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14. ABSTRACT Fatty acid binding protein 5 (FABP5) has been shown to coordinate metabolic and inflammatory responses. However, not much is known about its role in breast cancer. We have shown that FABP5 may play an important role in breast cancer progression, as FABP5 knockout in the host significantly decreased tumor volume in orthotopic models of breast cancer. We also have shown higher expression of FABP5 in highly metastatic triple negative breast cancer (TNBC) cell lines compared to low metastatic cell lines. FABP5 shRNA knockdown in TNBC exhibited less migration compared to control. We also analyzed FABP5 expression in tissue microarray containing 423 primary breast cancer patient samples with a median followup of 8 years and documented pathological type, grade, demographic, treatment and disease-free and overall survival patient outcome data. We found high FABP5 expression correlates with tumor grade, TNBCs, and lower disease-free survival. This suggests that high FABP5 in patient samples correlates with an overall worse prognosis.					
15. SUBJECT TERMS FABP5, knockout mice, PyMT transgenic mice, breast cancer, protein expression					
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1. INTRODUCTION: Fatty acid binding protein 5 (FABP5) is a small, 14-15 kDa, molecular weight intracellular fatty acid shuttle. FABP5 functions to shuttle long chain fatty acids from the extracellular environment to the nucleus. These fatty acids are then used as ligands for nuclear receptors and activate transcription. FABP5 has been shown to coordinate metabolic and inflammatory responses in adipocytes and may play a role in the development of obesity-mediated breast cancer. Both FABP5 and S100A7 proteins are over-expressed in the inflammatory skin disease, psoriasis. Additionally, there is evidence that these two proteins associate with one another. Previously reported by our lab, S100A7 enhances tumor progression in orthotopic breast cancer mouse models. Taking together the evidence of S100A7 in breast cancer progression and the support that FABP5 is involved in breast cancer development and progression. We have shown overexpression of FABP5 in mammary glands of mice overexpressing mS100a7a15, the mouse homolog of human S100A7. FABP5 was also shown to be expressed higher in S100A7 overexpressing triple negative breast cancer cells compared to vector control. Additionally, FABP5 has higher expression in a highly metastatic breast cancer cell line compared to a low metastatic breast cancer cell line. We determined FABP5 knockout in the host significantly decreases tumor volume in an orthotopic model of breast cancer. We showed that shRNA mediated FABP5 knockdown in triple negative breast cancer cells results in less migration compared to scrambled shRNA. Additional cell lines are being evaluated and used to validate these results. FABP5 expression was examined in a tumor microarray and found that FABP5 expression is correlated with higher tumor grade and triple negative breast cancer. Additionally, high FABP5 protein expression is associated with shorter disease free recurrence. The role of FABP5 in obesity and breast cancer is currently being studied using an orthotopic breast cancer mouse model and high fat diet treatment.

2. KEYWORDS: FABP5, knockout mice, PyMT transgenic mice, breast cancer, protein expression

3. OVERALL PROJECT SUMMARY: Our lab developed bi-transgenic mice expressing MMTV-Tet-O/rtTA-mS100a7a15 transgene, that upon treatment with doxycycline will overexpress mS100a7a15 in the mammary glands. These mice overexpressing mS100a7a15 developed an obese phenotype when fed doxycycline diet (1 g/kg, Harlan Laboratories) for 3 months (Figure 1). We asked whether FABP5 protein expression was enhanced in the mammary glands of MMTV-rtTA/TetO-mS100a7a15 mice on doxycycline diet (1 g/kg) and normal chow for 3 months. Mammary glands were isolated from MMTV-rtTA/TetO-mS100a7a15 mice fed doxycycline diet or normal chow and protein expression was analyzed from the homogenized mammary glands. The doxycycline treated mice were shown overexpress mS100a7a15 (1:1000, Santa Cruz) compared to untreated mice (Figure 2). In addition doxycycline treated mice expressed higher levels of FABP5 (1:1000, R & D Systems) in the mammary glands of mS100a7a15 compared to untreated mice (Figure 2).

Single cell progeny (SCP) cell lines derived from MDA-MB-231 triple negative breast cancer cells were analyzed for FABP5 protein expression. The highly metastatic SCP2 cells showed high expression of FABP5, the low metastatic SCP6 cells showed a decrease of FABP5 protein expression. SCP6 cells stably overexpressing S100A7 showed an increase in FABP5 protein expression compared to parental SCP6 cells (Figure 3). This supports the *in vivo* data from mouse mammary glands. It also leads to an excellent *in vitro* breast cancer cell line system to study the effects of FABP5 on breast cancer cell function and mechanism. In this proposal, SCP2 and SCP6 cell lines will be used to alter the expression of FABP5 and study the role it plays in processes of the cancer cell. Stable cell lines of SCP6 cells over-expressing FABP5 are

being generated and the FABP5 over-expressing construct (pIRES-FABP5-GFP) has been confirmed in SCP6 cell lines (Figure 3).

FABP5 knockdown cell lines (FABP5 Human shRNA, Origene) have been established in the single cell progeny from MDA-MB-231 breast cancer cell lines, SCP2 (Figure 4). Additionally, single cell clones were picked and validated for FABP5 knockdown through western blotting techniques (Figure 4). Migration capacity of FABP5 shRNA and FABP5 scrambled cell lines was assayed and found that FABP5 knockdown significantly decreases migration capacity of the cell line when compared to scrambled cells. 25,000 cells were allowed to migrate for twelve hours in a Boyden Chamber (Corning) in the presence or absence of 0.1% fetal bovine serum. Additional functional assays are ongoing to fully characterize the effect of FABP5 knockdown in these cells. Three other triple negative breast cancer cell lines; MDA-MB-436, MDA-MB-468, and SUM 159 PT, were analyzed for FABP5 expression through western blot (Figure 4). Additional cell lines will also be analyzed through western blotting techniques and FABP5 protein expression will be modified in these cell lines to validate the findings in the SCP2 cell lines.

