

Colony-stimulating Factor-1 Antisense Treatment Suppresses Growth of Human Tumor Xenografts in Mice¹

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ABSTRACT

Matrix metalloproteinases (MMPs) foster cellular invasion by disrupting extracellular matrix barriers and thereby facilitate tumor development. MMPs are synthesized by both cancer cells and adjacent stromal cells, primarily macrophages. The production of macrophages is regulated by colony-stimulating factor-1 (CSF-1). Tissue CSF-1 expression increased significantly in embryonic and colon cancer xenografts. We, therefore, hypothesized that blocking CSF-1 may suppress tumor growth by decelerating macrophage-mediated extracellular matrix breakdown. Cells expressing CSF-1 and mice xenografted with CSF-1 receptor (*c-fms*)- and CSF-1-negative malignant human embryonic or colon cancer cells were treated with mouse CSF-1 antisense oligonucleotides. Two weeks of CSF-1 antisense treatment selectively down-regulated CSF-1 mRNA and protein tissue expression in tumor lysates. CSF-1 blockade suppressed the growth of embryonic tumors to dormant levels and the growth of the colon carcinoma by 50%. In addition, tumor vascularity and the expression of MMP-2 and angiogenic factors were reduced. Six-month survival was observed in colon carcinoma mice only after CSF-1 blockade, whereas controls were all dead at day 65. These results suggest that human embryonic and colon cancer cells up-regulate host CSF-1 and MMP-2 expression. Because the cancer cells used were CSF-1 negative, CSF-1 antisense targeted tumor stromal cell CSF-1 production. CSF-1 blockade could be a novel strategy in treatment of solid tumors.

INTRODUCTION

The multistage process underlying the development of cancer is complex, during which a normal cell undergoes genetic changes that result in phenotypic alterations and the acquisition of the ability to invade and colonize distant sites (1, 2). Interactions between neoplastic cells and the surrounding microenvironment are crucial to each step of tumorigenesis. One group of proteins that plays an essential role in the dynamics of maintaining the cellular microenvironment is the MMP² family (3). The MMPs are associated with degradation of the ECM, including the basement membrane. Disruption of basement membrane integrity allows invasive tumors to spread locally and distantly (3, 4). In addition to fostering cellular invasion by disrupting ECM barriers, MMPs can also influence the microenvironment by altering cellular signals (5, 6).

Growing evidence supports an expanded role of MMPs in creating and maintaining a microenvironment that facilitates the initial stages of tumor development (7–9). More importantly, most MMPs are synthesized not only by the genetically altered cancer cells but also by

adjacent and intervening stromal cells (10). Macrophages are frequent components in the stroma of neoplastic tissues (11) and modify the ECM and angiogenesis (12–15). The production of macrophages is regulated by CSF-1 mediated through its receptor, the *c-fms* proto-oncoprotein (16). CSF-1 is produced by a variety of cells including endothelial cells (17), fibroblasts (18), and epithelial cells (19). We have shown that CSF-1, which stimulates the proliferation, differentiation, and survival of cells of the mononuclear lineage (20), also accelerates angiogenesis *in vivo* (21).

Serum levels of CSF-1 have been reported as markedly elevated in patients with endometrial (22), ovarian (23), and lung cancer (24). Moreover, evidence exists that CSF-1 promotes tissue invasion of lung cancer cells by enhancing MMP-2 production (25). On the basis of these facts, we hypothesized that blocking CSF-1 expression would decelerate ECM degradation and subsequently retard tumor growth. To block CSF-1, we designed CSF-1 PT-modified antisense ODNs. Antisense ODNs are chemically modified stretches of single-stranded DNA complementary to pre-mRNA and mRNA regions of a target gene that are capable of inhibiting gene expression. These constructs hold great promise as agents for the specific manipulation of gene expression (26, 27). To examine the efficacy of the CSF-1 antisense ODNs, they were first tested on cells expressing CSF-1 and then in immune-deficient mice xenografted with human embryonic (28) and colon cancer cells (29). We show that CSF-1 PT-modified antisense ODNs significantly down-regulated CSF-1 gene and protein expression *in vitro*. We provide evidence that CSF-1-negative human embryonic and colon cancer cells induced up-regulated host (mouse) CSF-1 and MMP-2 expression. Moreover, we demonstrate that 2 weeks of antisense treatment directed against mouse CSF-1 selectively suppressed the growth of the human embryonic and colon carcinoma xenografts, their increased MMP-2 protein expression, and their angiogenic activity.

MATERIALS AND METHODS

ODNs. Three PT-modified CSF-1 antisense ODNs and one scrambled ODN were obtained from VBC Genomics (Vienna, Austria). The antisense and scrambled control sequences were: ODN-181 (around CSF-1 start codon), 5'-ACGGGCAGCTGGGTCCCG-3'; ODN-196 (around CSF-1 start codon), 5'-GCCCGCGCGGTCAT-3'; ODN-16 (upstream of CSF-1 start codon in the untranslated region), 5'-GGCCGCGCTCGAGCTC-3'; and scrambled ODN, 5'-CGAGAGCCGCGCACGC-3'.

Analysis of CSF-1 Antisense ODN Effects *in Vitro*. Mouse epithelial F-9 tumor cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10% FCS, until they reached 50% confluency. Cells were rinsed with PBS, then treated with antisense ODNs or scrambled ODN (5, 15, and 25 μ g/ml in 2 ml of serum-free medium) for 1 h before supplementation with FCS. Untreated cells served as additional controls. Experiments were performed in triplicate. At 24 h after incubation, mRNA was isolated for RT-PCR, and the supernatant was filtered and used for ELISA. Real-time RT-PCR (Light Cycler; Roche, Mannheim, Germany) was performed as described earlier (30). The primer sequences for mouse CSF-1, *c-fms*, and β -actin were: CSF-1, sense 5'-CATCTCCATCCCTAAATCAAC-3', antisense 5'-ACTTGCTGATCCTCTCC-3'; *c-fms*, sense 5'-GCGATGTGTGAGCAA-

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³ The abbreviations used are: MMP, matrix metalloproteinase; ECM, extracellular matrix; CSF-1, colony-stimulating factor-1; *c-fms*, CSF-1 receptor; PT, phosphorothioate; ODN, oligonucleotide; CBC, complete blood count; IVM, intravital video microscopy; SCID, severe combined immunodeficient; VEGF, vascular endothelial growth factor.

