sciences) according to the manufacturer's instructions.

FGF2 mRNA Expression. Northern blot analysis of total RNA (40 μg/sample) was performed according to standard procedures using the human 1108 bp FGF2 cDNA as a probe (44). Uniform loading of the gels was assessed by ethidium bromide staining.

Conditioned Medium of Tet-FGF2 Transfectants. Cultures of the different Tet-FGF2 clones were grown in the absence or in the presence of tetracycline for 3 days. Then, all of the cultures were incubated for 3 more days in serum-free medium in the absence of tetracycline. Conditioned media were collected, clarified by centrifugation, concentrated 10 times with M_r 10,000 cutoff centrifugal concentrators (Centriplus; Amicon, Beverly, MA), and stored at −20°C until use.

Cell Growth Assay. Cell proliferation assay on GM 7373 cells was performed as described previously (49). Briefly, GM 7373 cells were seeded at 75,000 cells/cm^2 in 24-well dishes. Plating efficiency was higher than 90%. After overnight incubation, cells were incubated for 24 h in fresh medium containing 0.4% FBS in the absence or in the presence of aliquots (100 μg/ml) of the conditioned medium of Tet-FGF2 clones. At the end of the incubation, cells were trypsinized and counted in a Burker chamber. Control cultures incubated with no addition or with 10 ng/ml of recombinant FGF2 underwent 0.1–0.2 and 0.7–0.8 cell population doublings, respectively. Cells grown in 10% FBS underwent 1.0 cell population doublings (49).

uPA Up-Regulation Assay. Confluent cultures of GM 7373 cells were incubated for 18–20 h in fresh medium containing 0.4% FBS and aliquots (100 μg/ml) of the conditioned medium of Tet-FGF2 clones. After incubation, cell layers were washed twice with PBS, and uPA activity was measured in the cell extracts (49) by using the plasmin chromogenic substrate H-D-norleucyl-hexahydrotyrosil-lysine-p-nitroanilide acetate (American Diagnostic, Greenwich, CT).

Migration Assay. A modification of the Boyden chamber technique was used to evaluate cell migration (49). Briefly, the Neuroprobe 48-well microchemotaxis chamber was used. The two wells were separated by a polyvinyl pyrrolidone-free Nucleopore filter, 8-μm pore size, coated with gelatin (5 μg/ml). Aliquots (10 μg of protein) of the conditioned medium were dissolved in fresh medium containing 0.1% heat-inactivated FBS and placed in the lower wells, and the filters were adjusted over the holes. After the top plate was applied and mounted, 50 μl of GM 7373 cell suspension (10^6 cells/ml) were added to each upper well. After 4 h of incubation at 37°C, the filters were removed and fixed in methanol. The cells on the upper surface of the filter were removed with a cotton swab, and the cells that migrated across the filter were stained with Diff-Quick and counted in 10 different fields for each well at $\times$ 40 magnification.

Endothelial Cell-sprouting Assay. Fibrinogen (2.5 mg/ml) was dissolved in calcium-free medium containing aliquots (150 μg of protein) of the conditioned medium of Tet-FGF2 transfectants. Then, mouse aortic endothelial cell aggregates, prepared as described previously(48), were resuspended in the fibrinogen solution, and clotting was started by the addition of thrombin (250 milliunits/ml). The mixture was transferred into 24-well plates and allowed to gel at 37°C. Trasylol (200 KIU/ml) was added to the gel and to the culture medium to prevent the dissolution of the substrate. Cell aggregates were maintained for 2–3 days in DMEM containing the same dilution of conditioned medium present within the gel and were evaluated for the capacity to originate endothelial cell sprouts radiating out from the aggregates (48).

Tumorigenicity Assay. Female NCr-*nu/nu* mice were obtained from the animal production colony of the National Cancer Institute, Frederick Cancer Research and Development Center (Frederick, MD) and used when 6–8 weeks of age. Cells were harvested by brief exposure to 0.25% trypsin/0.02% EDTA, washed twice, and suspended in HBSS. Mice received a s.c. injection in the dorsal scapular region of 1×10^6 cells suspended in 0.2 ml HBSS. The tumor mass was measured twice a week with calipers, and tumor weight was estimated by the formula: (length $\times$ width^2)/2 (51). When indicated, animals received tetracycline in the drinking water (2 mg/ml) with a change every other day throughout the whole experimental period starting 4 days before or 20 days after cell implantation. At different time points, animals were sacrificed,

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sera were collected, and tumors were harvested and processed for FGF2 antigen and mRNA expression analysis and blood vessel immunostaining (see below). In some experiments, frozen biopsies of the xenografts corresponding to 500 μg of protein were sonicated in PBS, and proteins were analyzed for FGF2 content by the Quantikine FGF basic Immunoassay (R&D Systems Inc., Minneapolis, MN).

FGF2 Immunohistochemistry and *in Situ* Hybridization. Xenograft specimens were embedded in OCT compound and frozen, and 5-μm sections were obtained with a cryostat microtome. Then, sections were processed for immunohistochemical analysis by using anti-FGF2 antibody. To this purpose, sections were rinsed in PBS and incubated for 20 min with 0.3% H_2O_2 in absolute methanol to block endogenous peroxidase and for 20 min further with 0.2% Triton X-100 in PBS. Then, a 30-min preincubation with diluted normal serum was followed by incubation at 4°C with anti-FGF2 antibody (1:10 dilution) in a humidified chamber. Sections were then exposed to biotinylated secondary antibody (Vector Laboratories) and to avidin-biotin-peroxidase complex (Dako ABComplex HRP) for 30 min. Peroxidase color reaction was developed with 3-amino-9-ethyl-carbazole (Sigma), and the sections were lightly counterstained with Mayer's hematoxylin.

