Angiogenesis, Metastasis, and the Cellular Microenvironment

Control of VEGF Expression in Triple-Negative Breast Carcinoma Cells by Suppression of SAF-1 Transcription Factor Activity

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Abstract

Angiogenesis plays a significant role in cancer by providing increased blood supply to the affected tissues and thus bringing in growth factors, cytokines, and various nutrients for tumor growth. VEGF is the most prominent angiogenic agent that is markedly induced in cancer. Induction of VEGF has been widely studied but as cancer cells are quite adept at acquiring new alternative processes to circumvent surrounding environmental pressures, our understanding of the molecular mechanisms regulating VEGF expression in cancer, especially in triple-negative breast cancer cells, remains incomplete. Here, we present evidence of a novel mode of VEGF induction in triple-negative MDA-MB-231 breast cancer cells that is regulated by serum amyloid A activating factor 1 (SAF-1) transcription factor. Inhibition of SAF-1 by antisense short hairpin RNA profoundly reduces VEGF expression along with reduction in endothelial cell proliferation and migration. By both in vitro and in vivo molecular studies, we show that the effect of SAF-1 is mediated through its direct interaction with the VEGF promoter. In correlation, DNA-binding activity of SAF-1 is found to be significantly higher in MDA-MB-231 breast cancer cells. Examination of several breast cancer samples further revealed that SAF-1 is overexpressed in clinical breast cancer tissues. Taken together, these findings reveal that SAF-1 is a hitherto unrecognized participant in inducing VEGF expression in triple-negative breast cancer cells, an aggressive form of breast cancer that currently lacks effective treatment options. Suppression of SAF-1 activity in these cells can inhibit VEGF expression, providing a possible new method to control angiogenesis. Mol Cancer Res; 9(8); 1030–41. ©2011 AACR.

Introduction

Resistance of human cancers, including breast cancer, to current treatment protocols remains a major concern for cancer treatment. To address this shortcoming, much effort has been focused on identifying novel molecular pathways associated with the growth, proliferation, and metastasis of cancer cells. Breast cancer is a heterogeneous disease composed of several subtypes, of which triple-negative breast cancer that does not express estrogen receptor (ER), progesterone receptor (PR), and HER2 is particularly dreadful. Patients with triple-negative breast cancer have exceptionally poor prognosis, and this effect is more frequent among younger women, African-American and Hispanic women, and women with elevated waist/hip ratio.

An essential step in the growth and metastasis of breast cancer is angiogenesis. Distinct angiogenic patterns have been linked with high-grade in situ ductal carcinoma of the breast, and poor breast cancer prognosis correlates with increasing microvascular density (1). One of the most prominent factors that regulate angiogenesis is VEGF (2, 3). Role of VEGF in breast cancer is evident from the observation of increased synthesis of VEGF in breast cancer cells (4, 5) as well as in inflammatory breast cancer tissues (6). Moreover, reports of increased risk of developing invasive breast cancer with 2 VEGF gene polymorphisms that lead to increased VEGF expression (7) and reduction of the growth of some solid tumors by VEGF receptor–neutralizing antibodies (8, 9) lend support for a vital role of VEGF in breast cancer development. In cancer cells, large array of signals as well as several oncoproteins act as inducers of VEGF, creating a challenge for blocking VEGF production in tumor. Hypoxia-inducible factor-1α (HIF-1α) has been identified as a potent regulator of VEGF transcription (10–13). However, accumulating evidence suggests that HIF-1α is not the sole regulator of VEGF expression (14–19). Indeed, involvement of a concert of transcription factors has been proposed for VEGF expression (19). Thus, the mechanism regulating VEGF expression during the angiogenic switch in tumors is far from being resolved. Recently, a novel mode of VEGF regulation has been
identified by which synovial inflammation and angiogenesis in the arthritic joints are maintained (20). Serum amyloid A activating factor 1 (SAF-1), a zinc finger transcription factor, was shown to regulate VEGF induction via direct interaction with the VEGF proximal promoter region. SAF-1, and its human orthologue MAZ (21), is a member of a family of multiple Cys-His-type zinc finger proteins that are activated in response to various inflammatory signals (22–24). MAZ is shown to regulate c-myc proto-oncogene expression (21). The expression of c-myc mRNA is elevated in human primary breast carcinoma (25). Subsequent studies showed that MAZ drives tumor-specific expression of PPARγ1 gene in breast cancer cells (26) and Prox-1 gene expression in hepatocellular carcinoma (27). Studies in our laboratory indicate that phosphorylation of SAF-1/MAZ by protein kinase C (28), MAP kinase (29), and protein kinase A (30) leads to a marked enhancement of its DNA-binding ability. In addition, casein kinase II has been shown to phosphorylate MAZ and increase its DNA-binding and transcriptional activities (31).

To assess whether SAF-1/MAZ plays a role in inducing VEGF mRNA level in triple-negative breast cancer cells, we initiated a study. Here, we show that inhibition of SAF-1/MAZ expression in triple-negative MDA-MB-231 breast cancer cells results in marked reduction of VEGF expression, resulting in reduced proliferation and migration of human umbilical vein endothelial cells (HUVEC). Increased DNA-binding activity of SAF-1 and its interaction with the VEGF promoter, both in vivo and in vitro, provide evidence that SAF-1/MAZ regulates VEGF expression in breast cancer. In correlation, SAF-1 was detected in high abundance in breast cancer tissues.

