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VEGF-A and Tenascin-C produced by S100A4⁺ stromal cells are important for metastatic colonization

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Increased numbers of S100A4⁺ cells are associated with poor prognosis in patients who have cancer. Although the metastatic capabilities of S100A4⁺ cancer cells have been examined, the functional role of S100A4⁺ stromal cells in metastasis is largely unknown. To study the contribution of S100A4⁺ stromal cells in metastasis, we used transgenic mice that express viral thymidine kinase under control of the S100A4 promoter to specifically ablate S100A4⁺ stromal cells. Depletion of S100A4⁺ stromal cells significantly reduced metastatic colonization without affecting primary tumor growth. Multiple bone marrow transplantation studies demonstrated that these effects of S100A4⁺ stromal cells are attributable to local non-bone marrow-derived S100A4⁺ cells, which are likely fibroblasts in this setting. Reduction in metastasis due to the loss of S100A4⁺ fibroblasts correlated with a concomitant decrease in the expression of several ECM molecules and growth factors, particularly Tenascin-C and VEGF-A. The functional importance of stromal Tenascin-C and S100A4⁺ fibroblast-derived VEGF-A in metastasis was established by examining *Tenascin-C* null mice and transgenic mice expressing Cre recombinase under control of the S100A4 promoter crossed with mice carrying *VEGF-A* alleles flanked by loxP sites, which exhibited a significant decrease in metastatic colonization without effects on primary tumor growth. In particular, S100A4⁺ fibroblast-derived VEGF-A plays an important role in the establishment of an angiogenic microenvironment at the metastatic site to facilitate colonization, whereas stromal Tenascin-C may provide protection from apoptosis. Our study demonstrates a crucial role for local S100A4⁺ fibroblasts in providing the permissive "soil" for metastatic colonization, a challenging step in the metastatic cascade.

stromal fibroblasts | metastasis-associated fibroblasts | tumor microenvironment | metastatic microenvironment

About 90% of cancer deaths are attributable to systemic disease associated with metastasis (1). Among the steps involved in metastasis, the colonization step is considered the most challenging for an invading cancer cell (2). With metastatic disease as the leading cause of death among patients who have cancer (3), a greater need is emphasized for a better understanding of the metastatic process so as to identify efficacious cancer therapies.

S100A4 (also known as CAPL, p9Ka, 42A, pEL98, mts1, metastasin, calvasculin, 18A2, or FSP1) is a member of the S100 calcium-binding family, which has a high prognostic significance for metastasis in patients with cancer (4). Several studies have demonstrated a correlation between increased numbers of S100A4⁺ cells and poor prognosis of patients for a variety of cancer types, including colorectal adenocarcinoma, non-small cell lung cancer, breast adenocarcinoma, gastric cancer, esophageal squamous carcinoma, bladder cancer, prostate adenocarcinoma, melanoma, and ovarian carcinoma. Although S100A4⁺ cells encompass a variety of cell types, including malignant cancer cells and host stromal cells (5), studies have primarily focused on the metastatic properties of S100A4⁺ cancer cells (6–9).

It has long been speculated that the metastatic microenvironment may be an important determinant in the establishment of metastases (10), but support for the importance of stromal interactions in metastasis is still largely lacking. Others have demonstrated that the metastatic microenvironment contains greater numbers of S100A4⁺ stromal cells than the primary tumor microenvironment (11). In light of this, the present study investigated the functional contribution of S100A4⁺ stromal cells in metastasis.

Results

S100A4⁺ Stromal Cells in the Tumor and Metastatic Microenvironments.

4T1 breast cancer cells, when injected into the mammary fat pad of syngeneic female BALB/c mice, readily metastasize to the lung. Using this orthotopic cancer model allows us to distinguish between S100A4⁺ cancer cells and S100A4⁺ stromal cells, and also to study stromal interactions within a relevant microenvironment. To determine whether an influx of S100A4⁺ stromal cells correlates with metastasis, we used *S100A4-GFP* transgenic mice (6) in which the *S100A4* promoter drives expression of GFP, allowing us to track any S100A4⁺ stromal cells. In normal breast tissue and normal lung tissue, we observed few S100A4⁺ stromal cells. In the setting of cancer, S100A4⁺ stromal cells increased slightly in number within the tumor microenvironment (Fig. 1A), while there was a significant increase in S100A4⁺ stromal cells associated with metastasis (Fig. 1B). This accumulation of S100A4⁺ stromal cells in the tumor and metastatic microenvironments corresponds with observations previously seen in human cancers. We further confirmed that S100A4⁺ cells accumulate in the stromal regions surrounding metastatic nodules of patients with cancer (Fig. S1).

Ablation of S100A4⁺ Stromal Cells Attenuates Metastasis.

To functionally assess the role of S100A4⁺ stromal cells in metastasis, we used transgenic mice expressing viral thymidine kinase under control of the *S100A4* promoter (*S100A4-tk* mice), in which ganciclovir (GCV) treatment results in the selective ablation of S100A4⁺ stromal cells (12). In the physiological setting, few S100A4⁺ stromal cells reside in the normal lung; however, many S100A4⁺ stromal cells proliferate in response to invading cancer cells in the metastatic lung (Fig. S2A). The *S100A4-tk* model can

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The authors declare no conflict of interest.

