

Tumor-Derived Osteopontin Reprograms Normal Mammary Fibroblasts to Promote Inflammation and Tumor Growth in Breast Cancer

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Abstract

Breast tumors are characterized by an extensive desmoplastic stroma, abundantly populated by fibroblasts. Cancer-associated fibroblasts (CAF) support tumorigenesis by stimulating angiogenesis, cancer cell proliferation, and invasion. CAF also orchestrate tumor-promoting inflammation in multiple tumor types, including breast cancer. However, the mechanisms through which normal tissue fibroblasts are reprogrammed to tumor-promoting CAFs are mainly obscure. Here, we show that mammary fibroblasts can be educated by breast cancer cells to become activated to a proinflammatory state that supports malignant progression. Proteomic analysis of breast cancer cell-secreted factors identified the secreted proinflammatory mediator osteopontin, which has been implicated in inflammation, tumor progression, and metas-

tasis. Osteopontin was highly secreted by mouse and human breast cancer cells, and tumor cell-secreted osteopontin activated a CAF phenotypes in normal mammary fibroblasts *in vitro* and *in vivo*. Osteopontin was sufficient to induce fibroblast reprogramming and neutralizing antibodies against osteopontin-blocked fibroblast activation induced by tumor cells. The ability of secreted osteopontin to activate mammary fibroblasts relied upon its known receptors CD44 and $\alpha_v\beta_3$ integrin. Strikingly, osteopontin silencing in tumor cells *in vivo* attenuated stromal activation and inhibited tumor growth. Our findings establish a critical functional role for paracrine signaling by tumor-derived osteopontin in reprogramming normal fibroblasts into tumor-promoting CAFs. *Cancer Res*; 75(6); 963–73. ©2015 AACR.

Introduction

Breast cancer continues to be one of the leading causes of cancer-related deaths in women. Cancer-associated fibroblasts (CAF) are a heterogeneous population of fibroblastic cells found in the microenvironment of solid tumors. In some cancer types, including breast and pancreatic carcinomas, CAFs are the most prominent stromal cell type (1). CAFs include several subpopulations with diverse origins, including myofibroblasts [characterized by α -smooth muscle actin (α SMA) expression], activated local tissue fibroblasts, and bone marrow-derived progenitor cells (2, 3). CAFs have been shown to promote tumor growth by directly stimulating tumor cell proliferation, by modifying the components and architecture of the extracellular matrix, and by enhancing angiogenesis (4, 5). We previously demonstrated that CAFs enhance tumor growth also by mediating tumor-promoting inflammation: CAFs isolated from dysplastic skin in a mouse model of skin carcinogenesis

expressed a proinflammatory gene signature and promoted macrophage recruitment, neovascularization, and tumor growth *in vivo*, in an NF- κ B-dependent manner (6). This proinflammatory gene signature was maintained in CAFs from skin carcinomas, and evident in mammary and pancreatic tumors in mice, and in cognate human cancers (6). Moreover, we recently demonstrated that CAFs express proinflammatory factors in human breast tumors, and this expression is enhanced with tumor progression (7). Nevertheless, despite growing evidence on the central role of CAFs in facilitating tumor progression, very little is known about the mechanisms by which resident fibroblasts become activated.

Osteopontin (OPN), also known as SPP1 (secreted phosphoprotein 1), is an integrin-binding glyco-phosphoprotein expressed in a variety of cells and tissues. Its normal physiologic functions include developmental processes, vascular remodeling, and wound repair (8). Osteopontin is also implicated in inflammation, tumor progression, and metastasis (8), and is overexpressed in a variety of human carcinomas, including breast, lung, colorectal, stomach, ovarian and melanoma (8, 9). Elevated tumor and plasma levels of osteopontin have been associated with poor prognosis and with reduced survival in patients with breast cancer (10). The functional mechanisms linked to the tumor-promoting activities of osteopontin include increased survival of tumor cells, enhanced invasion and angiogenesis (11), upregulation of epithelial-mesenchymal transition (12), and systemic tumor-promoting effects such as facilitating the growth of indolent tumors and recruitment of bone marrow-derived cells (13). Osteopontin exerts its multifunctional roles via binding to several known cell surface receptors, including CD44 and $\alpha_v\beta_3$ integrin (8, 14).

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In addition to its secretion by tumor cells, osteopontin is also expressed by a variety of cells in the tumor microenvironment, including CAFs (6, 15). Several studies have demonstrated growth-promoting effects of tumor cell-derived osteopontin on various cells in the tumor microenvironment, such as macrophages, natural killer cells, and endothelial cells (8). However, the paracrine crosstalk between tumor-secreted osteopontin and CAFs remains unresolved.

We performed proteomic analysis that identified osteopontin as a factor secreted by mammary carcinoma cells. We show that breast cancer cell-secreted osteopontin can functionally activate normal mammary fibroblasts. Tumor cell-derived osteopontin activated a proinflammatory gene-expression signature and enhanced motility of normal mammary fibroblasts (NMF), characteristic of CAFs. This activation of mammary fibroblasts by secreted osteopontin depends on both of its known receptors: CD44 and $\alpha_v\beta_3$. Moreover, knockdown of osteopontin in tumor cells resulted in a remarkably attenuated tumor growth and reduced functional activation of fibroblasts, as evident by a dramatic reduction in collagen deposition and in immune cell recruitment. Our data demonstrate a novel functional role for tumor-secreted osteopontin in reprogramming resident tissue fibroblasts to become tumor-promoting, proinflammatory CAFs. This novel interaction suggests that targeting osteopontin in anticancer therapeutics has the advantage of simultaneously attacking tumor cells as well as their interactions with tumor-promoting fibroblasts in the microenvironment.

Materials and Methods

Primary fibroblasts

NMFs and OPN^{-/-} fibroblasts were isolated from mammary glands of 8-weeks-old female FVB/n or OPN^{-/-} mice as previously described (6). OPN^{-/-} mice were a gift from Prof. Eli Pikarsky (Hebrew University of Jerusalem, Jerusalem, Israel). Fibroblasts were cultured in DMEM supplemented with 10% FCS (Biological Industries) at 37°C and 5% CO₂. All experiments were performed with low passage (p2–4) fibroblasts. Multiple batches of fibroblasts were used.

Cell lines

Met-1 mouse breast carcinoma cells were a gift from Prof. Jeffrey Pollard, Hebrew University of Jerusalem, Jerusalem, Israel.

For shOPN Met-1, Met-1 cells were infected with lentivirus-expressing shRNA for mouse osteopontin, or with control vector containing a scrambled shRNA sequence (OpenBiosystems).

C18 primary breast carcinoma cells were prepared in our laboratory from fresh tumor tissue of MMTV-PyMT. To remove contaminant fibroblasts, short trypsinization was performed with trypsin-EDTA, followed by washing of detached fibroblasts. FACS analysis confirmed purity of the remaining cells.

MCF7, MCF10A, and HCC1143 cells were obtained from Prof. Matthias Mann (Max Planck Institute of Biochemistry, Germany); MDA-MB-231 cells were obtained from Prof. Benjamin Geiger (Weizmann Institute of Science, Israel); MDA-MB-468 and HCC70 were obtained from Dr. Ittai Ben-Porath (Hebrew University of Jerusalem, Jerusalem, Israel). All human cell lines were authenticated in the laboratory of T. Geiger, in the last year, using STR technology (BioRap Technologies). All cells were routinely tested for *Mycoplasma* using the EZ-PCR Mycoplasma Test Kit (Beit Haemek).

