Microenvironment and Immunology

An Ets2-Driven Transcriptional Program in Tumor-Associated Macrophages Promotes Tumor Metastasis

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Abstract

Tumor-associated macrophages (TAM) are implicated in breast cancer metastasis, but relatively little is known about the underlying genes and pathways that are involved. The transcription factor Ets2 is a direct target of signaling pathways involving in regulating macrophage functions during inflammation. We conditionally deleted Ets in TAMs to determine its function at this level on mouse mammary tumor growth and metastasis. Ets2 deletion in TAMs decreased the frequency and size of lung metastases in three different mouse models of breast cancer metastasis. Expression profiling and chromatin immunoprecipitation assays in isolated TAMs established that Ets2 repressed a gene program that included several well-characterized inhibitors of angiogenesis. Consistent with these results, Ets2 ablation in TAMs led to decreased angiogenesis and decreased growth of tumors. An Ets2-TAM expression signature consisting of 133 genes was identified within human breast cancer expression data which could retrospectively predict overall survival of patients with breast cancer in two independent data sets. In summary, we identified Ets2 as a central driver of a transcriptional program in TAMs that acts to promote lung metastasis of breast tumors. Cancer Res 70(4); 1323–33. ©2010 AACR.

Introduction

Sporadic human cancer results from somatic gene mutations that lead to aberrant growth, survival, genetic instability, and increased motility of tumor cells (1). In addition to genetic complexity, it is increasingly apparent that cellular complexity inherent in the tumor stroma plays an active role in promoting all stages of tumor progression (2). Among the many cell types in the tumor stroma, the tumor-associated macrophage (TAM) is a broadly defined myeloid cell type that has been implicated in tumor progression (2). TAMs are thought to be a polarized M2 subtype of macrophage that promotes tumor growth, invasion, and angiogenesis (3). Alternatively, the pleiotropic effects of macrophages within the tumor microenvironment may be mediated by distinct subpopulations of TAMs that can selectively affect distinct processes such as tumor angiogenesis or invasion (4,5).

The link between TAMs and tumor progression is especially well-established in breast cancer. Human clinical studies have shown that a high focal infiltration of TAMs in primary human breast tumors correlates directly with tumor cell invasion, increased vascularization, axillary lymph node involvement, and reduced relapse-free survival of patients (6–9). In a mouse mammary tumor model, genetic ablation of colony-stimulating factor-1 (Csf-1), a growth factor critical for macrophage growth, differentiation, and survival, results in a reduction in mammary TAMs and a lower incidence of lung metastasis (10).

Ets2, a member of the Ets family of transcription factors, is a direct effector of Csf-1 signaling pathways that modulates macrophage functions and survival during inflammation (11,12). ETS2 activates or represses the transcription of target genes in a context-dependent manner (13,14). Elevated expression of ETS2 has been correlated with human breast cancer (15). However, in mouse mammary tumor models, Ets2 promotes tumor progression from the stroma and not the tumor epithelial cell (16).

In the current study, a genetic approach was used to define the action of Ets2 in mouse mammary TAMs. The results show that Ets2 in TAMs decreased the growth rate of the primary tumor and tumor metastases as well as the mechanism involved repressing genes that are inhibitors of angiogenesis. One hundred and thirty-three human genes orthologous to the Ets2-TAM profile could retrospectively predict disease-free survival among patients present in two human breast cancer microarray data sets (17,18). These results identify...
an Ets2-regulated transcriptional program in TAMs that regulates the growth and spread of breast tumors.

Materials and Methods

Mice. The Ets2\textsuperscript{loxP} allele, Ets2\textsuperscript{th} knockout allele, MMTV-PyMT transgenic mice, and Lys-Cre knockin allele have been previously described (19–22). The c-fms-YFP construct was identical to the published c-fms-EGFP construct except for the substitution of YFP (23). Transgenic mice were produced by standard DNA microinjection procedures. All alleles used were >10 generations FVB/N background. Use and care of mice were approved by the Ohio State University Institutional Animal Care and Use Committee.

