 Colony-Stimulating Factor-1 Blockade by Antisense Oligonucleotides and Small Interfering RNAs Suppresses Growth of Human Mammary Tumor Xenografts in Mice

Seyedhossein Aharinejad, 1 Patrick Paulus, 1 Mouldy Sioud, 2 Michael Hofmann, 1 Karin Zins, 1 Romana Schäfer, 1 E. Richard Stanley, 1 and Dietmar Abraham 1

1 Laboratory for Cardiovascular Research, Department of Anatomy and Cell Biology, Vienna Medical University, Vienna, Austria; 2 Department of Immunology, Molecular Medicine Group, The Norwegian Radium Hospital, Oslo, Norway; and 3 Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York

ABSTRACT

Colony-stimulating factor (CSF-1) is the primary regulator of tissue macrophage production. CSF-1 expression is correlated with poor prognosis in breast cancer and is believed to enhance mammary tumor progression and metastasis through the recruitment and regulation of tumor-associated macrophages. Macrophages produce matrix metalloproteases (MMPs) and vascular endothelial growth factor, which are crucial for tumor invasion and angiogenesis. Given the important role of CSF-1, we hypothesized that blockade of CSF-1 or the CSF-1 receptor (the product of the c-fms proto-oncogene) would suppress macrophage infiltration and mammary tumor growth. Human MCF-7 mammary carcinoma cell xenografts in mice were treated with either mouse CSF-1 antisense oligonucleotide for 2 weeks or five intratumoral injections of either CSF-1 small interfering RNAs or c-fms small interfering RNAs. These treatments suppressed mammary tumor growth by 50%, 45%, and 40%, respectively, and selectively down-regulated target protein expression in tumor lysates. Host macrophage infiltration; host MMP-12, MMP-2, and vascular endothelial growth factor A expression; and endothelial cell proliferation within tumors of treated mice were decreased compared with tumors in control mice. In addition, mouse survival significantly increased after CSF-1 blockade. These studies demonstrate that CSF-1 and CSF-1 receptor are potential therapeutic targets for the treatment of mammary cancer.

INTRODUCTION

Mammary carcinoma has the highest incidence of all cancer types in American women, and its incidence rates have continued to increase since 1986 (1). The mechanism by which mammary epithelial cells undergo genetic changes that result in acquisition of the ability to invade and colonize distant sites is complex (2, 3). Normal and malignant mammary epithelium and the surrounding stromal cells produce and respond to various growth factors, including transforming growth factor α and β (4) and fibroblast growth factors (5). Among the stromal cells, macrophages play a unique role because they are recruited into mammary gland carcinomas (6, 7), and, in the absence of such tumor-associated macrophages, metastatic progression of mammary gland tumors is profoundly reduced (8). The macrophages probably enhance tumor progression through paracrine circuits involving the production of colony-stimulating factor (CSF-1) by tumor cells (9) or other host-derived stromal cells and by extracellular matrix (ECM)-modulating functions, mediated by matrix metalloproteases (MMPs; Refs. 10–12). Thus, a causal link exists between tumor-associated macrophages and the malignant potential of breast epithelial cells.

The production of macrophages is regulated by CSF-1, also known as macrophage CSF (13), via its receptor, the c-fms proto-oncprotein (14). CSF-1 is produced by a variety of cells (15–17), including mammary epithelium (17), and we have shown that CSF-1 stimulates the proliferation, differentiation, and survival of cells of the mononuclear phagocytic lineage (13, 16, 18) and accelerates angiogenesis in vivo (19). Consistent with the angiogenic effects of CSF-1, recent work suggests that CSF-1 stimulates monocytes, progenitors of macrophages, to secrete biologically active vascular endothelial growth factor (VEGF; Ref. 20). Collectively, the fact that CSF-1 promotes tissue invasion by lung cancer cells that also exhibit CSF-1-induced MMP production (21) and enhances metastasis of mammary tumors (8) and the fact that CSF-1 blockade suppresses tumor growth, MMP production, and macrophage recruitment (22) support the paradigm that CSF-1 enhances progression of malignancies via its effects on the recruitment and control of macrophages that regulate tumor cell growth, angiogenesis, and the ECM.