For *in situ* hybridization studies, the human 1108 bp FGF2 cDNA fragment was labeled with biotin-labeled dATP using the Random Primer Biotin Labeling kit with streptavidin-horseradish peroxidase (NEN Life Science Products, Boston, MA). After rehydration, tissue sections were prepared for hybridization by flooding them with 50% formamide in 2 × SSC buffer and warming up to 70°C. FGF2 probe (20 ng) was dissolved in 100% formamide, boiled for 10 min, mixed to a final volume of 15 μl of hybridization mixture (10% dextran sulfate, 2 × SSC, 500 μg/ml tRNA, 0.2 mg/ml BSA, and 10 mM DTT in diethyl pyrocarbonate-treated water), and placed onto each tissue section. Specimens were sealed and incubated overnight at 45°C in a humidified chamber. After hybridization, specimens were stained using the Tyramide Signal Amplification-Indirect *In Situ* Hybridization kit (NEN Life Science Products) according to the manufacturer's instructions, counterstained in hematoxylin, and mounted for microscopy.

Assessment of Microvessel Density. To evaluate microvessel density, frozen sections from each tumor were immunostained with a rat antimurine CD31 antibody for the detection of blood vessels, according to the procedure described in detail previously (44). Sections were examined at low-power magnification to identify the areas with the highest density of CD31-positive vessels. In each case, the most vascularized area was selected, and microvessels in a × 400-field were counted. Because all of the HEC-1-B-derived xenografts were characterized by a poorly vascularized central zone of necrosis, CD31-positive vessels were counted within the tumor parenchyma at the periphery of the lesion where angiogenesis is more robust (52). No significant differences in microvessel counts were observed between paired sections of individual tumors.

RESULTS

Control of FGF2 Expression by a Tetracycline-regulated Expression Vector. The single expression vector version of the Tet-off system (45) was used to modulate FGF2 production into human endometrial adenocarcinoma HEC-1-B cells that constitutively express low levels of FGF2 (44). To this purpose, the human FGF2 cDNA was cloned in the SIN-RetroTet expression vector (45) forming pTet-FGF2. Then, pTet-FGF2 was transduced into HEC-1-B cells. Stable transfectants were selected and compared with parental cells for FGF2 expression in the absence or in the presence of tetracycline. Also, transfectants were compared with FGF2-B9 cells generated in our laboratory by transfection of HEC-1-B cells with an expression vector that harbored the human FGF2 cDNA under the control of the β-actin promoter (44).

In the absence of tetracycline, Tet-FGF2 clones express high levels of FGF2 mRNA and of low and high molecular weight FGF2 protein when compared with parental HEC-1-B cells by Northern blot and Western blot analysis. These levels are similar to those measured in FGF2-B9 cells. In the presence of tetracycline, FGF2 expression is
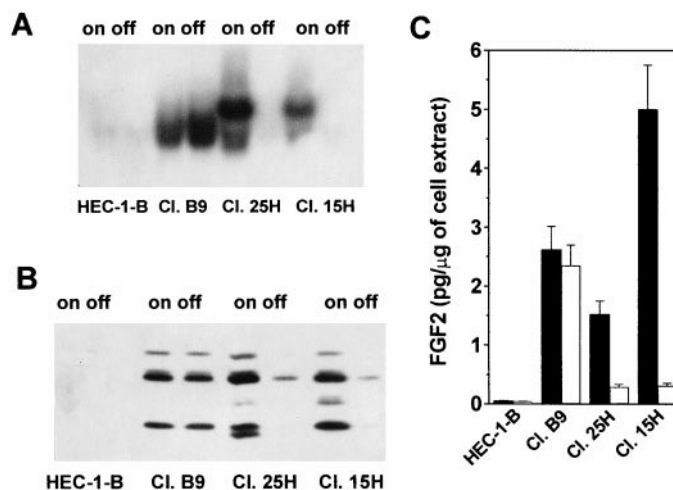

Fig. 1. Control of FGF2 expression by a tetracycline-off regulated expression vector. HEC-1-B cells were transfected with Tet-FGF2 expression vector. Parental cells (HEC-1-B), stable Tet-FGF2 25H transfectants (Cl. 25H) and Tet-FGF2 15H transfectants (Cl. 15H), and FGF2-B9 cells (Cl. B9) that constitutively overexpress FGF2 under the control of the β-actin promoter were grown then for 3 days in the absence (on) or in the presence (off) of 10 ng/ml tetracycline. Next, cells were analyzed for FGF2 mRNA steady state levels by Northern blot analysis (*A*) and for FGF2 protein expression by Western blotting of the cell lysate (*B*) as described in "Materials and Methods." All of the FGF2 transfectants express the different FGF2 isoforms with molecular weight ranging between 25,000 and 18,000 (*B*). FGF2 released by the different cell lines in the absence (*closed bar*) and in the presence (*open bars*) of tetracycline treatment was measured by ELISA (*C*). Data (mean ± SD of three replicates) were normalized for the protein content of the cell extracts.

dramatically suppressed in Tet-FGF2 25H and 15H transfectants, whereas it remains unchanged in FGF2-B9 cells (Fig. 1 *A* and *B*).

In all of the Tet-FGF2 clones tested, dose-response experiments demonstrated that the maximal suppression of FGF2 expression is observed at doses of tetracycline or of its analogue doxycycline (45) equal to or higher than 1.0 ng/ml. Also, inhibition was already apparent 24 h after the beginning of the antibiotic treatment and reached its maximum (>95% inhibition) at 72 h (data not shown).