Orthotopic and tail vein injection assays. Two breast cancer cell lines, Met-1 (MMTV-PyMT) and MVT-1 (MMTV-c-Myr; MMTV-VEGF) were used (24, 25). The cell lines were cultured in DMEM containing 10% fetal bovine serum at 37°C in a 5% CO2 incubator. Cultured tumor cells were harvested at 80% to 90% confluence and suspended in filtered cold 0.9% NaCl. Three million Met-1 cells or 200,000 MVT-1 cells were injected into the tail vein or mammary gland, respectively. Tail vein and orthotopically injected animals were dissected 18 and 35 d postinjection, respectively.

Isolation of TAMs. Minced mammary glands or lungs with metastatic tumors were digested with 20 mg of collagenase type 2 (Worthington), 480 units of DNase (Boehringer), and 1 mmol/L of MgCl\textsubscript{2} at 37°C in a 5% CO2 incubator. Cultured tumor cells were harvested at 80% to 90% confluence and suspended in filtered cold 0.9% NaCl. Three million Met-1 cells or 200,000 MVT-1 cells were injected into the tail vein or mammary gland, respectively. Tail vein and orthotopically injected animals were dissected 18 and 35 d postinjection, respectively.

RNA extraction and quantitative real-time PCR. RNA extraction and cDNA preparation were done as described previously (27). For samples used in microarray analysis, RNA was extracted with the RNeasy Stratagene micro-prep column (Stratagene) according to the instructions of the manufacturer. Two independent sets of RNA isolated from different TAMs/mice other than RNA used for the microarrays were used for verification.

Real-time quantitative reverse transcription-PCR was conducted using the Roche Universal Probe Library system (Roche Diagnostics) in an iCycler iQ Real-time Detection System (Bio-Rad) as described previously (27). Primer-probe combinations are available upon request.

Histology and immunohistochemistry. Tumor tissues were fixed in formalin overnight, processed, paraffin-embedded, and 5 μmol/L sections were prepared. For immunostaining, rat α-mouse F4/80 (1:40 dilution; Caltag Labs), rat α-mouse CD31 (1:50 dilution; Abcam), mouse α-human THBS1 (1:50 dilution; Abcam), mouse α-mouse THBS2 (1:50 dilution; BD Biosciences), goat α-mouse SPARC (1:100 dilution; BD Biosciences), and mouse α-mouse bmodemoeurydine (1:50 dilution; DAKO) primary antibodies were used. Biotinylated goat α-rat, goat α-mouse, or donkey α-goat (BD Biosciences) were the secondary antibodies used for immunohistochemical analyses. Images were acquired using an Axioscope 40 microscope (Zeiss) equipped with an Axioscam HRc camera (Zeiss). Immunohistochemical data was quantified by calculating the area of antibody staining per unit area of tumor using Metamorph 6.0 software. Whole mount hematoxylin staining of lungs was performed as described (28).

For colocalization studies, frozen sections of mammary tumors fixed in 4% paraformaldehyde were double-immunostained with α-F4/80 antibody (Alexa-594 secondary antibody; Invitrogen) and either α-THBS2, α-THBS1, or α-SPARC antibody (Alexa-488 secondary antibody; Invitrogen). Nuclei were stained with DRAQ5. Images of stained mammary tumors were acquired using a Zeiss 510 META laser scanning confocal microscope. Results are presented as the percentage of F4/80-positive or -negative cells that had colocalized staining in or around (extracellular space) for α-THBS2, α-THBS1, or α-SPARC, respectively.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChiP) assays were performed as described (27). Immunoprecipitation was carried out with 2.5 μg of antibodies. The ETS2 antibody has been previously described (19). Rabbit α-mouse HDAC1 and rabbit-IgG were purchased from Santa Cruz Biotechnology and Upstate, respectively. For lung TAMS, the immunoprecipitated chromatin was amplified using an unbiased genome amplification kit (Sigma Aldrich). Samples were analyzed by real-time PCR using the Roche Universal Probe Library (Roche Diagnostics) and the FastStart TaqMan master kit (Roche Diagnostics).

Microarray and survival analysis. Microarrays were performed on the Mouse Affymetrix 130A.2 platform. The primary data was analyzed by a modified robust multi-array average method to yield an average gene expression value (29, 30). The detailed description of the experiment and subsequent data analysis is presented in Supplementary Table S1A.