Based on this reasoning, we hypothesized that blocking CSF-1 using a small interfering RNA (siRNA) approach would suppress breast cancer development. siRNAs are RNA duplexes of 21–23 nucleotides that specifically inhibit gene expression in vitro and in vivo (23–25). Treatment of mice bearing human breast cancer xenografts with an established mouse CSF-1 antisense oligodeoxynucleotide (ODN; Ref. 22) and siRNAs directed specifically against host (mouse) CSF-1 or the CSF-1 receptor (CSF-1R, the product of the c-fms proto-oncogene) mRNA suppressed tumor growth by 40–50% and increased mouse survival. A selective decrease in expression of MMPs and VEGF-A was observed in association with this tumor suppression.

MATERIALS AND METHODS

ODNs and siRNAs. The sequences of phosphorothiate-modified mouse ODNs (VBC Genomics, Vienna, Austria) were as follows: CSF-1 ODN-196, 5′-GCCCGGCGGGTCTAGT-3′; and scrambled ODN, 5′-CGAGACGGCGG-CAGCG-3′. The sequences of siRNAs directed against target mouse molecules were as follows: CSF-1 sequences 1 and 2, 5′-GACCCUGUCAGACAAA-GAGTT-3′ and 5′-GCCAUAUGACUGAUAU TT-3′; c-fms sequences 1 and 2, 5′-GGCAUCUGCGUUAAGGGUGATT-3′ and 5′-GUGAUCUAG-UAGCAGCCTG-3′; and scrambled siRNA sequences 1 and 2, 5′-GAAGCAGACCGCUUUCCUUU CCAGCAGCTT-3′. Only the sequences for the sense strands are shown. Screening experiments were performed with in vitro transcribed siRNAs (23). Chemically synthesized and purified siRNAs (Eurogentec, Philadelphia, PA) were used for all other experiments.
Analysis of siRNA Effects in Vitro. Mouse epithelial F-9 tumor cells expressing CSF-1 and CSF-1R (American Type Culture Collection, Manassas, VA), were cultured in DMEM containing 10% FCS until they reached 50% confluence. Cells were rinsed with PBS, refed with serum-free DMEM, and then transfected with 25, 50, or 100 nm CSF-1, c-fms, or scrambled siRNA using Effectene (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Untreated cells served as additional controls. Experiments were performed in triplicate. After incubation for 48 h, mRNA was isolated for reverse transcription-PCR (RT-PCR), and the supernatant was filtered and used for ELISA. Real time RT-PCR (Light Cycler; Roche, Mannheim, Germany) was performed as described previously (22). The primer sequences for mouse CSF-1, c-fms, and β-actin were as follows: CSF-1 sense, 5'-CATCTCCATCTCCCCAAC-3'; CSF-1 antisense, 5'-ACTGGCTGATCTCCTCC-3'; c-fms sense, 5'-GGCATGCTGAGCGAAATGCA-3'; c-fms antisense, 5'-CCGATAATCGACCTGGCA-3'; β-actin sense, 5'-GGCTGACATCAGAAAGAAG-3'; and β-actin antisense, 5'-AGGAGCCAGACAGTAATC-3'. ELISA was performed to detect mouse CSF-1 protein in culture medium (using a monoclonal antibody, Quantikine, R&D, Minneapolis, MN). The results of CSF-1 and CSF-1R mRNA and protein expression were normalized to the corresponding β-actin signal. Measurements were performed in triplicate.

Tumor Models and Antisense and siRNA Treatment. The experiments performed in this study were approved by the Institutional Animal Care and Use Committee at the Vienna Medical University. Established human metastatic mammary carcinoma cells (MCF-7; American Type Culture Collection) were grown in immunodeficient mice as described previously (26). Pathogen-free female 5-week-old BALB/c-nu/nu (nude) mice (Harlan-Winkelmann, Borchern, Germany) were weighed, coded, and divided into experimental groups of N = 10 at random. Mice were anesthetized (ketamine hydrochloride/xylazine at 55/7.5 mg/kg, s.c.), and 2 × 10^6 MCF-7 cells/100 μl culture medium were injected s.c. into the left flank (22, 26). The growth of the tumor xenograft was evaluated in a pilot study by determining the tumor weight every other day (n = 12). Twenty-four days after cell injection, one group of animals was killed and evaluated for tumor weight. In the present study, mice developed human mammary carcinomas of similar weight at 24 days (288 ± 67 mg), 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 ODN, scrambled ODN, or Ringer’s solution (control) were inserted s.c. into a paraspinal pocket. The pumps delivered their contents (100 μl) constantly over 2 weeks, and all animals were killed 14 days after the beginning of the treatment. The selected dosage of 5 mg/kg per day for the antisense ODN over 14 days was based on pilot studies in mice (n = 18). The CSF-1, c-fms, and scrambled siRNA intratumoral treatment was started on day 24 at a dose of 10 μg/injection and cycled every three days. The selected dosage of 10 μg/injection was based on pilot studies in mice (n = 24).