In agreement with previous observations on FGF2-transfected HEC-1-B cells (44), the *in vitro* replication rate of Tet-FGF2 transfectants did not differ from that of parental cells and was not affected by the presence of tetracycline (data not shown), thus confirming the inability of FGF2 overexpression to affect the *in vitro* growth of HEC-1-B cells.

Tet-FGF2 Transfectants Release Biologically Active FGF2. Previous observations in our laboratory had shown that FGF2 transfection in HEC-1-B cells results in the production of clones characterized by a different capacity to release FGF2 and that FGF2 export decides the biological behavior of these clones (44). When FGF2-B9 cells and Tet-FGF2 25 H and 15H transfectants were evaluated for their capacity to export FGF2 by an ELISA, the results demonstrated that all of the tested clones release significant amounts of FGF2 (Fig. 1*C*). In agreement with previous observations on different FGF2 transfectants (28, 48, 53), released FGF2 represents ~2–5% of the total cell-associated growth factor. Tetracycline treatment caused a 90–95% reduction of the levels of released FGF2 in Tet-FGF2 cells without affecting FGF2 release in FGF2-B9 cells (Fig. 1*C*).

To assess whether extracellular FGF2 produced by the different Tet-FGF2 clones is biologically active, cells were grown in the absence or in the presence of tetracycline for 3 days. Then, all of the cultures were incubated for 3 more days in serum-free medium in the absence of the antibiotic. During this period, no significant reexpression of FGF2 was observed in tetracycline-pretreated Tet-FGF2 cells (data not shown). The conditioned medium was collected and

Fig. 2. Biological activity of the conditioned medium of Tet-FGF2 transfectants. Cultures of parental HEC-1-B cells, FGF2-B9 cells, and Tet-FGF2 25H and 15H transfectants were grown in the absence (*black bar*) or in the presence (*open bar*) of tetracycline for 3 days. All of the cultures were then incubated for 3 more days in serum-free medium in the absence of tetracycline. Conditioned medium was collected and evaluated for the capacity to stimulate cell proliferation (*A*), migration (*B*), and cell-associated uPA up-regulation (*C*) in endothelial GM 7373 cells as described in "Materials and Methods." Conditioned medium of Tet-FGF2–15H cells grown in the absence (*D*) or in the presence (*E*) of tetracycline was tested also for the capacity to induce endothelial sprouts in mouse aortic endothelial cell aggregates grown in tridimensional fibrin gel. Data in *A-C* are the mean ± SE of two to three experiments in duplicate. Data in *D-E* are representative of three independent experiments. **, statistically different from the conditioned medium of corresponding tetracycline-untreated cells (*P* < 0.01, Student's *t* test).
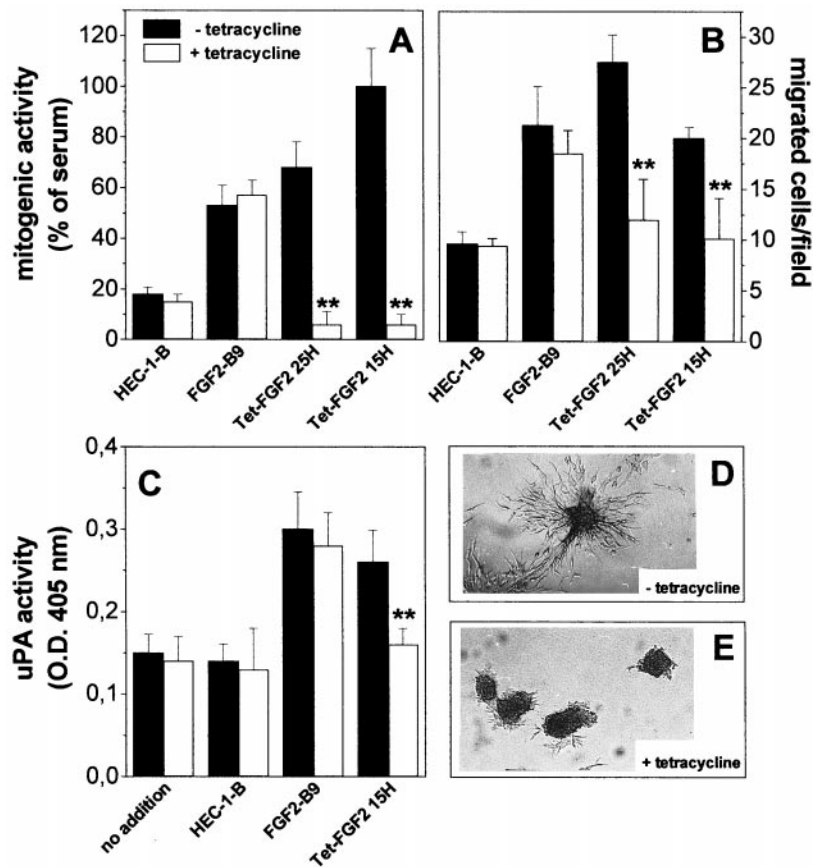
evaluated for the capacity to affect endothelial cell functions *in vitro*. As shown in Fig. 2*A*, only the conditioned medium of the tetracycline-untreated clones induced a significant mitogenic response in cultured GM 7373 endothelial cells. Accordingly, the conditioned medium of untreated Tet-FGF2 clones stimulated uPA production and migration in GM 7373 cells and caused endothelial cell sprouting when added to murine aortic endothelial cell aggregates grown in tridimensional fibrin gel (Fig. 2 *B-E*). Again, the conditioned medium collected from tetracycline-pretreated Tet-FGF2 clones was ineffective on endothelial cell response. It must be pointed out that tetracycline pretreatment did not affect the biological activity of the conditioned medium of B9-FGF2 cells that constitutively express the growth factor (Fig. 2).