Materials and Methods

Cell culture

MDA-MB-231, MCF-7, and MDA-MB-468 breast cancer cells and HUVEC-CS human umbilical vein endothelial cell lines were obtained from American Type Culture Collection and maintained according to supplier’s protocol. Normal human mammary epithelial cells (HMEC) were obtained from Cambrex/Clonetics and maintained in the Cambrex/Clonetics MGEM mammary epithelial cell growth media according to supplier’s protocol. For conditioned medium (CM), first the cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) for 24 hours. Next, the culture medium was replaced with DMEM containing 0.5% FCS for 24 hours. Next, the culture medium (CM), first the cells were grown in growth media according to supplier’s protocol. For conditioned medium (CM), first the cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) for 24 hours. Next, the culture medium was replaced with DMEM containing 0.5% FCS for 24 hours. Next, the culture medium (CM), first the cells were grown in growth media according to supplier’s protocol. For conditioned medium (CM), first the cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS) for 24 hours. Next, the culture medium was replaced with DMEM containing 0.5% FCS for 24 hours.

Plasmid constructs

The reporter plasmid 1.2VEGF-CAT3 was constructed by cloning VEGF promoter sequences from −1,179 to +21 into pBLCAT3 plasmid vector (20). Progressively deleted VEGF-CAT reporter constructs were prepared by cloning various segments of VEGF promoter that were generated by PCR amplification. Mutant reporters were prepared by megaprimer PCR, in which the individual SAF-1 elements were mutated by using mutant oligonucleotide sequences. The sequences used were SAF-1 site I (5′-CCCCCTATCCTGCGCCGCCCTGCTCGGGTGT-3′) and SAF-1 site II (5′-ATGCCCTCTCCTCCTAGTT-3′); underlined bases represent altered sequences. In the double mutant, both sites were altered.

Transfection analysis

The cells were transfected with reporter plasmid DNA (0.5 μg) together with pSVβ-gal (Promega Corporation) plasmid DNA (0.5 μg) by using METAFECTENE PRO (Biontex Laboratories). The pSVβ-gal DNA was used to monitor the efficiency of transfection and to normalize the cell extracts used for chloramphenicol acetyltransferase (CAT) assay. After 24 hours, the cells were harvested, extracts were prepared, and CAT and β-galactosidase activities were measured as described (32). All transfection experiments were carried out at least 3 times. For generating stably transduced cells, HMECs, MDA-MB-231 cells, and MDA-MB-468 cells were transduced with MAZ short hairpin RNA (shRNA) lentiviral particles (Santa Cruz Biotechnologies) or the empty vector virus, following the manufacturer’s protocol. MAZ is the human orthologue of SAF-1 (33). The stably transduced cells were isolated by puromycin selection and maintained in this selection medium.

Nuclear extract preparation, electrophoretic mobility shift assay, and DNase I footprint

Nuclear extracts were prepared from mammary cancer and normal mammary cells following a method as described earlier (22), and electrophoretic mobility shift assay was carried out by using a method described by Ray and colleagues (20). A double-stranded oligonucleotide with upper strand sequence 5′-CCCCCTATCCTGCGCCGCCCTGCTCGGGTGT-3′, containing the SAF-1–binding element of SA4 promoter (33), was labeled and used as a bona fide probe for the binding of SAF-1 transcription factor. In some reactions, VEGF DNA–containing sequences from −135 to +29 was end-labeled by using 32P-dCTP and used as the probe. Some binding reactions contained antibody (Ab) against SAF-1 (20), which was added to the binding reaction mixture during a preincubation assay for 30 minutes on ice. As a competitor of SAF-1 binding, some binding reactions contained 50 pmol of a canonical SAF-binding double-stranded oligonucleotide having an upper strand sequence of 5′-CCCCCTATCCTGCGCCGCCCTGCTCGGGTGT-3′ (17). In some reactions, 50 pmol of a nonspecific oligonucleotide containing a random sequence of 5′-TCGACTGAAGTTGAG-3′ was added as a competitor of binding reaction. For DNase I footprint analysis, DNA–protein binding reaction was carried out by using 5′–end–labeled VEGF DNA (−135 to +29), which was incubated with nuclear extracts (30 μg of protein) and subsequently treated with diluted DNase I as described previously (20). The resultant DNA fragments were resolved using an 8% polyacrylamide–8 mol/L urea sequencing gel.
Chromatin immunoprecipitation assay

This assay was conducted using a chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology) following standard protocol. Briefly, cells (2 × 10⁶ each) were plated in 60-mm dishes and grown for 24 hours. After formaldehyde treatment, cells were lysed and sonicated to generate on average 500 bp long DNA fragments. After incubation of the solutions with anti-SAF-1 Ab or control IgG at 4°C for 16 hours with rotation, immune complexes were extracted and cross-linking was reversed by heating at 65°C for 4 hours. The elutes were then digested with proteinase K and RNase A, and the DNA was phenol extracted and ethanol precipitated. Purified DNA was subjected to PCR, using primers covering the SAF-1 binding region of VEGF promoter. Primers used for amplification were sense (5’-GAGGTCCTCCTTTACTGGG-3’) and antisense (5’-CGGCTGCCCAGGCCTC-3’), which yields a 219-bp product.