Proteomic analysis

Conditioned media were diluted 1:1 in 8 mol/L urea in Tris-HCl pH 8.0. Samples (10 µg), reduced with 1 mmol/L DTT, and alkylated with 5 mmol/L iodoacetamide, followed by 3 hours incubation with endoproteinase Lys-C (1:100 enzyme to protein). Subsequently, samples were diluted 1:1 in 50 mmol/L ammonium bicarbonate and trypsin-digested overnight (1:50 enzyme to protein) at room temperature. Resulting peptides were purified and concentrated on C18 stageTips. LC/MS-MS analysis was performed on EASY-nLC1000 UHPLC coupled with Q-Exactive mass spectrometer (Thermo Scientific). Peptides were separated on a 50 cm Pepmap column (Thermo Scientific) using a 4-hour gradient of water-acetonitrile. Raw mass spectrometric data were analyzed with the MaxQuant software (16) ensuring 1% FDR on the peptide and protein levels.

Immunohistochemistry/immunofluorescence

Paraffin-embedded tissue sections were processed as previously described (6). Tissue sections were incubated overnight at 4°C with the following antibodies: anti-osteopontin (Santa Cruz Biotechnology; sc-21742), anti-mouse osteopontin (Abcam; ab11503), anti-CD44 (Novus Biologicals; NBP1-50704), anti-F4/80 (AbD Serotec; MCA497), and anti-CD3 (AbD Serotec; MCA1477), biotinylated secondary antibodies for 2 hours (Vector Laboratories), and 3,3'-diaminobenzidine (DAB; Sigma). Counterstaining was performed with Mayer Hematoxylin. Human formalin-fixed paraffin-embedded breast tumor sections were obtained from the Tel Aviv Sourasky Medical Center. Experiments were approved by the Institutional Helsinki Committee of the Tel Aviv Sourasky Medical Center.

Immunofluorescence was performed with an antibody for α SMA (Abcam, ab5694). PicroSirius Red staining was performed in picro-sirius red solution (Direct Red 80" #365548; Picric acid-P6744-1 GA; Sigma-Aldrich) for 1 hour. Sections were washed with acidified water, dehydrated in ethanol, cleared in xylene, and mounted in a resinous medium.

Microscopy, image capture, and analysis

Samples were visualized and analyzed using Leica DM4000B microscope and digital camera (Leica DFC 360 FX), using the Leica Application Suite software. Brightness and contrast were adjusted equally in all images presented.

Orthotopic tumors

Mice were maintained within the Tel Aviv University SPF facility. All experiments involving animals were approved by the TAU Institutional Animal Care and Use Committee.

A total of 2×10^5 Met-1 or shOPN Met-1 cells were injected into the right inguinal mammary gland of 8-weeks-old FVB/n female mice. Cells were resuspended in PBS and mixed 1:1 in volume with growth factor reduced Matrigel (BD Biosciences) immediately before injection.

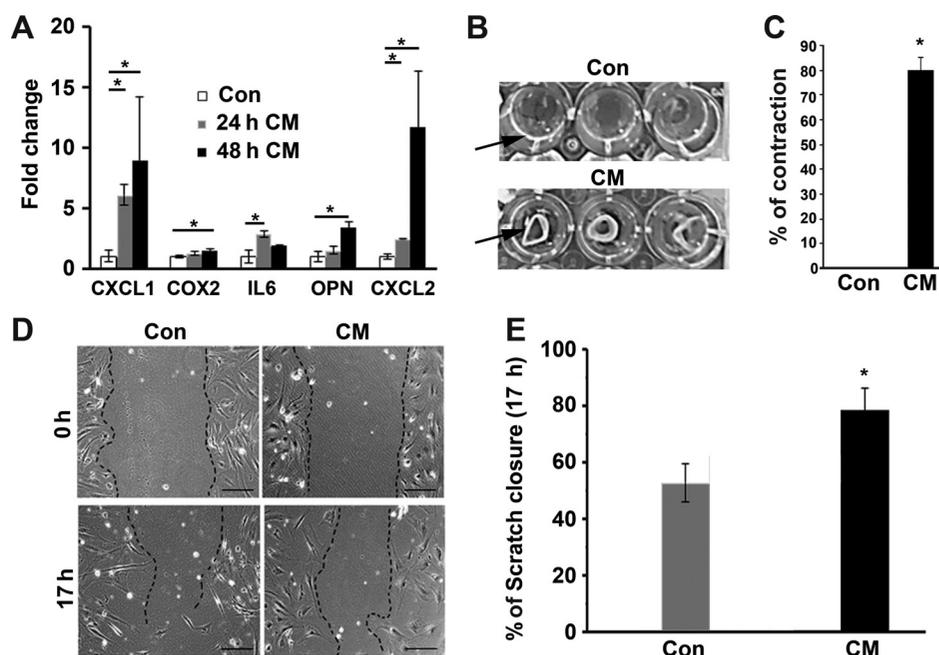
Tumors were measured twice weekly with calipers, and tumor volumes were calculated using the formula $X^2 \times Y \times 0.52$ (where X , smaller diameter and Y , larger diameter). Mice were euthanized when tumors reached a size of 10 mm in any diameter. Tumors were weighed and embedded in paraffin for histology.

Statistical analysis

Data were analyzed using the Student t test, and considered significant when the P value was less than 0.05. All statistical tests

Figure 1.

NMFs are activated by breast tumor cell CM. A, qRT-PCR analysis of proinflammatory genes expression in NMFs treated with serum-free medium (control) or with MET-1 CM for 24 or 48 hours. Results are normalized to the housekeeping gene GAPDH. Data, fold change from SFM (*, $P < 0.05$ as compared with control, $n = 3$). B, fibroblast contraction of collagen matrices. NMFs treated with SFM (Con) or CM for 24 hours were assessed for their ability to contract collagen. C, quantification of collagen contraction seen in B; *, $P < 0.05$, $n = 3$. D, scratch closure assay was conducted with SFM or with NMFs that were pretreated with CM for 24 hours. Scratch area was quantified by the ImageJ software; scale bar, 200 μm . E, quantification of scratch closure seen in D; *, $P < 0.05$, $n = 4$.



were two-sided. Bar graphs represent the mean and SD across multiple independent experimental repeats, unless otherwise stated.