Taken together, these data demonstrate that Tet-FGF2 transfectants overexpress FGF2 mRNA and protein to values similar to those measured in FGF2-B9 cells. Tetracycline rapidly suppresses FGF2 expression in these cells but not in FGF2-B9 cells. Accordingly, Tet-FGF2 clones release significant amounts of biologically active FGF2 only when grown in the absence of the antibiotic.

Modulation of *in Vivo* FGF2 Expression by Tetracycline. The tumorigenicity of Tet-FGF2 and FGF2-B9 cells was evaluated in nude mice. To this purpose, Tet-FGF2 clones 15H and 25H and FGF2-B9 cells were injected s.c. in the flank of nude mice (five animals/group) at 1×10^6 cells/implant, and their rate of growth was monitored. All of the cell lines grew at a similar rate *in vivo*, with the median time to reach tumor weight of 200 mg being 25 days for FGF2-B9 xenografts and 24 and 26 days for Tet-FGF2 15H and 25 H clones, respectively. Clone Tet-FGF2 15H was chosen for additional studies.

To assess the capacity of tetracycline to suppress FGF2 expression in Tet-FGF2 transfectants *in vivo*, nude mice that received s.c. injections with 1×10^6 Tet-FGF2 15H cells were given the antibiotic dissolved in the drinking water throughout the whole experimental

period. Control animals that received injections with the same tumor cell preparation were never exposed to the antibiotic. Tetracycline did not affect the health status of the mice as evaluated by lack of body weight loss compared with control mice. After 4 weeks, animals were sacrificed, their serum was collected, and xenograft specimens were analyzed for FGF2 expression by *in situ* hybridization and immuno-histochemistry. As shown in Fig. 3 *A* and *C*, Tet-FGF2 15H cells express high levels of FGF2 mRNA and protein in tumor tissue. At variance, FGF2 expression in tumor parenchyma is dramatically reduced in animals given tetracycline, with limited FGF2 immunoreactivity being detectable in blood vessel endothelial cells (Fig. 3 *B* and *D*). ELISA confirmed the significant decrease of FGF2 protein levels in Tet-FGF2 tumor extracts from tetracycline-treated animals (2.7 ± 0.4 and 1.1 ± 0.2 pg FGF2/μg of tumor protein in tetracycline-untreated and -treated animals, respectively). At variance, FGF2 mRNA and protein levels were not affected by tetracycline treatment in tumors derived from FGF2-B9 cells (data not shown).

Accordingly, the sera ($n = 4$) of tetracycline-treated animals added *in vitro* to the culture of Tet-FGF2 15H cells for 3 days (5% v/v final concentration) caused a 85–90% suppression of FGF2 protein production. Sera of control animals were instead ineffective. Specificity of the effect was demonstrated also by the inability of the serum of tetracycline-treated animals to affect FGF2 expression in cultured FGF2-B9 cells (data not shown).

Taken together, these data demonstrate that the tetracycline regimen in the drinking water was able to cause a long-lasting decrease of FGF2 expression in Tet-FGF2 transfectants, with limited amounts of FGF2 protein being still detectable within the lesion and possibly produced by infiltrating host stromal cells, including endothelial cells.

Growth and Neovascularization of Tet-FGF2 Xenografts. To evaluate the effect of FGF2 expression on tumor growth, Tet-FGF2
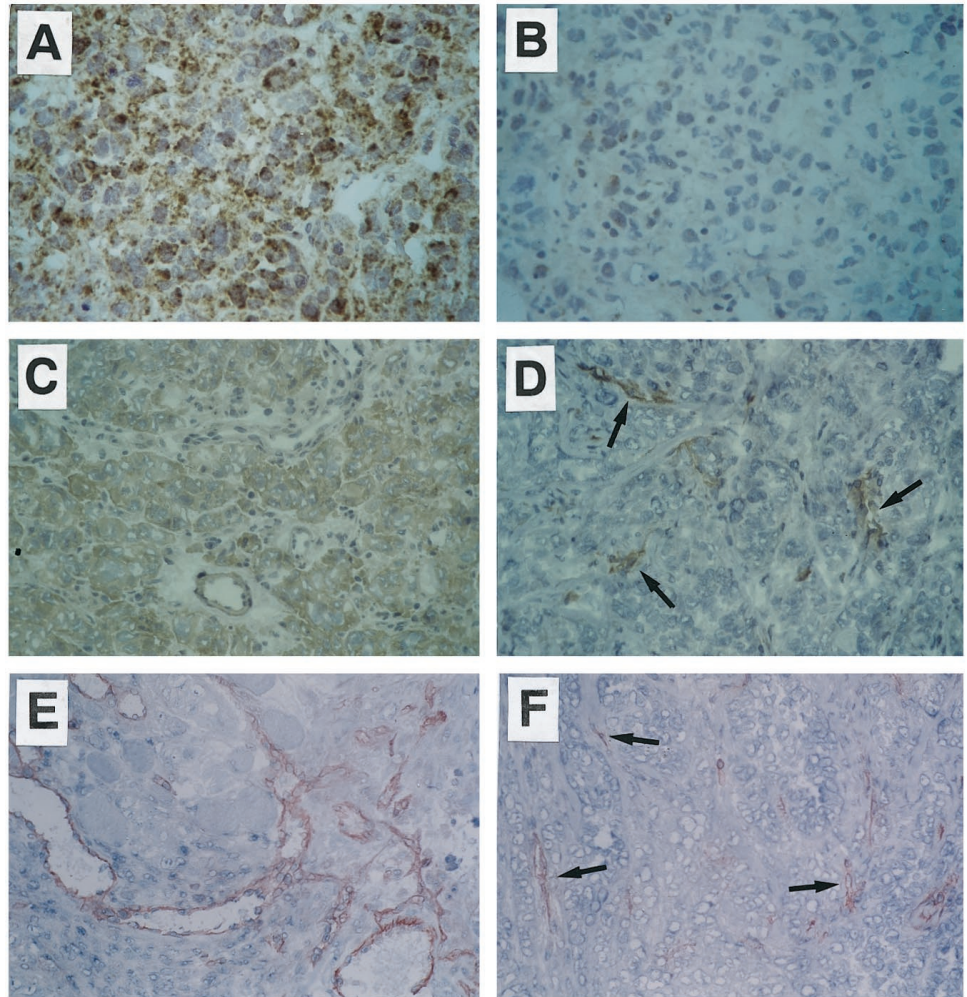
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Fig. 3. *In vivo* modulation of FGF2 expression. Nude mice that received transplantations s.c. with 1×10^6 Tet-FGF2 15H cells were left untreated (*A*, *C*, and *E*) or received 2 mg/ml tetracycline in the drinking water throughout the entire experimental period (*B*, *D*, and *F*). After 4 weeks, animals were sacrificed and tumor sections were processed for FGF2 mRNA *in situ* hybridization (*A* and *B*), FGF2 immunostaining (*C* and *D*), or CD31 immunostaining (*E* and *F*). Note the disappearance of FGF2 mRNA and protein expression in tumors isolated from tetracycline-treated animals in which FGF2 immunoreactivity was detectable only in blood vessel endothelial cells (*arrows* in *D*). Also note the small-caliber microvessels in tetracycline-treated Tet-FGF2 15H lesions (*arrows* in *F*) when compared with large-sized vessels in untreated tumors (*E*). This difference in microvascular morphology between the two experimental groups was observed in all of the tumors examined (five to eight tumors/group) independent of the size of the lesion and time of sacrifice and was shared by parental HEC-1-B tumors when compared with FGF2-B9 lesions (not shown). Original magnification: $\times 400$ (*A-D*), $\times 200$ (*E* and *F*).