A high-confidence 142 probe set (P < 0.05) human Ets2-TAM signature was generated by comparing 407 mouse probe sets (357 genes, absolute INT > 1.5) to the 98 lymph node–negative Rosetta cohort (divided into two groups based on lymphocyte/leukocyte infiltration status; ref. 31). For survival analysis, the 142 probe set Ets2-TAM signature was used as a query to retrieve gene expression data from Stockholm (GSE1456) breast cancer microarrays (downloaded from the National Center for Biotechnology Information-Gene Expression Omnibus web page). Similarly, gene expression data was also extracted from total and lymph node–negative Rosetta microarrays. The resultant data sets were loaded onto BRB Array Tools as described in Supplementary Table S3. Briefly, unsupervised K-means clustering of each data set was performed by using Cluster 3.0 (32), and samples were assigned into two groups. Kaplan-Meier survival analysis was performed by using the Survival Analysis module of the software package StatsDirect (StatsDirect, Ltd.). Significance of survival analyses was performed by using the log rank (Peto) test.

Statistical analysis. For lung metastases data, a nonparametric Kruskal-Wallis test with no multiplicity adjustment was used to compare medians between experimental and

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control groups. A repeated measures ANOVA model was used to analyze mammary tumor progression between the genetic groups over a period of 42 d. This approach takes into consideration longitudinal data, and the following terms were included in the model: genetic group, time and interaction (genotype * time). For the statistical analysis of imaging data, an unpaired Student’s t test was used. All the tests were two-sided.

Results

Deletion of Ets2 in TAMs decreases lung metastasis in spontaneous and orthotopic breast tumor models. Cre/LoxP technology was used to conditionally delete Ets2 in TAMs in the PyMT model, a penetrant breast cancer model with a high frequency of lung metastasis (21). The conditional Ets2LoxP allele used for this study contained LoxP sites flanking exon3-exon5 so that Cre-mediated recombination of the region resulted in the generation of a null allele (19). The well-characterized Lys-Cre knockin allele was used to delete Ets2 specifically in the macrophage compartment (22). However, initial studies revealed that Cre recombination in Lys-Cre:Ets2LoxP/LoxP mice was only 30% to 50% efficient (data not shown). To circumvent this problem, we adopted a strategy whereby mice contained one conditional Ets2LoxP allele and one conventional knockout allele, Ets2db (20). In the final cross, PyMT;Lys-Cre;Ets2LoxP/+ females were crossed with Ets2LoxP/+ males to generate both the experimental genotype, PyMT;Lys-Cre;Ets2LoxP/+;Ets2db, and the control genotype, PyMT:Ets2LoxP/db (Supplementary Fig. S1A). The frequency of Ets2 deletion in isolated mammary tumor macrophages varied between 70% and 90% with this allele configuration (Supplementary Fig. S1B).

Tumor progression was monitored in females of the two genotypes. Tumor initiation was identical between experimental and control mice (data not shown). A small, but statistically significant, decrease in overall tumor growth was observed in the experimental group (Supplementary Fig. S1C). This difference in tumor growth was not significant in the early carcinoma stage of progression, but was more pronounced during the late carcinoma stage (days 21–35 postinitiation; Supplementary Fig. S1C). However, the final tumor burden and tumor volume were similar in both PyMT;Lys-Cre;Ets2LoxP/db and PyMT;Ets2LoxP/db mice (Supplementary Fig. S1D).

Lung metastasis in both genetic groups was studied by whole mount analysis (Fig. 1A; Supplementary Fig. S1E). After image acquisition, the size of the tumors relative to total lung area and the total number of metastases in PyMT;Lys-Cre;Ets2LoxP/db versus PyMT;Ets2LoxP/db mice were quantified. The results showed that both the size and number of lung metastases were significantly reduced in PyMT;Lys-Cre;Ets2LoxP/db mice compared with controls (Fig. 1A; right; size decreased 3- to 4-fold; P = 0.001; Supplementary Fig. S2A, number decreased 2-fold; P = 0.02).