Results

Paracrine signaling from breast tumor cells reprograms mammary fibroblasts

We previously demonstrated that dermal fibroblasts could be educated by squamous carcinoma cells *in vitro* and *in vivo* to become proinflammatory CAFs (6). Here, we set out to test whether NMFs could become activated by paracrine signaling from breast carcinoma cells. To that end, primary fibroblasts, isolated from mammary glands of normal female mice were incubated with conditioned medium (CM) prepared from the MMTV-PyMT-derived breast carcinoma cell line Met-1 (17). Following incubation for 24 or 48 hours, analysis of the expression of proinflammatory genes revealed that tumor cell-secreted factors induced a significant upregulation in the expression of the proinflammatory factors *Cxcl1*, *Cxcl2*, *IL6*, *Cox-2*, and *OPN* (Fig. 1A). We chose to focus on these genes as they were previously shown to be part of a proinflammatory gene signature expressed by CAFs in skin, breast, and pancreatic carcinomas (6, 7, 18), as well as by senescent fibroblasts (19, 20). Moreover, their expression was shown to be functionally important for tumor progression supported by fibroblasts (6). To test whether reprogrammed NMFs gained additional CAF-like features, we further tested their activation status. Activated myofibroblasts are known to have enhanced matrix adhesions, leading to increased contraction of collagen gel matrices in an integrin-dependent manner (21, 22). We therefore investigated the effect of tumor-secreted factors on fibroblast-mediated collagen contraction. Fibroblasts were suspended in collagen matrices in serum-free conditions and incubated in Met-1 conditioned media. Addition of tumor cell-conditioned media induced fibroblasts to contract the collagen gels by $80.28\% \pm 5.04\%$ over 24 hours. In contrast, nonactivated

NMFs suspended in serum-free medium (SFM) did not contract the collagen matrix (Fig. 1B and C). CAFs, characterized also by their expression of αSMA , are known to be similar to myofibroblasts that are active in wound repair, where their coordinated motility and contraction is crucial in the process of wound closure (23–25). We therefore performed an *in vitro* wound closure assay to test the activation of NMFs by tumor cell-secreted factors. Quantification of wound area indicated that NMFs incubated with tumor cell conditioned media showed significantly higher scratch closure as compared with control mammary fibroblasts (Fig. 1D and E). This enhanced scratch closure activity was a result of enhanced motility rather than increased proliferation, as there was no increase in fibroblasts proliferation following incubation with tumor cell CM (Supplementary Fig. S1). Taken together, these results confirm that breast tumor cell-secreted factors activate a proinflammatory CAF-like phenotype in NMFs.

Osteopontin is highly expressed and secreted by breast tumor cells

We next wanted to identify tumor-secreted factors capable of activating NMFs. To that end, we profiled the proteome of the CM using high-resolution mass spectrometry. Duplicate analyses of the medium (with two technical replicates in each experiment) identified overall 1,297 proteins. Gene ontology analysis revealed 227 proteins that were annotated as extracellular and showed highly significant enrichment ($\text{FDR} = 9.8\text{E}-23$) of this group (Supplementary Table S1).

One of the most highly secreted proteins by Met-1 breast carcinoma cells was found to be osteopontin (Supplementary Table S2; Fig. 2A). To further assess the relevance of osteopontin secretion to human breast cancer, we surveyed expression data from multiple human breast tumors compared with normal breast samples, available via the Oncomine database. Bioinformatic analysis confirmed that osteopontin is highly expressed in multiple human breast carcinomas expression datasets (Fig. 2B). Moreover, immunostaining of tissue sections from human

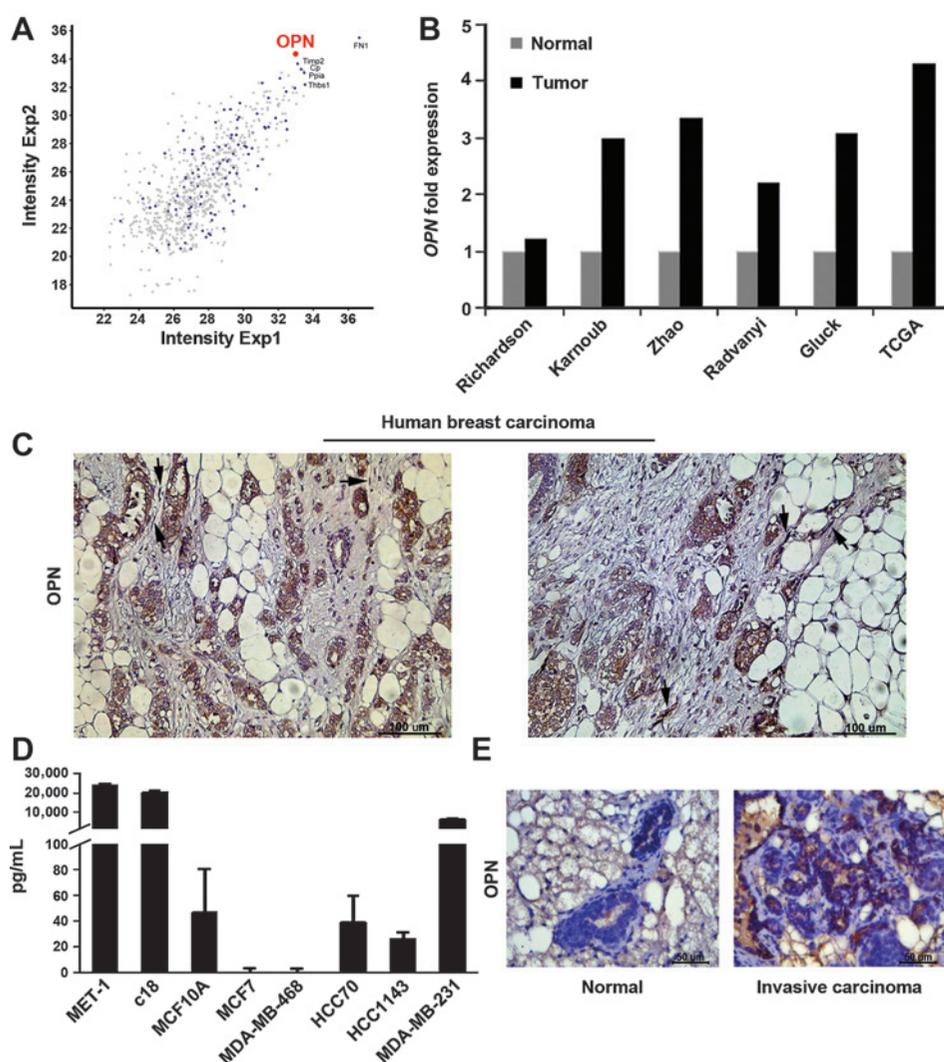


Figure 2. Osteopontin (OPN) is highly expressed in breast tumors. A, replicate LC/MS-MS analysis of MET-1 CM identified 1,297 proteins. Proteins annotated as extracellular are highlighted. B, osteopontin is upregulated in human breast tumors. Median-centered ratio of osteopontin in different datasets of human invasive ductal carcinoma, as published in Oncomine. Data from six different studies of human breast cancers (detailed in Materials and Methods). Values are presented as relative expression compared with normal tissues. C, IHC of osteopontin in tissue sections from human patients with breast cancer. Representative picture of 20 fields analyzed from two patients. Arrows, osteopontin-expressing fibroblasts; scale bar, 100 μ m. D, ELISA assay of human and mouse breast cancer cell lines. Cells were incubated in SFM for 48 hours ($n = 3$). E, IHC of osteopontin in tissue sections of normal mammary glands or tumor tissue from end-stage MMTV-PyMT female mice. Representative picture of 30 fields analyzed from three mice; scale bar, 50 μ m.

patients with breast cancer demonstrated that osteopontin is highly expressed by both tumor cells and CAFs in the tumor microenvironment (Fig. 2C). To assess the secretion of osteopontin by breast tumor cells, we performed ELISA assays of multiple mouse and human breast cancer cell lines, including Met-1, C18 (a primary PyMT-derived tumor cell line, prepared in our laboratory), MCF7, MDA-MB-231, MDA-MB-468, HCC70, HCC1143, and the mammary epithelial cell line MCF10A. Data analysis revealed that osteopontin is secreted at various levels by most breast cell lines tested, with the highest levels of osteopontin secreted by the most aggressive and invasive cell lines, for example, MDA-MB-231, Met-1, and C18 (Fig. 2D). Furthermore, IHC of mammary carcinoma tissue sections from transgenic MMTV-PyMT mice showed strong expression of osteopontin in tumor tissue, but not in normal mammary glands (Fig. 2E).