15H and FGF2-B9 cells were transplanted in nude mice receiving either tetracycline or regular drinking water throughout the whole experimental period starting 4 days before tumor cell injection (Fig. 4). In mice that received injections with Tet-FGF2 15H cells, tumor
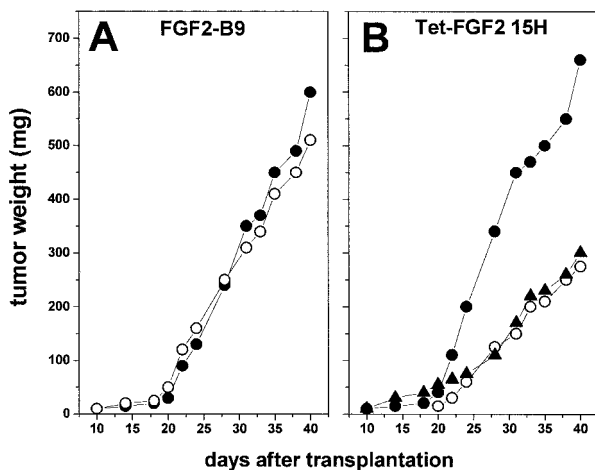

Fig. 4. Growth of Tet-FGF2 tumors: effect of early FGF2 down-regulation. Nude mice were randomized into two groups: the control group (●) and the group receiving 2 mg/ml tetracycline in the drinking water throughout the entire experimental period starting 4 days before cell injection (○). Within each group, two subgroups of mice (*n* = 8) received s.c. injections with 1×10^6 FGF2-B9 cells (*A*) or with 1×10^6 Tet-FGF2 15H cells (*B*). One additional group of untreated animals (*n* = 5) received injections with 1×10^6 parental HEC-1-B cells (▲ in *B*).

take at day 10 was 100%, and tumor lesions reached the size of 200 mg at day 24 (range = 23–29 days). Tetracycline treatment caused a 10-day delay in Tet-FGF2 15H tumor take (100% of tumors being palpable at day 20) and a marked decrease in their growth rate with tumor lesions reaching the size of 200 mg at day 37 (range = 28–47 days). The rate of growth of Tet-FGF2 15H tumors in the presence of tetracycline was similar to that observed for lesions generated by parental HEC-1-B cells that do not express FGF2 (Fig. 4*B*). In contrast, tumor take and growth of FGF2-B9 lesions were not affected by tetracycline. In the presence or absence of the antibiotic, tumors showed in fact a similar latency (100% of tumors being palpable at day 10) and reached the size of 200 mg at day 25 (range = 24–36 days and 23–39 days, respectively; Fig. 4*A*).

No morphological differences were observed in H&E-stained sections between Tet-FGF2 15H and FGF2-B9 tumors that showed the features of poorly differentiated adenocarcinomas with papillary and adenomatous pattern (44) both in the absence and in the presence of tetracycline treatment (data not shown).