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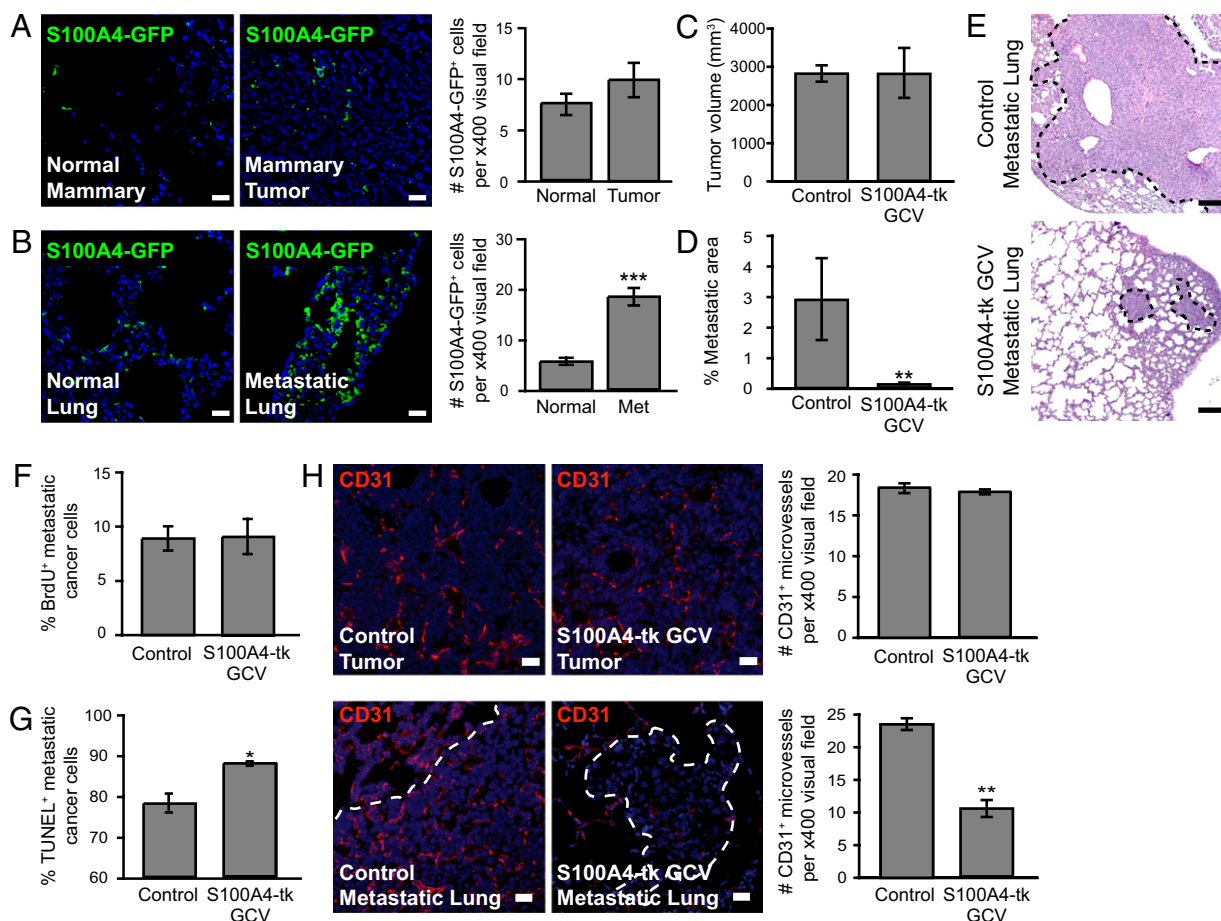


Fig. 1. Ablation of S100A4⁺ stromal cells inhibits metastatic colonization of orthotopically implanted 4T1 cancer cells. S100A4⁺ stromal cells (green) with DAPI nuclear counterstain (blue) in normal mammary tissue vs. 4T1 mammary tumor tissue (A) and normal lung tissue vs. 4T1 metastatic lung tissue (B) from S100A4-GFP transgenic mice. (Scale bars: 50 μ m.) Primary tumor volume (C) and percent metastatic area in lung tissue (D) of GCV-treated S100A4-tk mice ($n = 10$) and control littermates ($n = 10$) 24 d after orthotopic implantation of 4T1 cancer cells. (E) Representative H&E-stained lung sections with dotted lines encircling metastatic lesions. (Scale bars: 400 μ m.) Quantification of BrdU⁺ (F) and TUNEL⁺ (G) cancer cells within metastatic nodules. (H) Microvessel density was quantified by CD31 staining (red) with DAPI nuclear counterstain (blue) in primary tumors and within metastatic nodules. (Scale bars: 20 μ m.) Mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