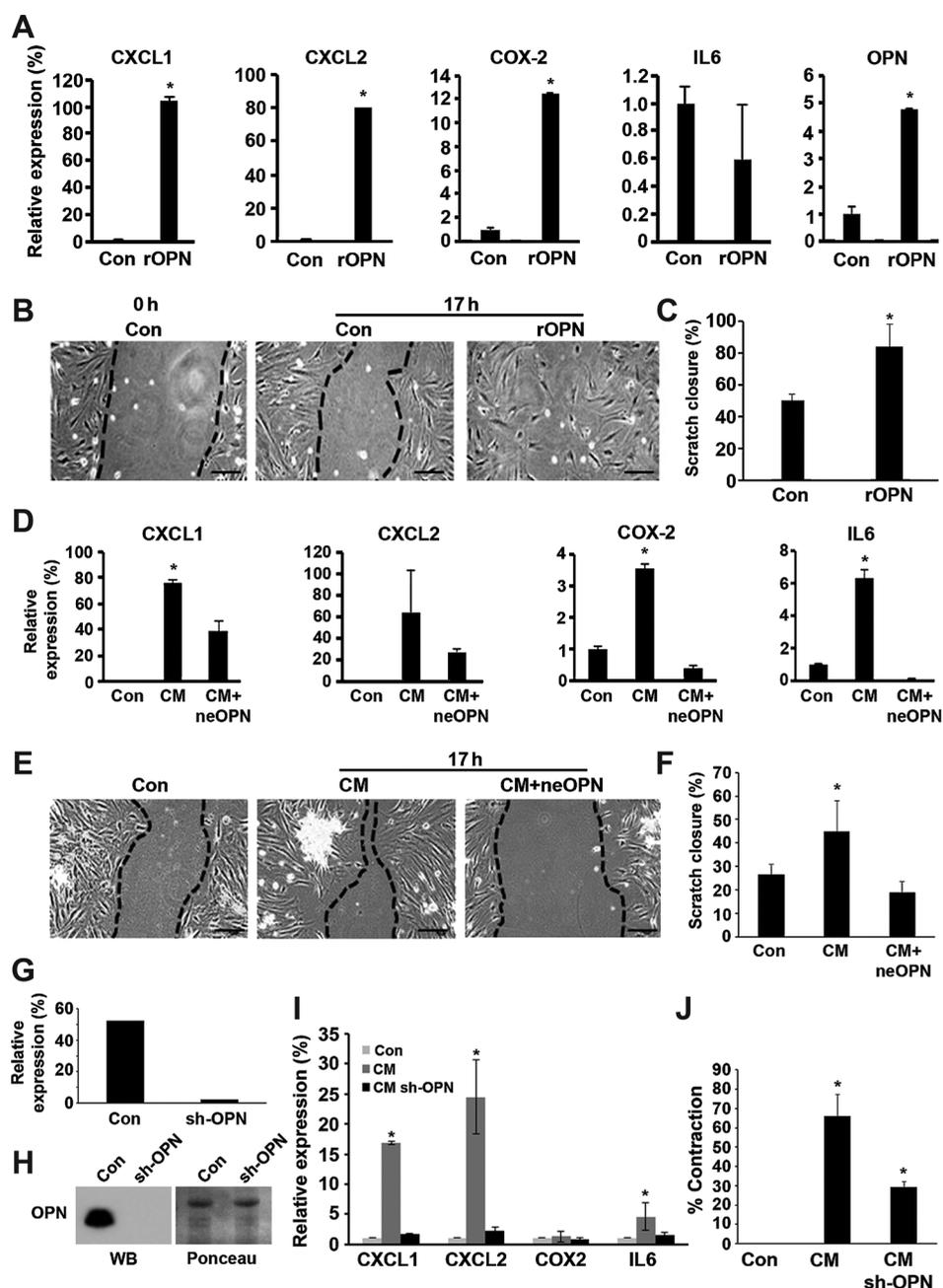
Osteopontin is necessary and sufficient to activate mammary fibroblasts

We next tested whether osteopontin was sufficient for the induction of a proinflammatory CAF phenotype in mammary fibroblasts. NMFs were incubated with SFM supplemented with

recombinant osteopontin (rOPN) for 48 hours, or left untreated. Analysis of the expression of proinflammatory genes revealed that osteopontin is sufficient to induce a strong upregulation in the expression of the proinflammatory gene signature (excluding IL6; Fig. 3A). Moreover, osteopontin was also sufficient for inducing the myofibroblasts phenotype, as reflected by significant enhancement of the motility of fibroblasts in a scratch closure assay (Fig. 3B and C). To test whether osteopontin in tumor cell CM is necessary for the induction of a CAF phenotype, we supplemented tumor cell-conditioned media with neutralizing antibodies to osteopontin. Data analysis revealed that inhibiting osteopontin in tumor cell CM strongly reduced the induction of the proinflammatory gene signature (Fig. 3D). Furthermore, neutralizing tumor cell-secreted osteopontin significantly inhibited the motility of fibroblasts in a scratch closure assay, implying that osteopontin is necessary for paracrine reprogramming of NMFs into CAFs by breast tumor cells (Fig. 3E and F). To further validate the functional role of tumor-derived osteopontin in reprogramming of fibroblasts, we knocked down the expression of osteopontin in breast tumor cells using shRNA (Fig. 3G and H). When NMFs were incubated

Figure 3.

Tumor cell-secreted osteopontin (OPN) activates NMFs. A, qRT-PCR analysis of NMFs that were incubated in SFM for 24 hours, followed by 48 hours incubation in SFM supplemented with 3 $\mu\text{g}/\text{mL}$ rOPN or left untreated (control). B, scratch closure assay conducted as above in the presence or absence of rOPN; scale bar, 200 μm . C, quantification of scratch closure seen in B; *, $P < 0.05$, $n = 4$. D, qRT-PCR analysis of proinflammatory genes. NMFs were incubated in SFM for 24 hours, followed by 48 hours incubation in Met-1 CM, CM + 3 $\mu\text{g}/\text{mL}$ anti-osteopontin-neutralizing antibodies (CM + neOPN), or SFM (Con). E, scratch closure assay. NMFs were treated as indicated; scale bar, 200 μm . F, quantification of scratch closure seen in E; *, $P < 0.05$, $n = 4$. G, qRT-PCR analysis of osteopontin expression in control Met1 cells or in shOPN Met1 cells. Results are normalized to GAPDH. H, Western blot analysis of concentrated tumor cell media from Met-1 cells, or shOPN Met-1 cells. Ponceau staining is shown as loading control. I, qRT-PCR analysis. NMFs were incubated in SFM for 24 hours, followed by 48 hours incubation in MET-1 CM (CM), Met-1 shOPN (CM shOPN), or NMFs CM (Con). Results were normalized to GAPDH; *, $P < 0.05$, $n = 2$. J, collagen contraction assay. NMFs were treated as above and collagen contraction was quantified; *, $P < 0.05$, $n = 3$.



with CM from tumor cells in which the expression of osteopontin was knocked down (CM shOPN), upregulation of the proinflammatory gene signature was almost completely abolished and activation of contractility was significantly reduced, further demonstrating that osteopontin is functionally required for fibroblast activation (Fig. 3I and J).