In a second experiment, to assess the impact of the modulation of FGF2 expression on vascularization of Tet-FGF2 xenografts, nude mice given or not given tetracycline were transplanted with Tet-FGF2 15H or FGF2-B9 cells as above, and vascular density was evaluated in CD31 immunostained tumor sections. For all of the groups, animals were sacrificed at 6–7 weeks when the tumors reached an average size of 800 mg, with the exception of Tet-FGF2 15H tumors from mice given tetracycline that at the same time measured ~300 mg. To avoid a bias attributable to possible differences in vascularity relative to
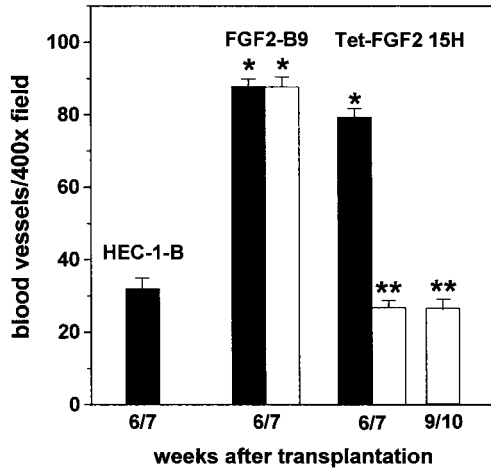
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Fig. 5. Vascularization of Tet-FGF2 tumors: effect of early FGF2 down-regulation. Parental HEC-1-B cells, FGF2-B9 cells, or Tet-FGF2 15H cells were transplanted in control (*closed bars*) and tetracycline-treated (*open bars*) nude mice as in Fig. 4. For each group, animals ($n = 5$) were sacrificed at 6–7 weeks. Additional animals ($n = 5$) that received injections with Tet-FGF2 15H cells and receiving tetracycline were sacrificed at 9–10 weeks. Tumor vascularity was assessed by CD31 immunostaining of tissue sections as described in "Materials and Methods." Statistically different ($P < 0.01$, Student's *t* test) from untreated parental HEC-1-B lesions (∗) or from the corresponding tetracycline-untreated group (∗∗).

tumor size, an additional group of tetracycline-treated Tet-FGF2 15H lesions was analyzed at 9–10 weeks when their average size was 800 mg. As shown in Fig. 5, tetracycline treatment causes an early 65–70% decrease of the vascular density in Tet-FGF2 15H lesions at 6–7 weeks to values similar to those observed in parental HEC-1-B cell tumors of similar size. Moreover, blood vessel formation remained suppressed in animals given tetracycline in large-sized Tet-FGF2 15H lesions at 9–10 weeks. At variance, the vascularity of FGF2-B9 tumors was not affected by the antibiotic treatment. It must be pointed out that, independent of the levels of FGF2 expression, all of the tumors were characterized by a poorly vascularized central zone of necrosis. Thus, CD31-positive vessels were counted within areas of viable tumor parenchyma at the periphery of the lesion where angiogenesis is more robust (52). In agreement with previous observations (54), striking differences in the morphology of CD31-positive microvessels were observed when FGF2-overexpressing lesions were compared with FGF2-less tumors (Fig. 3 *E* and *F*). Indeed, the microvasculature of all of the Tet-FGF2 15H and FGF2-B9 lesions examined showed a remarkable heterogeneity in lumen diameter with numerous large-caliber vessels. In contrast, all of the tumors originated by Tet-FGF2 15H cells in tetracycline-treated animals or by parental HEC-1-B cells in control animals were characterized by homogenous small-caliber vessels.

In a parallel experiment, to study whether FGF2 suppression also affects the vascular density associated with initial tumor growth, tetracycline-treated animals were sacrificed 3 weeks after cell injection (five animals/group). A 60% decrease of tumor vascularity was already present in these Tet-FGF2 15H lesions with a minimal tumor burden (50 mg), whereas no significant effects of tetracycline treatment were observed on the vascularity of FGF2-B9 lesions at comparable size (data not shown).

Taken together, these data indicate that the early suppression of FGF2 expression causes a significant decrease in tumor growth and vascularization in Tet-FGF2 transfectants. The extent of the inhibitory effect on blood vessel density is constant during tetracycline treatment, being therefore independent of the size of the tumor.

To assess whether FGF2 expression could also affect tumor growth and vascularization when the lesions have been established, animals

that received injections with Tet-FGF2 15H cells but not receiving tetracycline were randomized in two groups 20 days after cell transplantation (mean size of the tumor lesions equal to 127 ± 51 mg). At this point, one group of mice was given tetracycline in the drinking water, whereas the other group was left untreated. As shown in Fig. 6*A*, initiation of tetracycline treatment after tumors have been established causes only a minimal, not statistically significant delay of tumor growth. In contrast, evaluation of blood vessel density in tumors isolated from animals sacrificed 6 weeks after the beginning of tetracycline administration indicates that the late tetracycline treatment was still able to cause a significant decrease in tumor vascularity despite its lack of effect on the rate of tumor growth (Fig. 6*B*). Interestingly, in this trial the decrease in vascularity was already observed in representative tumors ($n = 4$) analyzed 2 weeks after the beginning of the tetracycline treatment (33 ± 4 blood vessels/ $\times 400$ field).

To compare the effect of early and late initiation of tetracycline treatment on Tet-FGF2 tumor growth and vascularization in the same experimental batch, one additional experiment was performed in which mice that received injections with Tet-FGF2 15H cells were randomized among one control untreated group and two experimental groups that received tetracycline in the drinking water starting 4 days before or 20 days after cell injection, respectively (five animals/group). All of the animals were sacrificed at day 47 when the average tumor size was 400 and 380 mg in control and late tetracycline treatment groups, respectively, and 200 mg in the early tetracycline treatment group. Again, evaluation of CD31-positive blood vessel density demonstrated a significant decrease of tumor vascularity ($P < 0.01$) in tetracycline-treated animals of both experimental groups (32 ± 6 and 34 ± 2 blood vessels/ $\times 400$ field for early and late tetracycline treatment, respectively) when compared with control animals (62 ± 3 blood vessels/ $\times 400$ field). These data confirm that early and late initiation of tetracycline treatment affect tumor vascularization to a similar extent, but only the early tetracycline regimen causes a significant decrease in the rate of tumor growth.