TGGCA-3', antisense 5'-CGGATAATCGAACCTCGCCA-3'; β -actin, sense 5'-GCGTGACATCAAAGAGAAG-3', antisense 5'-AGGAGCCAGAGCAGTAATC-3'. ELISA was performed to detect mouse CSF-1 protein in culture medium, using a CSF-1 monoclonal antibody according to the manufacturer's protocol (Quantikine; R&D; Minneapolis, MN). After incubation for 2 h, horseradish peroxidase-conjugated CSF-1 polyclonal antibody was added, followed by substrate solution. The absorbance was measured at 450 nm. The results of CSF-1 mRNA and protein expression were normalized to the number of cells. Measurements were performed in triplicate.

Tumor Models and CSF-1 Antisense Treatment. The experiments performed in this study were approved by the Institutional Animal Care and Use Committee at the University of Vienna. Established human metastatic embryonic (CRL-2073; American Type Culture Collection) and colon (SW-620; American Type Culture Collection) carcinoma cells were grown in immune-deficient mice as described earlier (28, 29). Pathogen-free male C.B-17 SCID/SCID (SCID) and BALB/*c-nu/nu* (nude) mice (Harlan-Winkelmann, Borcheln, Germany), 5 weeks of age, were weighed and coded and randomly assigned to experimental groups ($n = 8$). SCID mice were anesthetized (ketamine hydrochloride/xylazine at 55/7.5 mg/kg, s.c.), and 10^6 CRL-2073 cells/ $10 \mu\text{l}$ of culture medium were injected subcapsularly and bilaterally into the testis (28). Ten days after cell injection, one group of animals was killed and evaluated for tumor area and tumor weight ($17.9 \pm 3.9\%$ of total tissue area and 110 ± 27 mg, respectively). Animals in an earlier study had developed human embryonic tumors of similar size at 10 days (28), and treatment was initiated that same day. Nude mice were anesthetized as described above, and 8×10^6 SW-620 cells/ $80 \mu\text{l}$ of culture medium were grafted s.c. in the left flank (29). The growth of the human colon carcinoma xenograft was evaluated in a pilot study by determining the tumor weight every other day ($n = 4$). Animals in the present study developed human colon carcinomas of similar weight (117.5 ± 23.3 mg) at 8 days, and treatment was initiated that same day. Mice were anesthetized as described above, and constant infusion osmotic mini pumps (Alzet 1002; Alza, Palo Alto, CA) prefilled with antisense ODNs, scrambled ODN, or Ringer's solution (control) were inserted s.c. into a paraspinal pocket. Implanted pumps delivered their content ($100 \mu\text{l}$) at a rate of $0.25 \mu\text{l/h}$ over a period of 2 weeks, and all animals were killed 14 days after implantation of the pumps. The selected dosage of 5 mg/kg per day over 14 days was based on pilot studies in mice ($n = 24$). Untreated age-matched mice ($n = 16$) served as additional controls.

Analysis of the *in Vivo* Effects of CSF-1 Antisense ODNs in SCID Mice. On day 24, blood samples were obtained from the tail vein to examine CBC (28). Next, images of the right testicular microvasculature were recorded using fluorescence IVM, as described earlier (28). Before IVM, animals received FITC i.v. to enhance resolution and to allow recognition of vascular sprouts (31), and recorded images were transferred to a computer and analyzed off-line. In 10 microcirculatory units, the density of vascular sprouts was evaluated (28, 31). After IVM, the right testis was isolated and weighed. Tissue was processed for paraffin embedding, and serial sections were stained with H&E, Ki-67 antibody (tumor proliferation assay; DAKO, Glostrup, Denmark), MMP-2 (Santa Cruz Biotechnology, Santa Cruz, CA), or double-stained with von Willebrand (DAKO) and Ki-67 antibodies (to evaluate the density of proliferating endothelial cells). Briefly, deparaffinized sections were preincubated with 3% H_2O_2 and horse serum to block endogenous peroxidase and antigen activity, before incubation with the primary antibodies (MMP-2, 1:100 dilution; Ki-67, 1:50 dilution; von Willebrand, 1:1500 dilution). Biotinylated secondary antibodies (Vector Laboratories, Inc., Burlingame, CA) were used to detect positive staining, followed by streptavidin-biotin peroxidase complex. Negative controls were made by omitting the first antibody. Digitalized images were generated, and morphometry was carried out (28). The left testis was weighed, frozen in liquid nitrogen, and used for real-time RT-PCR and RIA analyses as described below.

Analysis of the *in Vivo* Effects of CSF-1 Antisense ODNs in Nude Mice. On day 22, the CBC was evaluated (28) and the colon carcinoma xenografts were isolated and weighed. One portion of the tissue was processed for histology as described above, and the remainder processed for RT-PCR, RIA, and Western blotting as described below.

Analysis of the Effects of CSF-1 Antisense ODNs on Survival in Nude Mice. The survival study, involving 28 mice bearing colon carcinoma xenografts, was set for 6 months. Fourteen mice were treated with CSF-1 ODN 196, seven with Ringer's solution and seven with scrambled ODN.

Quantitative Real-Time RT-PCR. Samples were homogenized in liquid nitrogen and processed for PCR as described earlier (30). The primer sequences for mouse CSF-1, *c-fms*, and β -actin were as described above. The primer sequences for mouse angiogenic molecules were: VEGF-A, sense 5'-TACTGCTGTACTCCACC-3', antisense 5'-GCTCATTCTCTATGTGCTG-3'; Flt-1, sense 5'-AAACCTGTCCAACACTACCTC-3', antisense 5'-TAATCCTCGTCTCCTTCC-3'; KDR-flk-1, sense 5'-GGAGATTGAAA-GAAGGAAC-3', antisense 5'-ACTTCTCTTCTCCATAC-3'; Ang-1, sense 5'-GCAACTTCTCCAACAGAC-3', antisense 5'-TCTTCTTTTTCTCC-3'; Tie-2, sense 5'-AACAGCGTCTTCTTCTACC-3', antisense 5'-ATCTCCGAACAATCAGCC-3'. The primer sequences for human molecules were: CSF-1, sense 5'-GCTGTTGGTGTCTGCTC-3', antisense 5'-CATGCTTTCATAATCCTTG-3'; *c-fms*, sense 5'-TGCTGCTCCGCTGC-TATTG-3', antisense 5'-TCAGCATTTACAGCCACC-3'; β -actin, sense 5'-TGCCATCCTAAAAGCCAC-3', antisense 5'-CAACTGGTCTCAAGT-CAGAGTG-3'. Measurements were performed three times.