We examined the effect of FABP5 knockout in the mouse (obtained from Dr. Jill Suttles) on breast cancer progression using an orthotopic PyMT injection model. We injected 1 million PyMT cells (obtained from Dr. Tsonwin Hai) into the fourth mammary gland of FABP5 knockout mice and wildtype mice. Tumors were measured weekly using external calipers. Tumor volume was calculated using the formula $V (\text{mm}^3) = L \cdot W^2 \cdot 0.52$. In a pilot study of n=3 mice per group, FABP5 knockout mice had significantly smaller tumor volumes at the endpoint compared to wildtype mice (Figure 5). Additionally, protein from whole tumor lysates were analyzed through western blot and found that FABP5 was expressed more in the wildtype tumors compared to FABP5 knockout mice, however FABP4 (1:1000, R & D Systems) is expressed in all tumors (Figure 5). From this pilot experiment, we increased our experimental number and repeated this orthotopic breast cancer experiment. Tumor volume was calculated weekly and FABP5 knockout mice had significantly smaller tumors compared to wildtype mice (Figure 6). Additionally, at the end of the experiment, FABP5 knockout mice had significantly smaller tumors compared to wildtype mice (Figure 6). FABP5 knockout MMTV-PyMT mice have been generated and will be used for spontaneous breast cancer mouse studies (Figure 7).

We determined the FABP5 expression in tissue microarrays (TMA) by immunohistochemistry using an anti-FABP5 antibody (1:100, R & D Systems). We obtained TMA from Dr. Shapiro that contains 423 primary breast cancer patient samples with a median follow-up of 8 years and documented pathologic type and grade, demographic, treatment, and disease-free and overall survival patient outcome data (Table 1 and Figure 8). These TMAs contain different stages, grades, ER⁺, ER⁻, TN, and HER2⁺ breast cancer tissue samples. We correlated expression of FABP5 with receptor status, grade, and disease free survival (Figure 8). High FABP5 expression is significantly correlated to high grade tumors (P=0.0036), negative PR status (P<0.0001), negative ER status (P<0.0001), and negative Her2 status (P=0.016). Additionally, high FABP5 expression is correlated with triple negative breast cancer (P<0.0001). FABP5 expression is also significantly correlated to disease free survival (P=0.002) (Figure 8).

To study the effect of high fat diet on tumor development in FABP5 knockout mice, we initiated an orthotopic tumor model mouse study. FABP5 knockout and wildtype (Charles River) mice are being fed high fat diet, 60% calories from fat (Research Diets), for four weeks. Body weight is being measured weekly (Figure 9). One million PyMT cells were injected into the fourth mammary pad of these mice after four weeks of high fat diet. Mice will continue to be fed high fat diet through the duration of the experiment and tumor volume will be analyzed as described

above.

4. KEY RESEARCH ACCOMPLISHMENTS:

Establishment of FABP5 knockout colony and generation of FABP5 knockout/PyMT transgenic mice.

Higher expression of FABP5 was obtained in transgenic mice that showed higher expression of mS100a7a15 in mammary glands. These mice show ductal hyperplasia and become obese.

We have shown higher expression of FABP5 in highly metastatic triple-negative breast cancer cell lines compared to low metastatic cell lines.

Initial studies suggest reduced breast tumor growth in FABP5 knockout mice compared to wildtype.

Generated FABP5 overexpressing triple-negative breast cancer cell lines

Host FABP5 knockout significantly decreased orthotopic PyMT breast tumors compared to wild-type controls

FABP5 shRNA knockdown in triple negative breast cancer cell lines exhibit less migration compared to scrambled control

A panel of triple negative breast cancer cell lines have been evaluated for FABP5 and selected for further validation of FABP5 *in vitro*

FABP5 knockout mice develop significantly smaller tumors compared to wildtype mice in an orthotopic injection model

shRNA FABP5 cell lines were generated and FABP5 knockdown in cell lines exhibit less migration compared to scrambled control

Breast cancer tissue microarray data stained for FABP5 shows FABP5 is highly correlated with tumor grade, receptor status and disease free survival

High FABP5 expression correlates with high tumor grade (P=0.0036)

High FABP5 expression correlates with negative PR status (P<0.0001), ER status (P<0.0001), and Her2 status (P=0.016)

High FABP5 Expression correlates with triple negative breast cancers (P<0.0001)

High FABP5 expression in patient samples is correlated with lower disease free survival

We have shown higher expression of FABP5 in highly metastatic triple-negative breast cancer cell lines compared to low metastatic cell lines.

FABP5 mice on high fat diet exhibit less weight compared to wildtype controls and orthotopic injection model has been initiated

5. CONCLUSION: FABP5 is playing a significant role in breast cancer progression in our orthotopic mouse model as well as *in vitro* cell line data. Excitingly, our data in the mouse and cell lines supports the data from our TMA. High FABP5 in breast cancer patient samples provides an overall worse prognosis with higher grade and worse disease free survival.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project.

(1) Lay Press: Nothing to report

(2) Peer-Reviewed Scientific Journals:

Nasser MW, Qamri Z, Deol YS, Ravi J, Powell CA, Trikha P, Shilo K, Leone G, Bai X-F, Zou X, Wolf R, Yuspa S, Ganju RK. S100A7 enhances mammary tumorigenesis through upregulation of inflammatory pathways. *Cancer Res.* 2012. 72(3):604-15.

Sneh A, Deol YS, Ganju A, Shilo K, Rosol TJ, Nasser MW, Ganju RK. Differential role of Psoriasin (S100A7) in Estrogen Receptor α Positive and Negative breast cancer cells occur through Actin remodeling. *Breast Cancer Research and Treatment* 2013 (In press).

(3) Invited Articles: Nothing to report

(4) Abstracts: Nothing to report

- b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Nothing to report

7. INVENTIONS, PATENTS AND LICENSES:

Nothing to report

8. REPORTABLE OUTCOMES:

Nasser MW, Qamri Z, Deol YS, Ravi J, Powell CA, Trikha P, Shilo K, Leone G, Bai X-F, Zou X, Wolf R, Yuspa S, Ganju RK. S100A7 enhances mammary tumorigenesis through upregulation of inflammatory pathways. *Cancer Res.* 2012. 72(3):604-15.

Sneh A, Deol YS, Ganju A, Shilo K, Rosol TJ, Nasser MW, Ganju RK. Differential role of Psoriasin (S100A7) in Estrogen Receptor α Positive and Negative breast cancer cells occur through Actin remodeling. *Breast Cancer Research and Treatment* 2013 (In press).

