Macrophage-Derived SPARC Bridges Tumor Cell-Extracellular Matrix Interactions toward Metastasis

Sabina Sangaletti,[1] Emma Di Carlo,[3] Silvia Gariboldi,[2] Silvia Miotti,[1] Barbara Cappetti,[1] Mariella Parenza,[1] Cristiano Rumio,[2] Rolf A. Brekken,[4] Claudia Chiodoni,[1] and Mario P. Colombo[1]

[1]Department of Experimental Oncology, Immunotherapy and Gene Therapy Unit, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico, Istituto Nazionale dei Tumori; [2]Department of Human Morphology, Universita degli Studi di Milano, Milan, Italy; [3]Department of Oncology and Neurosciences, Surgical Pathology Section, "G. d'Annunzio" University and Ce.S.I. Aging Research Center, "G. d'Annunzio" University Foundation, Chieti, Italy; and [4]Departments of Surgery and Pharmacology, Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, Texas

Abstract

Other than genetic imprinting and epithelial to mesenchymal transition, cancer cells need interaction with the nearby stroma toward metastasis. Secreted protein acidic and rich in cysteine (SPARC) is a matricellular protein known to regulate extracellular matrix (ECM) deposition and cell-ECM interaction. Gene expression profiles associate SPARC to malignant progression. Using reciprocal bone marrow chimeras between SPARC knockout and wild-type mice, we show that SPARC produced by inflammatory cells is necessary for spontaneous, but not experimental, i.v. metastasis. Macrophage-derived SPARC induces cancer cell migration and enhances their migration to other ECM proteins at least through $\alpha_v\beta_5$ integrin. Indeed, RNA interference knockdown of β_5 integrin expression reduces cell migration *in vitro* and metastasis *in vivo*. Together these results show that macrophage-derived SPARC takes part in metastasis, acting at the step of integrin-mediated migration of invasive cells. [Cancer Res 2008;68(21):9050–9]

Introduction

Metastatic cells leaving the primary tumor gain access to blood vessels through processes that require de-adhesion from the tumor mass, invasion into surrounding tissue, and intravasation. Extravasation is then required to seed the secondary organ. To successfully complete this complex cascade, tumor cells require a permissive microenvironment and productive heterotypic interactions with host cell (1, 2). Macrophages can affect the tumor microenvironment by providing trophic factors and promoting tumor cell migration and intravasation (3). Macrophages, recruited by tumor-derived colony-stimulating factor 1 (CSF-1), produce epidermal growth factor (EGF) that activates tumor cell migration (3, 4). Indeed, in many human cancers, macrophage infiltration correlates with poor prognosis (5).

Secreted protein acidic and rich in cysteine (SPARC), also known as osteonectin or BM-40, is a matricellular glycoprotein involved in tissue remodeling and repair that regulates cell-extracellular matrix (ECM) interaction (6). Gene expression profiling applied to breast cancer identified SPARC as a marker of poor prognosis and metastasis (7, 8). In general, SPARC has been associated with advanced breast, head and neck, stomach, and prostate cancers, melanoma, and glioma.

Different results come from studies showing the effect of SPARC produced autonomously by cancer cells. SPARC inhibits proliferation of the MDA-MB-231 carcinoma cell line, whereas its metastatic clone LM2 requires SPARC for lung virulence (9). On the contrary, forced SPARC expression by adenoviral infection in the same cell line results in metastasis inhibition (10).

Colon carcinoma cell lines that down-regulate SPARC expressions acquire resistance to chemotherapy, and addition of recombinant SPARC restores susceptibility to chemotherapy and apoptosis via caspase-8 (11). Accordingly, colon carcinomas negative for SPARC expression are associated with a poor prognosis (12). Similarly, ovarian, lung, and pancreatic carcinomas down-regulate SPARC by promoter methylation during progression (13–15). The reason why SPARC alternatively enhances or halts tumor progression is largely unknown. Complexity may stem from the cell origin and the complex interplay between SPARC produced by tumor and nearby stroma cells. Host-derived SPARC has been described as tumor suppressor for ovarian cancer via modulation of cell surface clustering and expression of α_v integrins, thus altering growth factor–stimulated survival signaling pathways including focal adhesion kinase, mitogen-activated protein kinase, and AKT (16). In this regard, a study on glioma shows that down-regulation of SPARC expression by short interfering RNA decreases tumor cell survival and invasion, reducing focal adhesion kinase and AKT activation (17). In contrast to ovarian cancer, melanoma is not influenced by host-derived SPARC (18).

No better insight comes from mouse studies. In mice lacking SPARC, Lewis lung carcinoma cells grow faster whereas N2C mammary carcinoma cells grow slower in N4 backcrosses to B6 and fully congenic BALB/c knockout (KO) mice, respectively (19, 20). Although these contrasting results have been explained on the basis of the different type of collagen whose proteolysis generates fragments favoring or inhibiting tumor angiogenesis in Lewis lung carcinoma and N2C tumors, respectively, other mechanisms are likely in place. Cell motility is related to collagen density. For example, dendritic cells can move quickly to draining lymph nodes and activate an early immune response because of scarce and loose collagen present in SPARC KO mice (21). With this premise, it might also be possible that tumor cells move easily in the SPARC-null environment, increasing their metastatic potential.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Mario P. Colombo, Immunotherapy and Gene Therapy Unit, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico, Istituto Nazionale dei Tumori, Via Venezian 1, 20133 Milan, Italy. Phone: 39-2-2390-2252; Fax: 39-2-2390-2630; E-mail: mario.colombo@istitutotumori.mi.it.

doi:10.1158/0008-5472.CAN-08-1327

Few studies have linked SPARC to metastasis. One reported an association between SPARC expression and lung colonization after i.v. injection of breast carcinoma cells (9). Another clearly showed a role of SPARC in promoting prostate carcinoma cell growth into purified bone matrix (22). To study the function of SPARC, in metastasis, we used the 4T1 mammary carcinoma, perhaps the most compelling model of spontaneous metastasis (23). We found that i.v. injection of 4T1 cells into SPARC KO mice resulted in significant lung colonization. In contrast, spontaneous lung metastases from mammary fat pad were reduced in SPARC KO mice compared with wild-type (wt) animals. We investigated the mechanism responsible for such a difference. We report here the characterization of the cell type producing the relevant SPARC and provide evidence that SPARC promotes cell migration and metastasis by modulating integrin-ECM interaction.

Materials and Methods

Cell lines, mice, and tumors. 4T1 (CRL-2539, LGC-Promochem) is a thioguanine-resistant carcinoma cell line (24). Female BALB/cAnNCrl mice, 8 to 10 wk old, were purchased from Charles River Laboratories. Congenic BALB/c Thy 1.1 mice were kindly provided by H. Levitsky (John Hopkins University, Baltimore, MD). SPARC KO mice on a BALB/c background have been described previously (20). Mice were maintained at Istituto Nazionale Tumori under standard conditions according to institutional guidelines; experiments were authorized by the Institute Ethical Committee for animal use.

Chimeric SPARC$^{-/-}$ (Thy 1.2) > SPARC$^{+/+}$ (Thy 1.1) as well as SPARC$^{+/+}$ (Thy 1.1) > SPARC$^{-/-}$ (Thy 1.2) mice (from here and thereafter indicated as wt > KO and KO > wt) were obtained as described (20). Engraftment was verified 8 wk after bone marrow transplant by staining peripheral blood mononuclear cells with FITC-conjugated mouse anti-mouse Thy 1.1 and phycoerythrin-conjugated mouse anti-mouse Thy 1.2, as well as isotype control FITC- and phycoerythrin-conjugated mouse IgG2a.

SPARC KO and mouse chimeras were injected orthotopically or i.v. with 7×10^3 4T1 cells. The lungs and primary tumor were harvested 28 d after tumor cell injection (14 d if injected i.v.) and evaluated by clonogenic assay and histology, respectively. For histology, tumors were embedded in optimal cutting medium, rapidly frozen, and sectioned at 5 μm, or fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned.

Clonogenic assay. Lungs were removed, minced, and digested with a collagenase IV/elastase (Worthington) solution for 140 min 4°C. The suspension was filtered with a cell strainer (BD) and centrifuged. Cells were washed and resuspended in DMEM containing thioguanine (10 μg/mL) and seeded in 100-mm^3 Petri dishes at three dilutions (1:2, 1:10, and 1:100). Colonies were allowed to grow for 2 wk and then fixed with methanol (BDH) and stained with methylene blue.