To confirm and extend the results obtained in the genetic PyMT model, a syngeneic model was used. The highly metastatic cell line, MVT-1, derived from mice doubly transgenic for MMTV-c-Myc and MMTV-VEGF (25) was injected into the mammary fat pads of Lys-Cre:Ets2LoxP/db and control Ets2LoxP/db female mice. After 35 days, mice were euthanized and examined. Although there were no differences in the final tumor burden for the primary tumors (data not shown), the size of metastases per total lung area was reduced 3-fold in the experimental Lys-Cre:Ets2LoxP/db group compared with the control group (Fig. 1B). These results indicate that the effect of Ets2 deletion was independent of the PyMT oncogene and also show that haploinsufficiency of Ets2 in the PyMT model is not a confounding factor.

Ets2 in lung macrophages is required for breast tumor metastasis. To firmly establish that the effect of Ets2 in TAMs on metastasis was independent of effects at the primary mammary tumors, a tail vein injection model was used. A metastatic PyMT cell line, Met-1 (24), was injected into the circulation via the tail vein in the same two genetic groups as above. After 18 days, mice were euthanized and metastases to lungs were quantified in H&E-stained sections (Fig. 1C). The results showed that the size of lung metastases were significantly reduced more than 3-fold in the Lys-Cre;Ets2LoxP/db mice compared with controls.

A potential explanation for the lower levels of metastasis observed in all three models might be that Ets2-regulated genes were required for macrophage survival and/or motility (11, 12). Immunostaining of tumor sections with F4/80 antibody, a marker for mature macrophages, revealed that Ets2 deletion did not result in a decrease in F4/80-positive macrophages associated with either primary or metastatic tumors (Supplementary Fig. S2B–C, respectively).

Identification of Ets2 target genes in TAMs. To address the mechanism of Ets2 function in TAMs, mammary TAMs were isolated and subjected to gene expression profiling using the Affymetrix platform. To accomplish this, mammary TAMs were tagged using a c-fms-YFP transgene (ref. 23; Supplementary Fig. S3A). This transgene was incorporated into the breeding scheme outlined above to produce experimental PyMT;Lys-Cre;Ets2LoxP/db;c-fms-YFP and control PyMT;Ets2LoxP/db;c-fms-YFP mice. YFP-positive cells isolated from collagenase-digested tissue by high-speed digital fluorescence-activated cell sorting represented ~10% to 15% of the total cells from the primary mammary tumor site (Supplementary Fig. S3B). Greater than 90% of these YFP-positive cells coexpressed macrophage markers such as F4/80 (Supplementary Fig. S3C). Typically, 3 × 10^5 to 5 × 10^5 YFP-positive TAMs could be isolated from a single mouse.

YFP-positive TAMs were isolated from both genetic groups at the stage when early carcinoma was initially detected in the PyMT model (21). The percentage of YFP-positive cells per mammary gland isolated by fluorescence-activated cell sorting was similar in both genetic groups, supporting the conclusion that a reduction in tumor macrophages was not responsible for the observed effects (Supplementary Fig. S3B). Because macrophages have also been shown to play a central role in tissue remodeling during mammary gland development (33), YFP-positive macrophages were extracted from the mammary gland of Lys-Cre:Ets2LoxP/db;c-fms-YFP and Ets2LoxP/db;c-fms-YFP females ~14 days after the onset...
of puberty. We reasoned that the role of macrophages in tissue remodeling during mammary gland development would provide a useful comparison to unmask the tumor-specific effects of \( Ets2 \).

Expression profiling was performed on the resulting four sets of RNA samples. Comparisons between all four sets of expression data were used to identify 357 genes (407 probe sets), the expressions of which depended on both loss of \( Ets2 \) and the presence of tumor (see Supplementary Table S1 for details). Approximately 25% of these genes were negatively regulated in the tumor microenvironment and the expression of these genes increased when \( Ets2 \) was deleted in TAMs.