The graduate student on this grant successfully defended this project to complete The Ohio State University Graduate School Candidacy Exam. This grant will support the graduate student's thesis and Ph.D.

9. OTHER ACHIEVEMENTS:

Nothing to report



Figure 1-mS100a7a15 overexpression in mammary glands enhances obesity in MMTV-rtTA bi-transgenic mice. Age matched MMTV-rtTA mS100a7a15 bi-transgenic mice were fed with or without Dox diet (1 g/kg) for 6 months. Figure is representative picture of mice showing differences in body weight and visceral fat in presence and absence of Dox.

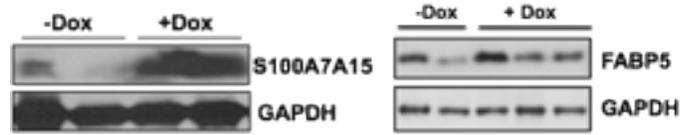


Figure 2-Expression of FABP5 in mS100a7a15 bi-transgenic mouse model. MMTV-rtTA mS100a7a15 mice were fed either doxycycline (+Dox) diet (1 g/kg) or normal chow (-Dox) for 3 months. Protein from mammary glands were isolated and Western blotting performed for indicated antibodies.

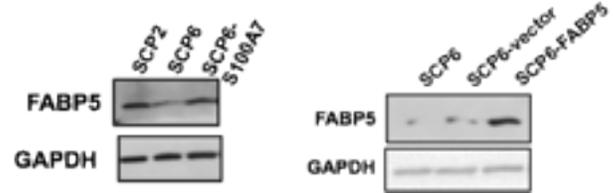


Figure 3-FABP5 protein expression in different cell lines. Cell lysates from SCP2, SCP6, S100A7 overexpressing SCP6 cells, SCP6 vector and SCP6 FABP5 overexpressing cells were immunoblotted for FABP5. Equal loading was observed through immunoblotting for GAPDH.

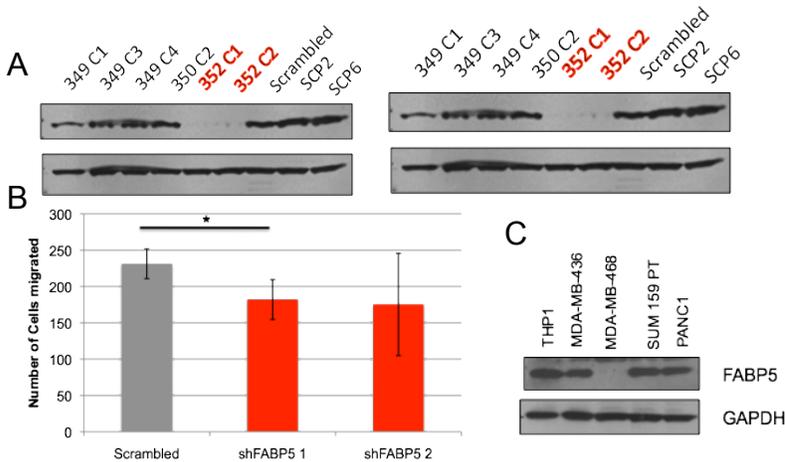


Figure 4-FABP5 knockdown in triple negative breast cancer cell lines, SCP2 and its effect on migration. **A)** Parental (left panel) and clones (right panel) of shRNA FABP5 in SCP2 cell lines. **B)** Migration of shRNA FABP5 SCP2 cell lines compared to Scrambled shRNA. 25,000 cells allowed to migrate for 12 h in the presence of 0.1% FBS. Four replicates were performed. **C)** Expression of FABP5 in triple negative breast cancer cell lines. Western blot FABP5 expression in human monocyte cell line (THP1), three triple negative breast cancer cell lines (MDA-MB-436, MDA-MB-468, SUM 159 PT) and a pancreatic cancer cell line (PANC 1). GAPDH expression was used as a loading control. *P=0.03. Student's t-test

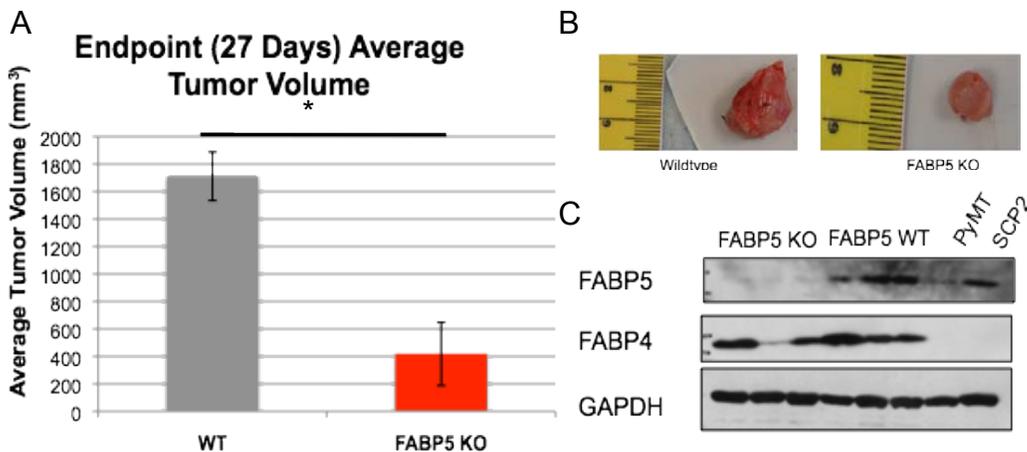


Figure 5-Host FABP5 knockout significantly decreases orthotopic breast tumors. **A)** Endpoint tumor volume of wildtype and FABP5 knockout mice injected orthotopically with 1×10^6 PyMT cells. **B)** Representative image of orthotopic PyMT tumors in wildtype (left panel) and FABP5 KO (right panel) *ex vivo*. **C)** Western blot of FABP5 and FABP4 protein from whole tumor lysate compared to PyMT injection cell line and SCP2 for positive control. GAPDH is referenced as loading control. n=3/group. *P=0.006. Student's t-test.