Plasmids for short interfering RNA of β_5-integrin and cell transfection. Targets for RNA interference were selected using the prediction software available from Ambion, Inc. The following oligonucleotide sequences were annealed and cloned into the pSilencer 1.0-U6 vector (ambion): 400 forward, 5′-GGCAAACCTCATCCGGAATTTCAAGAGAATTCCGGATGAGGTTTGCCTTTTTT; 400 reverse, 5′-AATTAAAAAAGGCAAACCTCATCCGGAATTCTCTTGAAATTCCGGATGAGGTTTGCCGGCC; 754 forward, 5′-GGACATCTCTCCTTTCTCCTTCAAGAGAGGAGAAAGGAGAGATGTCCTTTTTT; 754 reverse, 5′-GGACATCTCTCCTTTCTCCTTCAAGAGAGGAGAAAGGAGAGATGTCCTTTTTT; 1381 forward, 5′-TCTCTTCTTCACTGCCACCTTCAAGAGAGGTGGCAGTGAAGAAGAGATTTTTT; and 1381 reverse, 5′-TCTCTTCTTCACTGCCACCTTCAAGAGAGGTGGCAGTGAAGAAGAGATTTTTT. The annealed oligos were cloned into *Apa*I and *Eco*RI sites. All plasmids were sequenced to verify accuracy.

For transfection, 4T1 cells were grown to 70% confluence and transfected using Lipofectamine 2000 (Invitrogen Corporation) according to the manufacturer's instructions. To create stable lines, the cells were selected with 600 μg/mL puromycin for 3 wk. The RNA interference effect was tested by flow cytometry for expression of β_5-integrin by using a polyclonal rabbit anti–β_5 integrin.

Antibodies, histology, and immunohistochemistry. The following anti-SPARC antibodies have been used: monoclonal antibody (mAb) 303 and mAb 293 (25), a commercial monoclonal raised in rat (clone 124413), and a polyclonal raised in goat (R&D Systems Europe). To detect integrin β_5, we used a polyclonal rabbit antibody from Abcam (ab15459) and a monoclonal antihuman antibody (cross-reacting with mouse) from e-Bioscience (clone KN52, functional grade endotoxin free) as blocking antibody in migration assays. Also from Abcam were the polyclonal rabbit antibody to N-cadherin (ab12221), the polyclonal goat anti–phosphorylated vascular endothelial growth factor receptor 2 (ab38473), and the mAb to vimentin (clone RV202). α-Smooth muscle actin (α-SMA) mAb (clone 1A4) was from Sigma. From Calbiochem (Merck KGaA) we obtained the mAb to E-cadherin (clone ECCD-2). To detect or block integrin β_1 (CD29) we used a mAb from Biolegend (clone HMB1-1, functional grade). To detect integrin β_3 (CD61), we used a mAb from Becton Dickinson (clone 2C9-G2). Also from Becton Dickinson was the mAb to CD31 (clone MEC 13.3). To visualize SPARC, paraffin sections were boiled for 5 min in a pressure cooker. Pan-cytokeratin, F4/80, and α-SMA immunostaining required an antigen retrival in citrate buffer (DakoCytomation; 10 min). Immunostaining of frozen sections was done as previously described (20).

Double immunofluorescent staining and laser scanning confocal analyses. Acetone-fixed frozen sections were rehydrated in PBS and incubated for 30 min with the first primary antibody. The sections were washed in PBS and then incubated for 30 min with biotinylated secondary antibody, washed, and incubated with Alexa Fluor 488–conjugated streptavidin (Molecular Probes) for 20 to 30 min. After washing, sections were incubated for 30 min with the second primary antibody, washed again, and incubated for 30 min with biotinylated secondary antibody. After washing, sections were incubated with Alexa Fluor 594–conjugated streptavidin for 20 to 30 min and then washed. Cross-reaction between the first secondary antibody and Alexa Fluor 594 was prevented by saturation of all its binding sites with Alexa Fluor 488. Slides were mounted with Vectashield medium (Vector Laboratories) and examined with a Zeiss LSM 510 Meta laser scanning confocal microscope.

Flow cytometry. Flask-cultured 4T1 and 4T1β_5^{sil} cells were gently detached and stained for 60 min at room temperature with β_5 integrin, CD61, and SPARC antibodies diluted in PBS for cell surface staining or in saponin buffer 0.2% saponin and 0.5% bovine serum albumin (Sigma-Aldrich) for intracellular staining. Isotype-matched IgGs were used as controls. Cells were analyzed with a FACSCalibur (Becton Dickinson).

Migration assays. The underside of the Transwell filter inserts (6.5 mm; Costar) were coated with 10 μg/mL of fibronectin (Sigma), multimeric vitronectin from bovine plasma (Oxford Biomedical Research, UK), rat tail collagen type I (Becton Dickinson), and/or 200 ng of mouse SPARC purified from parietal yolk sac (Sigma) or recombinant human SPARC (ProSci) overnight at 4°C. The inserts were air-dried, and freshly trypsinized 4T1 or 4T1β_5^{sil} cells were added in serum-free media to the upper chamber of the transwell insert. The lower chamber was filled with 10% FCS supplemented medium. In some cases, cells were incubated with 5 μg/mL of functional grade endotoxin-free antibodies against α_v (Chemicon, CD51, RMV-7 clone), β_5 integrin (e-Bioscence, KN52 clone), or SPARC (mAb 303), or with RGD or RGES peptides (Sigma).

Cells were allowed to migrate overnight at 37°C. Nonmigrated cells were gently removed from the topside of the filter by scrubbing twice with cotton swab moistened with FCS-free medium. Cells on transwell insert were stained with Diff-Quik stain kit (PBI international) and washed with distilled water. Finally filters were removed from the insert and mounted on cover glass. The number of migrated cells was counted under a microscope in 10 randomly chosen high-power fields by two different observers.

Bone marrow macrophage preparation. Bone marrow from wt and SPARC KO mice was harvested by flushing femurs and tibias with medium. Cells were resuspended at 2×10^6/mL in cRPMI 1640 (Life Technologies, Inc.) supplemented with 5 ng/mL of macrophage colony-stimulating factor (M-CSF). On day 5 of culture, medium was replaced with fresh medium containing M-CSF. On day 6, adherent cells were harvested and

phenotypically characterized by mAbs to F4/80 and CD40 at flow cytometry. For *in vitro* migration experiments, 5×10^5 macrophages from wt and SPARC KO were allowed to attach for 2 h at 37°C to the underside face of a 24-mm transwell insert (Costar). 4T1cells (5×10^5) were labeled with the PKH-26 vital dye, following the manufacturer's instructions (Sigma), and added in serum-free medium to the upper chamber of the transwell insert. The lower chamber was filled with serum-free DMEM added with lipopolysaccharide (LPS; 200 ng/mL; from Sigma) and IFN-γ (500 units/mL; Peprotech). 4T1 cells were allowed to migrate for 24 h, then cells were harvested with trypsin. Harvested cells (macrophages + migrated 4T1 cells) were counted with a hemocytometer. The number of migrated PKH-26$^+$ 4T1 cells was determined as percentage of PKH-26$^+$ cells on total migrated cells by flow cytometry.

Results

SPARC requirement for metastasis. The 4T1 mammary carcinoma cell line, once injected into the mammary fat pad, spreads to several distant organs. 4T1 cells possess 6-thioguanine resistance, and their metastases are easily detected easily by plating dissociated organs under drug selection; resistant colonies are considered representative of individual clonogenic tumor cells. We injected 4T1 carcinoma cell lines into wt and SPARC KO mice. Although the growth of the primary tumor was similar in the two mouse lines (Fig. 1*A*), clonogenic assay revealed numerous metastases in the lungs of wt but a paucity in the lungs of SPARC KO mice (Fig. 1*B*). We also observed a significant decrease of 4T1 metastases in liver, lymph nodes, and brain of SPARC KO, but not wt, mice (Supplementary Table S1). Injection of the same cells into the tail vein, however, gave rise to lung metastasis without differences between wt and KO mice either counted in a clonogenic assay at day 14 (Fig. 1*C*) or as macrometastases at day 28 (data not shown). These results indicate that 4T1 cells require host SPARC to leave the primary tumor but not to seed in distant organs.

Bone marrow–derived SPARC is required for metastasis. Immunohistochemical analysis of 4T1 tumors grown in either wt or SPARC KO mice shows neoplastic cells that express SPARC at low level. Accordingly, *in vitro* 4T1 cells produce a low amount of SPARC in comparison with other carcinoma cell lines as tested by ELISA (4T1, 20 ng/mL; CT26 colon carcinoma, 177 ng/mL; and N2C mammary carcinoma, 200 ng/mL) or immunofluorescence. However, inside tumors from wt mice, SPARC was strongly expressed by infiltrating leukocytes endowed with widespread cytoplasmic processes that encircled tumor cells (Fig. 2*A, a* and *b*). Double immunofluorescence and confocal microscopy analyses revealed that most of these SPARC-expressing inflammatory cells were macrophages (Mac-1$^+$ cells; Fig. 2*A*, *c–e*) and, to a little extent, were lymphocytes (Fig. 2*A*, *f–h*). Accordingly, Western blot analysis of primary tumors from SPARC KO mice revealed negligible SPARC expression in such tumors (data not shown), thus confirming the major role of inflammatory cells as source of SPARC. To attribute a functional role to SPARC produced by inflammatory cells in invasion, bone marrow from SPARC KO mice was transplanted into irradiated wt mice (KO > wt) and wt bone marrow was transferred into SPARC KO mice (wt > KO); in both combinations, host and donor were congenic for the Thy 1 marker. Eight weeks after bone marrow transplantation, 4T1 cells were injected into the mammary fat pad of fully engrafted mice. SPARC KO mice that received wt bone marrow showed numerous metastasis whereas wt mice that received SPARC KO bone marrow had few metastases (Fig. 2*B*). Considering that leukocyte infiltration is not affected in tumors from SPARC KO mice (Fig. 2*C*), it is likely that the SPARC they produce, rather than their number, is responsible for the metastatic phenotype.