Analysis of the in Vitro Effects of CSF-1 and c-fms Blockade. On day 38, blood samples were obtained to examine complete blood count (22). Next, the tumors were isolated and weighed, and the animals were sacrificed. One portion of the tissue was processed for paraffin embedding, and the remainder was processed for real-time RT-PCR, Western blotting, and radioimmunoassay as described below. Paraffin-infiltrated serial sections were stained with H&E or Ki-67 antibody (tumor proliferation assay; Dako, Glostrup, Denmark) or double-stained with von Willebrand (Dako) and Ki-67 antibodies to evaluate the density of proliferating endothelial cells as described previously (22). Macrophages were immunostained with anti-F4/80 rat monoclonal antibody (Caltag Laboratories, Burlingame, CA; Ref. 8). Digitalized images were generated, and morphometry was carried out (22).

Analysis of the Effects of CSF-1 and c-fms Blockade on Survival. The survival study (N = 54) was set for 6 months. Mice were treated with CSF-1 ODN-196 (n = 12), CSF-1 siRNA (n = 12), c-fms siRNA (n = 12), Ringer’s solution (n = 6), scrambled ODN (n = 6), and scrambled siRNA 1 (n = 6).

Quantitative Real-Time RT-PCR. Tissue was processed for PCR as described previously (27). The primer sequences for mouse angiogenic factors were as follows: VEGF-A sense, 5'-TACCTCGTAGACTCCAC-3'; VEGF-A antisense, 5'-CTCGATTCTCCTATGGTTC-3'; Flt-1 sense, 5'-AACAATTCTGCAACTACTC-3'; Flt-1 antisense, 5'-TAATCCTCGTCTCCATTC-3'; KDR-flk-1 sense, 5'-GGAGATGGAAGAAGCAGAC-3'; and KDR-flk-1 antisense, 5'-ACTTCCCTCCTCCACAT-3'. The primer sequences for the primers were as follows: CSF-1 sense, 5'-CGGCTGTGTCGTCCTGTCTCCG-3'; CSF-1 antisense, 5'-CATGCCTTCATACTCTTG-3'; c-fms sense, 5'-TGCTGCTCCTGCTATGTG-3'; c-fms antisense, 5'-TCGAGTCCTCATCAGGC-3'; β-actin sense, 5'-TGCCCATCCTAAAAGGAC-3'; and β-actin antisense, 5'-CAACTTTATCTGAGATGTC-3'. Measurements were performed three times.

CSF-1 Radioimmunoassay. Preweighed tissue and cell samples were homogenized, heat inactivated, and centrifuged, and the supernatant was saved for assay as described previously (28). CSF-1 was measured in duplicate on April 15, 2017. © 2004 American Association for Cancer Research.
three samples from each mouse using a radioimmunoassay that only detects biologically active mouse CSF-1 (28, 29).

**Western Blotting.** Tissue lysates were prepared as described previously (22) and were separated (50 μg/lane) by 10% SDS-PAGE before electrophoretic transfer onto Hybond C super (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). The blots were probed with antibodies against MMP-2 (polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA), MMP-12 (polyclonal antibody, Santa Cruz Biotechnology), tissue inhibitor of metalloproteases (TIMP-1) (polyclonal antibody; Calbiochem, Temecula, CA), TIMP-2 (polyclonal antibody; Calbiochem), and TIMP-3 (polyclonal antibody; Calbiochem) before incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech). Proteins were immunodetected by chemiluminescence (Supersignal-West-Femto; Pierce) and quantified by Easy Plus Win 32 software (Herolab, Wiesloch, Germany).