Gene ontology indicated that genes encoding extracellular components were principally affected by \( Ets2 \) deletion (Fig. 2A). The major biological process represented was angiogenesis, with 34% of the genes annotated as having a role in this process (Fig. 2A). Many of the genes in the angiogenesis class were classified as inhibitors of angiogenesis.
Quantitative reverse transcription-PCR using RNA from independently isolated mammary TAMs representing early (first palpable tumor) and late (6 weeks after tumor initiation) carcinoma stages were used to verify the microarray results (Fig. 2B). Of 31 genes tested, 25 were confirmed to be differentially expressed in TAMs with or without Ets2 (Supplementary Table S2). Data for 14 of the genes classified as encoding inhibitors of angiogenesis are shown (Fig. 2B; Supplementary Fig. S3D). Expression of these genes in both early and late tumors was increased when Ets2 was deleted. In contrast, potential ETS2 targets known to be involved in inflammation such as Mmp9 and Tnfα (12), and other genes associated with inflammation such as Il6, were not significantly affected by Ets2 deletion in TAMs, emphasizing that the analysis identified tumor-specific targets of ETS2 (Supplementary Fig. S3D).

The same 31 genes were also studied in lung TAMs isolated following tail vein injection of Met-1 cells (bottom, Fig. 2B; Supplementary Table S2). In these TAMs, 25 of 31 genes were differentially expressed when Ets2 was deleted, including the angiogenic gene set, indicating the Ets2 targets were similar in mammary or lung TAMs.

**ETS2 directly regulates antiangiogenic genes in isolated TAMs.** Examination of 1 kb of the proximal promoter regions of four candidate genes not previously reported as ETS2 targets (Thbs1, Thbs2, Timp1, and Timp3) revealed conserved ETS binding motifs in their proximal promoter regions (Supplementary Fig. S4A). Based on these conserved sequences, ChIP experiments were performed on lung TAMs from mice with or without Ets2. For the experiments, ~50,000 YFP-tagged, F4/80-positive cells were isolated from lungs containing metastases following tail vein injection of Met-1 cells. Antibodies against ETS2 and its corepressor HDAC1 (14) were used in the ChIP assays (Fig. 3).

The ChIP experiments revealed that in wild-type cells, ETS2 and HDAC1 were both enriched at all four of these promoter

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**Figure 2.** Ets2 represses the expression of extracellular matrix-modifying genes in TAMs. A, gene ontology based on cellular localization (left pie chart) and biological process (right pie chart) of the genes differentially regulated in TAMs with or without Ets2. B, confirmation of genes identified by the microarray analysis using real-time quantitative PCR. RNA was extracted from independently isolated sets of TAMs derived from mammary glands (top) or from lungs of mice injected with the Met-1 cell line (bottom). Genotypes and stage of tumor development are indicated. Data is represented as average fold induction in samples analyzed in duplicates.
sequences (Fig. 3). In contrast, when Ets2 was conditionally deleted, both the levels of ETS2 and HDAC1 were significantly reduced at each of the four promoters. Similar results were obtained for the Thbs1 promoter in TAMs isolated from the primary mammary tumor (Supplementary Fig. S4B).

Expression of angiogenesis inhibitors in TAMs lacking Ets2 correlates with reduced tumor angiogenesis and proliferation. To verify the expression of ETS2 targets in situ, we performed immunohistochemical staining on paraffin-embedded samples prepared from metastatic lung tumors using commercially available antibodies. This analysis showed robust expression of THBS2, THBS1, and SPARC within tumors from mice with Ets2 deletions in TAMs compared with Ets2+ controls (Fig. 4A; Supplementary Fig. S5A–B).

To confirm that the tumor macrophages were expressing these proteins, frozen mammary tumor sections were analyzed by double immunofluorescent staining using F4/80 to identify TAMs. The MVT-1 orthotopic mammary fat pad injection model was used for this analysis. Staining with α-F4/80 and α-THBS2 showed extensive overlap between the two proteins in sections obtained from tumors with Ets2 deletions (Fig. 4B, top; Supplementary Movie 1). Because THBS2 is an extracellular protein, expression was found both intracellularly and in the adjacent extracellular space in ∼75% of F4/80-positive cells, as clearly evident in confocal reconstructions of 15-μm sections (see Supplementary Movie 1). In contrast, coexpression of THBS2 in F4/80-positive cells was 10-fold lower in tumors with Ets2 deletions (Fig. 4B, bottom). Importantly, expression of THBS2 in F4/80-negative cells was not affected by deletion of Ets2 in TAMs (Fig. 4B, bottom bar graph). Identical results were obtained for THBS1 and SPARC (Supplementary Fig. S6A–B and Supplementary Movies 2–3, respectively).