Host SPARC does not influence epithelial to mesenchymal transition. To study the mechanism underlying SPARC activity in metastasis, we tested the possibility that SPARC may induce epithelial to mesenchymal transition (EMT) in 4T1 cells, which is a key step toward metastasis. During EMT, epithelial cells lose the epithelial E-cadherin and acquire the mesenchymal markers N-cadherin and vimentin (26). Immunohistochemistry and immunofluorescence done on primary tumors, either sectioned or dissociated into cell suspension, showed no difference in the expression of E-cadherin, N-cadherin (Fig. 3), and vimentin (data not shown) between tumors collected from wt and SPARC KO mice. E-cadherin, in particular, was expressed on cell surface or within cytoplasm inside the tumor while absent at the tumor edge

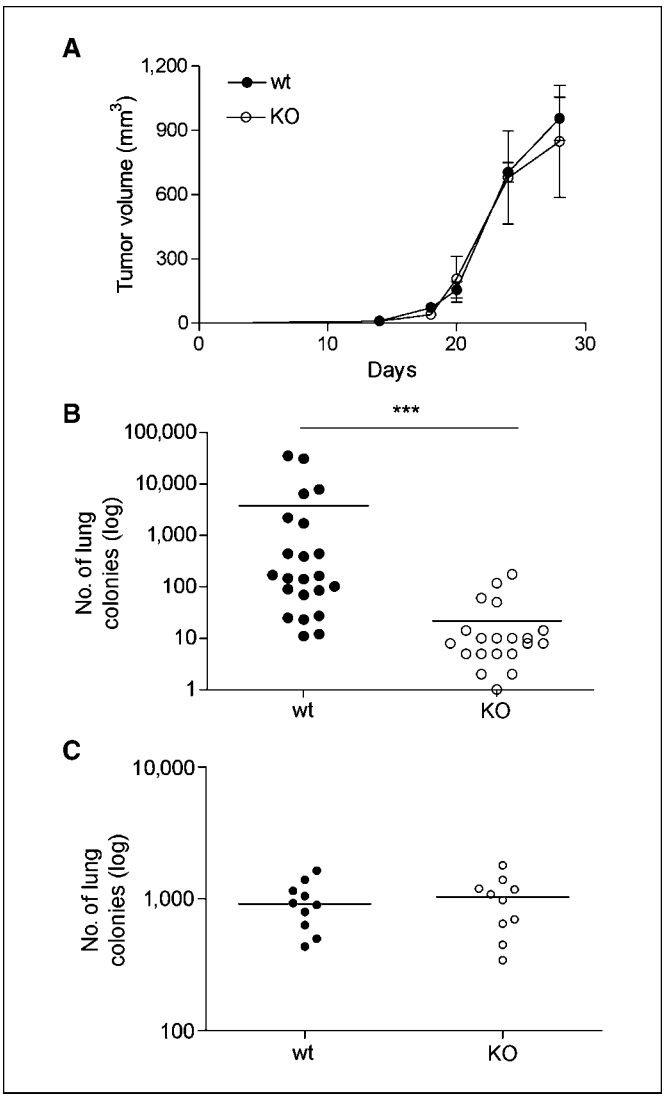

Figure 1. SPARC is required for spontaneous metastasis. 4T1 cells were injected into the mammary fat pad (*A* and *B*) or i.v. (*C*) of wt (●) or SPARC KO (○) mice. *A,* growth of primary tumor following mammary fat pad injection. *Points,* mean volume ($n = 20$); *bars,* SD. *B,* spontaneous lung metastases from tumors shown in *A. Bars,* median number of lung colonies. Clonogenic assay was done 28 d after mammary fat pad cell injection. ***, $P < 0.001$, two-tailed Mann-Whitney *t* test. *C,* experimental lung colonies after i.v. cell injection. *Bars,* median number of lung metastasis. Clonogenic assay was done 14 d after i.v. cell injection.

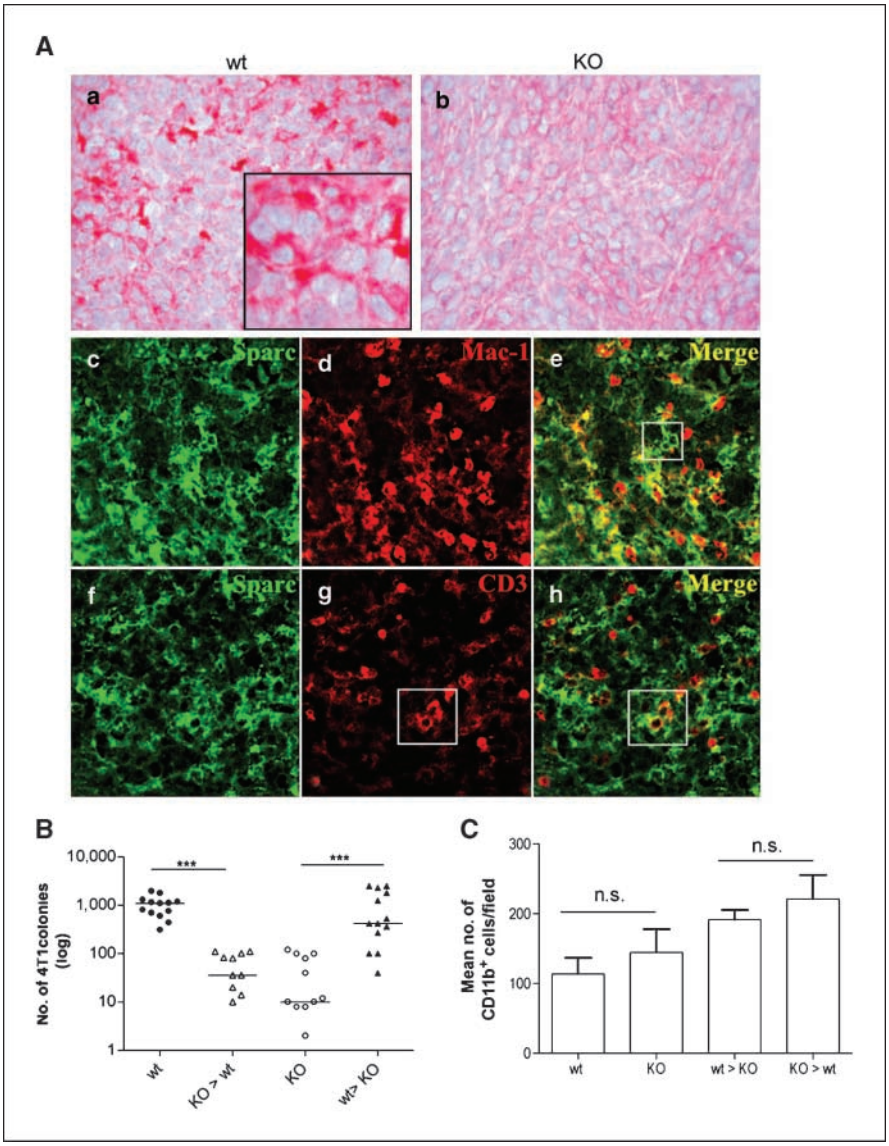

Figure 2. Bone marrow–derived SPARC affects 4T1 metastasis *in vivo*. *A,* SPARC is barely expressed by neoplastic cells (faint staining of the tumor cell cytoplasm) in 4T1 tumors grown in either wt (*a*) or SPARC KO (*b*) mice; additional SPARC expression in wt mice was found in cells that appear as tumor-infiltrating inflammatory cells mostly endowed with widespread cytoplasmic processes that encircled the tumor cells (*a* and *b*, ×630; *inset*, ×1,000). Accordingly, immunofluorescence and confocal microscopy show that, in tumors from wt mice, SPARC was mostly produced by Mac-1$^+$ cells (*c–e*), but additional infiltrating cells with small round morphology not overlapping Mac-1 (*e, white square*) were identified as CD3$^+$ T cells (*g* and *h, white squares*) and found to contribute to the overall SPARC production (*f–h*). Magnification, ×630 (*a–c* and *f–h*). *Bar,* 100 μm. *B,* spontaneous lung metastasis in wt, SPARC KO mice, and wt > KO and KO > wt chimeras. *Bars,* median number of lung colonies. ***, $P < 0.001$, two-tailed Mann-Whitney *t* test. *C,* mean number of CD11b$^+$ cells infiltrating tumors from wt, KO, and the indicated bone marrow chimeras. The increased number of infiltrating macrophages regardless of SPARC expression in bone marrow chimeras is likely due to a rebound effect after total body irradiation. *n.s.,* not significant ($P > 0.05$).