take advantage of the activation of S100A4⁺ stromal cells in response to metastasis and target these proliferating cells for selective ablation. 4T1 cancer cells were orthotopically implanted into GCV-treated S100A4-tk mice and control littermates. S100A4-tk⁺ stromal cells were significantly depleted in the mammary tumor and metastatic lung of GCV-treated S100A4-tk mice (Fig. S2B). Ablation of S100A4⁺ stromal cells had no effects on primary tumor growth (Fig. 1C); however, metastatic colonization was significantly attenuated (Fig. 1D and E). Administration of GCV had no effect on tumor growth or metastasis in WT mice (Fig. S3), confirming that decreased metastatic colonization in GCV-treated S100A4-tk mice was not attributable to nonspecific GCV toxicity. The percentage of proliferating cancer cells within metastatic nodules was not significantly affected by the ablation of S100A4⁺ stromal cells (Fig. 1F); however, analysis of apoptosis demonstrated a significant increase in the percentage of apoptotic cancer cells within the metastatic nodules of GCV-treated S100A4-tk mice (Fig. 1G). Increased apoptosis in GCV-treated S100A4-tk mice was further associated with decreased angiogenesis at the metastatic site, whereas angiogenesis at the primary tumor site was not significantly affected (Fig. 1H). These results implicate a specific role for S100A4⁺ stromal cells in metastatic colonization.

S100A4⁺ Stromal Cells Act Locally at the Metastatic Site. Greater numbers of S100A4⁺ stromal cells were observed in the meta-

static microenvironment than in the primary tumor microenvironment. In addition, the ablation of S100A4⁺ stromal cells only inhibited angiogenesis at the metastatic site and not at the primary tumor site. Therefore, we speculated that S100A4⁺ stromal cells may act directly at the metastatic site to facilitate metastatic colonization. To bypass any primary tumor contribution, we injected 4T1 cancer cells i.v. into GCV-treated S100A4-tk mice and control littermates. Even in the absence of a primary tumor, metastatic colonization was still significantly impaired after ablation of S100A4⁺ stromal cells (Fig. 2A). Although the percentage of proliferating cancer cells within the metastatic nodules was not significantly affected (Fig. 2B), the percentage of apoptotic cancer cells was significantly increased in the metastatic nodules of GCV-treated S100A4-tk mice (Fig. 2C). This increased apoptosis was again associated with reduced angiogenesis within the metastatic nodules of GCV-treated S100A4-tk mice (Fig. 2D). Collectively, these results support the notion that S100A4⁺ stromal cells act locally at the metastatic site to facilitate metastatic colonization via the establishment of an angiogenic microenvironment to support the survival and propagation of metastasizing cancer cells.

Ablation of S100A4⁺ Stromal Cells Also Attenuates Liver Metastasis of CT26 Colorectal Cancer Cells. To assess the role of S100A4⁺ stromal cells in an alternative model of metastasis, we used the CT26 colorectal cancer cell line (Fig. S4). CT26 colorectal cancer cells,

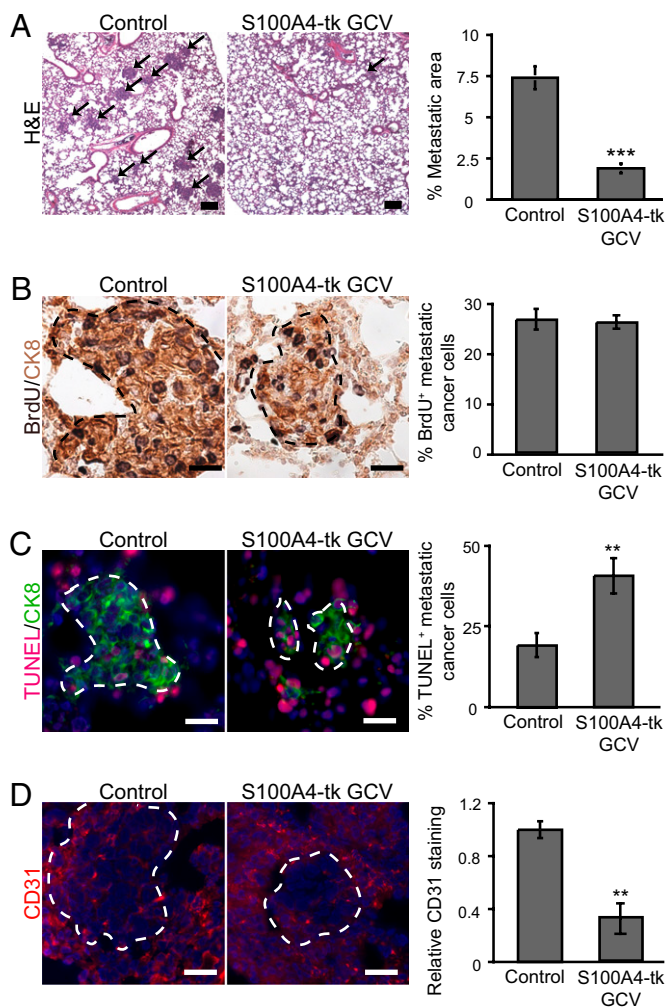


Fig. 2. Ablation of $S100A4^+$ stromal cells inhibits metastatic colonization of i.v. inoculated 4T1 cancer cells. (A) Percent metastatic area in lung tissue of GCV-treated $S100A4-tk$ mice ($n = 6$) and control littermates ($n = 6$). Representative H&E-stained lung sections are displayed. Arrows point to metastatic lesions. (Scale bars: 160 μm .) Quantification of BrdU⁺ (B) and TUNEL⁺ (C) cancer cells within metastatic nodules. (D) Quantification of microvessel density by CD31 staining (red) with DAPI nuclear counterstain (blue) within metastatic nodules. Dotted lines encircle metastatic lesions. (Scale bars: 20 μm .) Mean \pm SEM. ** $P < 0.01$; *** $P < 0.001$.