DISCUSSION

In the present study, the Tet-off system has been used to modulate FGF2 production in the human endometrial adenocarcinoma HEC-
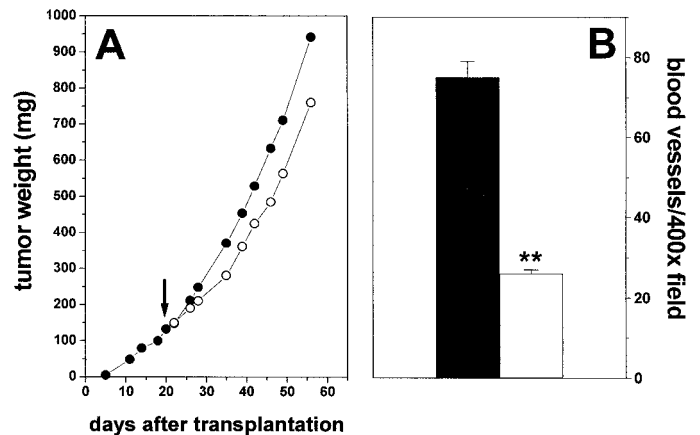

Fig. 6. Growth and neovascularization of Tet-FGF2 tumors: effect of late FGF2 down-regulation. Nude mice received s.c. injections with 1×10^6 Tet-FGF2 15H cells. After 20 days, animals were randomized into two groups (10 mice/group): one control group (●, ■) and one group receiving 2 mg/ml tetracycline in the drinking water (○, □). In *A*, tumor growth was monitored with calipers. In *B*, mice were sacrificed at 9 weeks after cell injection, and tumor vascularity was assessed by CD31 immunostaining of tissue sections as described in "Materials and Methods." ∗∗, statistically different from control group ($P < 0.01$, Student's *t* test).

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1-B cell line (44). Tet-FGF2 transfectants express significant levels of FGF2 partly released in the extracellular environment. Accordingly, the conditioned medium of different Tet-FGF2 clones stimulates endothelial cell proliferation, migration, protease production, and morphogenesis. Tetracycline treatment effectively switches off FGF2 overexpression in these cells at both mRNA and protein levels and abolishes the biological activity of their conditioned medium. Tetracycline had no effect on FGF2 expression in FGF2-B9 cells, a HEC-1-B clone that overexpresses FGF2 under the control of a constitutive β-actin promoter, and on the biological activity of their conditioned medium, thus confirming the specificity of the effect of the antibiotic on Tet-FGF2 clones.

When injected s.c. in nude mice, Tet-FGF2 cells and FGF2-B9 cells originate highly vascularized tumors that grow at a similar rate, significantly higher than that of parental HEC-1-B cell xenografts. Tetracycline administration in the drinking water throughout the whole experimental period caused a significant delay in tumor take and a decrease in the growth rate of Tet-FGF2 xenografts during the early stages of tumor development. This was paralleled by a significant decrease in tumor vascularization observed in the early development of lesions (tumor burden = 50 mg) and maintained in large-sized tumors (800 mg). Several experimental evidences indicate that the inhibitory effect of tetracycline on early Tet-FGF2 tumor growth is because of its ability to switch off FGF2 expression with a consequent decrease in tumor vascularization: *a*) tetracycline administration in the drinking water suppresses FGF2 expression in Tet-FGF2 lesions at both mRNA and protein levels; *b*) the serum of tetracycline-treated animals is able to inhibit FGF2 expression in Tet-FGF2 cells *in vitro*; and *c*) tetracycline administration does not exert a significant effect on FGF2 expression, tumor growth, and vascularization of FGF2-B9 lesions constitutively expressing FGF2. It must be pointed out that FGF2 expression and/or tetracycline treatment do not affect Tet-FGF2 cell proliferation *in vitro*. This is in keeping with previous observation on the lack of effect of FGF2 overexpression on FGF2-B9 cell proliferation (44). Nevertheless, we cannot rule out the possibility that modulation of FGF2 expression in transfected HEC-1-B cells may affect other biological properties of this cell line with possible consequences on its tumorigenic potential. HEC-1-B cells express FGFR-1 and FGFR-4, and both exogenous and endogenous FGF2 can modulate uPA production in these cells (44).

Tet-FGF2 lesions eventually reach a large tumor burden also under the continuous presence of tetracycline, thus indicating that FGF2 up-regulation does not represent an absolute requirement for tumor growth and vascularization in this experimental system. Indeed, parental HEC-1-B cells that do not express significant levels of FGF2 are tumorigenic when injected in nude mice, with growth and vascularization of HEC-1-B xenografts being similar to that observed for tetracycline-treated Tet-FGF2 lesions (present study and Ref. 44). We have observed that HEC-1-B cells secrete significant amounts of VEGF (54). VEGF levels are not affected by FGF2 expression in these cells, as shown by ELISA of the conditioned medium of parental, FGF2-B9, and Tet-FGF2 cells grown in the absence and in the presence of tetracycline. Also, ELISA of FGF2-B9 and Tet-FGF2 tumor extracts showed that intratumor VEGF levels remain constant throughout the experimental period both in the absence and in the presence of tetracycline treatment.[4] These data suggest that the production of VEGF and possibly of other as yet unidentified angiogenesis factors are sufficient to allow a basal level of vascularization able to sustain tumor growth in HEC-1-B cells. FGF2 up-regulation causes a further induction of tumor vascularization that results in an earlier

appearance and a faster rate of growth of the lesion during the early stages of tumor development. It is interesting to note that the levels of VEGF released by FGF2-B9 and Tet-FGF2 cells (range between 1.9 and 2.6 ng of immunoreactive VEGF/ml of conditioned medium) are significantly higher than the levels of released FGF2. Thus, in agreement with previous observations (54), the data suggest that the release of limited amounts of FGF2 may deeply affect early tumor growth and vascularization even in the presence of a high background of secreted VEGF. This hypothesis is supported by the synergistic action exerted by the two growth factors in stimulating angiogenesis (55, 56) and by the possibility to inhibit the *in vivo* growth and angiogenic activity of various human tumor cell lines by either anti-FGF2 (39) or anti-VEGF (25, 57) approaches (see Ref. 40 for further discussion). The concomitant block of more than one angiogenic factor (*e.g.,* VEGF and FGF2) to impair tumor progression in the model system here described deserves further investigation.