(Fig. 3*A*). This pattern of expression indicates that cells at the leading edge acquire the mesenchymal phenotype regardless of whether recipient mice produce SPARC. Should the low amount of SPARC produced directly by 4T1 cells be sufficient to induce EMT (27), it is, however, insufficient to move the metastatic process forward.

Bone marrow–derived SPARC regulates fibronectin fiber deposition. A well-known SPARC activity is the regulation of ECM deposition (19, 20), an effect that we analyzed by Masson trichrome, silver staining, or immunohistochemistry. A well-developed or scant stroma characterized 4T1 tumors from wt or SPARC KO mice, respectively. Distinct collagen deposition and a robust fiber network characterized tumors from wt mice, whereas faint collagen and a thin and fragile fiber network characterized tumors from SPARC KO mice (Fig. 4*A, a* and *b*). In addition, 4T1 tumors grown in wt > KO chimeras showed a collagen content and a fiber network similar to those of tumors grown in wt mice (Fig. 4*A, a* and *b*), whereas tumors from KO > wt chimeras phenocopied those from SPARC KO mice (data not shown). In agreement to the fiber network, fibronectin staining was much more prominent in tumors from wt or wt > KO chimeras than in tumors from SPARC KO or KO > wt chimeras (Fig. 4*A–c* and data not shown). Additional confocal microscopy analysis shows macrophages spreading and colocalizing with fibronectin fibers in tumors from wt, but not KO, mice. These data suggest that macrophages can be a source of fibronectin in tumors (Supplementary Fig. S1), an extension of what has been shown for fibroblasts (28), which, in the absence of SPARC, cannot organize fibronectin fibers.

SPARC affects cell migration and spreading on fibronectin fibers. Cell-matrix adhesive interactions involving basement membrane components and fibronectin fibers play an active role in tumor cell migration and metastasis (29). To test whether SPARC affects cell migration to fibronectin, we combined fibronectin with different amounts of SPARC before coating them onto the underside face of transwell inserts. We found that SPARC enhanced fibronectin-induced 4T1 cell migration in a dose-dependent manner (Fig. 4*B*) and that such effect was inhibited by mAbs to CD29 (β_1-integrin), CD51 (α_v-integrin), and integrin β_5 (Fig. 4*C*), indicating that a fibronectin receptor is likely involved (30, 31). Moreover, SPARC enhanced 4T1 cell adhesion and spreading on

fibronectin as revealed by the staining of the actin cytoskeleton of 4T1 cells seeded onto glasses coated with fibronectin or fibronectin + SPARC (data not shown). Thus, bone marrow–derived SPARC influences ECM protein availability and further increases 4T1 cell migration and spreading.

Macrophage-derived SPARC affects 4T1 cell migration. Macrophages producing SPARC are the main infiltrating cells in 4T1 tumors. We tested whether SPARC produced by macrophages, in addition to matrix deposition, has a role in inducing 4T1 cell migration. By using a modified *in vitro* transwell assay, we found that macrophages producing SPARC (wt), but not SPARC KO macrophages, enhance migration of 4T1 cells *in vitro*. In this assay, bone marrow–derived macrophages were seeded onto the underside face of a transwell insert, and 4T1 cells, labeled with PKH-26 vital dye, were added in the upper chamber. Migration, tested 24 hours later, and expressed as percentages of $PKH\text{-}26^+$ cells on total number of cells collected form the underside transwell, was significantly higher when macrophages were from wt mice, and the difference between wt and KO was even greater if macrophages were preactivated with LPS plus IFNγ (Fig. 5*A*), a stimulus that increases the production of SPARC as well as other proinflammatory cytokines. Accordingly to a role of SPARC in migration, a mAb to SPARC (mAb 303) significantly reduced the boost of migration induced by LPS + IFNγ on wt macrophages, but was ineffective on the same boost on KO macrophages. No migration was detected in the absence of macrophages, whereas the limited but significant migration toward SPARC KO macrophages indicates that SPARC works in addition to other macrophage-derived factors in inducing cancer cell migration (3).

To test the distinct role of SPARC in inducing 4T1 cell migration, purified murine SPARC (or rhSPARC) was coated onto the underside face of transwell or given as soluble protein. Figure 5*B* shows that coated, but not soluble, SPARC induces haptotactic migration of 4T1 cells in a dose-dependent manner. Haptotactic migration requires integrin engagement (32). SPARC has been described to mediate prostate cancer cell adhesion to bone matrix through $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (22). In our hands, SPARC-induced migration was inhibited by the addition of a specific mAb against α_v integrin (CD51) or RGD peptide but not by mAb to β_1 integrin (CD29) or control RGES peptide (Fig. 5*C*), suggesting that 4T1 cells respond to SPARC through an α_v integrin that is not β_1. Because SPARC does not contain a canonical RGD sequence, it is possible that such effect could be mediated through the interaction between SPARC and a β_3 or β_5 integrin ligand directly produced by the tumor cells, like vitronectin. Accordingly, SPARC enhanced the migration of 4T1 cells to multimeric vitronectin (Fig. 5*D*). Differently from fibronectin, tumors from wt and KO mice show a similar vitronectin content, thus excluding the possibility that a different vitronectin deposition determines the metastatic phenotype (data not shown).

Effect of β_5-integrin silencing on 4T1 cell migration and metastasis. The above experiments suggested $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins as candidate target for a direct SPARC activity. We analyzed their expression in 4T1 cells and tumors. Although 4T1 cells express both β_3 and β_5 integrins at the mRNA (data not shown) and protein (flow cytometry) levels *in vitro* (Fig. 6*A*), the expression of β_3 integrin *in vivo* was undetectable by immunohistochemistry on tumor cells being restricted to blood vessels (Fig. 6*B*). On the contrary, a mAb to β_5 integrin stained 4T1 cells (Fig. 6*B*), thus suggesting the silencing of β_5 integrin as an approach to test its function in SPARC-induced migration of 4T1 cells. Expression plasmids containing three different putative β_5-RNA interference were cotransfected with a neo resistance gene,

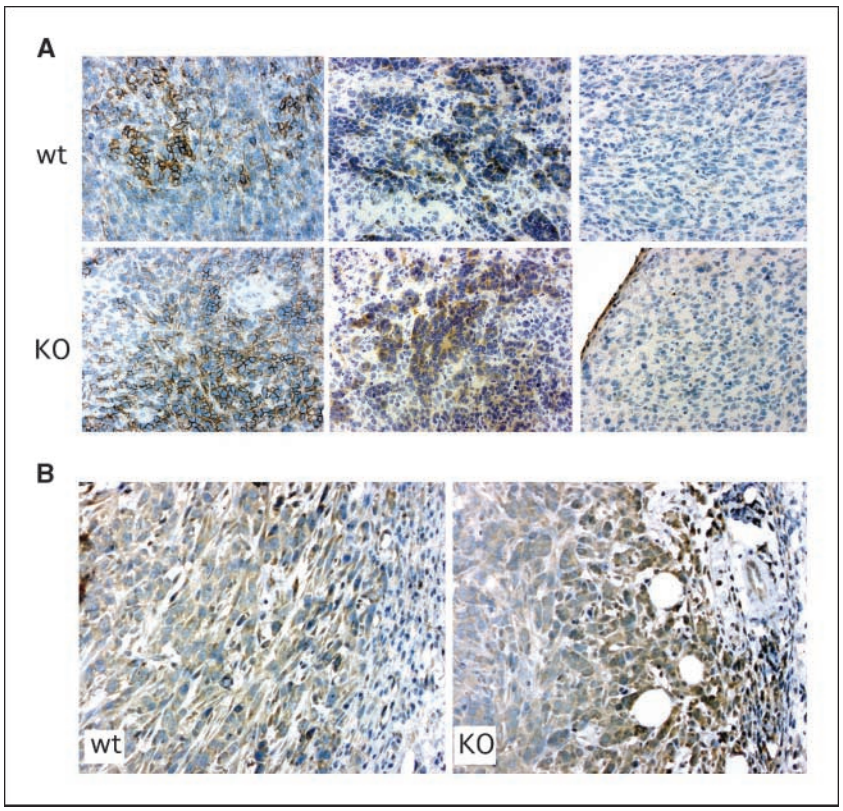

Figure 3. Tumors from wt and SPARC KO mice show similar distribution of epithelial and mesenchymal markers. Immunohistochemical analysis of E-cadherin (*A*) and N-cadherin (*B*) in 4T1 tumors from wt and SPARC KO mice. Three different tumor areas from wt and KO mice have been selected to show the distinctive features of E-cadherin expression going from a functional membrane stain (*left*) to a nonfunctional intracellular (*center*) or negative stain (*right*). Tumors from both wt and KO mice show the same pattern of expression of both E-cadherin (*A*) and N-cadherin (*B*).