Reverse transcriptase PCR analysis

Total RNA was extracted by using by the guanidinium thiocyanate method (34) and reverse-transcribed by using a reverse transcriptase PCR (RT-PCR) kit (Invitrogen) and following the manufacturer’s protocol. DNase-treated RNA (1 μg) was used in the reverse transcription reaction, with random hexamer and oligo dT₁₂₋₁₈ as extension primers. PCR was done using following gene-specific primers: VEGF: 5’-GGATGTCTATCAGCGCAGCTAC-3’ and 5’-GGCTCACCAGCTCGGCTTTGTCAC-3’; SAF-1: 5’-AGCGCACGTGGTCCGACACGAGGAG-3’ and 5’-ATTGGACAAACCTCACCAGTAC-3’; GAPDH: 5’-TGACACCACCACTGCTTAG-3’ and 5’-TACAGG-CAGGGATGTGCTTC-3’. Resulting DNA products were resolved in a 1.5% agarose gel.

Frozen human clinical breast tumor samples were purchased from ProteoGenex, Inc. For semi-quantitative RT-PCR analysis of SAF-1 transcripts, DNA-free RNA samples were reverse transcribed to cDNA as described above. The cDNA products were amplified by PCR in 4 replicate reactions containing ³²P-labeled dNTPs. One of the replicate reactions of each sample was removed from the thermal cycler at the end of cycles 15, 20, 25, and 30. PCR products were fractionated using a 1.5% agarose gel, and the bands were detected by autoradiography and the radioactivity in each band was measured. The data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification, and minus reverse transcription control was used for each sample to ensure specificity of the RT-PCR amplification.

Cell proliferation assay

HUVECs were plated (in triplicates) at a density of 2 × 10⁴/well in 96-well tissue culture plates and grown for 24 hours in the growth medium. Each well was washed gently with serum-free medium, and the culture medium was replaced with fresh medium containing 10% (v/v) concentrated CM derived from vector-transfected or SAF-1 shRNA–transfected MDA-MB-231 breast cancer cells, as indicated. Twenty four hours later, cell numbers were determined using the MTT cell proliferation assay Kit (Promega Corporation).

Cell migration assay

Cell migration was assayed using a QCM assay kit (Millipore). Briefly, HUVECs (2 × 10⁴ cells) were placed on the upper side of the fibronectin-coated Boyden chambers. The bottom chambers were filled with CM from vector-transfected or SAF-1 shRNA–transfected MDA-MB-231 breast cancer cells. In addition, some chambers received CM that were preincubated for 1 hour at room temperature with a neutralizing anti-hVEGF monoclonal antibody (mAb; 1 μg/mL; R&D systems), as indicated. After 24 hours, the nonmigrated cells were removed by cotton swab and the membrane was placed in cell staining solution, following supplier’s protocol. The cells that migrated onto the filter were counted manually by examination under a microscope.

Tube formation assay

Twenty-four well plates were coated with 500 μL per well of 1:1 diluted growth factor–reduced Matrigel (BD Biosciences) and incubated for 60 minutes at 37°C. HUVECs suspended in 500 μL aliquots of sample CM or no serum medium, as indicated, were added to each well to bring the final concentration of cells to approximately 5 × 10⁴ cells/well. In some wells, CM was preincubated with a neutralizing anti-hVEGF mAb (1 μg/mL; R&D systems). After 24-hour incubation, tube formation was evaluated by phase-contrast microscopy and photographed by a digital camera. All experiments were repeated in triplicate.

Statistical analyses

To compare multiple sets of data, a 1-way ANOVA with post hoc Fisher’s least significant difference test was used. For paired data sets, a 2-tailed t test was used. Values of P < 0.05 were considered to represent a significant difference.

Immunohistochemistry and Western immunoblot analysis

Tissue microarrays (TMA) containing 60 samples, 30 breast cancer and 30 normal adjacent tissues (diameter, 2.0 mm; thickness, 4 μm), were purchased from IMGENEX Corporation. Deparaffinized sections were stained using anti-SAF-1 Ab at 1:500 dilution as described previously (35). Antigen retrieval was done by heating deparaffinized tissues in sodium citrate buffer, pH 6.0, for 30 minutes in a commercially available vegetable steamer. Preimmune rabbit IgG was used as a control. Horseradish peroxidase–conjugated goat anti-rabbit IgG was used as the secondary Ab. Color was developed with 3,3′-diaminobenzidine, and slides were counterstained with hematoxylin solution. Hematoxylin and eosin–stained image of each tissue was obtained from IMGENEX Corporation.

For Western blotting, cultured cells were lysed in 50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, 0.5 mmol/L...
dithiothreitol, 1% SDS, 1 mmol/L phenylmethylsulfonyl-fluoride, and 0.5 mg/mL of benzamidine buffer followed by sonication. The extracts (50 µg protein) were fractionated in a 5%/11% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. To evaluate relative amount of proteins in each lane, proteins were stained with Ponceau S solution (Sigma–Aldrich). Immunoblotting was carried out at a 1:3,000 dilution of anti-SAF-1 Ab, prepared as described earlier (35) or anti-GAPDH Ab (Santa Cruz Biotechnology). Bands were detected using a chemiluminescence detection kit (Amerham Biosciences).