when injected into the spleen, establish metastases in the liver. In the $S100A4-GFP$ mice, $S100A4^+$ stromal cells are detected in negligible numbers in normal liver tissue. After implantation of CT26 cancer cells into the spleen, $S100A4^+$ stromal cells significantly increased in number within the metastatic microenvironment of the liver. CT26 colorectal cancer cells were then injected into the spleen of GCV-treated $S100A4-tk$ mice and control littermates. Ablation of $S100A4^+$ stromal cells in GCV-treated $S100A4-tk$ mice significantly impaired liver metastasis. These results demonstrate the importance of $S100A4^+$ stromal cells in metastatic colonization of different cancer cell types at distinct organ sites.

$S100A4^+$ Immune Cells in the Metastatic Microenvironment Derive from the Bone Marrow. $S100A4^+$ stromal cells have been identified as either fibroblasts or immune cells in the breast tumor microenvironment (5). Our analysis of the metastatic microenvironment in patients with breast cancer determined that antibody staining for $S100A4$ exhibited a staining pattern different from that of the immune cell marker CD45 (Fig. S5); within the

stromal compartment of metastatic lesions, $S100A4^+$ cells are more prevalent than $CD45^+$ cells. In the 4T1 breast cancer model, $\sim 40\%$ of $S100A4^+$ stromal cells expressed CD45 in the metastatic microenvironment, indicating the presence of both $S100A4^+$ immune cells and $S100A4^+$ fibroblasts. Bone marrow transplantation from $S100A4-GFP^+$ transgenic donors to WT recipients demonstrated that the entire population of $S100A4^+$ immune cells is primarily derived from the bone marrow, as the number of $S100A4-GFP^+/CD45^+$ cells remains the same between the total $S100A4-GFP$ transgenic mice and WT mice bearing an $S100A4-GFP$ bone marrow transplant (Fig. 3A).

$S100A4^+$ Immune Cells Do Not Have a Significant Impact on Metastasis. To functionally distinguish between $S100A4^+$ immune cells and $S100A4^+$ fibroblasts in metastasis, we performed various bone marrow transplant experiments with the $S100A4-tk$ transgenic mice. Because $S100A4^+$ immune cells primarily derive from the bone marrow, $S100A4^+$ immune cells can be targeted for selective ablation via the GCV treatment of WT mice transplanted with $S100A4-tk$ bone marrow. Alternatively, ablation can be limited to mostly $S100A4^+$ fibroblasts in GCV-treated $S100A4-tk$ transgenic mice transplanted with WT bone marrow. Consistent with our earlier findings, the ablation of all $S100A4^+$ stromal cells in GCV-treated $S100A4-tk$ mice transplanted with $S100A4-tk$ bone marrow significantly reduced metastatic colonization (Fig. 3B). When the ablation of $S100A4^+$ stromal cells was limited to