CSF-1 RIA. Prewashed tissue and cell samples were homogenized, heat-inactivated, and centrifuged, and the supernatant was saved for assay as described previously (32). CSF-1 was measured in duplicate on three samples from each mouse using a RIA that only detects biologically active mouse CSF-1 (32, 33).

Western Blotting. Tissue samples were lysed in solubilization buffer and Complete-EDTA-free Protease Inhibitor Cocktail (Roche). Tissue lysates ($50 \mu\text{g/lane}$) were separated by 10% SDS-PAGE before electrophoretic transfer onto Hybond C super (Amersham Pharmacia Biotech, Buckinghamshire,

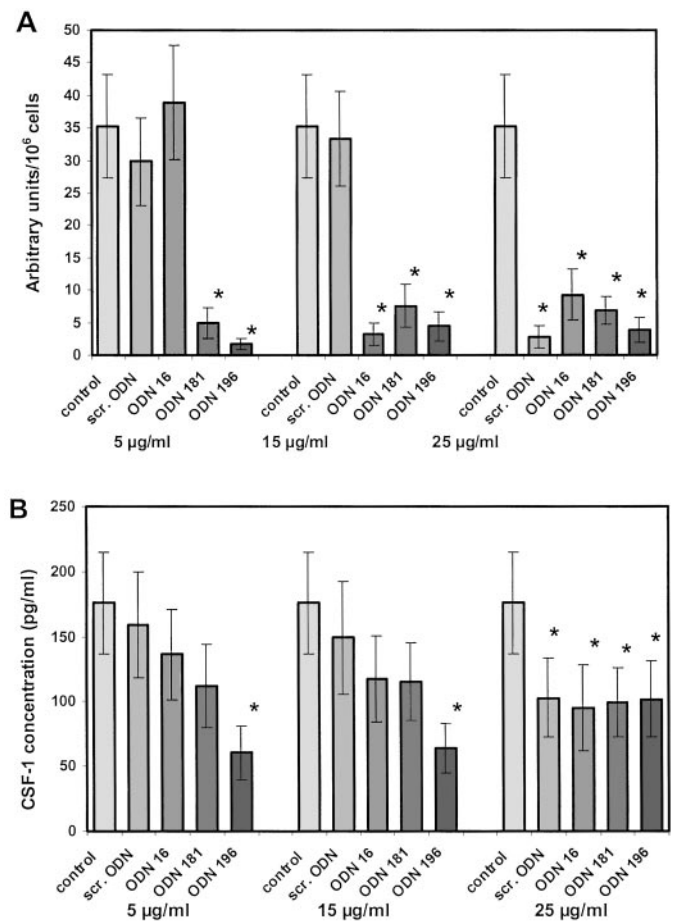


Fig. 1. CSF-1 antisense ODNs down-regulate CSF-1 gene and protein expression in F-9 cells. A, real-time RT-PCR measurements in cell lysates. CSF-1 mRNA expression decreases after 5, 15, and 25 $\mu\text{g/ml}$ antisense ODN-181 and ODN-196, whereas antisense ODN-16 and scrambled (*scr.*) ODN down-regulate CSF-1 gene expression only at 15 and 25 $\mu\text{g/ml}$ culture medium, respectively. B, ELISA measurements in cell supernatants. CSF-1 protein expression declines at 5 and 15 $\mu\text{g/ml}$ only in the case of CSF-1 antisense ODN-196, whereas all ODNs including the scrambled ODN decrease CSF-1 protein expression at 25 $\mu\text{g/ml}$ culture medium. *, significantly different from controls ($P < 0.001$, A; $P < 0.05$, B).

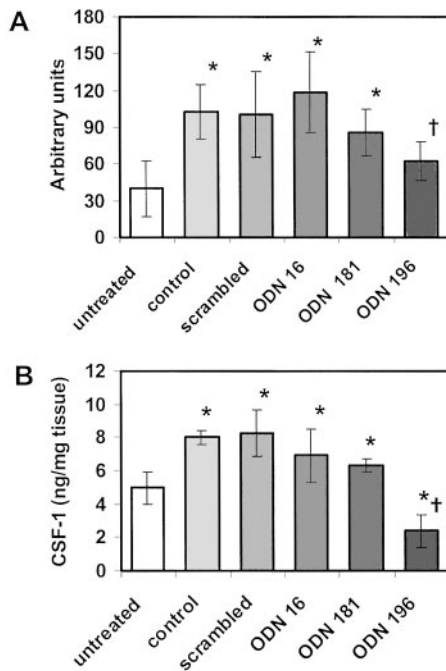


Fig. 2. Effect of 5 mg/kg/day CSF-1 antisense ODN on CSF-1 tissue expression in SCID mice bearing embryonic tumors is sequence dependent. *A*, real-time RT-PCR mRNA measurements in tumor and tissue lysates of SCID mice indicate that only CSF-1 antisense ODN-196 significantly suppresses CSF-1 mRNA expression in tumor lysates after 2 weeks of treatment. *B*, mouse CSF-1 RIA measurements in tissue and tumor lysates of SCID mice confirm the mRNA data. After CSF-1 antisense ODN-196 treatment, CSF-1 tissue protein levels decrease even below the baseline level of untreated SCID mice. *, significantly different from untreated ($P < 0.05$); †, significantly different from control ($P < 0.05$, *A*; $P < 0.004$, *B*).

United Kingdom). The blots were probed with polyclonal antibody against MMP-2 (Santa Cruz Biotechnology) before incubation with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech; Ref. 30). Proteins were immunodetected on the membrane using enhanced chemi-

luminescence (Amersham Pharmacia Biotech), and specific protein bands were quantified using Easy plus Win 32 software (Herolab, Wiesloch, Germany).