Results

Increased SAF-1 and VEGF expression in MDA-MB-231 breast cancer cells

Recently, a novel mode of induction of VEGF that is regulated by SAF-1 transcription factor was implicated for the escalation of synovial inflammation and angiogenesis under arthritic condition (20). Because VEGF is highly induced in many cancers, including breast cancer, we examined whether SAF-1 plays any role in this process. To this end, we first analyzed expression pattern of SAF-1 in triple-negative MDA-MB-231 breast cancer cells. As seen in Figure 1A, SAF-1 expression was markedly increased in MDA-MB-231 cells as compared with HMECs. VEGF mRNA level, under the same culturing condition, was significantly higher in MDA-MB-231 cells (Fig. 1B). Three VEGF bands, representing different isoforms derived from alternative splicing of the VEGF primary transcript (36, 37), were detected. SAF-1 mRNA level was also high in 2 other breast cancer lines, MCF-7 and MDA-MB-468 cells (Fig. 1C). To determine whether increase of SAF-1 mRNA level correlates with its promoter-binding activity, its binding to a consensus SAF-1–binding element was monitored. Although SAF-1–specific DNA–protein complex was barely visible in HMEC, a prominent DNA–protein complex was seen with the same amount of nuclear extracts of MDA-MB-231 cells (Fig. 1D, compare lanes 1 and 2). This complex was inhibited by a canonical SAF-binding oligonucleotide and anti-SAF-1 Ab but not by a nonspecific DNA or a nonspecific Ab (Fig. 1D, lanes 3–6). Promoter-binding activity of SAF-1 in MDA-MB-468 cells (Fig. 1D, lanes 7–11) was found to be comparable with that of MDA-MB-231. Together, these results showed a strong correlation between SAF-1 level and increased VEGF expression in breast cancer cells.

Figure 1. Increased SAF-1 and VEGF expression in triple-negative breast carcinoma cells. A, total RNA isolated from HMECs and triple-negative MDA-MB-231 cells was subjected to RT-PCR analysis by using SAF-1 gene-specific primers. As a loading control, GAPDH mRNA level was evaluated by using GAPDH-specific primers. B, total RNA, isolated from HMECs and MDA-MB-231 cells, was subjected to RT-PCR analysis by using VEGF–specific primers. As a loading control, GAPDH mRNA level was evaluated by using GAPDH–specific primers. C, total RNA, isolated from HMECs, MCF-7 cells, and MDA-MB-468 cells, was subjected to RT-PCR analysis by using SAF-1–gene–specific primers. As a loading control, GAPDH mRNA level was evaluated by RT-PCR. D, SAF-1 activity is increased in breast cancer cells. Nuclear extracts (10 µg of protein) prepared from HMECs (lane 1), MDA-MB-231 cells (lanes 2–6), and MDA-MB-468 (lanes 7–11) were incubated with 32P–labeled consensus SAF–binding oligonucleotide (1 pmol DNA/reaction) as probe. In addition, lanes 3, 4, 8, and 9 contain 50 pmol of either a competitor oligonucleotide (homologous oligo) or a nonspecific oligonucleotide (Nonsp. oligo) as described in Materials and Methods. Lanes 5 and 10 contain normal IgG (nonspecific Ab, Nonsp. Ab), whereas lanes 6 and 11 contain anti-SAF-1 Ab. DNA–protein complexes were resolved using a 6% nondenaturing polyacrylamide gel.
Regulation of VEGF in breast cancer cells

In the human VEGF promoter, between −130 and −30 nucleotides, a highly GC-rich sequence is present and two SAF-1 DNA-binding sites were located within this region. To determine the role of SAF-1, we considered whether deletion of the GC-rich sequence would impair VEGF promoter function in the breast cancer cells. A diagram representing the VEGF promoter and GC-rich region within is shown in Figure 2A. Deletion of sequences from −130 to −30 nucleotides significantly lowered VEGF expression in both breast cancer cells, MDA-MB-231 and MDA-MB-468. Even in low VEGF expressing HMEC cells, there was further reduction in expression (Fig. 2B). To provide evidence for a direct requirement of SAF-1 for VEGF promoter function, these breast-derived cells were cotransfected with wild-type 1.2VEGF-CAT reporter gene and different concentrations of SAF-1 expression plasmid, pcDNA-SAF-1. Overexpression of pcDNA-SAF-1 proportionately increased 1.2VEGF-CAT expression in both breast cancer cells (Fig. 2C).

To determine the role of two SAF-1–binding domains within the GC-rich sequence of VEGF in the expression of this gene, site-directed mutants at binding sites I and II were generated in the 1.2-kb VEGF promoter (Fig. 3). In addition, a double mutant was created in which both SAF-1–binding sites were mutated. When MDA-MB-231 cells were transfected with these promoter constructs, mutation of site I showed higher loss of VEGF promoter function and, when both elements were inactivated, VEGF-CAT reporter expression was highly compromised (Fig. 3B). Similar results were obtained when MDA-MB-468 cells were used in the transfection assays (data not shown). These results suggested a possible importance of both sites in the induction of VEGF in breast cancer cells.