Because many of the tumor-specific Ets2 targets detected, including THBS1, THBS2, and SPARC have been implicated in angiogenesis, blood vessel density was analyzed in experimental and control mice using α-CD31 immunostaining of paraffin-embedded tumor sections. For these experiments, both primary MVT-1 tumors and lung tumors formed by tail vein injection of Met-1 cells were studied (Fig. 5A). A significant 2- to 3-fold reduction in tumor vasculature was observed in both primary mammary tumors and lung metastases (Fig. 5A).

Bromodeoxyuridine incorporation was used to measure cell proliferation in lung metastases in the Met-1 tail vein injection model (Fig. 5B). The analysis showed a significant 2.5-fold decrease in bromodeoxyuridine-labeled tumor cells in mice with Ets2-deficient TAMs compared with controls. Tumor cell apoptosis, measured by staining with activated caspase-3 antibody, was not significantly affected by Ets2 deletion (Supplementary Fig. S2D).

The Ets2-TAM gene expression signature predicts survival of patients with breast cancer. To determine if the mouse genetic studies were relevant to human disease, the mouse expression data was compared with the Rosetta human breast cancer data set (31). Initially, 407 mouse probe sets that were differentially expressed in mouse TAMs with or without Ets2 were compared with the Rosetta array platform and 341 homologous human probe sets were identified (see
Supplementary Table S3 for details). These 341 probe sets were compared with 2,856 probe sets that represented genes differentially expressed in 117 human samples annotated as with or without lymphocyte/leukocyte infiltration (31). This comparison showed that 142 of the mouse Ets2-TAM probe sets, representing 133 genes, were significantly differentially expressed in lymphocyte/leukocyte infiltration–positive versus --negative human breast cancers ($P < 0.05$, see Supplementary Table S3).

Figure 4. Increased expression of thrombospondin-2 in TAMs that lack Ets2. A, lung sections from mice injected with the Met-1 cell line in the indicated genotypes, immunostained with α-THBS2. Bottom panels, high-magnification images of insets from the respective top panels (B). Bars, 100 μm. Quantification of antibody staining is presented as the average area of staining per tumor area (graphs, bottom). Five different tumor areas from five different mice in each group were analyzed. B, images of mammary tumor sections from mice injected with the MVT-1 cell line harvested 1 wk postinjection. Double-immunostained with α-F4/80 (red, left) and α-THBS2 (green, middle), and merged F4/80-THBS2 images (yellow, right). Quantification of antibody staining is presented as the average percentage of F4/80-positive cells that are also positive for THBS2 in the mammary tumors (graph at right). Bar at the top right corner, 20 μm. Five different tumor areas from four different tumors in each group were analyzed. Statistical significance ($P$ value evaluated by unpaired Student’s $t$ test) is shown.
and Supplementary Fig. S7A). Gene ontology analysis of these human genes showed that extracellular matrix components and angiogenesis were predominantly affected, just as for the mouse Ets2-TAMs genes (Supplementary Fig. S7B). A subset of 70 genes differentially expressed with high significance ($P < 0.001$) is represented in the heat map presented in Fig. 6A. Interestingly, Ets2 expression itself was on average 8-fold higher in lymphocyte/leukocyte infiltration–positive patients when compared with the negative group (Fig. 6A, bar graph; $P = 0.0002$).

To determine if the TAM gene signature correlated with the clinical outcome of patients, the 133 human Ets2-TAM gene signature was used for unsupervised clustering of expression data obtained from 159 patients with sporadic breast cancer in the Stockholm data set (ref. 18; see Supplementary Table S3). Expression of the Ets2-TAM signature predicted overall survival in this group with high confidence (Fig. 6B; $P = 0.0007$; hazard ratio, 3.1). Similar results were obtained with the entire 295-patient Rosetta sample set (Fig. 6C; $P = 0.0003$; hazard ratio, 2.31).