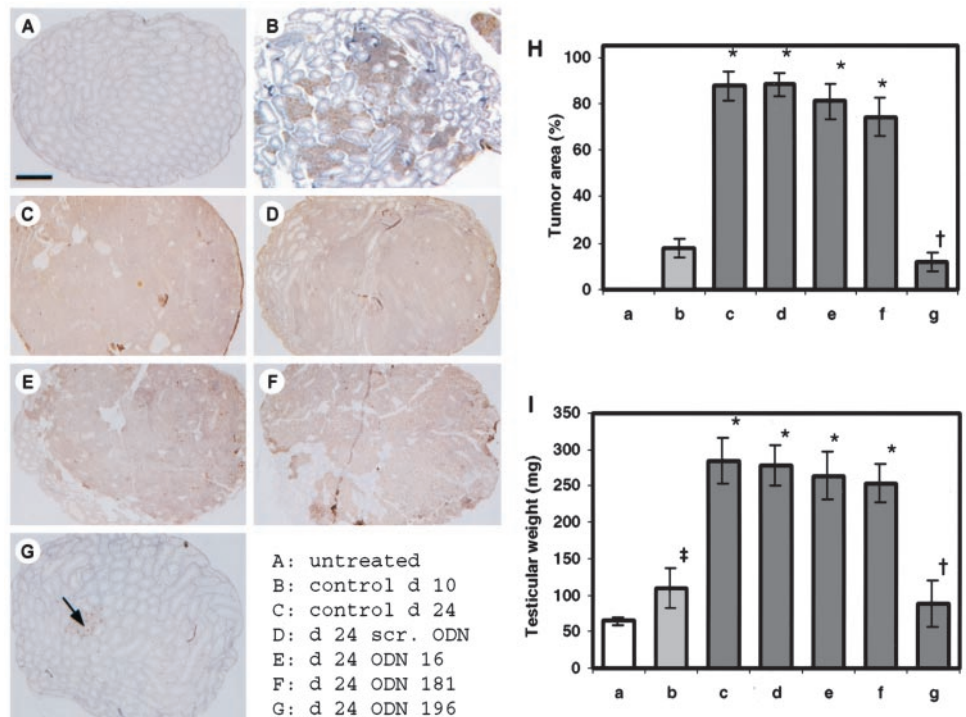
Statistical Analysis. ANOVA with Bonferroni *t* test was used to compare the data between the groups. For analysis of the number of animals with long-term survival (6 months), related to the number of mice that died, the χ^2 test was used to compare the groups. The overall survival curves after treatment were analyzed by the Kaplan-Meier survival test. Data are expressed as means \pm SD. *P*s of <0.05 were considered to indicate statistical significance.

RESULTS

CSF-1 Antisense PT-ODNs Down-Regulate CSF-1 Expression *in Vitro*. To examine the efficacy of the designed ODNs, we used CSF-1-expressing mouse epithelial cells and treated them with 5, 15, or 25 $\mu\text{g/ml}$ of CSF-1 antisense PT-ODN-16, ODN-181, ODN-196, and scrambled ODN. Treatment with 5, 15, and 25 $\mu\text{g/ml}$ ODN-181 and ODN-196 decreased CSF-1 mRNA expression significantly ($P < 0.001$; Fig. 1A). CSF-1 gene expression declined ($P < 0.001$) after treatment with 15 and 25 $\mu\text{g/ml}$ ODN-16, but not 5 $\mu\text{g/ml}$ ODN-16. Although treatment with 5 and 15 $\mu\text{g/ml}$ scrambled ODN did not change CSF-1 mRNA expression significantly, scrambled ODN at 25 $\mu\text{g/ml}$ down-regulated CSF-1 mRNA compared with controls ($P < 0.001$). At 5 and 15 $\mu\text{g/ml}$, only ODN-196 significantly down-regulated CSF-1 protein expression ($P < 0.05$), whereas at 25 $\mu\text{g/ml}$, all ODNs decreased CSF-1 protein expression ($P < 0.05$; Fig. 1B). These experiments indicated that the effect of the applied CSF-1 antisense ODNs was sequence and dose dependent, and that the down-regulated CSF-1 gene and protein expression observed at 25 $\mu\text{g/ml}$ for all ODNs, including the mismatch control ODN, was nonspecific and most likely caused by the higher PT dosage.

Human Embryonic Cancer Cells Up-Regulate Host CSF-1 Production. Before initiating *in vivo* experiments, we performed real-time RT-PCR and ELISA analyses of CRL-2073 cells and found that they do not express detectable mRNA or protein for human CSF-1 or its receptor *c-fms* (data not shown). When these cells were xenografted to SCID mice, however, mouse tissue CSF-1 gene and

Fig. 3. CSF-1 antisense ODN-196 significantly suppresses embryonic tumor growth in SCID mice. Representative photomicrographs of sections of testis stained with Ki-67 antibody show tumor cell proliferation. *A*, untreated mouse. *B*, tumor in a control mouse on day 10. *C*, tumor in a control mouse on day 24. *D*, tumor on day 24 in a mouse after treatment with scrambled (*scr.*) ODN. *E*, tumor on day 24 in a mouse after treatment with ODN-16. *F*, tumor on day 24 in a mouse after treatment with ODN-181. *G*, tumor (arrow) shrank to a dormant level on day 24 in a mouse after treatment with ODN-196. Bar, 800 μm . *H*, mean tumor area as a percentage of the total tissue area. *I*, mean testicular weight. CSF-1 antisense ODN-196 significantly reduces tumor area and testicular weight in SCID mice (*g* in *H* and *I*). *a-g* in *H* and *I* refer to corresponding experimental groups (and/or panels). *, significantly different from *b* ($P < 0.005$); †, significantly different from *c-f* ($P < 0.01$); ‡, significantly different from *a* ($P < 0.05$).