SAF-1 is present in the DNA–protein complexes formed by the VEGF promoter and the nuclear extracts of breast cancer cells

Next, we analyzed whether nuclear factors present in HMECs and MDA-MB-231 cells differ in terms of their binding to the VEGF promoter.
interaction with this critical region of VEGF promoter to regulate its expression. DNA-binding assay conducted using a radiolabeled VEGF promoter (−135 to +29) as the probe indicated the formation of 2 DNA–protein complexes a and b (Fig. 4A). MDA-MB-231 breast cancer cells formed predominantly complex b (Fig. 4A, lane 3), which is composed of 3 closely migrating DNA–protein complexes. Almost identical pattern of complexes were formed by the nuclear extracts of MDA-MB-468 cells (data not shown). HMECs formed very little complex b but a moderate level of complex a (Fig. 4A, lane 2). To determine whether any of these complexes is composed of SAF-1 protein, anti-SAF-1 Ab was added in the DNA-binding assay, which severely inhibited all 3 DNA–protein complexes within complex b but had no effect on complex a (Fig. 4A, lane 2). To determine whether any of these complexes is composed of SAF-1 protein, anti-SAF-1 Ab was added in the DNA-binding assay, which severely inhibited all 3 DNA–protein complexes within complex b but had no effect on complex a (Fig. 4A, lane 2). Appearance of 3 bands as part of complex b could be the result of binding of several members of the SAF family that often bind as heteromeric complexes (38, 39). Interestingly, the intensity of complex a was increased when SAF-1 interaction with the probe was inhibited by anti-SAF-1 Ab (Fig. 4B, compare lanes 2 and 4, and lanes 3 and 5). At present, identity of the protein(s) in complex a remains unknown because its formation was not affected by Ab against several transcription factors tested, including Sp1, AP-1, AP-2, and NF-κB (data not shown). To determine the boundaries of DNA-binding regions of factors forming these complexes, DNase I footprint analysis was conducted. Two protected regions, spanning nucleotide position −96 to −64 and −47 to −30, where nuclear factors in MDA-MB-231 cells bound avidly were detected (Fig. 4C, lane 2). A third binding region, from nucleotide position −14 to +10, was detected to be formed by factors in HMEC extract (Fig. 4C, lane 1) but not in MDA-MB-231 extract (Fig. 4C, lane 2). These results suggested that the DNA–protein complex a, as seen earlier in Figure 4A, lane 2, could be formed by a nuclear factor predominantly present in the normal mammary epithelial cells and possibly interacts with the VEGF probe at nucleotide position −14 to +10. The complex a most likely has no stimulatory effect on VEGF expression since HMEC expresses very low level of VEGF. In contrary, complex b is abundantly formed by breast cancer cells, MDA-MB-231 and MDA-MB-468 cells, which produce copious amount of VEGF.

SAF-1 is actively engaged at the VEGF promoter in MDA-MB-231 cancer cells

We carried out ChIP assays to determine whether SAF-1 is engaged in vivo with VEGF promoter in breast cancer cells. As seen in Figure 5, the SAF-1–binding region of VEGF promoter was successfully amplified by PCR when chromatin DNA from MDA-MB-231 cells was isolated by precipitation with anti-SAF-1 Ab (Fig. 5, lane 6). Similar results were obtained when we used MDA-MB-468 cells, but for brevity those data are not shown. In contrast, no
described in Materials and Methods with 32P-labeled HMECs (lane 2) and MDA-MB-231 cells (lane 3) were incubated as

231 breast cancer cells. A, nuclear extracts (10 μg of protein) of HMECs (lanes 2 and 4) and MDA-MB-231 cells (lane 3) were incubated as
described in Materials and Methods with 32P-labeled VEGF DNA containing sequences from –135 to +29. Resulting DNA–protein complexes were fractionated in a 6% nondenaturing polyacrylamide gel. Two groups of complexes, a and a, are indicated.

B, characterization of VEGF promoter–binding factors. Nuclear extracts (10 μg of protein) of HMECs (lanes 2 and 4) and MDA-MB-231 cells (lanes 3 and 5) were incubated with 32P-labeled VEGF DNA containing sequences from –135 to +29 as described above. In addition, lanes 4 and 5 contained anti-SAF-1 Ab. C, a single 5′-end-labeled DNA fragment of VEGF DNA that contains sequences from –135 to +29 was incubated, as described above, with nuclear extracts (50 μg of protein) from HMECs (lane 1) and MDA-MB-231 cancer cells (lanes 2 and 3). In lane 3, nuclear extract was preincubated with anti-SAF-1 Ab. DNA–protein complexes were briefly exposed to low concentration of DNase I, and resultant DNA fragments were resolved using a 8% polyacrylamide gel containing 8 mol/L urea, as described in Materials and Methods. In lane 4, the probe was incubated without any added nuclear extract. In lane 5, a G-specific reaction was carried out as size markers. The protected regions are indicated by vertical bars and their respective nucleotide positions in the VEGF DNA probe were identified from the size marker.