Figure 5. Impaired tumor angiogenesis and proliferation with Ets2 deletion in TAMs. A, images of mammary (top) and lung (bottom) sections from mice injected with the MVT-1 and Met-1 cells, respectively, genotypes as indicated. Immunostained with α-CD31. B, micrographs of lung sections from mice injected with the Met-1 cell line in the indicated genotypes, immunostained with α-bromodeoxyuridine. Panels 2 and 4 (A) and bottom panels (B), high-magnification images of insets from the respective top panels. Bar, 100 μm. Quantification of antibody staining is presented as the average area of staining per tumor area (graphs, right). Five different tumor areas from five different mice in each group were analyzed. Statistical significance ($P$ value evaluated by unpaired Student’s $t$ test) is shown.
Discussion

The influence of the microenvironment, particularly macrophages, on tumor growth and metastasis have long been recognized, but relatively little is known of the gene pathways and mechanisms macrophages use to promote tumor malignancy (34). The results presented here show that in mouse models, Ets2 in tumor macrophages promotes angiogenesis and growth of both primary tumors and lung metastases. The mechanism of action of ETS2 in TAMs involved direct repression of genes encoding predominantly extracellular products, including well-characterized inhibitors of angiogenesis. Recently, an independent report of global gene profiling in TAMs also observed the expression of several antiangiogenic genes along with well-known positive regulators such as Vegf-a, results consistent with our data (35).

Figure 6. The Ets2-TAM gene signature predicts survival in human breast cancer patients. A, heat map of differential expression of the Ets2-TAM 70 gene profile ($P < 0.001$, see text) in 98 breast cancer samples distinguished by lymphocyte/leukocyte infiltration. Bar graph (top), the level of Ets2 expression in each of the 98 cancer samples. B and C, Kaplan-Meier analysis of overall survival in Stockholm and Rosetta breast tumor cohorts, respectively.

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However, the antiangiogenic effect of TAMs lacking *Ets2* is dominant even in the context of MVT-1 tumor cells that overexpress *VEGF-A*. Additionally, the presumed role of VEGF-A produced by TAMs in triggering the angiogenic switch have been challenged by recent findings showing that deletion of VEGF-A in TAMs actually results in increased tumor growth (36, 37). Thus, *Ets2* has a previously unappreciated role in TAMs in controlling the balance between positive and negative regulators of angiogenesis necessary for tumor metastasis.

*Ets2* in TAMs increased the growth of primary and metastatic tumors. *Ets2* could indirectly affect tumor growth by modulating angiogenesis, or directly through paracrine mechanisms. The *Ets2* targets identified would favor the former possibility, as obvious paracrine candidates such as *Ile* or *Egf* were not differentially expressed. In either case, the results are consistent with the *Ets2* pathway playing a role in some activities associated with the alternatively activated M2 macrophage population (2, 3). M2 macrophages are believed to modulate inflammatory response and to promote tissue remodeling and angiogenesis; in the context of tumor progression, M2-like cells are believed to promote immune suppression as well as tumor angiogenesis, invasion, and metastasis (2, 3). Extracellular function and angiogenesis are the major *Ets2* targets identified in our studies, providing a molecular mechanism by which M2-like tumor macrophages modulate the extracellular microenvironment to promote tumor growth and angiogenesis at both primary and tumor sites.

A key finding is that a portion of the mouse *Ets2*-TAM gene expression signature was present in human breast cancer expression data and that it could retrospectively predict overall survival in two independent cohorts of patients with sporadic breast cancer. This 133-gene signature is independent of other breast tumor signatures capable of predicting patient outcome, including stromal gene signatures (38, 39). Although further efforts will be required to fully implement these findings and determine their significance to human disease, the results validate the relevance of our hypothesized-driven mouse modeling approach for dissecting TAM functions in tumor growth and metastasis.

Dispersed tumor cells are present in many patients with breast cancer and may be the mediators of tumor recurrence (40). Breast tumor micrometastases are genetically distinct from the primary tumor indicating that they are disseminated early in tumor progression (41, 42). Results obtained in the *PyMT* and *Her2/Neu* mouse models show an early spread of mammary epithelial cells before the carcinoma stage, providing experimental verification of the human data (42). Thus, understanding how dispersed dormant cells progress to growing metastases is a problem with considerable clinical relevance. Further studies on *Ets2* and its downstream targets could provide unique insights into understanding how the microenvironment modulates the growth of tumor cells at metastatic sites.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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