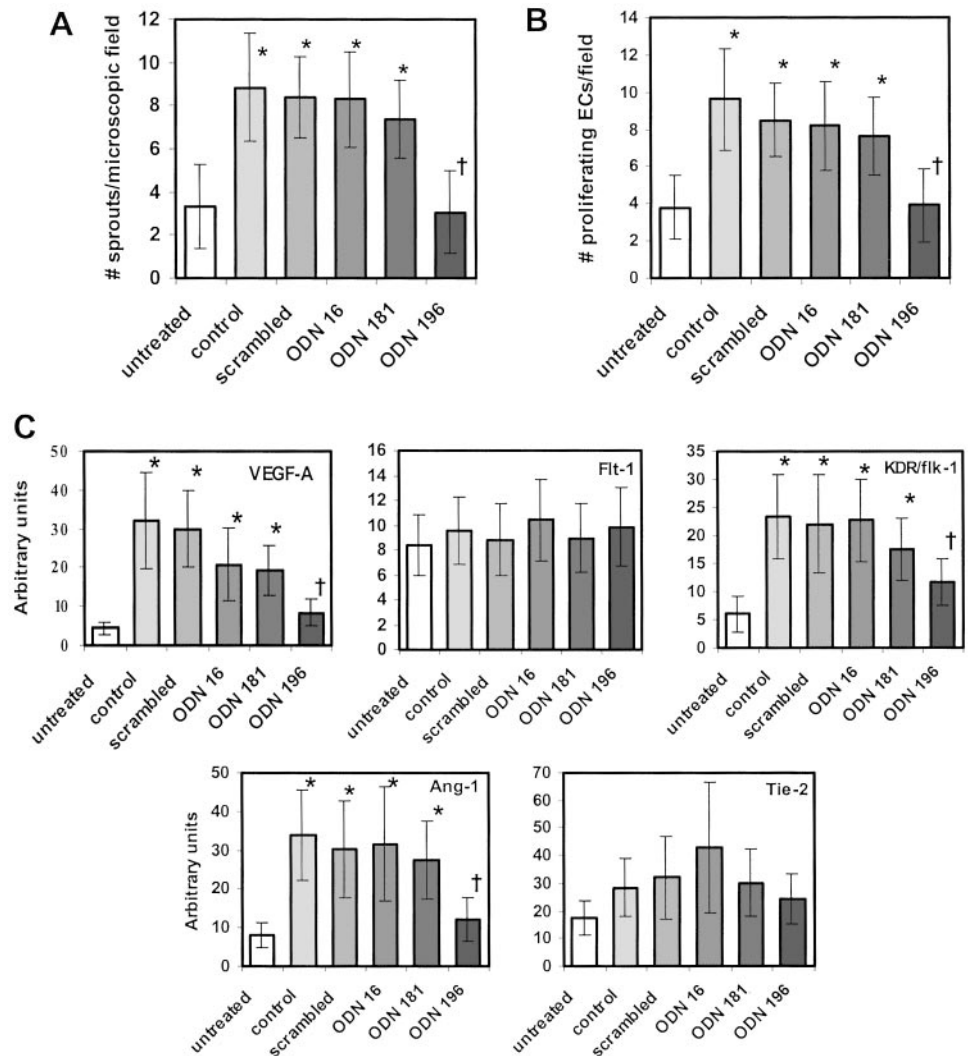


Fig. 4. CSF-1 antisense ODN-196 treatment decreases vascularity and expression of angiogenic molecules in embryonic tumor xenografts. *A*, quantitative analysis of the density of capillary sprouts using IVM. *B*, quantitative histomorphometric analysis, showing the density of capillaries with proliferating endothelial cells (ECs). The density of capillary sprouts increases in control animals, and only ODN-196 treatment reduces their density significantly. *C*, real-time RT-PCR measurements in embryonic tumor and tissue lysates. Consistent with the changes in vascularity, the mRNA expression of both VEGF-A and its receptor-2, KDR/flk-1, as well as Ang-1, decreases after ODN-196 treatment, whereas the mRNA levels of VEGF receptor 1, Flt1, and Ang-1 receptor Tie-2 are not significantly changed. *, significantly different from untreated ($P < 0.05$; *A*, *B*, and *C* (KDR/flk-1); $P < 0.004$, *C* (VEGF-A and Ang-1); †, significantly different from control ($P < 0.05$).

protein expression increased significantly ($P < 0.05$) compared with untreated mice (Fig. 2). Associated with increasing CSF-1 tissue expression, enhanced infiltration of macrophages around and into the tumor tissue was detected (histology not shown). These experiments indicated that human embryonic cancer cells stimulate increased host tissue expression of CSF-1. Correlated with these results, RT-PCR conclusively showed increased mouse *c-fms* expression in tumor lysates (data not shown).

CSF-1 Antisense ODN-196 Suppresses CSF-1 Expression and the Growth of Embryonic Tumor Xenografts. Mice bearing established human embryonic tumors were treated with CSF-1 antisense PT-ODNs, scrambled ODN, or Ringer's solution. Antisense ODN treatment was well tolerated. Local inflammatory reactions were not observed, nor were there significant changes in the CBC of treated mice (results not shown). After CSF-1 antisense ODN-196 treatment, CSF-1 mRNA levels were reduced compared with controls ($P < 0.05$). CSF-1 gene expression, however, was not significantly changed after treatment with ODN-16, ODN-181, and scrambled ODN (Fig. 2A). Consistent with the changes in CSF-1 mRNA levels, treatment with CSF-1 antisense ODN-196 significantly down-regulated tissue CSF-1 protein levels ($P < 0.004$), whereas treatment with ODN-16, ODN-181, and scrambled ODN did not (Fig. 2B). Marked differences were found in the tumor area and testicular weight between SCID mice with human embryonic tumors treated with CSF-1

ODN-196 for 2 weeks ($11.9 \pm 3.7\%$ tumor area; 89 ± 32 mg tumor weight) and those treated with Ringer's solution ($87.7 \pm 6.2\%$ tumor area; 285 ± 31 mg tumor weight; $P < 0.01$) or scrambled ODN ($88.2 \pm 5\%$ tumor area; 278 ± 27 mg tumor weight; $P < 0.01$; Fig. 3). Treatment with CSF-1 antisense ODN-16 and ODN-181 did not result in a significant change of tumor area ($81.1 \pm 7.6\%$ and $74.1 \pm 8.3\%$, respectively) or tumor weight (264 ± 33 mg and 254 ± 26 mg, respectively; Fig. 3).