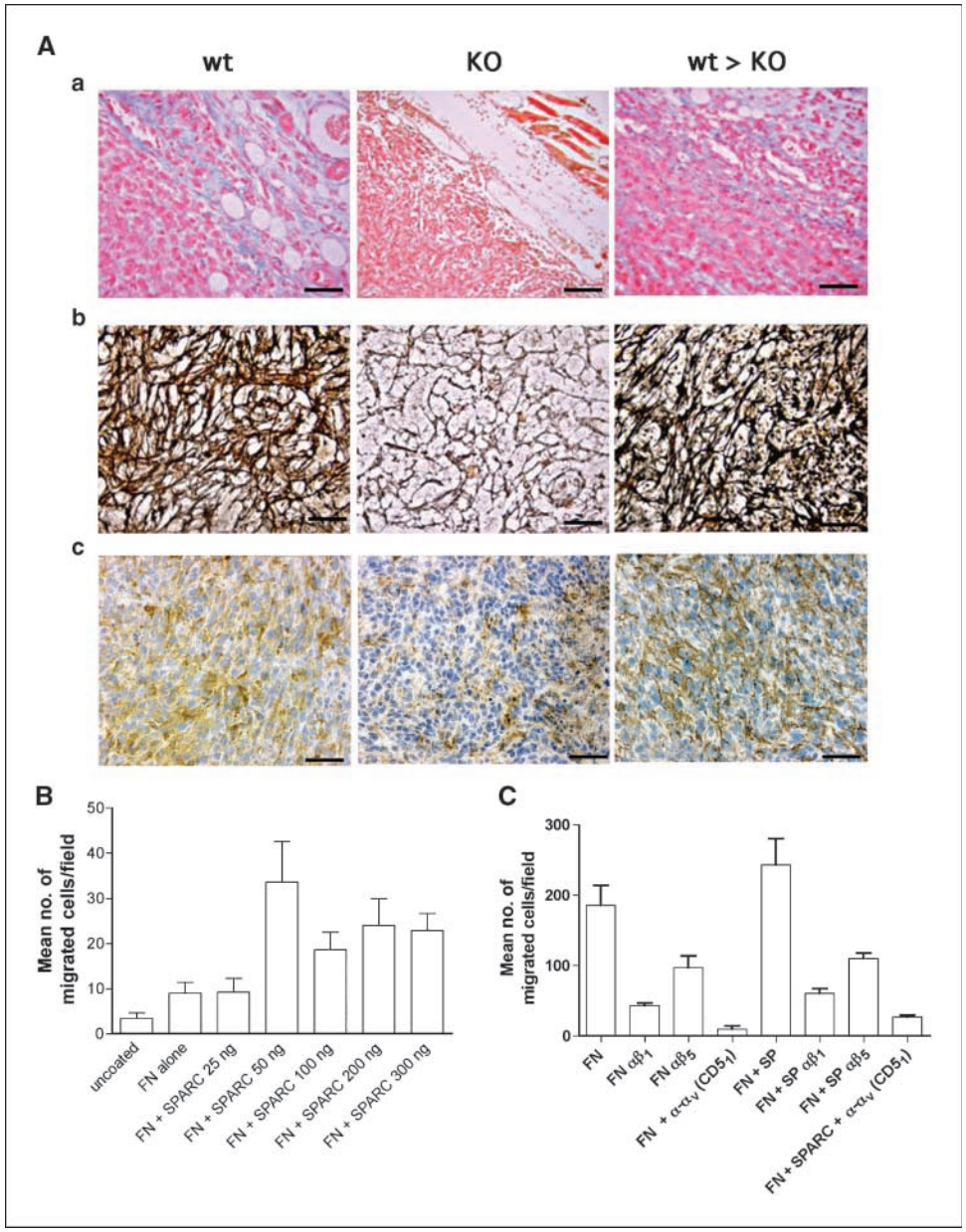

Figure 4. SPARC modulation of ECM deposition. *A,* Masson trichrome (*a*) and silver staining (*b*) of 4T1 tumors from wt, SPARC KO, and wt > KO chimeras. *c,* immunohistochemical analysis of fibronectin (*FN*) in tumors from wt, SPARC KO, and wt > KO chimeras showing a paucity of fibronectin fibers in tumors from SPARC KO mice. *B,* SPARC synergizes with fibronectin in inducing 4T1 cell migration (*B*) and such effect is inhibited by mAbs to β_1, α_v, and β_5 integrins (*C*).

and the best stable β_5-silenced 4T1 cell clones (754^{sil} and 1381^{sil}; Fig. 6*B*) were selected for further *in vivo* and *in vitro* experiments. Silenced clones were tested for migration toward SPARC (Fig. 6*C*) and for their metastatic capacity into wt mice (Fig. 6*D*). We found that both the migration toward SPARC and the number of lung metastasis of 754^{sil} and 1381^{sil} cells were reduced compared with control nonsilenced cells (400^{sil}, 4T1empty, and parental 4T1 cells). In addition, migration to multimeric vitronectin, which specifically binds β_5 integrin, was reduced in β_5-silenced cells (754^{sil}) but not in parental 4T1 cells (data not shown). Confirming the role of β_5 integrin as a SPARC counter-receptor, a blocking antibody to β_5 integrin (clone KN52) inhibited 4T1 cell migration toward wt macrophages, as well as antibodies to α_v (CD51) or SPARC (mAb 303). Accordingly, the migration of $4T1\beta_5^{sil}$ cells (754^{sil}) to wt macrophages was as low as that of parental 4T1 cells to SPARC KO macrophages, indicating that SPARC-independent macrophage-induced migration does not require β_5 integrin (Fig. 6*E*).

Based on the above-described experiments, we reasoned that SPARC affects metastasis in two ways. One is by regulation of ECM fiber deposition and promotion of their interaction with the cognate receptors ($\alpha_v\beta_1$ and $\alpha_v\beta_5$), and the other is by a direct promigratory effect via the β_5-integrin. As shown in Fig. 4, bone marrow–derived SPARC reverts the paucity of fibronectin fibers in recipient KO mice and offers the possibility of testing the role of β_5 integrin in metastasis by injecting $4T1\beta_5^{sil}$ cells into wt > KO chimeras. In this setting, $4T1\beta_5^{sil}$ (754^{sil}) produced less metastasis than parental 4T1 cells injected into the same recipient, indicating that the promigratory signals transduced by β_5 integrin have a true role in metastasis (Fig. 6*F*).

Impaired metastasis was not because of reduced cell proliferation or primary tumor take of silenced clones because both

proliferation (*in vitro*) and tumor size (*in vivo*) were not different from those of parental cells (data not shown).

Discussion

Here we have shown that SPARC produced by host cells, most likely macrophages, is an important factor in spontaneous 4T1 mammary carcinoma cell metastasis. The lack of host SPARC has no role in experimental lung metastasis (i.v. injection) nor does it have a role on EMT. Reciprocal bone marrow chimeras between wt and SPARC KO mice and immunolocalization experiments established that macrophages of donor origin are the source of SPARC. *In vitro* migration assays showed that macrophages induce 4T1 cell migration especially if SPARC is produced. We provided evidence that SPARC favors fibronectin and vitronectin interaction with 4T1 cells through integrins. Silencing experiments indicate that the β_5 integrin was needed for *in vitro* migration and partially for metastasis *in vivo*.