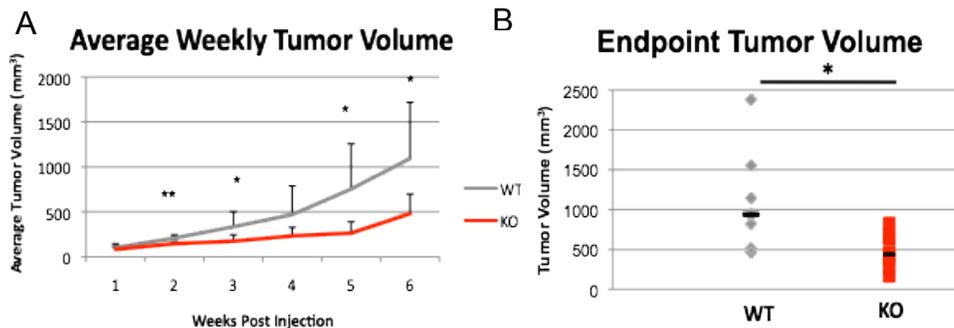


Figure 6-Host FABP5 knockout significantly decreases orthotopic breast tumors. **A)** Average tumor volume was measured weekly for wildtype and FABP5 knockout mice orthotopically injected with 1×10^6 PyMT cells. **B)** Endpoint orthotopic tumor volume of wildtype and FABP5 knockout mice. $n=7$ and $n=8$ for Wildtype and FABP5 KO, respectively. * $P=0.03$, ** $P=0.001$. Student's t-test.

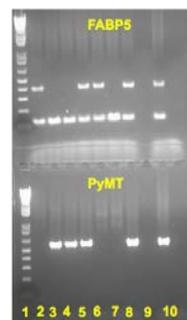
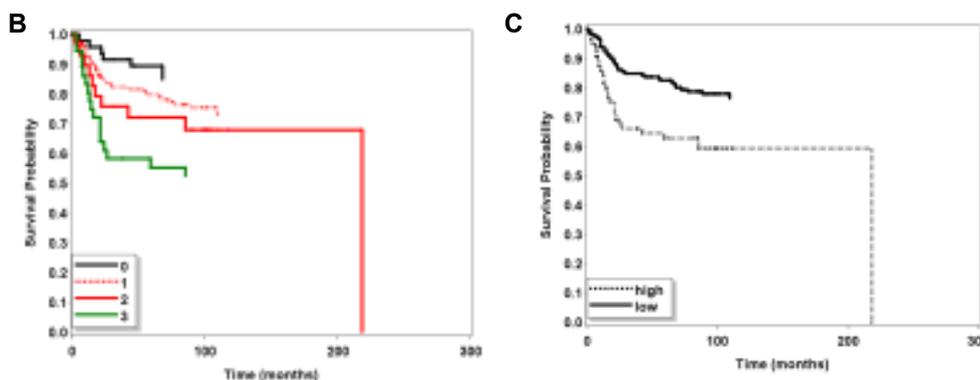
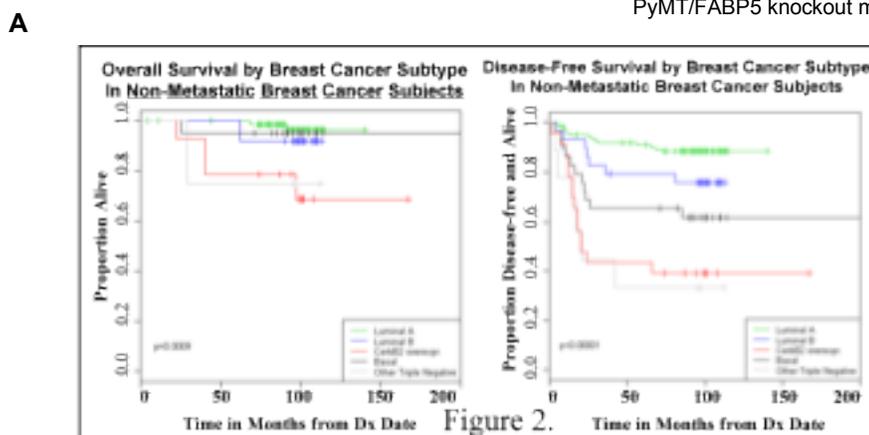


Figure 7-Genotyping of MMTV-PyMT/FABP5 knockout mice. Lane 1 is the ladder, Lane 2-9 MMTV-PyMT/FABP5 pups, Lane 10 is positive control for heterozygous FABP5 (top) and PyMT (bottom). Wild-type FABP5 band is 382 bp and KO band is 120 bp. PyMT band is ~550bp. Lane 3 and 4 indicate MMTV-PyMT/FABP5 knockout mice.

Median age (range)	53 (20-91)
Race/Ethnicity	
White	87%
Black	11%
Other	2%
Post-menopausal	54%
Stage	
I	29%
II	46%
III	13%
IV	6%
Grade	
I	8%
II	41%
III	41%
ER positive	73%
HER2 3+ or FISH+	18%
Triple Negative	11%
Luminal A	42%
Luminal B	11%

Table 1-Demographic profile of SFBCAS tumor microarray.



FABP5 expression correlates with shorter disease free survival in a tumor microarray of 423 patients. **A)** Kaplan-Meier survival curve of overall survival by breast cancer subtype (left panel) and disease free survival by breast cancer subtype (right panel). **B)** Kaplan-Meier survival curve of disease free survival by FABP5 expression level. $P=0.0006$ between 0 (black) and 3 (green). **C)** Kaplan-Meier survival curve of disease free survival by dichotomized FABP5 protein expression. $P=0.002$

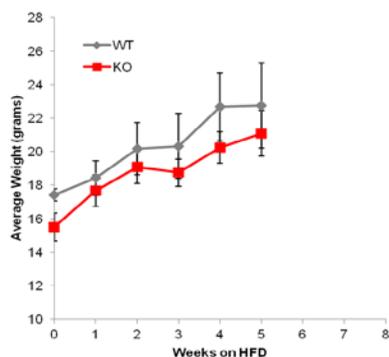


Figure 10-FABP5 KO mice weigh less than wildtype counterparts on high fat diet. Average weight of FABP5 KO and wildtype mice on high fat diet. $n=6$ /group. ** $P<0.001$, * $P=0.02$ Student's t-test