CSF-1 Antisense ODN-196 Decreases Angiogenic Activity in Embryonic Tumor Xenografts. Both IVM and histomorphometry of embryonic tumors showed significantly increased density of vascular sprouts in control compared with untreated mice ($P < 0.05$) that returned to the baseline level of untreated animals after CSF-1 antisense ODN-196 treatment. The sprout density was, however, unchanged in mice treated with ODN-16, ODN-181, and scrambled ODN (Fig. 4, *A* and *B*). Similarly, VEGF-A, its receptor KDR/flk-1, and Ang-1 mRNA levels were significantly reduced in CSF-1 antisense ODN-196-treated mice ($P < 0.05$), but not significantly decreased in ODN-16, ODN-181, and scrambled ODN-treated SCID mice (Fig. 4C). ODN-196 treatment did not change VEGF receptor 1 (Flt-1) and Ang-1 receptor (Tie-2) tissue mRNA expression levels significantly. VEGF and Ang receptor gene expression levels remained unchanged in the case of ODN-16-,

ODN-181-, and scrambled ODN-treated mice compared with controls (Fig. 4C).

CSF-1 Antisense ODN-196 Decreases MMP-2 Expression in Embryonic Tumor Xenografts. After xenografting of human embryonic cancer cells to the mouse testis, protein expression of MMP-2, a key molecule in mediating tumor metastasis and angiogenesis, increased significantly in control *versus* untreated animals ($P < 0.001$). Treatment with CSF-1 antisense ODN-196, but not ODN-16, ODN-181, or scrambled ODN, significantly down-regulated MMP-2 protein expression in testicular tissue ($P < 0.005$; Fig. 5). Positive MMP-2 antigen staining was primarily observed in the intertubular interstitium, in the capsule of testicular tubules, and less frequently in the wall of vessels in untreated and ODN-196-treated mice (Fig. 5, A and C), whereas in controls and ODN-16-treated mice MMP-2 expression was primarily intratubular (Fig. 5, B and D).

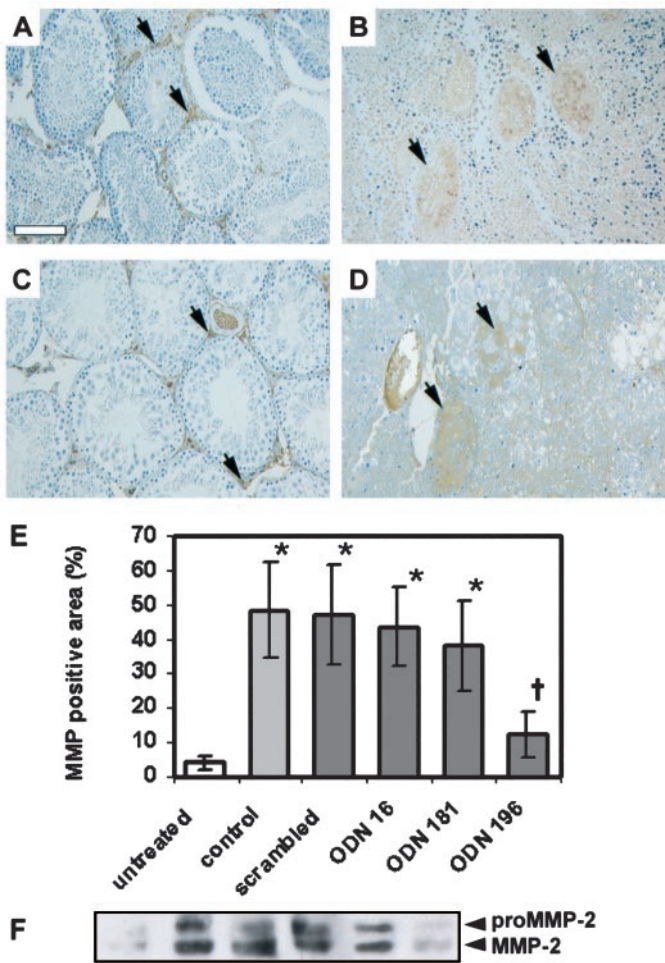


Fig. 5. CSF-1 antisense ODN-196 reduces MMP-2 protein expression in embryonic tumor xenografts. Representative immunocytochemistry images of specimens stained with MMP-2 antibody are shown. A, testis of an untreated mouse. MMP-2 expression is barely visible (arrowheads). B, control testis. MMP-2 expression is significantly up-regulated and is readily seen inside the tubuli (arrowheads). C, testis of a mouse after CSF-1 antisense ODN-196 treatment. The pattern of MMP-2 expression (arrowheads) is similar to the pattern in untreated testis. D, MMP-2 expression (arrowheads) in the testis of a mouse after ODN-16 treatment. Note similarity to the expression in control testis. Bar, 100 μ m. E, morphometry of MMP-2-positive areas (percentage of total testicular area) in experimental groups. Only in ODN-196-treated animals was the expression of MMP-2 reduced. F, representative Western blot images. The MMP-2 tissue protein expression is increased in control mice and mice treated with scrambled ODN, ODN-16, and ODN-181 compared with untreated mice. After ODN-196 treatment, MMP-2 protein expression decreases to untreated levels. *, significantly different from untreated ($P < 0.001$); †, significantly different from control ($P < 0.005$).