**Statistical Analysis.** ANOVA with Bonferroni’s t test was used to compare the data between the groups. The χ² test was used to compare groups in the analysis of long-term survival (6 months). The overall survival curves after treatment were analyzed by the Kaplan-Meier survival test. Data are expressed as means ± SD. P values of <0.05 were considered to indicate statistical significance.

**RESULTS**

**Small Interfering RNAs Down-Regulate CSF-1 and c-fms Expression in Vitro.** CSF-1- and c-fms-expressing mouse epithelial cells were treated with 25, 50, or 100 nM CSF-1, c-fms siRNA sequence 1 and 2, or scrambled siRNAs. Whereas CSF-1 siRNA sequence 2, regardless of the applied dose, and c-fms siRNA sequence 1 at 25 and 50 nM were not effective, CSF-1 siRNA sequence 1 and c-fms siRNA sequence 2 down-regulated CSF-1 and c-fms expression significantly. Treatment with 25, 50, and 100 nM CSF-1 siRNA sequence 1 decreased CSF-1 mRNA and protein expression compared with controls, whereas treatment with scrambled siRNA did not (Fig. 1A). CSF-1 mRNA expression decreased after treatment with 50 and 100 nM c-fms siRNA sequence 2 compared with controls, but not after treatment with scrambled siRNA (Fig. 1B). These experiments indicated that the effect of the CSF-1 and c-fms siRNAs was sequence and dose dependent.

**MCF-7 Cells Up-Regulate Mouse CSF-1 Production but Lose Their Ability to Express Human CSF-1 after Xenografting in Mice.** Before initiating *in vivo* experiments, we performed real-time RT-PCR and ELISA analyses of MCF-7 cells and found that they express both mRNA and protein for human CSF-1 and its receptor, CSF-1R (data not shown). When these cells were xenografted to mice, mouse tissue CSF-1 expression increased over time (Fig. 2, A and B), whereas MCF-7 cells lost their ability to express human CSF-1 (data not shown). In contrast, mouse CSF-1R mRNA was expressed throughout tumor development (Fig. 2C). These experiments indicated that human cancer cells lose their own (human) CSF-1 expression but stimulate host (mouse) cells to overexpress CSF-1.

**CSF-1 Antisense and siRNA against CSF-1 and c-fms Down-Regulate Target Proteins and Suppress the Mammary Tumor Growth.** Mice bearing human mammary tumor xenografts were treated with CSF-1 antisense ODN-196, scrambled ODN, CSF-1 siRNA sequence 1 and c-fms siRNA sequence 2, scrambled siRNA sequences 1 and 2, and Ringer’s solution (control). Both antisense and siRNA treatments were well tolerated, and we did not observe significant changes in the complete blood count of treated mice (results not shown). After CSF-1 antisense ODN-196 or siRNA but not scrambled ODN or scrambled siRNA treatment, tissue CSF-1 mRNA declined compared with controls (Fig. 2A). Likewise, treatment with CSF-1 antisense ODN-196 or CSF-1 siRNA sequence 1 down-regulated tissue CSF-1 protein levels, whereas treatment with scrambled ODN or scrambled siRNA sequence 1 did not (Fig. 2B). Treatment with mouse c-fms siRNA sequence 2, but not scrambled siRNA sequence 2, down-regulated tissue CSF-1 mRNA expression (Fig. 2C). Treatment with CSF-1 antisense ODN-196 or CSF-1 siRNA sequence 1 reduced mouse CSF-1R mRNA expression.

In line with tissue CSF-1 and CSF-1R mRNA down-regulation, the mean tumor weight was markedly reduced in mice with human mammary tumors treated with CSF-1 antisense ODN-196 for 2 weeks (943 ± 85 mg, with CSF-1 siRNA sequence 1 (1052 ± 153 mg), or with c-fms siRNA sequence 2 (1128 ± 204 mg), compared with mice treated with Ringer’s solution (1869 ± 291 mg), scrambled ODN (1713 ± 316 mg), scrambled siRNA sequence 1 (1821 ± 312 mg), or scrambled siRNA sequence 2 (1898 ± 396 mg; Fig. 3). Thus, treatment with CSF-1 antisense ODN-196, CSF-1 siRNA, and c-fms siRNA suppressed tumor development by 50%, 45%, and 40%, respectively. These experiments indicated that blocking CSF-1 or its receptor can significantly suppress the progression of mammary tumor xenografts in mice.