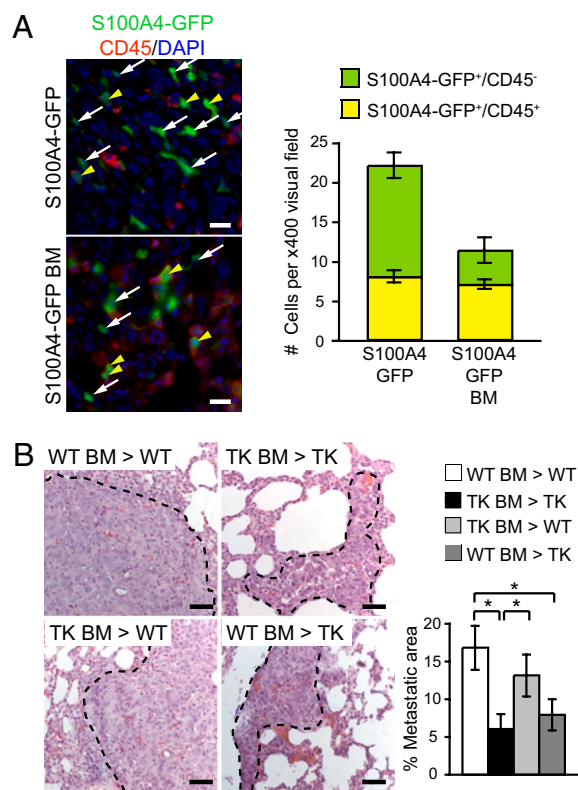


Fig. 3. Bone marrow-derived $S100A4^+$ stromal cells do not functionally contribute to metastatic colonization. (A) $S100A4^+$ (green) and $CD45^+$ (red) stromal cells in metastatic lung tissue of $S100A4-GFP$ mice and WT mice bearing an $S100A4-GFP$ bone marrow (BM) transplant. White arrows point to $S100A4-GFP^+$ stromal cells; yellow arrowheads point to $S100A4-GFP^+/CD45^+$ stromal cells. (Scale bars: 20 μm .) (B) Quantification of metastatic area in WT mice transplanted with WT bone marrow (WT BM > WT, $n = 13$), $S100A4-tk$ mice transplanted with $S100A4-tk$ bone marrow (TK BM > TK, $n = 13$), WT mice transplanted with $S100A4-tk$ bone marrow (TK BM > WT, $n = 10$), and $S100A4-tk$ mice transplanted with WT bone marrow (WT BM > TK, $n = 12$). Representative H&E-stained lung sections are displayed with dotted lines encircling metastatic lesions. (Scale bars: 50 μm .) Mean \pm SEM. * $P < 0.05$.

the bone marrow compartment in GCV-treated WT mice with *S100A4-tk* bone marrow, metastatic colonization was not significantly affected. In contrast, when only non-bone marrow-derived $S100A4^+$ stromal cells are ablated in GCV-treated *S100A4-tk* mice with WT bone marrow, a significant reduction in metastatic colonization is also observed. Thus, these results suggest that bone marrow-derived $S100A4^+$ stromal cells, which are primarily $S100A4^+$ immune cells, do not play a significant role in metastatic colonization in this setting.

$S100A4^+$ Stromal Cells Provide Tenascin-C in Support of Metastatic Colonization. Although $S100A4$ protein itself can contribute to metastatic progression (13, 14), $S100A4^+$ stromal cells may provide additional factors to foster the development of metastases. We have identified that the $S100A4^+$ stromal cells affecting metastasis are most likely fibroblasts; thus, we surveyed the literature and used expression profiling data produced by other research groups (15–17) to generate a putative list of fibroblast-derived ECM molecules reported to be important for metastasis. We identified 12 candidate ECM molecules and assessed their expression in normal lung tissue compared with control and GCV-treated *S100A4-tk* lung tissue (Fig. S6A). Although Collagen I, Collagen III, Collagen XVIII, and Thrombospondin 1 showed increased expression during metastasis to implicate their potential contribution to the metastatic process, their expression did not change significantly in GCV-treated *S100A4-tk* mice. On the other hand, the increased expression of Fibronectin extra domain-A (ED-A) in metastasis, which concurs with the results reported by Kaplan and colleagues (18), decreased upon ablation of $S100A4^+$ stromal cells, implicating a specific contribution of Fibronectin ED-A by $S100A4^+$ stromal cells. However, Tenascin-C demonstrated the most differential expression: Tenascin-C was not expressed in normal lung tissue, but significant expression was observed in the metastatic nodules of control mice, which diminished to control levels upon ablation of $S100A4^+$ stromal cells (Fig. 4A). This indicates that $S100A4^+$ stromal cells are likely to

be a primary source of Tenascin-C within the metastatic microenvironment. In addition, we performed gene expression profiling analysis comparing $S100A4^+$ stromal cells isolated from normal lungs and $S100A4^+$ stromal cells isolated from metastatic lungs, and Tenascin-C was among the top 20 ECM molecules up-regulated by metastasis-associated $S100A4^+$ stromal cells (Fig. S6B). Thus, Tenascin-C is regulated both on the transcript level and protein level by $S100A4^+$ stromal cells within the metastatic microenvironment, implicating its potential contribution to metastatic colonization.

To specifically assess the role of Tenascin-C in metastasis, we injected 4T1 cancer cells i.v. into *Tenascin-C* null (*TN-C KO*) mice and WT littermates. Immunofluorescence labeling with antibodies specific to Tenascin-C confirmed that Tenascin-C was substantially reduced in the metastatic lesions of *TN-C KO* mice, demonstrating a substantial contribution of stromal cells to Tenascin-C accumulation within the metastatic microenvironment (Fig. S6C). Some speculate that Tenascin-C may be produced by endothelial cells, but double immunofluorescence for Tenascin-C and the endothelial marker CD31 in WT mice demonstrated that Tenascin-C expression did not colocalize with endothelial cells (Fig. S6D). In addition, the significant decrease in Tenascin-C expression in GCV-treated *S100A4-tk* mice (Fig. 4A) indicates that $S100A4^+$ stromal cells are a primary source of Tenascin-C in the metastatic microenvironment. In the absence of stromal Tenascin-C, *TN-C KO* mice exhibited fewer and smaller metastatic nodules; however, no difference in angiogenesis was observed at the metastatic site (Fig. 4B). In addition, the growth of primary 4T1 tumors was not significantly altered in these mice (Fig. 4C). Together, these results suggest that stromal Tenascin-C, predominantly provided by $S100A4^+$ cells, facilitates metastatic colonization.