The low constitutive SPARC expression in 4T1 cells marks the difference from that produced by macrophages. A cell-autonomous function of SPARC is mostly unknown; nevertheless, SPARC can be found in the nucleus (33), and addition of exogenous SPARC results in phosphorylation of β-catenin (34). Furthermore, SPARC through the interaction with cyclin D1 can inhibit cell proliferation (35), thus explaining why many tumor cells down-modulate SPARC expression by promoter hypermethylation (15). Indeed, forced SPARC expression, by gene transduction, in 4T1 cells reduces cell proliferation *in vitro* and impairs tumor formation *in vivo*, rendering the assay on metastasis unfeasible (data not shown). Accordingly, doxycycline-induced SPARC expression in the human breast cancer cell line MDA-MB-231 results in growth inhibition (36), and adenoviral-mediated SPARC gene transfer into the same

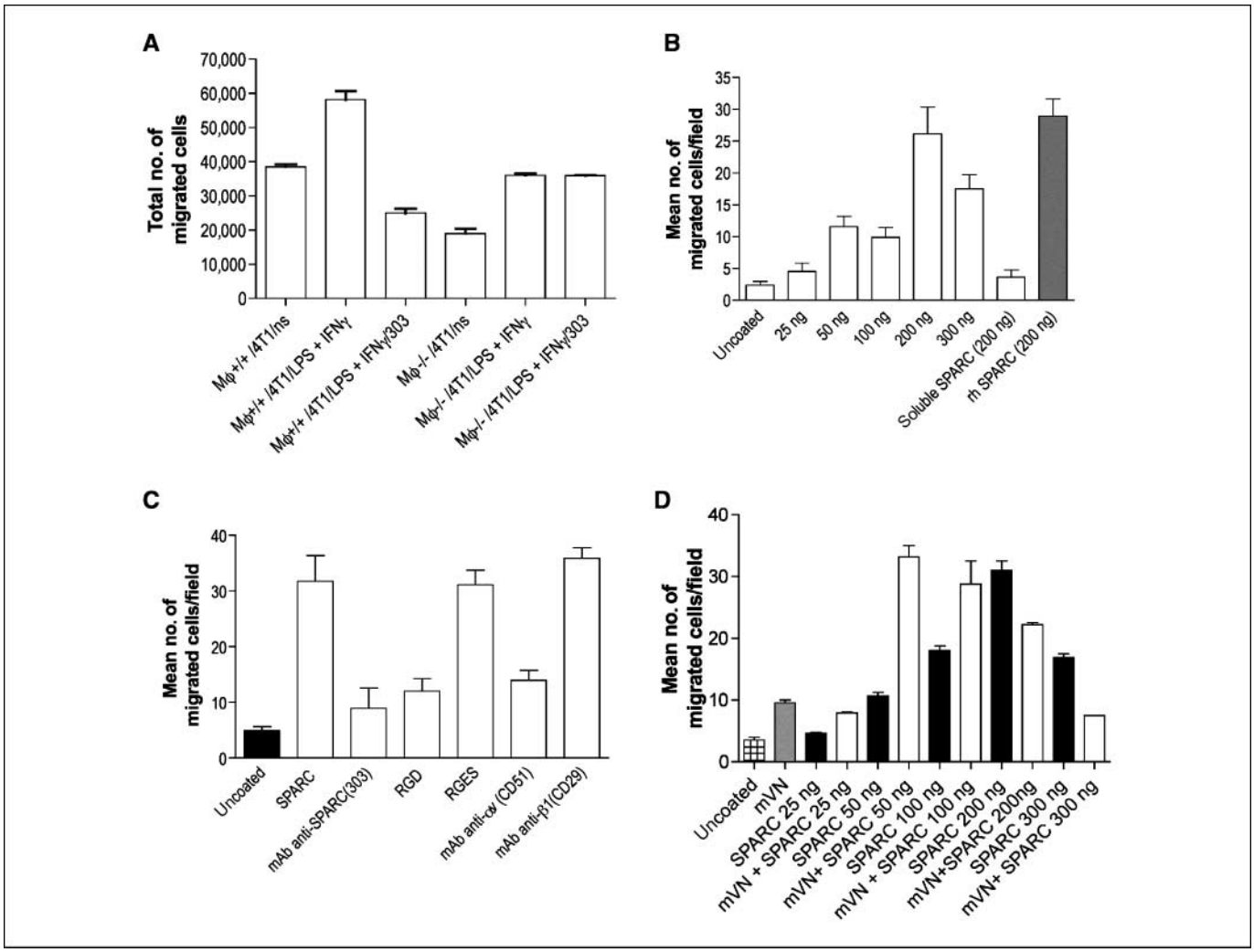

Figure 5. SPARC induces 4T1 cell migration. *A,* migration of PKH-26 labeled 4T1 cells in response to macrophages from wt and SPARC KO mice treated or not with LPS plus IFNγ. Migration to both wt and KO macrophages is enhanced by LPS + IFNγ but only the increased migration to wt macrophages is inhibited, in part, by a mAb to SPARC (mAb 303). *B,* dose-dependent SPARC-induced 4T1 cell migration. Different doses of purified murine SPARC were coated onto the underside face of the transwell insert. 4T1 cells were added to the upper chamber of the transwell, and nonmigrated cells were removed after overnight incubation. Migrated cells were counted on the underside of filters with a microscope in 10 randomly chosen fields. Recombinant human SPARC was also tested at the dose in which purified SPARC induces maximal migration. *C,* effect of integrin inhibitors on SPARC-induced 4T1 migration. 4T1 cells were preincubated with 5 μg/mL of functional grade endotoxin-free antibodies against integrin α_v (CD51), integrin β_1 (CD29), or SPARC (mAb 303) or mixed with RGD and control RGES peptides. *D,* SPARC synergizes with multimeric vitronectin (*mVN*) in promoting 4T1 cell migration. Multimeric vitronectin mixed with SPARC (*filled columns*) was more active in inducing 4T1 cell migration than either multimeric vitronectin (*grey column*) or SPARC given alone (*empty columns*) especially at a low SPARC concentration that mimics a physiologic setting. *Columns,* mean number of cells per field; *bars,* SD.