Because osteopontin is part of the CAF proinflammatory gene signature and is highly upregulated in mammary CAFs and in reprogrammed NMFs, we investigated whether fibroblast-derived osteopontin is necessary for NMF activation. To that end, we isolated mammary fibroblasts from $\text{OPN}^{-/-}$ mice and tested their activation by tumor cell-secreted factors.

Interestingly, although tumor-secreted osteopontin was sufficient for upregulating the expression of COX-2 and CXCL2, the upregulation of IL6 and CXCL1 was strongly attenuated in the absence of fibroblast-derived osteopontin (Fig. 4A), suggesting that autocrine osteopontin signaling is required for activation of these genes, and that pathway-specific mechanisms are operative in the reprogramming of normal fibroblasts into CAFs. Moreover, we found that CAF-derived osteopontin is required for CM-induced fibroblast migration (Fig. 4B) and for the activation of enhanced contractility (Fig. 4C). Thus, some aspects of the induced CAF phenotype are osteopontin dependent.

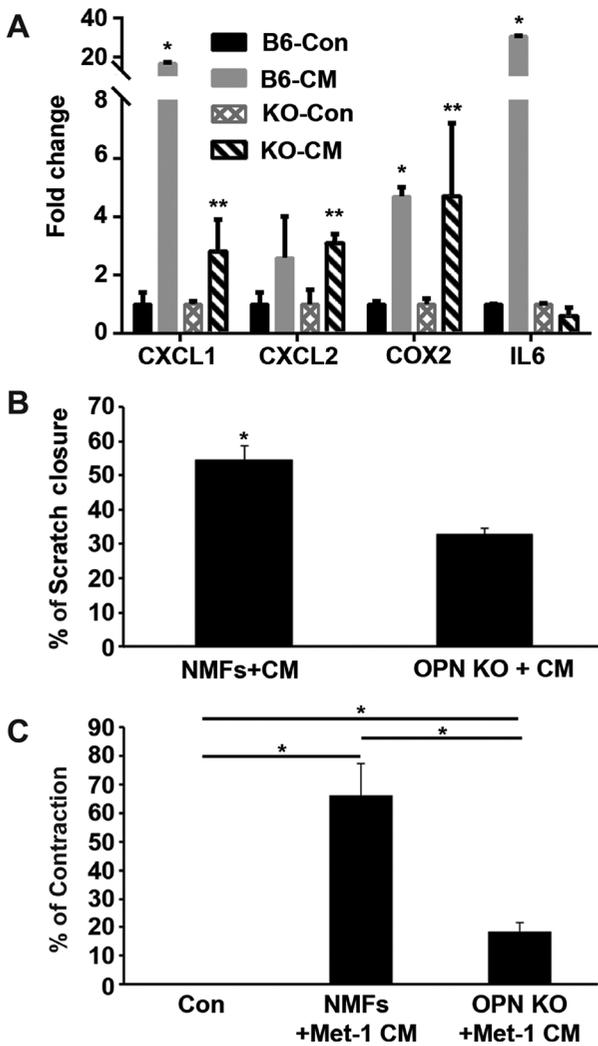


Figure 4. Fibroblasts-derived osteopontin (OPN) is required for NMFs activation. A, qRT-PCR analysis of pro-inflammatory genes by NMFs or *OPN*^{-/-} NMFs incubated in tumor cell CM for 48 hours. Results were normalized to GAPDH. *, *P* < 0.05 with respect to B6-Con; **, *P* < 0.05 with respect to KO-Con, *n* = 3. B, wild-type NMFs or *OPN*^{-/-} NMFs were incubated in tumor cell CM and their motility was assessed as above; *, *P* < 0.05, *n* = 2. C, wild-type NMFs or *OPN*^{-/-} NMFs were incubated in tumor cell CM and their motility was quantified as above; *, *P* < 0.05, *n* = 3.

Osteopontin signals to fibroblasts via CD44 and $\alpha_v\beta_3$ integrin

We next set out to find the molecular mechanism by which tumor secreted osteopontin signals to mammary fibroblasts. Osteopontin exerts its diverse physiologic roles via binding to several known cell surface receptors, including CD44 and multiple integrins (8, 14). $\alpha_v\beta_3$ integrin is the best characterized osteopontin receptor, via the RGD (Arg-Gly-Asp) sequence (26), and it is known to be expressed by fibroblasts (27). CD44 is a major cell surface receptor for hyaluronate that binds osteopontin in an RGD-independent manner. We first wanted to ascertain the expression of CD44 in mammary fibroblasts. To that end, we analyzed CD44 expression by immunostaining of cultured NMFs. Analysis revealed that CD44 is expressed in mammary fibroblasts (Supplementary Fig. S2A). Moreover, the

expression of CD44 is significantly upregulated in CAFs isolated from PyMT mammary tumors compared with NMFs (Supplementary Fig. S2B). In agreement with this observation, analysis of CD44 expression by FACS demonstrated that reprogrammed NMFs upregulated the expression of CD44 following incubation with tumor cell-secreted factors (Supplementary Fig. S2C). IHC of CD44 in tissue sections from PyMT mammary tumors confirmed the expression of CD44 in CAFs *in vivo* (Supplementary Fig. S2D). Thus, acquiring the CAF phenotype includes upregulation of the osteopontin receptor CD44.

To assess which of the known osteopontin receptors is operative in the activation of NMFs by tumor cell-secreted osteopontin, we inhibited their function by anti-CD44-neutralizing antibodies, and the RGD peptide, commonly used as a competitive inhibitor of $\alpha_v\beta_3$ (28). NMFs were incubated with tumor cell CM or with CM supplemented with anti-CD44 antibodies, or RGD, or both, and analyzed for the expression of proinflammatory genes. Analysis of the results indicated that inhibiting both receptors attenuated the upregulation of the proinflammatory gene signature, whereas inhibition of only CD44 or $\alpha_v\beta_3$ had a gene-specific effect. These results indicate that activation of NMFs by osteopontin is mediated by both CD44 and $\alpha_v\beta_3$, but the expression of different proinflammatory genes may be upregulated via distinct osteopontin receptors (Fig. 5A). Addition of anti-CD44 or RGD, or both, to SFM supplemented with rOPN resulted in a similar trend of inhibition, although to a lesser extent, possibly due to differences between the activity of exogenous versus cell-secreted osteopontin (Fig. 5B). We next tested the effect of inhibiting CD44 and $\alpha_v\beta_3$ on motility of NMFs in a scratch closure assay. Interestingly, inhibition of $\alpha_v\beta_3$ significantly reduced NMFs motility, whereas blockade of CD44 had no significant effect (Fig. 5C), suggesting that distinct osteopontin-responsive pathways in fibroblasts regulate their functions.