**CSF-1 and c-fms Blockade Down-Regulates Mouse MMP-12 and MMP-2 Expression in MCF-7 Mammary Tumor Xenografts.** After human MCF-7 cell xenografting in mice, macrophage invasion in the tumor xenografts was observed (Fig. 4A). In association with this, host (mouse) MMP-12, a macrophage-specific protease involved in ECM remodeling, was strongly expressed during tumor progression in control animals (Fig. 4B). Furthermore, host (mouse) MMP-2, a key molecule in tumor metastasis and angiogenesis, was up-regulated with tumor progression in control animals. Treatment with CSF-1...
These experiments showed that blockade of CSF-1 or its receptor significantly reduces macrophage recruitment toward the mammary tumor and that this effect is associated with down-regulation of MMP-2 and MMP-12, consistent with the possible involvement of MMP-2 and MMP-12 in the macrophage promotion of tumor progression in this breast cancer model.

CSF-1 and c-fms Blockade Decreases Angiogenic Activity in Mammary Tumor Xenografts. Histomorphometry of mammary tumors showed increased density of proliferating endothelial cells with tumor progression in controls that was decreased after CSF-1 antisense ODN-196, CSF-1 siRNA, and c-fms siRNA blockade. The density of proliferating endothelial cells was, however, unchanged in mice treated with scrambled ODN or scrambled siRNAs (Fig. 5A). VEGF-A mRNA levels increased with tumor progression and were reduced in CSF-1 antisense ODN-196-treated, CSF-1 siRNA-treated, and c-fms siRNA-treated mice, but not in scrambled ODN- or scrambled siRNA-treated mice (Fig. 5B). CSF-1 and c-fms blockade, however, did not significantly affect tissue mRNA expression of VEGF-A receptors Flt-1 and KDR (Fig. 5B). These data indicated that blocking CSF-1 or c-fms is associated with decreased VEGF-A expression and reduced angiogenic activity in mammary tumor xenografts.

CSF-1 Blockade Increases Survival in Mice with Mammary Tumor Xenografts. The median time to death of animals in the control group was 62 days, and all animals died between 48 and 72 days after tumor cell implantation. Survival was significantly increased in mice after treatment with CSF-1 antisense ODN-196 or CSF-1 siRNA sequence 1 and slightly (but not significantly) increased after treatment with c-fms siRNA sequence 2 versus control animals, and the median times to death were 127, 103, and 76 days, respectively (Fig. 6). At 72 days (at which time the last animal of the control groups died), 100% of CSF-1 antisense ODN-196-treated mice, 92% of CSF-1 siRNA-treated mice, and 58% of c-fms siRNA-treated mice were still alive (Fig. 6).

DISCUSSION

The mammary gland comprises stromal and epithelial cells that communicate with each other through the ECM. Although it is the mammary epithelium that proliferares and has the most tumorigenic potential, the mammary stroma contributes both instructive and permissive signals (30). The mammary stroma consists of multiple components, including adipocytes, pre-adipocytes, fibroblasts, blood vessels, inflammatory cells, and ECM. CSF-1 is synthesized by mouse mammary ductal epithelium (17). Macrophages, recruited by CSF-1, promote mammary ductal invasion during puberty (31) and are required during pregnancy for lobulo-alveolar differentiation (32). Consistent with a role for macrophages, CSF-1 is also necessary for the progression of mammary tumors to malignancy in mice (8). Macrophages produce molecules such as MMPs that can influence these processes. Indeed, excessive side branching and tumorigenesis occur when the stromal MMPs are overexpressed in the mouse mammary gland (33, 34), and side branching is reduced in mice deficient in MMP-3 (28). Accordingly, human breast hyperplasia, dysplasia, and carcinoma frequently show elevated stromal MMP activity (35).