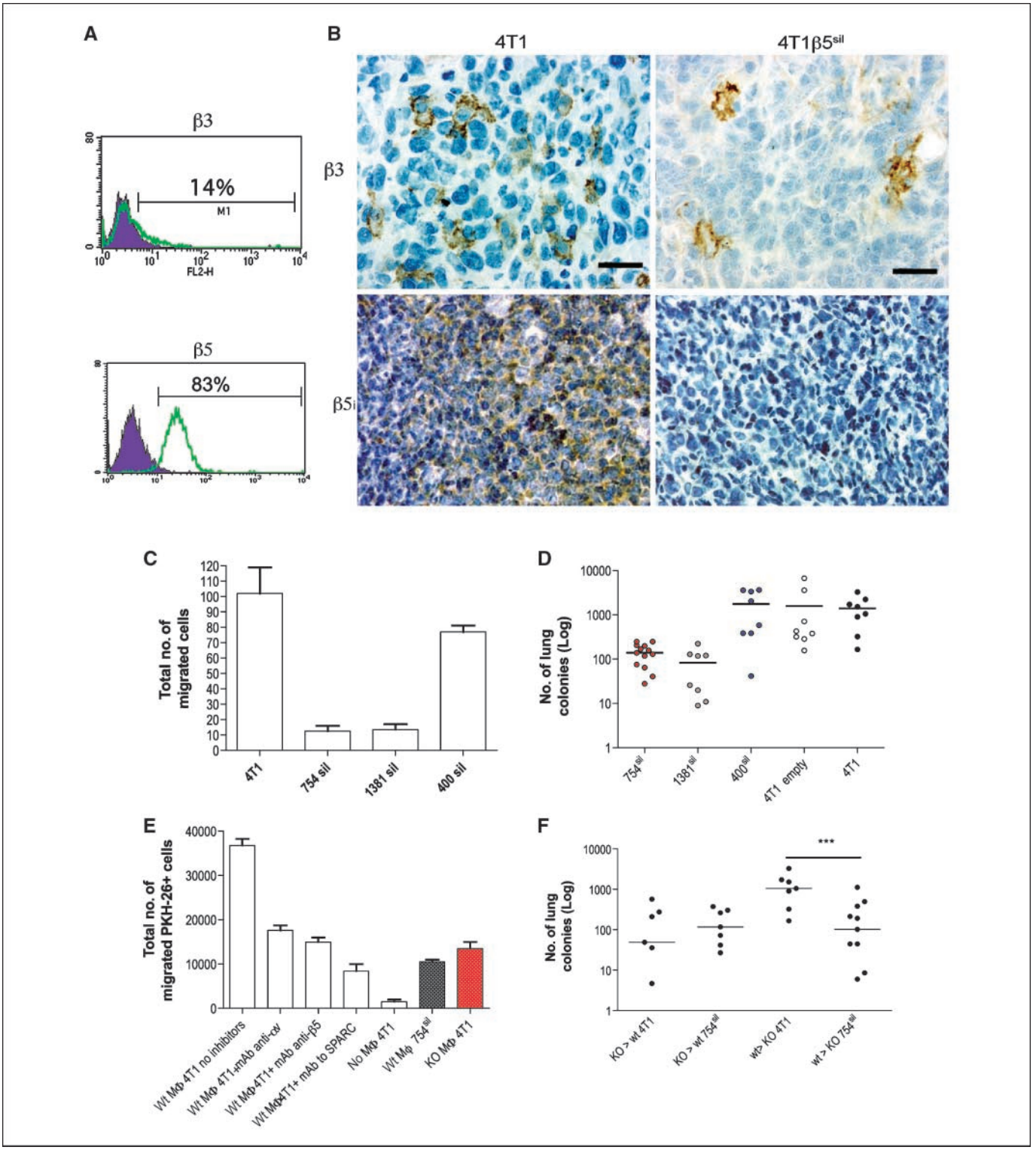

Figure 6. 4T1 cells express β_5 integrin, which is necessary for cell migration to SPARC and for *in vivo* metastasis. *A,* the level of expression of β_3 integrin (CD61) and β_5 integrin by 4T1 cells cultured *in vitro* was determined by flow cytometry. *Blue,* binding of the isotype-matched control antibody; *green,* target antibody binding. *B,* expression of β_3 and β_5 integrins on 4T1 tumor sections. Expression of β_3 integrin was confined to cells with endothelial morphology that coexpress CD31 (data not shown). On the contrary, almost all tumor parenchyma stained positive for β_5 integrin. Such β_5 expression is lost in tumors from 4T1β_5^{sil} cells that still remain negative for β_3 integrin. *Bar,* 100 μm. *C,* migration of 4T1β_5-integrin–stable silenced 4T1 clones (754^{sil} and 1381^{sil}), obtained from two different target sequences, in response to SPARC. *Columns,* mean number of migrating cells; *bars,* SD. *D,* reduced spontaneous lung metastasis by β_5 integrin silencing. Stable silenced 754^{sil} and 1381^{sil} were compared with a nonsilenced clone (400^{sil}; *blue dots*), empty vector–transduced (*open dots*), and parental 4T1 (*black dots*) cells. *E,* inhibition of 4T1 cell migration in response to wt macrophages depends on β_5 integrin. Addition of a mAb blocking β_5 integrin inhibited 4T1 cell migration to the same extent as antibodies blocking α_v (CD51) or SPARC (mAb 303). Accordingly, 4T1β_5^{sil} (754^{sil}) migrated to wt macrophages as 4T1 cells to SPARC KO macrophages. *F,* spontaneous lung metastasis of 4T1β_5^{sil} and parental 4T1 cells injected into wt > KO and KO > wt bone marrow chimeras. *Bars,* median number of lung colonies. ***, $P < 0.001$, two-tailed Mann-Whitney *t* test. Representative of three independent experiments.

cells reduced their invasive capacity and metastatic behavior (10). On the other hand, gene expression profile and RNA interference silencing pointed to SPARC as a key gene involved in the metastasis of MDA-MB-231 cells and clone variants (9). This discrepancy likely reflects the differences between cell-based (*in vitro*) and tissue-based (*in vivo*) analyses. Stroma cells are likely the most relevant source of SPARC in the tumor microenvironment; acting on the cell surface, SPARC might provide different signals to the tumor either directly or through bystander molecules (see below). Accordingly, leukocyte-derived rather than tumor-derived SPARC influenced the stroma density, composition, and outgrowth of N2C mammary carcinoma cell line (20).

Embedded in the ECM, macrophages are often the most represented cell type in tumor stroma (5). In addition to fostering tumor growth, macrophages have long been implicated in metastasis through several mechanisms (3).

4T1 tumors are largely infiltrated by SPARC-producing macrophages. Our data indicate that SPARC produced by macrophages is necessary to advance the metastatic process without affecting EMT. *In vitro* assay shows that macrophages from wt mice induce 4T1 cell migration more efficiently than macrophages from SPARC KO mice, a difference that is nullified by the addition of a mAb to SPARC (mAb 303). Stimulation with LPS and IFNγ similarly activates macrophages from the two strains, in terms of NO production and CD40 up-regulation, as well as increases 4T1 cell migration. The increased migration to SPARC KO activated macrophages was not inhibited by the mAb 303, suggesting that SPARC works in addition to other macrophage-derived factors in inducing tumor cell migration.

The best described role of macrophages in tumor cell migration and intravasation involves a loop in which tumor cells producing CSF-1 recruit macrophages, which in turn secrete EGF, promoting carcinoma cell migration along ECM fibers (4). Here, we have described a new and alternative mechanism of macrophage-tumor cell interaction that involves a matricellular protein (SPARC) able to affect both tumor cell migration and fiber availability toward metastasis.

It is well recognized that ECM is not a merely scaffold for tumor cell but provides signals fostering cell growth, survival, and motility (37). One of the SPARC-associated functions is to promote ECM deposition. Indeed, the absence of SPARC has been associated with decreased collagen deposition (19, 20) and fibronectin production (28). Fibronectin has a role in cell migration during embryonic development, wound healing, and malignancy (29, 38). In addition to acting as a substrate, fibronectin can be cleaved to provide bioactive fragments with chemotactic activity (39). Thus, reduced metastasis in the SPARC KO host can, in part, be explained by the paucity of fibronectin.

Tumor cell migration to ECM component is multifaceted and depends primarily on the reciprocal amount of integrins, their ligands, and affinity. At low ligand concentration, cells are rounded and unable to move (40). This condition likely mimics the absence of host SPARC. On the contrary, at high ligand concentration, cells are very spread while remaining static because of excessive adhesion. In three-dimensional systems, matrix stiffness is also a critical factor. Cells with amoeboid migration, such as dendritic cells and T lymphocytes, move independently from integrin-mediated focal contact while they are affected by matrix hindrance and stiffness. Accordingly, these immune cells move faster in SPARC KO mice, characterized by a loose collagen structure (21). In sharp contrast, tumor cell movement requires a traction force generated by integrin-ECM ligand interactions (41). In this setting, SPARC has a role in regulating ECM-ligand availability (20, 28) and their interaction with cognate integrin receptors.

The complete loss of integrin-mediated cell-ECM contacts induces anoikis (42). In our hands, physiologic amounts of SPARC (nanogram range) similar to those produced by macrophages induced migration without changing the adhesion to other ECM molecules. To completely detach cells from substrate, a higher amount of SPARC (>20 μg/mL) is required (data not shown; ref. 43). This seems to be compatible with the intermediate state of adhesion that allows cell migration (44). These data indicate the importance of a tight control of SPARC expression by cancer/stroma cells. Moreover, the fact that only coated, but not soluble, SPARC supports 4T1 cell migration further confirms that this effect is mediated by integrin receptors according to the notion that an integrin ligand presented in soluble form is not able to induce the recruitment of focal adhesion kinase complexes and cytoskeleton reorganization (45). For instance, plasma-born ECM proteins fibronectin and vitronectin do not work as integrin ligands in their native soluble form but have to undergo conformational changes associated with fiber deposition and denaturation, respectively, to expose integrin binding sites (46, 47). We have identified β_5 integrin as a potential SPARC receptor. In addition, SPARC binds to fibronectin present in the ECM and modulates its promigratory signal through its receptors ($\alpha_v\beta_1$ and $\alpha_v\beta_5$). RGD peptide blocks 4T1 cell migration to SPARC coated onto a transwell inserts, although SPARC has no RGD sequence. This suggests that SPARC effect requires a canonical integrin ligand, which, in this setting, might be vitronectin provided by 4T1 cells. Accordingly, cell migration induced by multimeric vitronectin is enhanced by the addition of SPARC.

Differently from breast cancer, ovarian carcinoma down-regulates SPARC expression during progression. However, data obtained by immunohistochemistry on tissue specimens indicate that SPARC is produced by the reactive stroma of invasive human ovarian carcinomas and lymph node metastases (48, 49), according to our view of stroma-derived SPARC fostering a permissive environment for cell migration.

The type and amount of integrin receptors expressed on the cell surface and their association with growth factor receptor might help to explain the different effects of SPARC in mammary versus ovarian carcinomas. It has been described that $\alpha_v\beta_3$ integrin triggers an apoptotic rather than a prosurvival signal, depending on the integrin/ligand ratio. If the ligand level is low or in the presence of ligand antagonist, $\alpha_v\beta_3$ integrin induces apoptosis (45). Moreover, accordingly to our observation of negligible β_3 expression on 4T1 cells, this classic fibronectin/vitronectin receptor has been reported to be absent from most human breast cancer cells and compensated with the presence of $\alpha_v\beta_1$ and $\alpha_v\beta_5$ as alternative receptors (50).

In summary, we provide evidence that SPARC is a factor that, once produced by bone marrow host cells, creates an environment that facilitates tumor cell metastasis mainly by modulating cell-matrix adhesive properties. The presence of EMT in tumors from SPARC KO mice has suggested that SPARC effect on metastasis is after EMT and located at the step of the integrin-mediated migration of the cells. This is supported by the fact that in the absence of SPARC, metastasis failed to occur both because of the reduced deposition of fibers and because of its effect on 4T1 cell migration to fibronectin or multimeric vitronectin. We cannot

exclude the possibility that this mechanism could also be important to seed distant organ, but the presence of metastasis in SPARC KO mice after i.v. injection of 4T1 cells is in contrast to host SPARC serving a major role in the metastatic target organ.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 4/9/2008; revised 7/11/2008; accepted 8/12/2008.