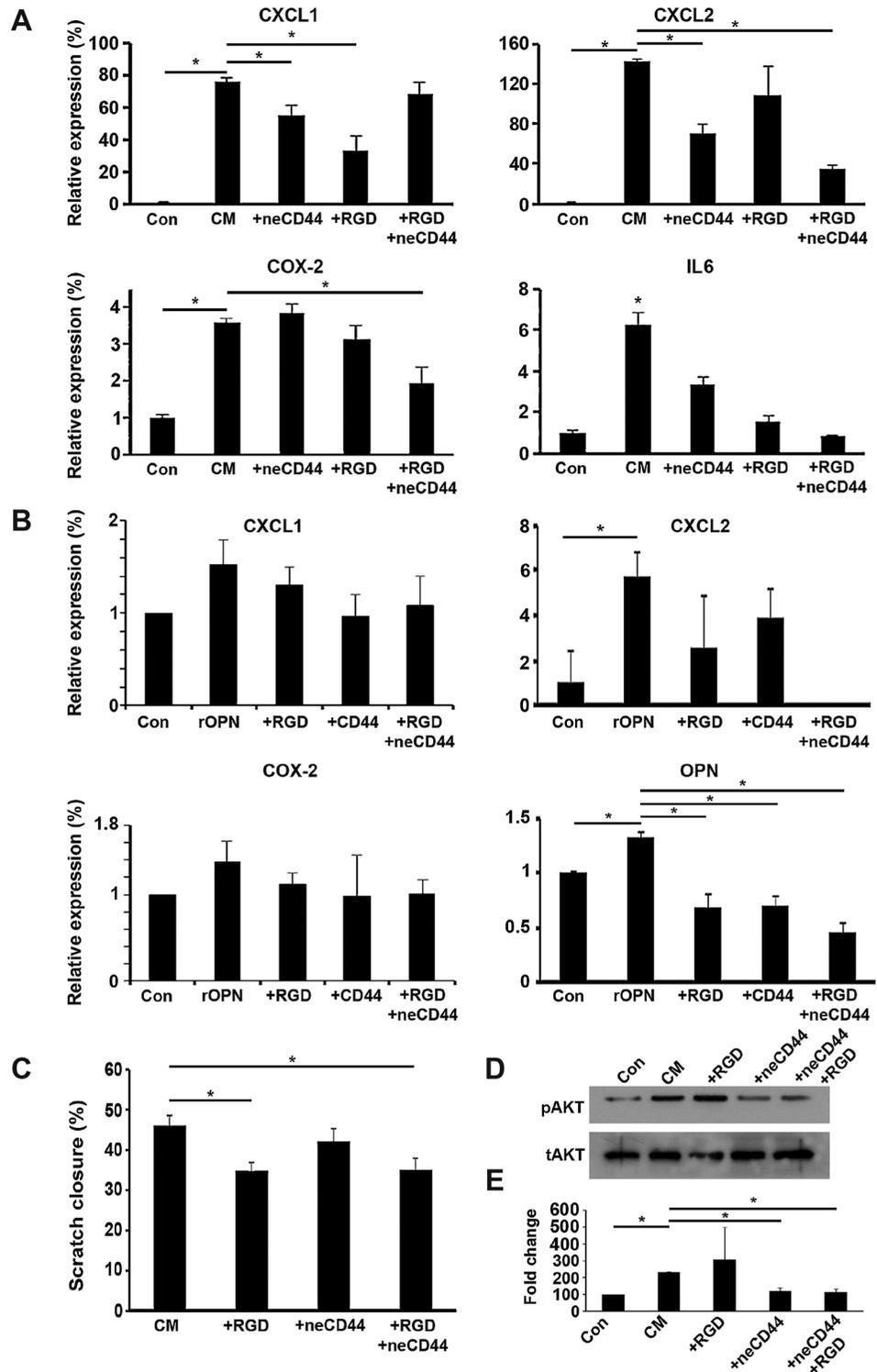
Seeking to get mechanistic insight into the signaling pathways induced in fibroblasts by osteopontin, we next tested the downstream intracellular pathways that are activated by osteopontin receptors. Binding of the $\alpha_v\beta_3$ integrin receptor by osteopontin is known to induce ERK1/2 phosphorylation (29). Binding of osteopontin to CD44 activates phosphorylation of AKT (30). To test the functional importance of each signaling pathway, NMFs were incubated in SFM for 24 hours followed by treatment with either tumor cell CM, CM supplemented with RGD, or neutralizing antibodies to CD44, or both. As expected, incubation with CM significantly upregulated phosphorylation of AKT, and neutralizing antibodies to CD44 inhibited this upregulation, indicating that activation of NMFs by CM occurs via the (PI3K)-Akt pathway (Fig. 5D and E). However, phosphorylation of ERK was not inhibited by addition of RGD to tumor cell CM, indicating that other factors in the CM were still operative in activating ERK phosphorylation (data not shown).

Osteopontin is functionally required for tumor growth *in vivo*

Finally, we tested the functional significance of tumor-derived osteopontin for tumor growth *in vivo*. To that end, cells in which osteopontin was knocked down (shOPN Met-1), or control cells, were injected orthotopically to mammary glands of FVB/n female mice. Of note, knockdown of osteopontin did not have an effect on tumor cell growth *in vitro* (Supplementary Fig. S3).

Strikingly, shOPN tumors grew significantly slower and were smaller than control tumors (Fig. 6A and B). Because

Figure 5. Tumor cell-secreted osteopontin (OPN) activates NMFs via binding to $\alpha_v\beta_3$ and CD44. A, qRT-PCR analysis of NMFs treated with CM supplemented with RGD, neCD44, or a combination of both inhibitors. Results were normalized to GAPDH; Con, SFM. Data, fold change from control (*, $P < 0.05$, $n = 3$). B, qRT-PCR analysis of NMFs treated with SFM supplemented with 400 ng/mL rOPN and 10 μ g/mL RGD, 10 μ g/mL anti-CD44-neutralizing antibodies (neCD44) or a combination of both, as indicated; Con, NMFs CM. Data, fold change from Con; *, $P < 0.05$, $n = 2$. C, quantification of scratch closure assay conducted with CM supplemented with neCD44, RGD, or both, as indicated; *, $P < 0.05$, $n = 3$. D, Western blot analysis of NMFs treated with tumor cells CM, CM supplemented with 10 μ g/mL RGD, 10 μ g/mL anti-CD44-neutralizing antibodies (neCD44), or a combination of both inhibitors for 30 minutes. Antibodies specific for phosphorylated AKT (pAkt) or total Akt (tAkt). E, quantification of results shown in D; *, $P < 0.05$, $n = 2$.



knockdown of osteopontin did not affect the proliferative rate of tumor cells, and based on our *in vitro* results demonstrating that tumor cell-secreted osteopontin activates NMFs, we hypothesized that the effect of osteopontin deletion reflects functional activation of CAFs *in vivo*. We therefore assessed collagen deposition and organization in tumor tissue sections.