detectable PCR product was seen in samples obtained from HMECs following immunoprecipitation with anti-SAF-1 Ab (Fig. 5, lane 3). In the control assay, nonspecific

IgG did not immunoprecipitate VEGF DNA (Fig. 5, lanes 2 and 5). As an additional control, amplification of an upstream region in the VEGF promoter was attempted by a different set of primers, which showed no PCR-amplified product (Fig. 5, lanes 8, 9, 11 and 12). The lanes containing 10% input DNA showed successful amplification of a specific product that indicated the efficacy of the primers used in PCR (Fig. 5, lanes 1, 4, 7, and 10). Together these results showed that SAF-1 DNA–binding element of VEGF is actively occupied by SAF-1 transcription factor in breast cancer cells. In contrast, this transcription factor is minimally engaged with VEGF promoter in the normal breast epithelial cells.

Reduction of endogenous SAF-1 level in MDA-MB-231 breast cancer cells reduces VEGF mRNA expression and affects HUVEC migration, proliferation, and tube formation

To test whether lowering of SAF-1 level in MDA-MB-231 breast cancer cells has any effect on VEGF expression, HMECs and MDA-MB-231 cells were stably transfected with a SAF-1/MAZ shRNA lentivirus or a control empty vector viral particle. Messenger RNA level for SAF-1 in these transfected cells was analyzed by RT-PCR. Results showed that SAF-1 shRNA can reduce SAF-1 mRNA level in both HMECs and MDA-MB-231 cells and the effect is profound in the latter cells (Fig. 6A). No change in GAPDH mRNA expression level indicated specificity of the SAF-1 shRNA action. In correlation to the reduction of SAF-1 mRNA, SAF-1 protein level was also reduced in SAF-1 shRNA–transfected cells as compared with vector-transfected cells (Fig. 6B). VEGF expression was also significantly inhibited in SAF-1 shRNA–transfected breast cancer cells (Fig. 6C). Similar inhibition was seen for VEGF121 (data not shown). Because HMECs express VEGF at a very low level, it became difficult to notice further reduction of VEGF165 level in SAF-1 shRNA–transfected HMECs. Together these results suggested that SAF-1 shRNA proficiently reduces endogenous SAF-1 level and can inhibit VEGF expression. When we tested the migration of HUVECs in the presence of CM derived from SAF-1 shRNA–transfected MDA-MB-231 breast cancer cells, a reduction of the HUVEC migration activity was observed when compared with the migration generated by CM from control vector–transfected cells (Fig. 6D). A as a control, when VEGF-A Ab was included in the CM to sequester available VEGF protein, migration of HUVECs was similarly restricted while control non-immune serum showed no such adverse effect. Proliferation rate of HUVECs was also affected by CM derived from SAF-1 shRNA–transfected MDA-MB-231 cells as compared with that of CM from control vector–transfected cells (Fig. 6E). Tube formation by HUVECs in Matrigel-coated plates was also inhibited by the CM of SAF-1 shRNA–transfected MDA-MB-231 cells (Fig. 6F). Together, these results suggested that ablation of SAF-1 in the breast cancer cells leads to diminished support for vascular endothelial cell function.
SAF-1 expression in human breast cancer and adjacent normal tissues

To determine whether SAF-1 is expressed in clinical breast cancer specimens, we evaluated level of this mRNA in patient-matched tumor and adjacent normal tissue pairs. Semiquantitative RT-PCR analysis of these human breast cancer tissues showed marked elevation of SAF-1 mRNA in the tumor versus the adjacent normal tissue (Fig. 7A). A TMA that contained formalin-fixed, paraffin-embedded 30 breast cancer and 30 normal adjacent tissues was used for monitoring SAF-1 protein expression. All slides of the tumors were reviewed by a pathologist. Tumor stage and grade were defined by using the modified Scarff-Bloom-Richardson system (40). Of the 30 infiltrating ductal carcinoma samples, 15 scored between 7 and 8 whereas remaining 15 scored between 8 and 9. These scores place majority of these tumors in grade 3 category and defined them as poorly differentiated with worst prognosis. Thirty normal breast tissue samples obtained from the adjacent region of the tumors scored between 3 and 5, representing well- to moderately differentiated conditions. Immunohistochemical (IHC) analysis of the serial sections of the abovementioned tissue samples indicated nominal but detectable levels of SAF-1 expression. All slides of the tumors were reviewed by a pathologist. Tumor stage and grade were defined by using the modified Scarff-Bloom-Richardson system (40). Of the 30 infiltrating ductal carcinoma samples, 15 scored between 7 and 8 whereas remaining 15 scored between 8 and 9. These scores place majority of these tumors in grade 3 category and defined them as poorly differentiated with worst prognosis. Thirty normal breast tissue samples obtained from the adjacent region of the tumors scored between 3 and 5, representing well- to moderately differentiated conditions. Immunohistochemical (IHC) analysis of the serial sections of the abovementioned tissue samples indicated nominal but detectable levels of SAF-1 expression in normal breast tissues, adjacent to tumors (Fig. 7B). In marked contrast, SAF-1 expression was very high in the tumor region (Fig. 7C). It is noteworthy that analysis of these 30 different types of tumor samples revealed no statistically significant association between SAF-1 expression and ER, PR, and p53 status of the tumors. A summary of histologic and IHC analyses of the results in Figure 7B and C is shown in Table 1.