$S100A4^+$ Stromal Cells Provide VEGF-A in Support of Metastatic Colonization. To identify factors secreted by $S100A4^+$ stromal cells that may aid in the establishment of an angiogenic microenvironment to support metastatic colonization, we then assessed

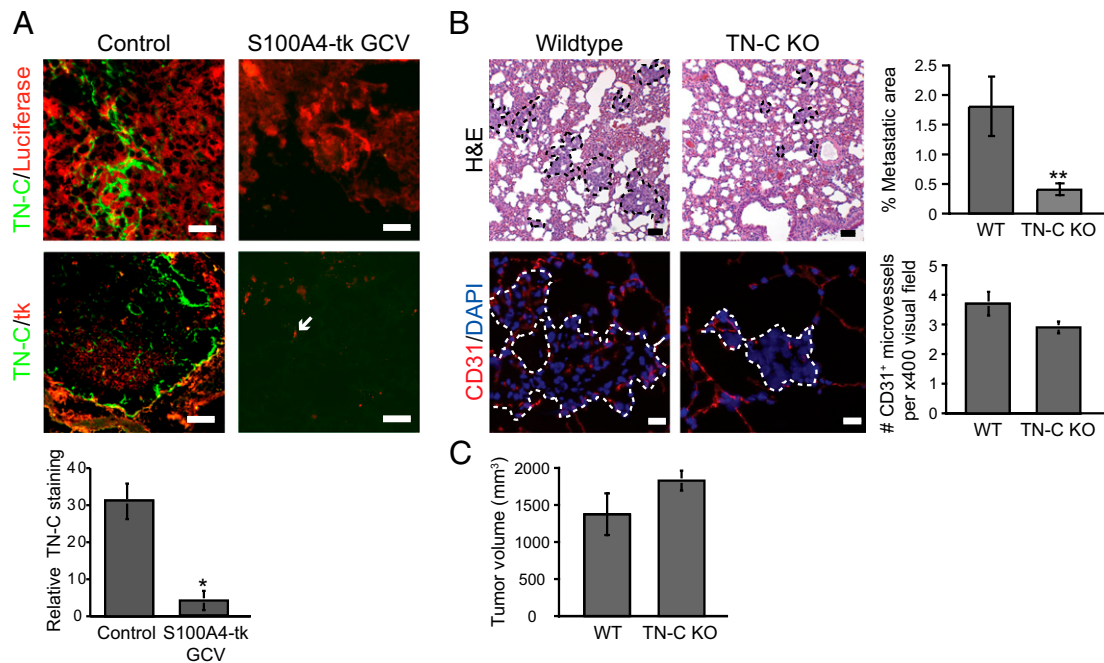


Fig. 4. Loss of stromal Tenascin-C inhibits metastatic colonization. (A) Representative images of the cancer cell marker luciferase (red) and Tenascin-C (green) as well as $S100A4-tk$ (red) and Tenascin-C (green) double staining within metastatic lung tissue of control and GCV-treated *S100A4-tk* mice. (Scale bars: 20 μ m.) The bar graph summarizes the relative Tenascin-C (TN-C) antibody staining between the two groups. (B) Percent metastatic area and microvessel density in lung tissue of *TN-C KO* ($n = 4$) and WT (Wildtype) littermates ($n = 6$) 4 d after i.v. inoculation of 4T1 cancer cells. Representative H&E-stained lung sections. (Scale bars: 50 μ m.) CD31 staining (red) with DAPI nuclear counterstain (blue). (Scale bars: 20 μ m.) Metastatic lesions are encircled by dotted lines. (C) Primary tumor volume of *TN-C KO* ($n = 6$) and WT littermates ($n = 4$) 24 d after orthotopic implantation of 4T1 cancer cells. Mean \pm SEM. * $P < 0.05$; ** $P < 0.01$.

the chemokines and growth factors up-regulated by metastasis-associated S100A4⁺ stromal cells within our gene expression profile (Fig. S7A). VEGF-A was the most up-regulated gene among chemokines and growth factors by S100A4⁺ stromal cells during metastasis, and it is a very promising candidate for the angiogenic effects observed in GCV-treated *S100A4-tk* mice. We confirmed that VEGF-A had mild expression in S100A4⁺ cells of uninvolved stroma, with enhanced expression in S100A4⁺ stromal cells associated with metastasis. Furthermore, elevation of VEGF-A/S100A4⁺ cells within metastatic nodules was down to baseline level upon ablation of S100A4⁺ stromal cells (Fig. 5A).