Differential role of psoriasin (S100A7) in estrogen receptor α positive and negative breast cancer cells occur through actin remodeling

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Abstract Psoriasin (S100A7) is a calcium-binding protein that has shown to be highly expressed in high-grade ductal carcinoma in situ (DCIS) and a subset of invasive breast cancers. However, its role in invasion and metastasis is not very well known. In this study, we have shown that S100A7 differentially regulates epidermal growth factor (EGF)-induced cell migration and invasion in ER α ⁻ MDA-MB-231 cells and ER α ⁺ MCF-7 and T47D breast cancer cells. Further signaling studies revealed that S100A7 enhances EGF-induced EGFR phosphorylation and actin remodeling that seems to favor lamellipodia formation in ER α ⁻ cells. In addition, S100A7 overexpression enhanced NF- κ B-mediated matrix metalloproteinase-9 (MMP-9) secretion in MDA-MB-231 cells indicating its role in

enhanced invasiveness. However, S100A7 overexpression inhibited migration and invasion of MCF-7 cells by inactivating Rac-1 pathway and MMP-9 secretion. Moreover, S100A7 overexpressing MDA-MB-231 cells showed enhanced metastasis compared to vector control in in vivo nude mice as detected by bioluminescence imaging. Our tissue microarray data also revealed predominant expression of S100A7 in ER α ⁻ metastatic carcinoma, especially in lymph node regions. Overall these studies suggest that S100A7 may enhance metastasis in ER α ⁻ breast cancer cells by a novel mechanism through regulation of actin cytoskeleton and MMP-9 secretion.

Keywords Breast cancer · S100A7 · Estrogen receptor- α · Actin remodeling · MMP-9

Amita Sneh and Yadwinder S. Deol contributed equally to this study.

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Abbreviations

S100A7	Psoriasin
ER α	Estrogen receptor α
EGF	Epidermal growth factor
DCIS	Ductal carcinoma in situ
IHC	Immunohistochemistry

Introduction

Psoriasin (S100A7) is a low molecular weight S100 gene family protein, originally isolated from skin psoriatic lesions [1]. The S100 gene family consisting of ~20 members is defined by calcium binding helix–loop–helix structural EF hand motif [2]. Apart from two calcium-binding sites, S100A7 has an additional zinc-binding site [3]. S100A7 expression has been reported in epithelial malignancies such as breast, lung, bladder, skin, esophageal, gastric, and head and neck [4–7]. In breast cancer, S100A7 expression is

highly associated with high-grade ductal carcinoma in situ (DCIS) and invasive carcinoma compared to normal breast tissues [4, 8, 9]. Differential expression of S100A7 in breast cancer was initially observed in primary breast cancer compared to normal tissue [10]. Previous studies have shown that S100A7 is expressed in ~50 % of ER α ⁻ and only ~20 % of ER α ⁺ cases of breast cancer [4, 8, 9, 11–13]. Interestingly, in terms of prognosis, both DCIS and invasive breast cancer forms showed consistent association of S100A7 with ER α ⁻ tumors [4]. Furthermore, S100A7 has been known to modulate tumor growth by activating several signaling pathways, including PI3K, NF- κ B, AP-1, and Jab1 [13–15]. However, recent studies have reported the tumor suppressive effects of S100A7 in ER α ⁺ breast cancer cells [16, 17].

Breast carcinoma is classified based on the expression of three receptors: EGFR, ER, and HER-2. EGFR expression is closely related to ER receptor status and has adverse association to overall patient survival with poor prognosis. One prominent feature of ER α ⁻ tumors, especially triple-negative basal-like subtype, is the expression of EGFR [18]. These basal-like tumors are associated with aggressive histological features, poor prognosis and are extremely difficult to treat. High EGFR expression is also associated with metastatic and invasive form of breast cancer [18]. EGFR activation provokes a plethora of signaling pathways that includes cell proliferation, adhesion and motility and promotes invasion and angiogenesis [19]. Recent studies in our laboratory and others have shown that S100A7 regulates epidermal growth factor (EGF)/EGFR-mediated signaling pathways [13–15]. Studies have also shown that S100A7 and EGFR are associated with ER α ⁻ tumors in a large unselected cohort of breast cancer patients [13].

Actin dynamics and remodeling have been identified as major determinant of metastasis and invasion that are the key basis of most cancer-related deaths. During cell motility, branched network of actin filaments are required to assemble beneath the plasma membrane to consistently progress the cell forward to form lamellipodia [20]. The recruitment of active Rac1, a small Rho GTPase at the leading edge, is itself sufficient for cell extension and further movement [21, 22]. Moreover, the influence of S100A7 in calcium-mediated signal transduction and cellular events through direct interactions with intermediate filaments also implies its role in modulation of the cytoskeleton [2].

Hence, present study investigated the influence of S100A7 on metastatic and invasive abilities of ER α ⁻ and ER α ⁺ breast cancer cells upon EGF stimulation. Our studies showed that S100A7 differentially regulates migration and invasion in ER α ⁻ and ER α ⁺ cells. S100A7 overexpression enhanced EGF-induced migration/invasion in ER α ⁻ cells, while its overexpression inhibited migration/invasion in ER α ⁺ cells. We also showed that S100A7

enhances metastasis in vivo and its predominant expression was observed in ER α ⁻ lymph node metastatic group of breast patient samples. In addition, actin polymerization pathway seems to play an important role in establishing the differential effect of S100A7 in ER α ⁻ and ER α ⁺ breast cancer cells.

Materials and methods

Cells, stable transfections, and antibodies

The vector information and generation of stable clones of MDA-MB-231, MCF7, and T47D breast carcinoma cells with stable vector and S100A7 overexpression used in the present study are as described earlier [16, 23]. Knockdown of p65-NF- κ B was performed using its siRNA transfection (50 nM; Dharmacon) for 72 h using lipofectamine 2000 as per manufacturer's protocol. Antibodies used were mostly from Cell Signaling, GAPDH (Santa Cruz Biotechnology) and Phalloidin-568 (Invitrogen).

Migration and invasion assay

Wound healing assay, chemotaxis assay, and invasion assay were performed and calculated as described previously [15, 16, 23, 24].