Although control tumors were characterized by massive deposition of collagen and collagen fibrils throughout the tumor mass, shOPN Met-1 tumors had clearly reduced collagen content and the number of collagen fibrils was significantly decreased (Fig. 6C-E). Interestingly, α SMA was expressed by CAFs in both control tumors and in tumors in which

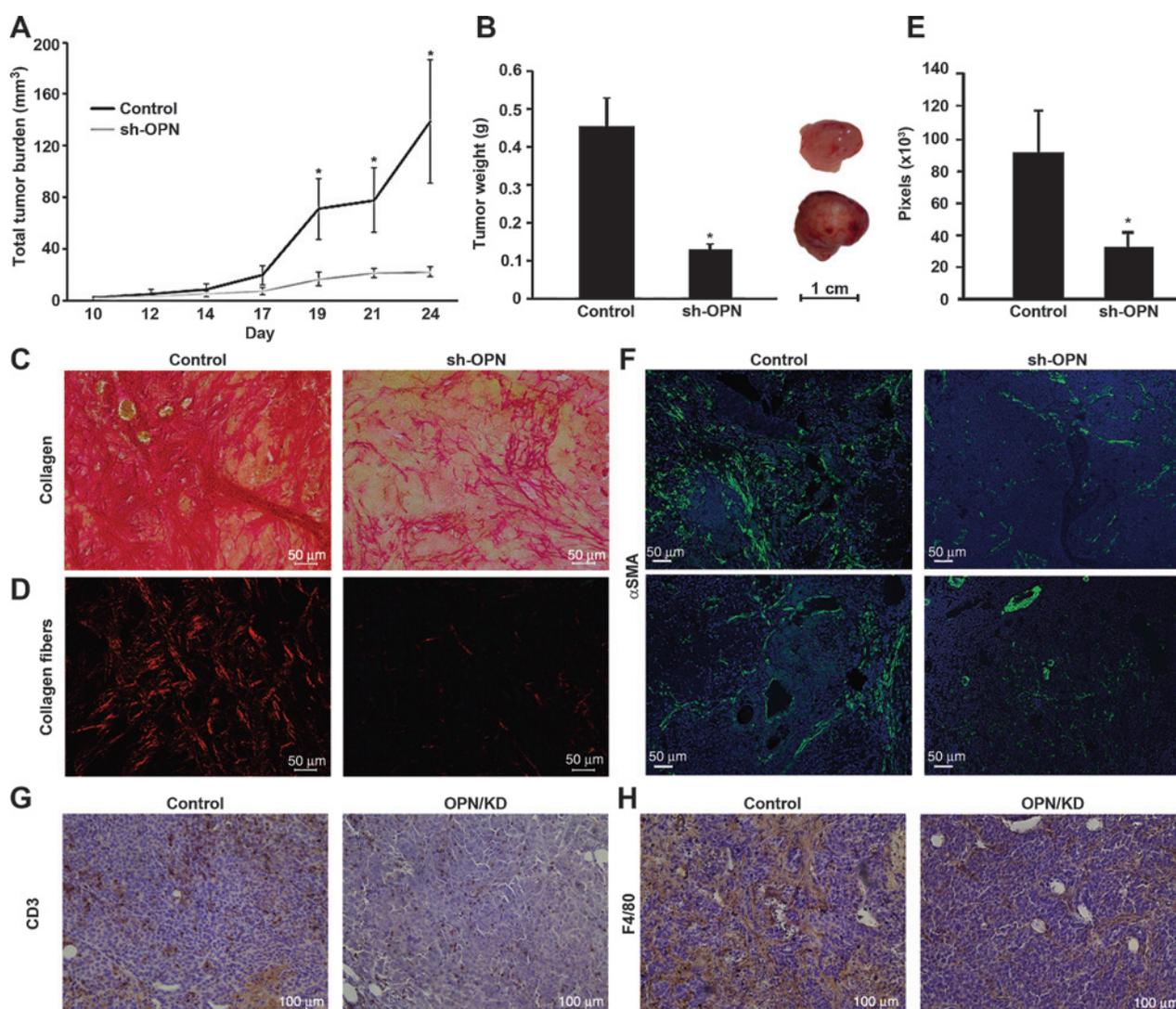


Figure 6.

Knocking down osteopontin (OPN) in tumor cells inhibits tumor growth. A, a total of 2×10^5 control Met-1 cells or shOPN Met-1 cells were injected into the right inguinal mammary gland of 8-week-old FVB/n female mice; error bars, SEM (*, $P < 0.05$, $n = 10$). B, average tumor weight (g) at termination of experiment. Right, representative tumor images at day 24. C, PicroSirius red staining. Representative images of multiple fields/mouse analyzed from 9 mice; scale bar, 50 μm . D, representative polarized light imaging of collagen fibers; scale bar, 50 μm . Twenty fields of 5 mice were analyzed from each group. E, quantification of images shown in D; error bars, SEM. F, representative images of αSMA staining of Met-1 or shOPN met-1 tumors. Multiple fields of 4 mice were analyzed from each group. G and H, representative images of T-cell (CD3) and macrophages (F4/80) staining in Met-1 and shOPN-Met1 tumors. Multiple fields of 3 mice were analyzed from each group; scale bar, 100 μm .

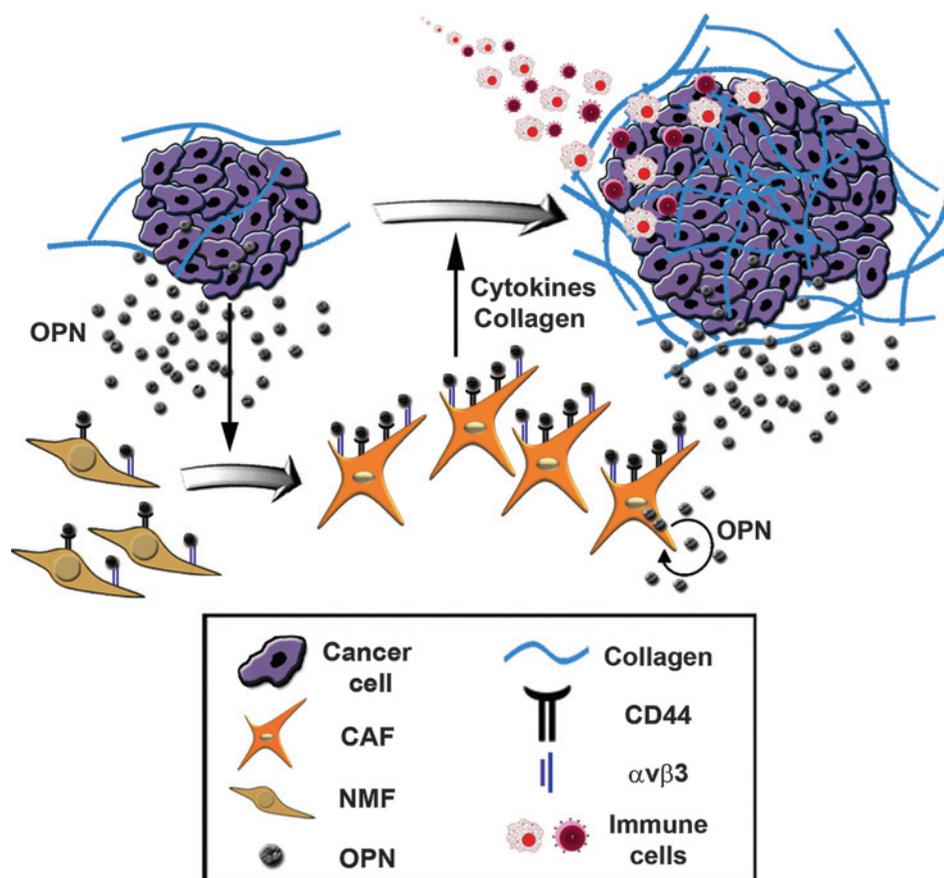
osteopontin was knocked down (Fig. 6F). These findings provide evidence that osteopontin is required for functional activation of collagen deposition in stromal fibroblasts in breast tumors, which facilitate tumor growth. Moreover, analysis of immune cell recruitment into shOPN tumors as compared with control tumors, revealed that shOPN tumors were less infiltrated by T cells and macrophages (Fig. 6G and H). This observation is in agreement with our *in vitro* data, which implicate tumor-derived osteopontin in proinflammatory activation of CAFs.