Discussion

VEGF-mediated signaling plays a pivotal role in tumor angiogenesis, tumor growth, and metastasis. Induction of VEGF is regarded as highly complex, involving many intricate mechanisms, some of which may be activated only during tumor initiation and/or progression. Clarification of the molecular mechanisms regulating increased synthesis of VEGF in triple-negative breast cancer cells is crucial for identification of new therapies. We report a novel finding that SAF-1/MAZ transcription factor, an inflammation responsive protein, is capable of modulating expression of VEGF in triple-negative breast cancer cells. We draw our conclusion from the following results that show (i) lack of VEGF induction in HMECs due to the low abundance of SAF-1 while increased VEGF expression in breast cancer cells due to highly abundant SAF-1 that occupies VEGF promoter, (ii) marked increase of SAF-1 functional activity in breast cancer cells and clinical breast cancer tissues, and (iii) reduction of VEGF expression in breast cancer cells as a result of inhibition of endogenous SAF-1 by shRNA-mediated gene silencing, which, in turn, impaired VEGF-induced angiogenesis.

Among multiple angiogenic factors in cancer tissues, VEGF is the most predominant. This growth factor is found to be essential for angiogenic switch or new blood vessel formation from the preexisting ones that is vital for tumor growth and metastasis. We show that SAF-1 is overexpressed in breast cancer cells (Fig. 1) and is highly abundant in clinical breast cancer specimens (Fig. 7). Furthermore, knockdown of SAF-1 in MDA-MB-231 cells by SAF-1/MAZ-specific shRNA results in inhibition of VEGF expression and VEGF-induced HUVEC migration, proliferation, and tube formation (Fig. 6). By using ChIP analysis, DNA-binding and reporter gene assays, 2 SAF-1 DNA-binding elements at a GC-rich region in the proximal promoter region of VEGF were identified (Figs. 2–5). We further verified by DNase I footprint analysis and specifically by using SAF-1

Figure 5. In vivo interaction of SAF-1 transcription factor with VEGF promoter in MDA-MB-231 cells. HMECs (lanes 1–3 and 7–9) and MDA-MB-231 cells (lanes 4–6 and 10–12) were fixed with formaldehyde and immunoprecipitated with SAF-1 Ab or nonspecific IgG (Nonsp. IgG) following the ChIP assay method as described in Materials and Methods. Immunoprecipitated DNAs were used as templates for PCR amplification of human VEGF promoter by using primers encompassing the SAF-1 DNA-binding element or an unrelated element in the VEGF promoter (20). Amplified products were resolved using a 1.5% agarose gel.
Ab–neutralized MDA-MB-231 breast cancer cell nuclear extract in this footprint assay that SAF-1 transcription factor interacts with the VEGF promoter (Fig. 4C). In correlation with our observation, previous unrelated studies have linked activation of MAZ/SAF-1 with several cancers. Notably, MAZ was shown to drive tissue-specific expression of PPARγ in breast cancer cells (26) and prospero-related homeobox I (Prox-I) gene expression in liver tumors (27). In these studies, both mRNA and protein levels of MAZ were shown to be increased (26, 27).

SAF-1 is the first identified member of a multiple Cys2-His2–type zinc finger protein transcription factor family, which presently contains 3 members, SAF-1/MAZ/Pur-1 (21, 33, 41), SAF-2 (38), and SAF-3 (39). SAF-1 is activated by a number of inflammatory stimuli predominantly via phosphorylation. Proto-oncogene c-src and activation of cSrc kinase are necessary for the increased expression of VEGF in breast cancer cells (42). In addition, oncogenes such as H-ras (Ras-V12) and KSHV-GPCR (Kaposi’s Sarcoma Herpes Virus-Gprotein coupled chemokine receptor) are known to induce angiogenesis by increasing VEGF secretion (43–45). Because ras and other dominant oncogenes activate protein kinases, it is likely that oncogenic transformation of normal breast epithelial cells during which VEGF is overexpressed might involve activation of SAF-1 protein.

The VEGF gene in both human and mouse does not contain a canonical TATA box-like promoter sequence, instead it contains highly GC-rich sequences at the proximal promoter region (46, 47). This GC-rich region was shown to be essential in regulating basal and TGF-α, hepatocyte growth factor (HGF), p53, and p42/p44 MAP kinase–mediated induction of VEGF (42, 48–51). Notably, among the 2 SAF-1 DNA-binding sites, the site I is located within this GC-rich region (Figs. 3 and 4).