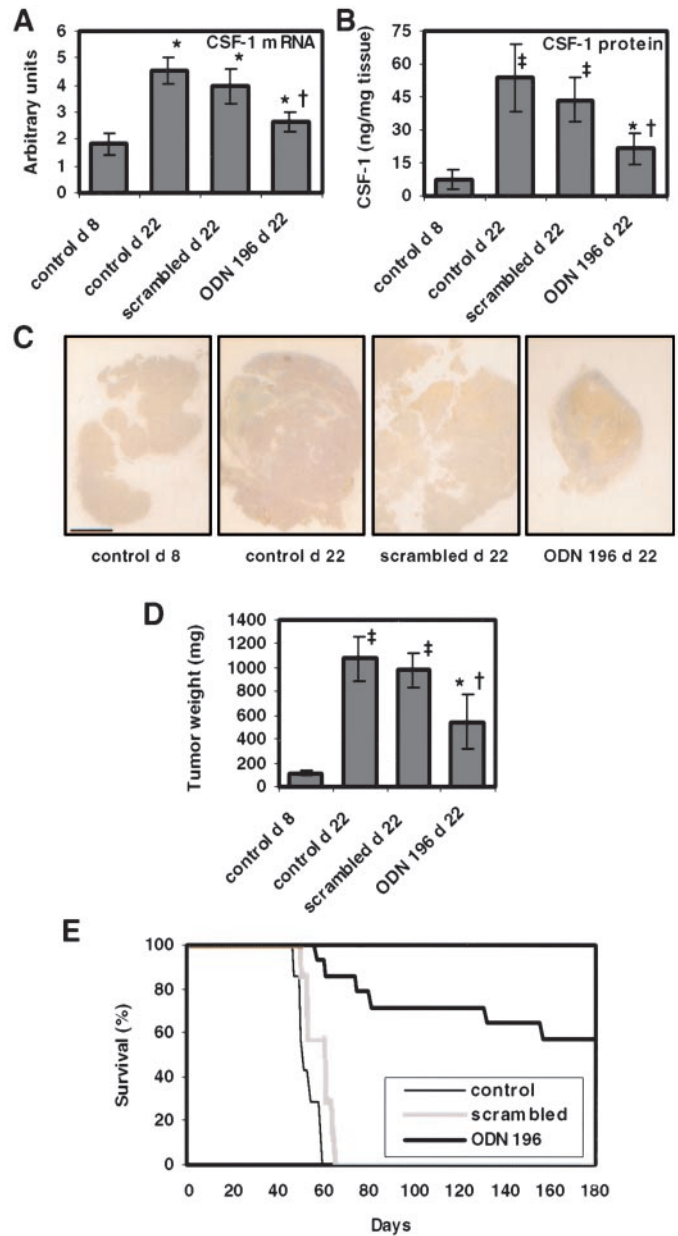


Fig. 6. CSF-1 antisense ODN-196 suppresses CSF-1 protein expression and markedly retards the growth of colon cancer xenografts in nude mice. Real-time RT-PCR (A) and RIA (B) measurements of CSF-1 in tumor lysates indicate that CSF-1 expression increases with tumor progression, and that ODN-196 treatment down-regulates increased CSF-1 mRNA and protein expression levels. C, representative photomicrographs of sections of colon carcinoma xenograft stained with Ki-67 antibody. Tumor size is markedly decreased after CSF-1 blockade as compared with control mice. Bar, 1 mm. D, mean colon carcinoma weight. CSF-1 antisense ODN-196 significantly reduces tumor weight. E, survival of mice bearing colon carcinoma. Whereas 8 of 14 mice receiving CSF-1 antisense survived 6 months, none of the animals in the control groups survived beyond day 65. *, significantly different from control day 8 ($P < 0.05$); †, significantly different from control day 22 and scrambled day 22 ($P < 0.05$); ‡, significantly different from control day 8 ($P < 0.005$).

CSF-1 Antisense ODN-196 Suppresses the Growth of a Human Colon Carcinoma Xenograft and Increases the Survival in Grafted Mice. The experiments with CSF-1 antisense treatment in the mouse model of human embryonic tumor suggested that ODN-196 was most effective in suppressing CSF-1 expression and tumor growth. These promising results encouraged us to test CSF-1 antisense treatment in other human tumor models. We chose colon carcinoma because of its poor prognosis, short median survival, and high incidence. We used SW-620 human colon carcinoma cells that did not

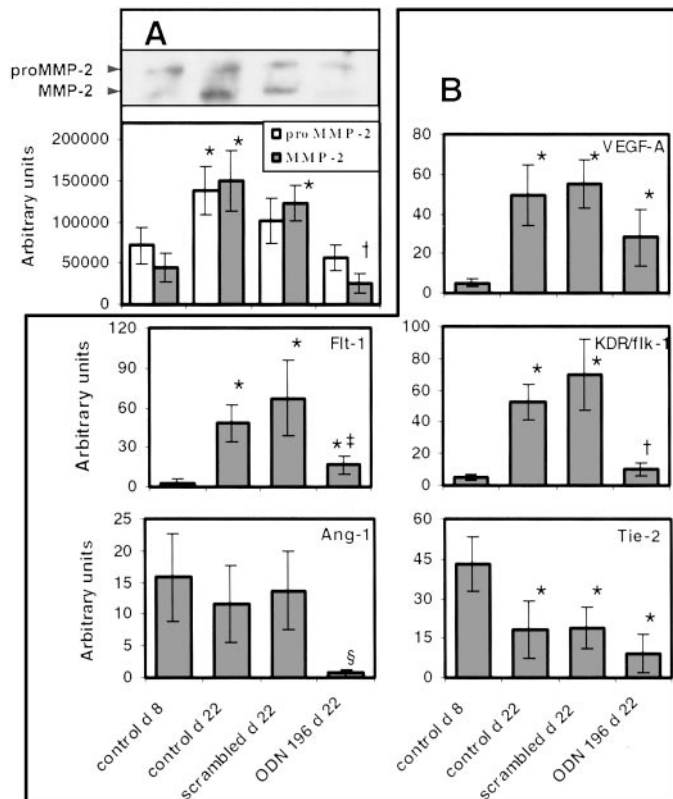


Fig. 7. CSF-1 antisense ODN-196 treatment down-regulates expression of MMP-2 and angiogenic molecules in colon carcinoma xenografts in nude mice. A, representative Western blot images of MMP-2 and quantification of MMP-2 protein expression levels in tumor lysates. MMP-2 expression increases with tumor progression and declines significantly following CSF-1 blockade. B, real-time RT-PCR measurements of angiogenic molecules in colon carcinoma lysates. The mRNA levels of VEGF-A receptors Flt-1 and KDR and of Ang-1 are down-regulated after ODN-196 treatment. VEGF-A and Tie-2 mRNA levels are not significantly changed. *, significantly different from control day 8 ($P < 0.05$); †, significantly different from control day 22 and scrambled day 22 ($P < 0.005$); ‡, significantly different from control day 22 and scrambled day 22 ($P < 0.05$); §, significantly different from controls ($P < 0.001$).

express CSF-1 and *c-fms* (data not shown). Using the established flank model in nude mice, we show that host CSF-1 tissue mRNA ($P < 0.05$) and protein levels ($P < 0.005$) increased with progressing tumor growth (Fig. 6, A and B). After 2 weeks of CSF-1 antisense ODN-196 treatment at 5 mg/kg/day, CSF-1 mRNA and protein expression was significantly ($P < 0.05$) down-regulated versus controls (Fig. 6, A and B). Tumor growth was markedly retarded in mice after CSF-1 blockade (Fig. 6C), and similarly their tumor weight was significantly decreased ($P < 0.05$) compared with the tumor weight of mice treated with Ringer's solution (Fig. 6D). Similar to mice bearing embryonic tumors, CSF-1 treatment was well tolerated in nude mice bearing colon carcinoma. The CBC was not significantly changed by the treatments (data not shown). Long-term (6-month) survival was observed in 8 of 14 mice after CSF-1 blockade, whereas all mice were dead after 65 days in the two control groups (7 of 7 mice in each group; $P < 0.01$; Fig. 6E). At sacrifice 6 months after the therapy, none of the mice had metastases. At 65 days (at which time the last animal of the control groups died) still 85.7% of CSF-1 antisense-treated mice were alive ($P < 0.01$; Fig. 6E).