To elucidate the role of S100A4⁺ stromal cell-derived VEGF-A in metastasis, we crossed mice expressing Cre recombinase under control of the *S100A4* promoter (*S100A4-Cre*) with mice carrying *VEGF-A* alleles flanked by loxP sites (*VEGF-A^{flx/flx}*), resulting in *S100A4-Cre;VEGF-A^{flx/flx}* progeny to delete VEGF-A expression specifically in S100A4⁺ stromal cells. The *S100A4-Cre* mice demonstrated ~85% recombination efficiency (Fig. S7B). *S100A4-Cre;VEGF-A^{flx/flx}* mice live normally and do not exhibit any spontaneous phenotype. 4T1 cancer cells were implanted into *S100A4-Cre;VEGF-A^{flx/flx}* mice and WT littermates either i.v. or orthotopically. Immunofluorescence labeling with an antibody specific to VEGF-A confirmed that VEGF-A was significantly reduced in metastatic lesions of *S100A4-Cre;VEGF-A^{flx/flx}* mice (Fig. S7C), demonstrating a substantial contribution of S100A4⁺ stromal cells to VEGF accumulation within the metastatic microenvironment. The growth of primary tumors was not impaired in *S100A4-Cre;VEGF-A^{flx/flx}* mice (Fig. 5B), although the metastatic burden was significantly reduced (Fig. 5C and D). Similar to GCV-treated *S100A4-tk* mice, *S100A4-Cre;VEGF-A^{flx/flx}* mice exhibited decreased angiogenesis at the metastatic site (Fig. 5E), indicating that S100A4⁺ stromal cell-derived VEGF-A facilitates metastatic colonization by favoring an angiogenic metastatic microenvironment.

To determine specifically whether VEGF-A affecting metastasis is derived from S100A4⁺ fibroblasts or immune cells, we performed various bone marrow transplant experiments with the *S100A4-Cre;VEGF-A^{flx/flx}* transgenic mice (Figs. S8 and S9). Consistent with our earlier findings, the loss of VEGF-A from all S100A4⁺ stromal cells in *S100A4-Cre;VEGF-A^{flx/flx}* mice transplanted with *S100A4-Cre;VEGF-A^{flx/flx}* bone marrow significantly reduced metastatic colonization. When VEGF-A expression was suppressed in only bone marrow-derived S100A4⁺ stromal cells in WT mice with *S100A4-Cre;VEGF-A^{flx/flx}* bone marrow, metastatic colonization was not significantly affected. In contrast, when VEGF-A expression was suppressed in only non-bone marrow-derived S100A4⁺ stromal cells in *S100A4-Cre;VEGF-A^{flx/flx}* mice with WT bone marrow, metastatic colonization appeared to be attenuated. Thus, the loss of VEGF-A from bone marrow-derived S100A4⁺ cells appears to have no effect on metastatic colonization, implicating that VEGF-A specifically derived from S100A4⁺ fibroblasts, and not S100A4⁺ immune cells, plays a role in metastatic colonization.

Discussion

S100A4 was independently cloned by several research teams (19–25) as a marker associated with cell growth and cell motility. Increased numbers of S100A4⁺ cells have been correlated with metastasis and poor prognosis in patients with cancer (26). The metastatic capabilities of S100A4⁺ cancer cells have been thoroughly studied (6–9, 11, 27); however, in the tumor microenvironment, S100A4 is also expressed by fibroblasts and immune cells, including macrophages (5). Studies using spontaneous models of cancer are unable to distinguish between the functions of S100A4⁺ cancer cells and S100A4⁺ stromal cells (6, 14). Others have assessed the function of S100A4 protein in S100A4⁺ stromal cells, in which the absence of S100A4 protein appears to prevent the recruitment of immune cells and fibroblasts to the tumor microenvironment (13). With S100A4's known function in cell motility, attenuation of metastasis attributable to the loss of S100A4 protein in S100A4⁺ stromal cells may simply reflect restricted migration of metastasis-promoting stromal cells to the site of metastasis.