Gelatin zymography

This method was used to compare MMP-2 and MMP-9 with gelatinase activity of MDA-MB-231 and MCF-7 cells upon S100A7 overexpression (24 h). Supernatants containing secreted form of MMPs were concentrated using centrifugal filter units (Millipore) and detected using Novex gelatin zymography. Renaturing, developing, and staining steps were followed to visualize active MMP bands according to the manufacturer's instructions (Life technologies).

Western blotting

Western blot analysis was done as previously described [23, 24].

Rac1 activation assay

Activation of Rac1 was determined using the Rac/Cdc42 activation assay kit as per manufacturer's protocol (Millipore). Briefly, cell lysates were incubated with 10 μ g/mL p21-activated kinase 1 agarose beads for 60 min at 4 °C. Agarose beads were collected by centrifugation followed by heat denaturation of samples and Rac1 activation was

evaluated by immunoblotting by anti-human Rac1 antibody.

G-actin/F-actin in vivo biochemical assay

This quantitative assay was performed to determine the relative effect of EGF on filamentous actin (G-actin) versus free globular actin (F-actin) content. Briefly, cells were suspended in F-actin stabilizing buffer and separated from G-actin by ultra-centrifugation as per manufacturer's directions (Cytoskeleton Inc.). The difference in G-actin and F-actin content was examined by western blot using G-actin antibody.

Confocal microscopy

Briefly, treated cells were fixed with 4 % paraformaldehyde at room temperature. Cells were washed with 1× PBS, blocked with 5 % BSA in 1× PBS for 60 min and incubated with Phalloidin-568 overnight at 4 °C. Cells were washed with 1× PBS and mounted using vectashield mounting medium containing DAPI and examined under Olympus FV1000 Filter confocal microscope. Images were acquired with 40× objective and modified using FV10-ASW2.0 software.

Bioluminescent imaging (BLI) and analysis

Nude mice obtained from Charles River Laboratories, were maintained at Ohio State University animal facility under IACUC rules and regulations. Nude mice ($n = 10$) were injected intracardially with MDA-MB-231-luc-D3H2LN-S100A7-luciferase or vector control ($1 \times 10^5/100 \mu\text{L}$) and were weekly assessed for tumor burden (IVIS System 200, Xenogen Corporation). Mice were anesthetized intraperitoneally with 0.15 mg/mL of D-luciferin (PBS) and bioluminescent images were collected between 2 and 5 min post-injection. The light intensity was detected by IVIS camera system, integrated, digitalized, and displayed for relative photon flux as calculated per mouse.

Tissue microarrays (TMA) and immunohistochemical analysis

TMA were obtained from Imgenex (San Diego, CA) and immunohistochemistry (IHC) analysis was performed on paraffin-embedded formalin fixed breast tissue specimens. TMAs were de-paraffinized according to manufacturer's recommendation and immunostained with S100A7 antibody at 1:50 dilution (Imgenex). Vectastain Elite ABC reagents (Vector Laboratories) using avidin DH:biotinylated horseradish peroxidase H complex, 3,3'-diaminobenzidine (Polysciences), and Mayer's hematoxylin

(Fisher Scientific) were used for detection of the bound antibodies.

Statistical analysis

All the experiments were performed at least three to four times to confirm the results. The results were then expressed as mean \pm SD of data obtained from these three or four experiments. The statistical significance was determined by the Student's *t* test and value of $p < 0.05$ was considered significant as denoted by asterisks.

Results

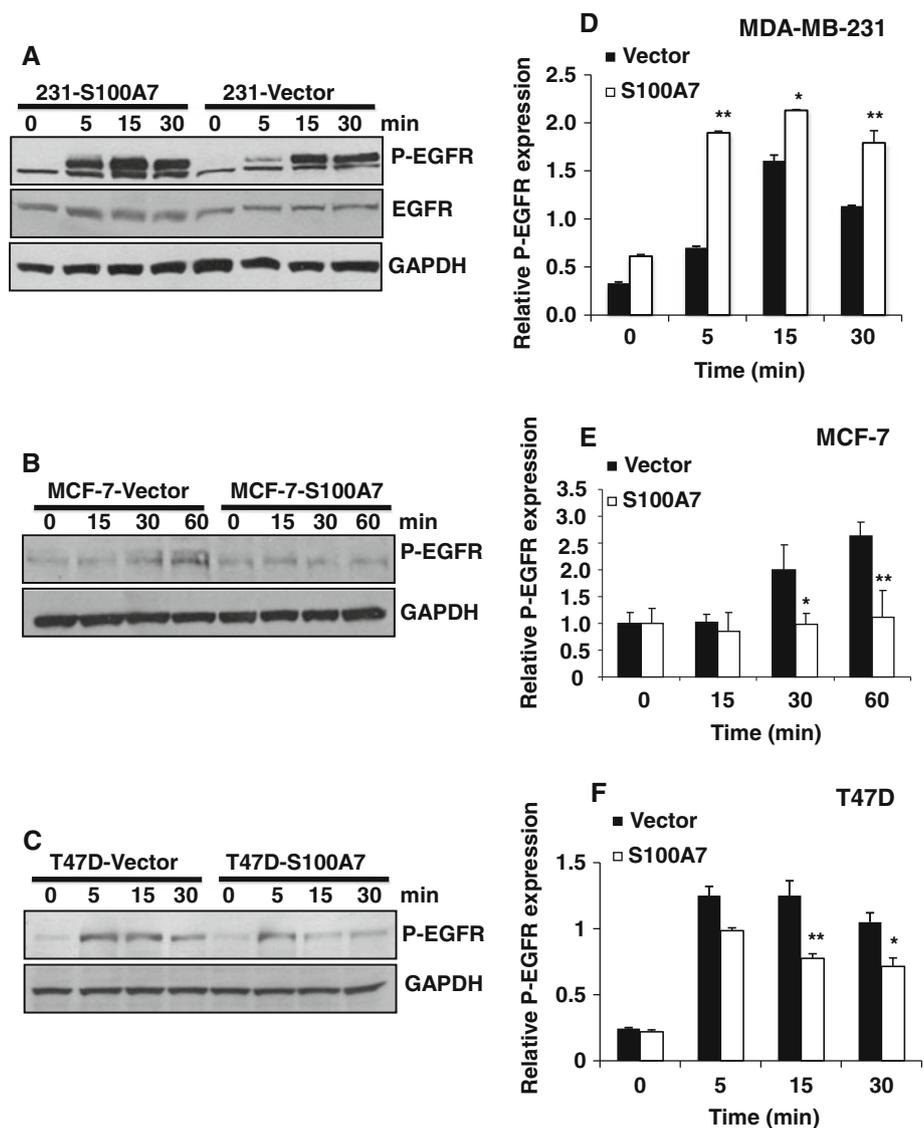
S100A7 overexpression differentially activates EGFR in ER α^- and ER α^+ breast cancer cells