Grant support: Associazione Italiana Ricerca sul Cancro, Italian Ministry of Health, Alleanza Contro il Cancro, Fondazione Cariplo (M.P. Colombo), and CariChieti (E. Di Carlo). R.A. Brekken is the Effie Marie Cain Scholar in Angiogenesis Research.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

1. Stetler-Stevenson WG, Aznavoorian S, Liotta LA. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annu Rev Cell Biol 1993;9:541–73.
2. Fidler IJ. The pathogenesis of cancer metastasis: the "seed and soil" hypothesis revisited. Nat Rev Cancer 2003;3:453–8.
3. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell 2006;124:263–6.
4. Wyckoff J, Wang W, Lin EY, et al. A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. Cancer Res 2004;64:7022–9.
5. Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res 1996;56:4625–9.
6. Bradshaw AD, Sage EH. SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury. J Clin Invest 2001;107:1049–54.
7. Mackay A, Jones C, Dexter T, et al. cDNA microarray analysis of genes associated with ERBB2 (HER2/neu) overexpression in human mammary luminal epithelial cells. Oncogene 2003;22:2680–8.
8. Jones C, Mackay A, Grigoriadis A, et al. Expression profiling of purified normal human luminal and myoepithelial breast cells: identification of novel prognostic markers for breast cancer. Cancer Res 2004;64:3037–45.
9. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. Nature 2005;436:518–24.
10. Koblinski JE, Kaplan-Singer BR, VanOsdol SJ, et al. Endogenous osteonectin/SPARC/BM-40 expression inhibits MDA-MB-231 breast cancer cell metastasis. Cancer Res 2005;65:7370–7.
11. Taghizadeh F, Tang MJ, Tai IT. Synergism between vitamin D and secreted protein acidic and rich in cysteine-induced apoptosis and growth inhibition results in increased susceptibility of therapy-resistant colorectal cancer cells to chemotherapy. Mol Cancer Ther 2007;6:309–17.
12. Yang E, Kang HJ, Koh KH, Rhee H, Kim NK, Kim H. Frequent inactivation of SPARC by promoter hypermethylation in colon cancers. Int J Cancer 2007;121:567–75.
13. Mok SC, Chan WY, Wong KK, Muto MG, Berkowitz RS. SPARC, an extracellular matrix protein with tumor-suppressing activity in human ovarian epithelial cells. Oncogene 1996;12:1895–901.
14. Suzuki M, Hao C, Takahashi T, et al. Aberrant methylation of SPARC in human lung cancers. Br J Cancer 2005;92:942–8.
15. Sato N, Fukushima N, Maehara N, et al. SPARC/osteonectin is a frequent target for aberrant methylation in pancreatic adenocarcinoma and a mediator of tumor-stromal interactions. Oncogene 2003;22:5021–30.
16. Said N, Najwer I, Motamed K. Secreted protein acidic and rich in cysteine (SPARC) inhibits integrin-mediated adhesion and growth factor-dependent survival signaling in ovarian cancer. Am J Pathol 2007;170:1054–63.
17. Shi Q, Bao S, Maxwell JA, et al. Secreted protein acidic, rich in cysteine (SPARC), mediates cellular survival of gliomas through AKT activation. J Biol Chem 2004;279:52200–9.
18. Prada F, Benedetti LG, Bravo AI, Alvarez MJ, Carbone C, Podhajcer OL. SPARC endogenous level, rather than fibroblast-produced SPARC or stroma reorganization induced by SPARC, is responsible for melanoma cell growth. J Invest Dermatol 2007;127:2618–28.
19. Brekken RA, Puolakkainen P, Graves DC, Workman G, Lubkin SR, Sage EH. Enhanced growth of tumors in SPARC null mice is associated with changes in the ECM. J Clin Invest 2003;111:487–95.
20. Sangaletti S, Stoppacciaro A, Guiducci C, Torrisi MR, Colombo MP. Leukocyte, rather than tumor-produced SPARC, determines stroma and collagen type IV deposition in mammary carcinoma. J Exp Med 2003;198:1475–85.
21. Sangaletti S, Gioiosa L, Guiducci C, et al. Accelerated dendritic-cell migration and T-cell priming in SPARC-deficient mice. J Cell Sci 2005;118:3685–94.
22. De S, Chen J, Narizhneva NV, et al. Molecular pathway for cancer metastasis to bone. J Biol Chem 2003;278:39044–50.
23. Heppner GH, Miller FR, Shekhar PM. Nontransgenic models of breast cancer. Breast Cancer Res 2000;2:331–4.
24. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 1992;52:1399–405.
25. Sweetwyne MT, Brekken RA, Workman G, et al. Functional analysis of the matricellular protein SPARC with novel monoclonal antibodies. J Histochem Cytochem 2004;52:723–33.
26. Savagner P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. Bioessays 2001;23:912–23.
27. Robert G, Gaggioli C, Bailet O, et al. SPARC represses E-cadherin and induces mesenchymal transition during melanoma development. Cancer Res 2006;66:7516–23.
28. Barker TH, Baneyx G, Cardo-Vila M, et al. SPARC regulates extracellular matrix organization through its modulation of integrin-linked kinase activity. J Biol Chem 2005;280:36483–93.
29. Ruoslahti E. Fibronectin and its integrin receptors in cancer. Adv Cancer Res 1999;76:1–20.
30. Pasqualini R, Hemler ME. Contrasting roles for integrin β 1 and β 5 cytoplasmic domains in subcellular localization, cell proliferation, and cell migration. J Cell Biol 1994;125:447–60.
31. Pankov R, Yamada KM. Fibronectin at a glance. J Cell Sci 2002;115:3861–3.
32. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002;110:673–87.
33. Yan Q, Weaver M, Perdue N, Sage EH. Matricellular protein SPARC is translocated to the nuclei of immortalized murine lens epithelial cells. J Cell Physiol 2005;203:286–94.
34. Young BA, Wang P, Goldblum SE. The counteradhesive protein SPARC regulates an endothelial paracellular pathway through protein tyrosine phosphorylation. Biochem Biophys Res Commun 1998;251:320–7.
35. Francki A, Motamed K, McClure TD, et al. SPARC regulates cell cycle progression in mesangial cells via its inhibition of IGF-dependent signaling. J Cell Biochem 2003;88:802–11.
36. Dhanesuan N, Sharp JA, Blick T, Price JT, Thompson EW. Doxycycline-inducible expression of SPARC/Osteonectin/BM40 in MDA-MB-231 human breast cancer cells results in growth inhibition. Breast Cancer Res Treat 2002;75:73–85.
37. Lukashev ME, Werb Z. ECM signalling: orchestrating cell behaviour and misbehaviour. Trends Cell Biol 1998;8:437–41.
38. Leiss M, Beckmann K, Giros A, Costell M, Fassler R. The role of integrin binding sites in fibronectin matrix assembly *in vivo*. Curr Opin Cell Biol 2008;20:502–7.
39. Yamada KM. Fibronectin peptides in cell migration and wound repair. J Clin Invest 2000;105:1507–9.
40. Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. Nature 1997;385:537–40.
41. Zaman MH, Trapani LM, Sieminski AL, et al. Migration of tumor cells in 3D matrices is governed by matrix stiffness along with cell-matrix adhesion and proteolysis. Proc Natl Acad Sci U S A 2006;103:10889–94.
42. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994;124:619–26.
43. Sage H, Vernon RB, Funk SE, Everitt EA, Angello J. SPARC, a secreted protein associated with cellular proliferation, inhibits cell spreading *in vitro* and exhibits Ca+2-dependent binding to the extracellular matrix. J Cell Biol 1989;109:341–56.
44. Murphy-Ullrich JE. The de-adhesive activity of matricellular proteins: is intermediate cell adhesion an adaptive state? J Clin Invest 2001;107:785–90.
45. Stupack DG, Cheresh DA. Get a ligand, get a life: integrins, signaling and cell survival. J Cell Sci 2002;115:3729–38.
46. Narasimhan C, Lai CS. Conformational changes of plasma fibronectin detected upon adsorption to solid substrates: a spin-label study. Biochemistry 1989;28:5041–6.
47. Tomasini BR, Mosher DF. Conformational states of vitronectin: preferential expression of an antigenic epitope when vitronectin is covalently and noncovalently complexed with thrombin-antithrombin III or treated with urea. Blood 1988;72:903–12.
48. Brown TJ, Shaw PA, Karp X, Huynh MH, Begley H, Ringuette MJ. Activation of SPARC expression in reactive stroma associated with human epithelial ovarian cancer. Gynecol Oncol 1999;75:25–33.
49. Paley PJ, Goff BA, Gown AM, Greer BE, Sage EH. Alterations in SPARC and VEGF immunoreactivity in epithelial ovarian cancer. Gynecol Oncol 2000;78:336–41.
50. Meyer T, Marshall JF, Hart IR. Expression of α_v integrins and vitronectin receptor identity in breast cancer cells. Br J Cancer 1998;77:530–6.

Macrophage-Derived SPARC Bridges Tumor Cell-Extracellular Matrix Interactions toward Metastasis

Sabina Sangaletti, Emma Di Carlo, Silvia Gariboldi, et al.

Cancer Res 2008;68:9050-9059.