**Figure 6.** Ablation of SAF-1 reduces VEGF expression and angiogenesis in vitro. A, RT-PCR analysis for SAF-1 and GAPDH expression in empty vector or SAF-1 shRNA–transfected MDA-MB-231 cells. RT-PCR of total RNA was carried out by using gene-specific primers for SAF-1 and GAPDH (used as a control). B, Western immunoblot analysis. Fifty micrograms of protein isolated from vector or SAF-1 shRNA–transfected MDA-MB-231 cells was probed with SAF-1 Ab and GAPDH Ab (used as a control). C, RT-PCR analysis of VEGF and GAPDH expression in vector or SAF-1 shRNA–transfected MDA-MB-231 cells. RT-PCR of total RNA was carried out by using gene-specific primers for VEGF and GAPDH (used as a control). D, cell migration assay. HUVECs were grown on top of fibronectin-coated surface in Transwell plate as described in Materials and Methods. CM from vector-transfected or SAF-1 shRNA–transfected MDA-MB-231 cells was used. In some wells, anti-VEGF Ab or nonspecific IgG were included, as indicated. Migrated cells, following 24-hour incubation, were quantitated as described in Materials and Methods. These results represent mean ± SE of 3 independent experiments. *, P < 0.05. E, Cell proliferation assay. HUVECs were grown in 96-well plate in the absence or presence of CM prepared from different cell lines as indicated. Proliferation assay was done as described in Materials and Methods. These results represent mean ± SE of 3 independent experiments. **, P < 0.05. F, tube formation of HUVECs on Matrigel. HUVECs (2 × 104) suspended in sample CM and plated on Matrigel-coated 24-well culture plates. After 24-hour incubation, cells were photographed using phase-contrast microscope. a, DMEM; b, DMEM + CM of vector-transfected MDA-MB-231 cells; c, DMEM + CM of SAF-1 shRNA–transfected MDA-MB-231 cells; d, DMEM + CM of vector-transfected MDA-MB-231 cells plus anti-VEGF Ab.
and 4), suggesting that binding of SAF-1 is crucial for both basal and inducible expression of VEGF. Indeed, our results on the effect of SAF-1 suppression, as shown in Figure 6, show a clear dependence of VEGF expression on the presence of SAF-1. Together, these interesting findings implicate that apparently harmful VEGF expression in breast cancer can be effectively regulated by controlling SAF-1 level and/or its activity. Further studies along these lines may, therefore, provide a new avenue for achieving breast cancer chemoprevention particularly for patients with triple-negative breast cancer, as the therapeutic options for these patients are quite limited.

In summary, we define a regulatory role of SAF-1/MAZ on the transcriptional regulation of VEGF in triple-negative breast cancer cells. Inhibition of VEGF expres-

Figure 7. SAF-1 expression in clinical breast cancer tissues. A, total RNA was isolated from breast cancer and adjacent pair-matched normal region tissues, and gene expression was measured by using semiquantitative RT-PCR. Expression was normalized to that of GAPDH as described in Materials and Methods. Data are representative of 3 experiments (mean ± SD). B and C, analysis of breast carcinoma and adjacent normal tissues by using H&E staining for histopathology and IHC procedure for evaluation of SAF-1 expression. The inset regions represent higher magnification of the boxed areas. A total of 60 breast tissue samples (30 carcinoma and 30 adjacent normal) were tested and some representative samples are shown here. Pathologic evaluation of the samples and the analysis of SAF-1 expression in B and C are summarized in Table 1.
Ferrara N. The role of vascular endothelial growth factor in pathologic angiogenesis by selective ablation of SAF-1 transcription factor raises an interesting possibility of blocking angiogenesis in breast cancer and a novel option for targeted treatment of triple-negative breast cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

15. Feldkamp MM, Lau N, Rak J, Kerbel RS, Guha A. Normoxic and hypoxic regulation of vascular endothelial growth factor (VEGF)

Table 1. SAF-1 expression in different stages of human breast cancer tissue

<table>
<thead>
<tr>
<th>Breast tissue</th>
<th>Histologic grade</th>
<th>Stage</th>
<th>SAF-1 level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltrating ductal carcinoma (n = 30)</td>
<td>C1</td>
<td>6</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>8</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>C3</td>
<td>9</td>
<td>+++</td>
</tr>
<tr>
<td>Adjacent normal tissue (n = 30)</td>
<td>B1</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>4</td>
<td>+</td>
</tr>
</tbody>
</table>

*Histologic grading of the samples was carried out by using modified the Scarff-Bloom-Richardson system (40), and the results of the representative samples that are shown in Figure 7 are summarized here. C1, C2, and C3 represent the left, middle, and right panels of Figure 7C, respectively. B1 and B2 represent top and bottom panels of Figure 7B, respectively.

Cancer stage was determined by following the American Joint Committee on Cancer (AJCC) staging system.

A semiquantitative estimate of SAF-1 expression level was based on combined score for the proportion of stained cells and the intensity of stain and following the criteria described elsewhere (52, 53). Proportion score represented the estimated percentage of stained cells (0, <10%; 1, between 10% and 25%; 2, between 25% and 50%; 3, between 50% and 75%; 4, between 75% and 90%; and 5, >90%). Intensity score corresponded to the average staining intensity of the cells (0, no staining; 1, weakly stained; 2, moderately stained; and 3, strongly stained). Levels of staining were derived as follows: samples with an intensity score of 0 or a proportion score of 0 were designated negative (–), and samples with intensity score of 1 and a proportion score of 1–2 were designated weak (+). Samples with an intensity score of 2 and 3, and combined scores of 2–3, 4–6, and 7–8 were designated as weak (+), moderate (++), and strong (+++) expression, respectively.

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Control of VEGF Expression in Triple-Negative Breast Carcinoma Cells by Suppression of SAF-1 Transcription Factor Activity

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