It has been shown that S100A7 downregulation inhibits EGFR-mediated signaling in ER α^- cells [15]. Here, we have analyzed the effect of S100A7 overexpression on EGF-induced receptor activation in ER α^- (MDA-MB-231) and ER α^+ (MCF-7 and T47D) cells by EGFR phosphorylation. We observed an increase in EGFR phosphorylation in S100A7 overexpressing MDA-MB-231 cells upon EGF treatment (Fig. 1a). However, S100A7 overexpression reduced EGF-induced EGFR phosphorylation in MCF7 cells compared to vector (Fig. 1b). In another ER α^+ cell line, T47D, we observed similar results of time-dependent inhibition of EGFR phosphorylation upon EGF stimulation (Fig. 1c). The quantitative analysis of all immunoblots showed consistent increase and decrease in EGFR phosphorylation of S100A7 overexpressing ER α^- and ER α^+ cells, respectively (Fig. 1d–f). Therefore, differential EGFR phosphorylation might play an important role in S100A7 overexpressing ER α^- and ER α^+ breast cancer cells.

S100A7 overexpression affects cell motility of ER α^- and ER α^+ cells

The motile ability of tumor cells determines their metastatic phenotype. In the present study, EGF-induced cell migration was performed to analyze the cell motility of ER α^- and ER α^+ cells upon S100A7 overexpression. The wound healing assay revealed the effect of S100A7 in directional cell migration of ER α^- and ER α^+ cells. The assay showed S100A7 to significantly increase EGF-mediated migratory abilities of S100A7 overexpressing MDA-MB-231 cells (Fig. 2a). We observed significant increase in wound closure of S100A7 overexpressing MDA-MB-231 cells compared to vector control. In contrast, S100A7 inhibited the directional cell migration of ER α^+ MCF-7

Fig. 1 EGF-induced differential phosphorylation of EGFR in ER α^{-} and ER α^{+} breast cancer cells with S100A7 overexpression. EGFR phosphorylation status was analyzed in S100A7 overexpressing ER α^{-} MDA-MB-231 cells (a) and ER α^{+} MCF-7 (b) and T47D cells (c) on EGF stimulation (100ng/mL) by western blotting. The relative EGFR phosphorylation levels in all cells are representative of three independent experiments, calculated with respect to GAPDH for MDA-MB-231, MCF-7, and T47D (d–f) at indicated time. The statistically significant *p* values were indicated as * <0.05 and ** <0.005



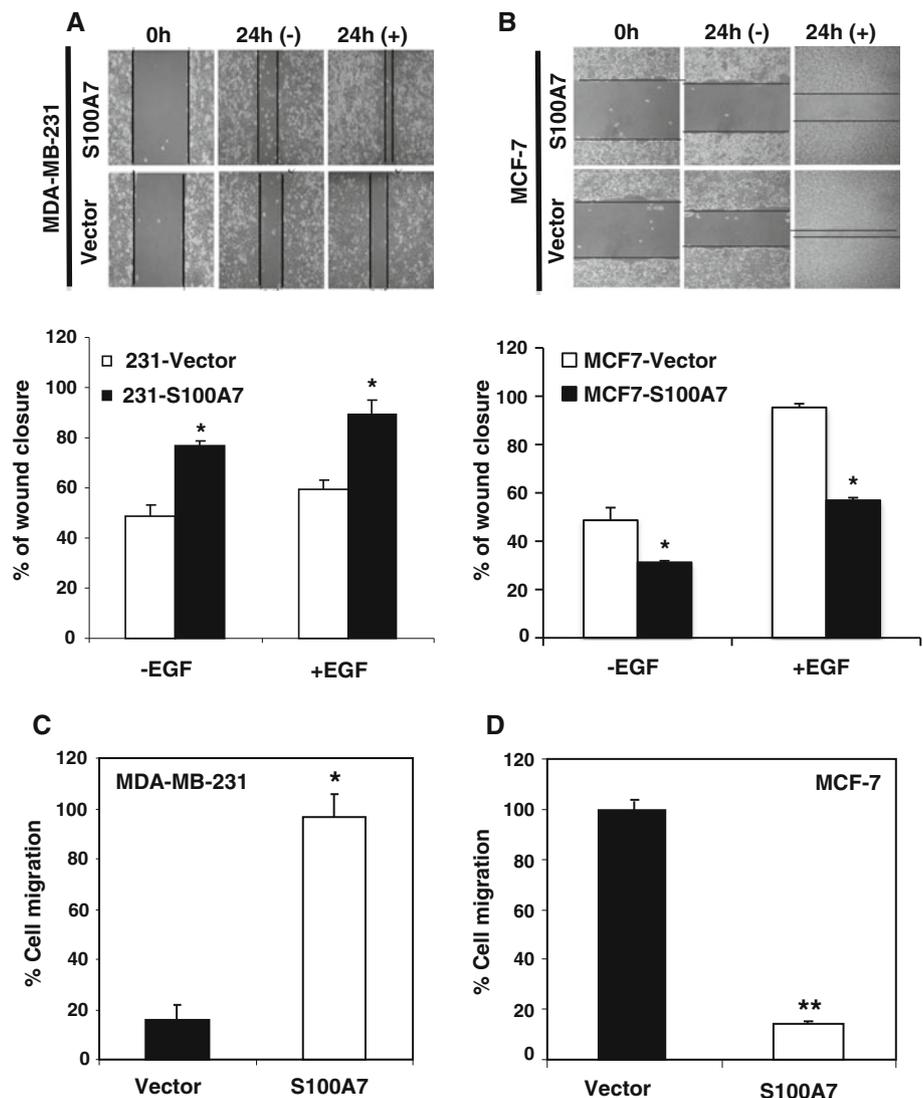
cells by relatively slowing down their wound closure compared to vector cells (Fig. 2b). Moreover, cell migration assay using transwell chambers showed ~five fold increase in EGF-induced migration of S100A7 overexpressing MDA-MB-231 cells compared to its vector control (Fig. 2c). SCP6, a single cell progeny of MDA-MB-231 cells, which has previously been characterized as a low metastatic cell line, was also analyzed to evaluate the effect of S100A7 overexpression on cell migration (Supplementary Fig. 1a) [25]. Importantly, S100A7 was able to promote EGF-induced cell migration in SCP6 as well. However, S100A7 overexpression in MCF-7 cells inhibited EGF-induced cell migration by ~eight fold (Fig. 2d). Similar results were seen on S100A7 overexpression in ER α^{+} T47D cell line, which showed lesser inhibition in cell migration compared to MCF-7 cells (Supplementary Fig. 1b). Taken together, these data reveal the differential