The present study shows that blocking mouse CSF-1 reduces macrophage recruitment to human mammary cancer xenografts in mice. Associated with this effect, we observed down-regulated host MMP-2 and macrophage-specific MMP-12 activities in the tumor lysates. CSF-1-mediated ECM modification by MMPs produced by macrophage may therefore contribute to tumor progression in this mouse model of human mammary cancer. These results are consistent with recent reports (12, 30, 31, 33, 34) on the role of MMPs and suggest that some of the CSF-1-mediated effect on tumor progression
may be transmitted through MMPs. Apart from regulation at the level of gene transcription, the activities of MMPs are tightly regulated by endogenous inhibitors, the TIMPs (12). However, TIMP expression is not affected by CSF-1 or c-fms blockade in mammary tumor xenografts, indicating that TIMP regulation is independent of CSF-1 and that an increase in local TIMP levels, coupled with inhibition of CSF-1 signaling, may enhance the tumor-inhibitory effect of CSF-1/c-fms blockade.

In our previous study, we concluded that the down-regulation of angiogenic VEGF might be mediated by the inhibition of macrophage production of MMPs and their alteration of the ECM to release angiogenic factors (22), and the induction of VEGF promoter activity in stromal fibroblasts by tumor implants in mice (36) supported this hypothesis. Since then, CSF-1 has been shown to induce VEGF production and angiogenic activity by monocytes in vitro (20), and in vivo studies indicate that the secretion of VEGF by tumor macrophages is essential for tumor-induced angiogenesis (37). Thus, the inhibition of VEGF mRNA expression and of proliferating endothelial cells in breast cancer xenografts after CSF-1 blockade is likely to involve inhibition of CSF-1-stimulated VEGF production by tumor-associated macrophages.

Overexpression of CSF-1 and its receptor are correlated with a poor prognosis in human breast cancer (38–40). A marked leukocytic infiltration in these tumors is also correlated with poor prognosis, and the majority of infiltrating cells are macrophages (41, 42). CSF-1 is the primary growth factor regulating tissue macrophage production in...
ated macrophages recruited by CSF-1 enhance tumor progression. Thus, it is likely that tumor-associated macrophages recruited by CSF-1 enhance tumor progression by several mechanisms (44). Human MCF-7 cells produce CSF-1, and because human CSF-1 is active on mouse macrophages, it is possible that tumor production of human CSF-1 plays a role in the initial recruitment of host macrophages. However, the loss of expression of human CSF-1 and increase in expression of mouse CSF-1 with tumor progression, coupled with the effectiveness of the mouse CSF-1 blockade, support the paradigm that the tumor-surrounding stroma and its cellular components play a key role in the regulation of tumorigenesis and angiogenesis. Because the reduced mouse c-fms expression after CSF-1 blockade is most likely due to the observed decrease in the number of tumor-associated macrophages, future clinical trials of CSF-1 blocking agents in human breast cancer could benefit from targeting the CSF-1-producing cells of both the stroma and the tumor.

We are unaware of other reports that have used RNA interference as a therapeutic modality in breast cancer. Small interfering RNAs mimic intermediates in the RNA interference pathway and can silence genes in somatic cells, without significant activation of the IFN pathway that can lead to global down-regulation of translation as well as global RNA degradation (23, 45). Our data provide the first evidence that siRNAs directed against mouse CSF-1 and c-fms, delivered directly into the tumor, have the ability to down-regulate the target genes and can, similar to CSF-1 antisense ODNs, suppress the progression of human mammary cancer xenografts in mice, significantly increasing mouse survival. The tumor-suppressive effect of the CSF-blocking agents was associated with a selective MMP down-regulation and reduced angiogenic activity in tumor lysates. This demonstration of a therapeutic effect of CSF-1/c-fms siRNA treatment in a xenograft model is encouraging. However, the immune response deficiency of the nude mouse might be a limiting factor in the use of this model. In future studies, it will be important to test efficacy in a syngeneic mouse model that is more representative of human disease, such as the polyoma middle T oncoprotein mouse breast cancer model (46). Nevertheless, the following conclusions can be drawn from the present findings. First, RNA interference might be a powerful means of blocking gene function. Second, the fact that five injections of siRNAs at a much lower dose than the dose of antisense ODNs required for a comparable effect can block the function of the target genes effectively favors the use of these nucleic acid-based constructs for large-scale human studies (47). Third, CSF-1 blockade as a paradigm for suppressing solid tumor progression is strongly supported by this study, which also supports its more general application to other tumor types. Fourth, our results, in the absence of treatment-related
hematological toxicity, call for additional studies to test whether stable constructs carrying siRNAs or chemically modified siRNAs that are optimized to target breast cancer tissue could be of benefit when used systemically.

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