An interesting observation in our study is that the down-regulation of FGF2 expression after the tumor had reached a certain size did not affect the further growth of the lesion. Similar results had been obtained by the conditional switching of VEGF- or FGF-binding protein expression in large lesions originated by the injection of tumor cells of different origin (42, 43), thus suggesting that our findings are not the consequence of unique properties of the cell line here adopted. In the previous studies (42, 43), late down-regulation of VEGF- or FGF-binding protein expression did not affect the growth of large tumors, although it resulted in a marked inhibition of small tumors. However, the effect of VEGF expression on tumor vasculature was not investigated, and the increased expression of FGF2 and interleukin-8 after the late VEGF down-regulation was hypothesized to be sufficient to sustain tumor growth and vascularization (42), whereas the modulation of FGF-binding protein did not appear to exert any effect on blood vessel density of the lesions (43). In the present study, down-regulation of FGF2 expression in established lesions caused instead a rapid and significant decrease of tumor vascularization, similar to that observed when tetracycline regimen was initiated before tumor cell implantation. Thus, our data indicate that a significant decrease in blood vessel density is not paralleled by a significant decrease of the growth of established tumors. This raises the possibility that a threshold effect may exist in the relationship between tumor growth and vascularization in established cancers. According to this hypothesis, an increase in vascularization above the threshold values (*e.g.,* by angiogenic growth factor overexpression) will not provide any advantage for established lesions, the growth of which will be inhibited only when vascularization falls below the threshold values. This hypothesis may explain the observation that early initiation of angiostatic therapies can be more efficacious than late initiation in reducing tumor growth (58–60). The possibility also exists that tumor microcirculation not lined by endothelial cells may contribute to established tumor growth (61). The impact of angiogenesis inhibitors on this form of tumor microcirculation remains to be established.

Although quantification of the number of CD31-positive microvessels in "vascular hot spots" represents a widely used procedure to assess tumor vascularization in experimental and clinical protocols, microvessel density may not necessarily reflect tumor perfusion and blood supply. However, we have observed that Tet-FGF2-overexpressing tumors are characterized by an increase in blood vessel density that is paralleled by a striking increase in blood vessel diameter when compared with parental HEC-1-B lesions. Remarkably, tetracycline treatment caused a significant reduction in both microvessel density and caliber. These findings support previous observations (54) on the modifications of the tumor microvascular architecture of HEC-1-B xenografts after FGF2 overexpression. Indeed, tridimen-

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[4] R. Giuliani and D. Coltrini, unpublished observations.

sional morphometric analysis of microvascular corrosion casts indicated that blood vessels of FGF2-overexpressing tumors are characterized by a wider average vascular diameter when compared with the microvasculature of parental HEC-1-B lesions and by an extreme variability of the diameter of each individual vessel (54). It is worth noting that the possibility to generate microvascular casts of these s.c. tumors after injection of the casting resin into the ascending aorta (54) suggests that both large- and small-caliber vessels are functional. Interestingly, VEGF overexpression also induces an increase in tumor vessel density and size (62). Accordingly, inhibition of tumor angiogenesis by anti-VEGF antibody causes a decrease of microvessel density and diameter, with a consequent decrease in blood supply (63). Similar conclusions have been drawn for the antiangiogenic action exerted by thrombospondin-1 or -2 overexpression in wound healing and tumor growth (64, 65). Additional experiments will be required to fully elucidate the impact of the modulation of FGF2 expression on tumor microvascular architecture, functional vascularity, and blood supply.

In the context of our experimental model, tetracycline mimics an antiangiogenic molecule by inhibiting FGF2 expression and exerting a significant effect on Tet-FGF2 tumor vascularization at both early and late stages of tumor progression. It is worth noting that tetracycline administration in the drinking water had no effect on FGF2-B9 tumor vascularization (see above) and on the angiogenic response elicited by human recombinant FGF2 in a murine Matrigel plug assay (66),[5] thus indicating that the antibiotic does not exert a direct antiangiogenic effect under our experimental conditions. However, despite the ability of both early and late tetracycline regimes to inhibit tumor vascularization, only small tumors respond also with an inhibition in the rate of growth. To this respect, two points are worth noting. First, antiangiogenic therapy may be more efficacious in preventing or delaying tumor relapse and/or the appearance of metastases by inducing tumor dormancy (67, 68) rather than in causing the regression of established tumor lesions (see also Refs. 69–71). Second, the evaluation of the efficacy of antiangiogenic therapy cannot be restricted to the assessment of its impact on tumor vascularization and, conversely, an antiangiogenic therapy able to significantly affect tumor vascularization may not impair the size of the tumor. Thus, our data expand previous observations on the different efficacy of antiangiogenic drugs in prevention, intervention, and regression trials in a transgenic mouse model of pancreatic islet carcinogenesis (60).

In conclusion, the results indicate that beyond the initial phases, the progressive growth of tumors is at least partly independent of FGF2 expression and vascular density. The possibility that a significant block of angiogenesis may not affect the expansion of large cancers needs to be considered in the design of clinical trials with antiangiogenic compounds.

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