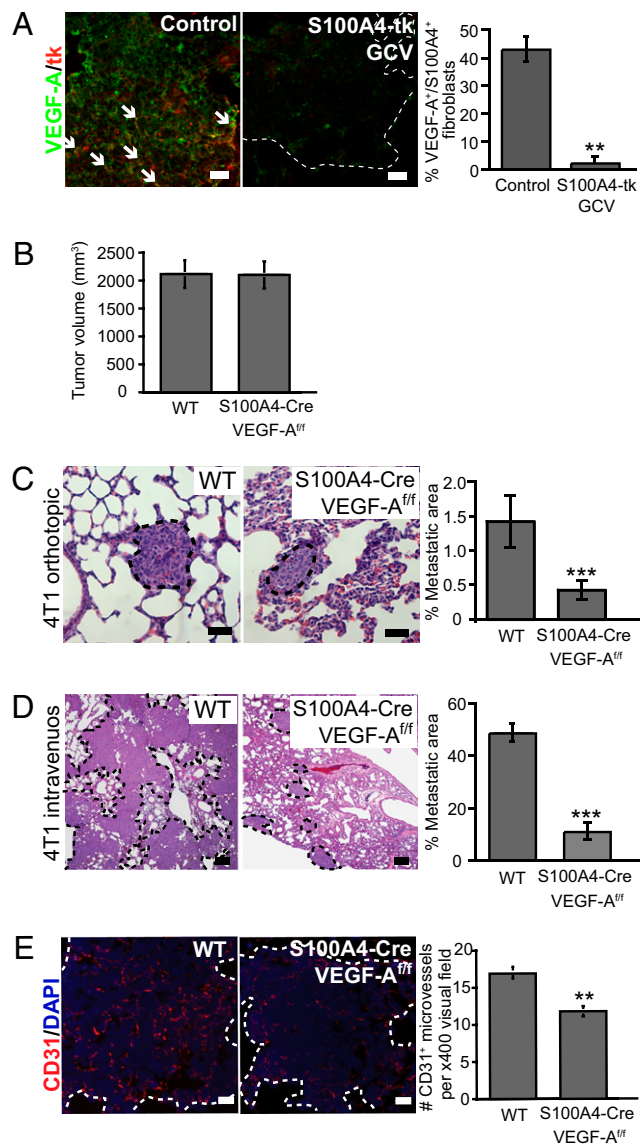


Fig. 5. S100A4⁺ stromal cells provide VEGF-A to the metastatic microenvironment to support metastatic colonization. (A) Representative images and quantification of VEGF-A (green) and S100A4-tk (red) immunostaining in metastatic lung tissue of control and GCV-treated *S100A4-tk* mice. (Scale bars: 20 μ m.) (B) Primary tumor volume of *S100A4-Cre;VEGF-A^{flx/flx}* mice and control littermates 24 d after 4T1 orthotopic implantation. Representative H&E-stained lung sections and quantification of metastatic area from *S100A4-Cre;VEGF-A^{flx/flx}* (*S100A4-Cre;VEGF-A^{flx/flx}*) mice and control littermates after 4T1 orthotopic (C) and 4T1 i.v. (D) injections ($n = 5-8$ in each group). (Scale bars: C, 50 μ m; D, 100 μ m). (E) Microvessel density was quantified by CD31 staining (red) with DAPI nuclear counterstain (blue) within metastatic lesions of these mice. (Scale bars: 50 μ m.) Dotted lines encircle metastatic lesions. Mean \pm SEM. ** $P < 0.01$; *** $P < 0.001$.

S100A4⁺ cells may have other functions in metastasis aside from functions related to S100A4 protein. We have demonstrated that S100A4⁺ stromal cells, predominantly fibroblasts, support metastatic colonization via production of ECM proteins and secreted growth factors, such as Tenascin-C and VEGF-A. S100A4⁺ stromal cell-derived VEGF-A appears important in the establishment of an angiogenic microenvironment at the site of metastasis, whereas stromal Tenascin-C may be important in protecting cancer cells from apoptotic stress in the metastatic microenvironment.

Tenascin-C has been reported to provide survival cues to cancer cells, and its expression is associated with metastasis and confers

poor prognosis in patients with cancer (28, 29). It is likely that Tenascin-C produced by the metastasis-associated fibroblasts provides protection against apoptosis via cooperative interaction with receptors, such as TGF- β receptor II. Alternatively, Tenascin-C may induce cancer stem cell markers to promote cancer cell survival. In addition, VEGF-A produced by S100A4⁺ fibroblasts likely induces the activation of lung capillary endothelial cells and helps to support the growing metastatic nodule. It is also possible that this source of VEGF-A provides endothelial survival cues via VEGF receptor 2-mediated up-regulation of Akt (30). Serum levels of VEGF-A can often predict which patients will develop metastatic disease (31). On a similar note, anti-VEGF therapy, such as bevacizumab, has been successful at extending the survival of patients with metastatic disease (32–35). We speculate that the scoring of S100A4⁺ stromal cells in patients with metastatic disease may help to identify those who may be more receptive to anti-VEGF therapy. Collectively, our results demonstrate that nonimmune S100A4⁺ stromal cells associated with metastatic nodules are important mediators of metastatic colonization and contribute to the permissive “soil” (10, 36) required by tumor cells to form organized metastatic lesions in secondary sites.

Materials and Methods

For 4T1 orthotopic injections, 1×10^6 4T1 mammary carcinoma cells were injected into the mammary fat pad; S100A4-*tk* mice received daily i.p. injections with 50 mg/kg of GCV starting on day 12, and mice were killed on day 24. For 4T1 i.v. injections, S100A4-*tk* mice received GCV starting 3 d before cancer cell inoculation; 5×10^5 4T1 mammary carcinoma cells were injected into the retroorbital venous plexus, and mice were killed 10 d afterward unless otherwise stated. For CT26 intrasplenic injections, 5×10^5 CT26 colorectal cancer cells were injected into the spleen and mice were killed 10 d afterward; S100A4-*tk* mice received GCV starting on the day of cancer cell implantation. Bone marrow transplants were performed at least

3 wk before cancer cell implantation, in which female recipient mice were lethally irradiated at 500 rad and retroorbitally injected with bone marrow cells obtained from male donor mice. Tumor volumes were measured using Vernier calipers and calculated using a standard formula ($\text{length} \times \text{width}^2 \times 0.52$). Mice were injected with 1 mg/mL BrdU 1 h before they were killed. All organs were collected as described in our previous publications (37). H&E staining of paraffin-embedded tissues was generated by the Beth Israel Deaconess Medical Center histology core. Metastases were identified via histopathological analysis, and metastatic area was quantified by National Institutes of Health ImageJ software as a percentage of total tissue area; this analysis was verified by a trained pathologist. Mouse studies were approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. Comparisons between groups were analyzed by a two-tailed unequal variance *t* test, with $P \leq 0.05$ considered to be statistically significant. Additional details are provided in *SI Materials and Methods*.

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