The MMP-2 protein expression in tumor lysates was markedly increased on day 22 compared with its expression in day 8 control mice ($P < 0.05$). After CSF-1 inhibition, MMP-2 protein expression declined significantly ($P < 0.005$; Fig. 7A). ODN-196, but not scrambled ODN treatment, resulted in down-regulation of mRNA levels of the VEGF-A receptors Flt-1 ($P < 0.05$) and KDR/flk-1 ($P < 0.005$),

and of Ang-1 ($P < 0.001$). mRNA expression of VEGF-A and the Ang-1 receptor Tie-2 were not significantly changed (Fig. 7B).

DISCUSSION

CSF-1 is the primary growth factor regulating tissue macrophages (34). Tumor-associated macrophages increase ECM breakdown and thereby enhance tumor growth and invasion (25, 35). MMPs are known to be crucial for degrading ECM components and for promoting both endothelial and tumor cell invasion *in vitro* and *in vivo* (15, 36). Metastatic tumor cell lines have been reported to express higher levels of gelatinolytic MMPs than do their nonmetastatic counterparts (4). MMP-2 and MMP-9 are most closely correlated with metastatic potential (4, 36, 37). Recent studies indicate (37) that inhibition of the extracellular activation of pro-MMP-2 is a major mechanism in the action of the antiangiogenic and antitumor protein endostatin (38). Our results show that human embryonic and colon cancer cells up-regulate host CSF-1, and that this up-regulation is correlated with increased MMP-2 expression. The fact that the human cancer cells used here do not express *c-fms* indicates that the host *c-fms*-expressing macrophages have been the targets of the therapy. Whether CSF-1 blockade affected the recruitment of macrophages or their local proliferation in these tumor models is not certain, although studies of CSF-1 biology suggest that both mechanisms are likely to occur (34, 39).

Because there is no direct evidence for expression of the CSF-1 receptor on endothelial cells, one likely possibility is that the reduced vascularity and down-regulation of angiogenic growth factors we observed are mediated by the inhibition of action of CSF-1 stimulation of macrophage MMP expression and subsequent alteration of the ECM, thereby affecting the expression of angiogenic factors. In this context, accumulating evidence shows that tumor implants in mice induce VEGF promoter activity in stromal fibroblasts (40). The latter mechanism benefits the treatment modality proposed in this study, which bypasses the genetically unstable cancer cells and instead targets the stromal cells. In any case, MMPs mediate selective proteolytic degradation of ECM, and this mechanism is required for invasion of both cancer and endothelial cells (12–15, 41, 42), underlying the significance of the observed MMP-2 suppression in our tumor models. Clearly, the little understood role of stromal elements in endothelial cell proliferation and migration deserves further study.

Several conclusions can be drawn concerning our observation that ODN-196 treatment down-regulated both KDR/flk-1 and Flt-1 mRNA expression in colon carcinoma xenografts and KDR/flk-1 expression in embryonic tumor xenografts, but did not change Tie-2 expression in both carcinomas. First, this finding is in agreement with the observation that KDR, but not Flt-1, is essential for tumor angiogenesis (43) and for VEGF-induced endothelial cell proliferation and migration (44). Second, it seems that the molecular regulation of Ang/Tie system is regulated at the ligand level in our tumor models, as indicated by up-regulated Ang-1 but not Tie-2 mRNA expression levels. Third, the different down-regulation pattern of VEGF receptors following CSF-1 blockade suggests a tumor-specific regulation of VEGF receptors, at least in the human tumor xenografts used in this study.

The sequence specificity of the effects observed in our studies indicates an antisense mechanism of action of CSF-1 antisense ODN-196 used, although additional therapeutically beneficial sequence-dependent non-antisense interactions cannot be ruled out (26, 27, 45, 46). Our data indicate that down-regulated CSF-1 protein expression after antisense ODN treatment is caused by reduced CSF-1 transcript levels, either through direct inhibition of transcription or through degradation of the transcript by ribonucleases. Also, the possibility

that translation of CSF-1 was hampered by binding of the antisense ODN to mRNA cannot be excluded and could be the reason for the more efficient *in vitro* down-regulation of CSF-1 (at both transcriptional and translational levels) after ODN-196 treatment compared with ODN-181 (only efficient at transcriptional level).

Our data provide the first evidence that reduction of CSF-1 in two human tumor xenografts by antisense ODN has the potential to suppress tumor growth, MMP expression, angiogenic activity, and to increase survival. These results support the assumption that altering gene function by antisense ODN is a therapeutic concept worth pursuing (26, 27, 47). For several reasons, the CSF-1 antisense ODN therapy approach described here might be extended to a variety of human solid malignancies. First, CSF-1 and its receptor are expressed in several tumor stromal cell systems and a variety of neoplasms of epithelial origin (48). Second, high levels of *c-fms* transcript correlate strongly with high-grade and advanced clinical presentations of prognostic poor outcome (49, 50). Third, MMP overexpression has been demonstrated in many cancer types (4, 15, 36, 37). Fourth, in transgenic mice susceptible to mammary cancer that were also homozygous for a null mutation in the *CSF-1* gene (*CSF-1^{op}*), neither the incidence nor the growth of the primary tumor was affected by the absence of CSF-1, yet the mice were delayed in the development of invasive metastatic carcinomas. Transgenic expression of CSF-1 in the mammary epithelium of either *CSF-1^{op}/CSF-1^{op}* or wild-type tumor-susceptible mice caused an acceleration of the late stages of carcinoma and a significant increase in pulmonary metastasis (35). Moreover, the absence of antisense-related hematological toxicity in the present human tumor models is promising. Further study is needed to examine whether CSF-1 antisense ODN approach can serve as a single treatment or adjuvant to chemotherapy or radiation therapy in different human cancer types.

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