Taken together, our data implicate tumor-secreted osteopontin in functional activation of mammary fibroblasts to proinflammatory, tumor-promoting CAFs in breast cancer.

Discussion

Our study identifies breast cancer–derived osteopontin as a novel inducer of an activated, proinflammatory CAF phenotype in mammary fibroblasts. We show that osteopontin is highly secreted by mouse and human breast cancer cells. Osteopontin drives a CAF phenotype in NMFs, as reflected by their increased ability to contract collagen matrices, enhanced motility, and upregulation of a proinflammatory gene signature, previously shown to be a hallmark of CAFs in mouse and human carcinomas (6, 7, 18). Osteopontin-induced activation of NMFs is mediated via binding of osteopontin to its receptors CD44 and $\alpha_v\beta_3$ on fibroblasts. Moreover, blocking of osteopontin signaling inhibited fibroblast reprogramming *in vitro* and remarkably attenuated tumor growth

Figure 7. Tumor-secreted osteopontin drives a proinflammatory and tumor-promoting CAF phenotype in mammary fibroblasts. Tumor cells secrete osteopontin (OPN), which activates mammary fibroblasts via CD44 and $\alpha_v\beta_3$ to express proinflammatory genes (including osteopontin) and deposit collagen in the tumor microenvironment, thus supporting tumor growth.



and fibroblast activation *in vivo*, implying that osteopontin is a key player in the crosstalk between tumor cells and stromal fibroblasts (Fig. 7).

Tissue-resident fibroblasts were suggested as precursors for CAFs, activated by tumor and immune cell-derived factors (6, 31–33). However, the heterotypic signaling networks that mediate the functional conversion of resident fibroblasts into CAFs are largely unresolved. Cancer cells take advantage of the enormous plasticity of stromal cells, such as fibroblasts and macrophages, and produce multiple signals that generate a tumor-promoting microenvironment. Previous studies demonstrated that coculturing or tumor cell CM could activate stromal fibroblasts (34–36). Moreover, several secreted factors were suggested to be capable of fibroblast activation. For example, Hsp90 alpha was suggested to initiate a proinflammatory phenotype in prostate fibroblasts (37), TGF β and SDF-1 were implicated as inducers of a CAF-like phenotype (23,38). We previously demonstrated that IL1 β is capable of reprogramming normal dermal fibroblasts into proinflammatory CAFs (6). Thus, reprogramming of normal fibroblasts to activated CAFs is a general phenomenon operative in multiple cancers, giving rise to heterogeneous intratumoral subpopulation of CAFs. Although this may be a general phenomenon, the specific tumor-derived mediators capable of inducing this education are likely tumor-type dependent. Moreover, even within different subtypes of the same cancer, variable tumor-derived factors give rise to distinct CAF phenotypes (39). Nevertheless, most of the tumor-derived factors that activate fibroblasts are unknown.

Our study, based on proteomics analysis of breast tumor cell secreted factors, implicates osteopontin in reprogramming of mammary fibroblasts into tumor-promoting CAFs.

Elevated osteopontin levels in tumors and in patients' blood, correlate with poor prognosis (8, 40, 41). Notably, although the link between overexpression of osteopontin and worse outcome is clear, meta-analysis of osteopontin in breast cancer concluded that osteopontin expression is independent of breast cancer subtypes (42). However, the role of tumor-derived osteopontin in modulating the tumor microenvironment is largely unresolved. Interestingly, immunohistochemical studies in hepatocellular carcinoma indicated that osteopontin-positive cancer cells were located in the tumor periphery, adjacent to stromal cells (43), suggesting its involvement in paracrine tumor–stroma interactions. We and others have previously shown that osteopontin is part of a proinflammatory gene signature upregulated in CAFs (6, 7, 15, 18). Others suggested that expression of osteopontin by skin fibroblasts is required for their differentiation to myofibroblasts (44). Our current results expand and complement these observations by providing a functional role for tumor-derived osteopontin in the reprogramming of mammary fibroblasts to CAFs. Importantly, we show that fibroblast-derived osteopontin was also important for fibroblasts activation, suggesting that osteopontin secreted from cancer cells initiates a signal that is then amplified by fibroblast-derived osteopontin. Thus, paracrine as well as autocrine signaling by osteopontin play a key role in tumor–stromal interactions in breast cancer.

We show that CD44, a known osteopontin receptor, was upregulated in mammary CAFs and in reprogrammed NMFs, suggesting that responsiveness to osteopontin may be part of acquiring the CAF phenotype. Indeed, blockade of the osteopontin receptors CD44 and $\alpha_v\beta_3$, resulted in an attenuated CAF phenotype. However, individual proinflammatory genes were differentially inhibited by blockade of CD44 or of $\alpha_v\beta_3$, implying that distinct signaling pathways downstream of osteopontin are involved in their regulation. This pathway specificity is further supported by the fact that blocking $\alpha_v\beta_3$, but not CD44, could inhibit osteopontin-induced motility of fibroblasts. Indeed, $\alpha_v\beta_3$ was shown to contribute to osteopontin-induced fibroblast motility (45).

Understanding of factors driving fibroblast activation is critical for development of stromal-targeted therapeutics. Our results, demonstrating that inhibition of osteopontin in tumor cells did not alter their growth rates *in vitro*, but significantly hindered their ability to form tumors *in vivo* support a key role for osteopontin in tumor–stroma communication. Functional activation of collagen deposition by CAFs contributes to tumor growth and progression (21, 46). Our study implicates osteopontin in functional activation of fibroblasts, leading to the formation of a growth-permissive desmoplastic stroma.

Previous studies demonstrated that inhibition of osteopontin in human xenograft models results in attenuated tumor growth and/or reduced metastasis (13, 47, 48). Moreover, tumor-secreted osteopontin was found to have systemic effects that support the establishment of distant indolent tumors, via osteopontin-induced mobilization of bone marrow cells (13). Our results expand these findings and suggest a fibroblast-dependent mechanism for osteopontin-mediated tumor progression: We show that genetic inhibition of osteopontin in a syngeneic, immunocompetent tumor model results in attenuated tumor growth due to osteopontin-mediated activation of stromal fibroblasts and immune cell recruitment.

Remarkably, knockdown of osteopontin in mouse models of wound healing resulted in a reduced inflammatory response and subsequent size of postsurgical adhesions. This attenuated inflammatory response was accompanied by a reduction in collagen deposition within the wound area (49, 50). These observa-

tions are in agreement with our study, suggesting that osteopontin is indeed a key player in fibroblast activation, not only in fibroblasts associated with carcinogenesis and tumor progression, but also in wound healing.

Taken together, our data provide insight to the multiple complex reciprocal interactions between tumor cells and CAFs, and shed light on a novel pathway by which resident fibroblasts are being reprogrammed by tumor cells to become proinflammatory, tumor-promoting CAFs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: Y. Sharon, Y. Raz, N. Cohen, N. Erez

Development of methodology: Y. Sharon, N. Cohen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Sharon, Y. Raz, T. Geiger

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Sharon, Y. Raz, T. Geiger

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