consequence of S100A7 on migratory response of breast cancer cells depending on their ER α status.

Role of MMP-9 activation in invasiveness of S100A7 overexpressing ER α^{-} and ER α^{+} cells

One of the hallmarks of tumor metastasis is its ability to degrade extracellular matrix to invade distant organs. Matrigel invasion assay was performed to analyze the EGF-induced invasion of S100A7 overexpressing ER α^{-} and ER α^{+} cells. We found that S100A7 overexpression has significantly increased EGF-induced invasive ability of MDA-MB-231 cells compared to vector control by approximately twofold (Fig. 3a). However, there was a considerable decrease in invaded population of S100A7 overexpressing MCF7 cells upon EGF stimulation (Fig. 3b). Similar EGF-mediated inhibition of cell invasion

Fig. 2 Effect of S100A7 on EGF-induced cell motility, migration, and invasion of ER α^- and ER α^+ cells. Wound healing assay images represent EGF-induced effect on cell motility of S100A7 overexpressing MDA-MB-231 and MCF-7 cells compared to their vector control (a, b). Their quantitative analysis represent one of the three independent experiments performed with *p* value denoted as $* < 0.05$. Relative migratory potential of S100A7 overexpression in MDA-MB-231 (c) and MCF-7 cells (d) was compared to vector by transwell migration assay, represented as percent cell migration with *P* value as $* < 0.05$ and $** < 0.005$. EGF-induced effects were analyzed at 100ng/mL concentration for cell motility assays



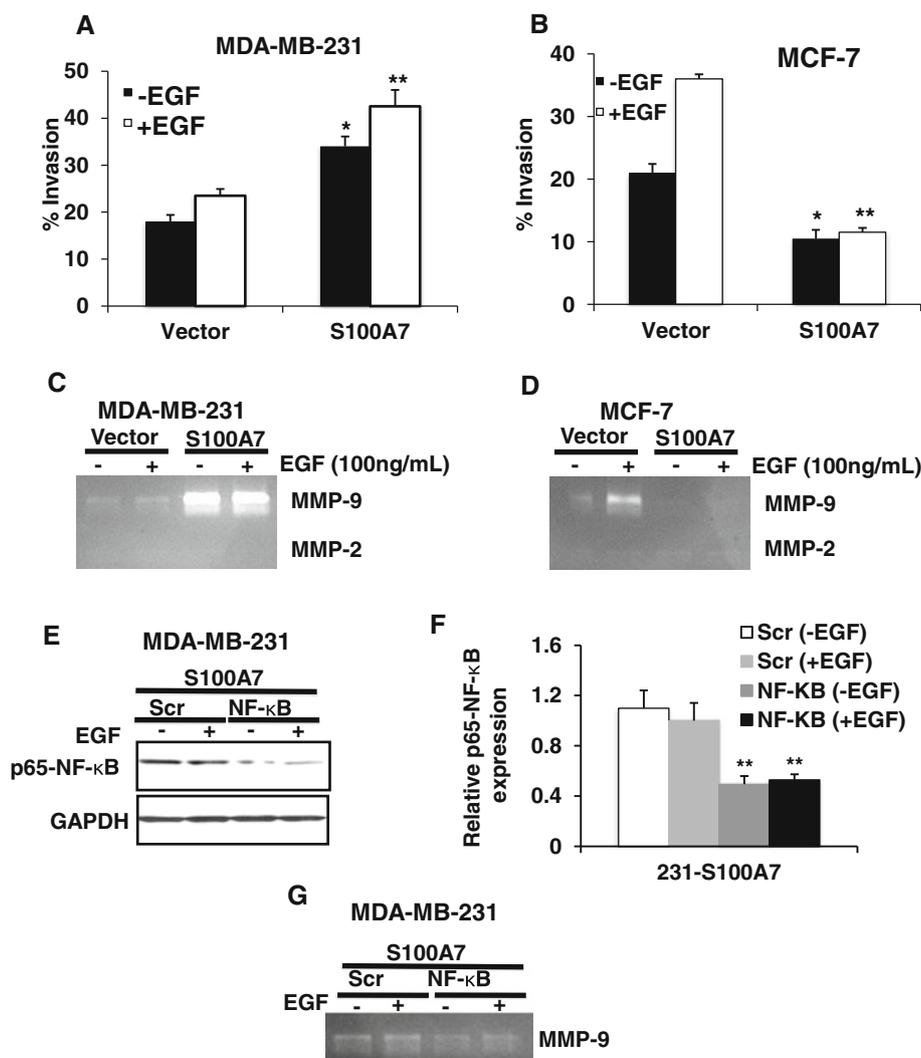
was observed in S100A7 overexpressing T47D cells compared to control cells (Supplementary Fig. 2). The difference in invasion of vector and S100A7 overexpressing cells has been expressed as percentage, which was statistically significant. It is known that cancer cells require matrix metalloproteinases (MMPs) to invade the extracellular matrix underlying their basement membrane and stroma. Hence, we have analyzed the presence of S100A7 expression on activation status of MMP-2 and -9 that have implications in the process of tumor invasion [26]. We observed an increase in active form of MMP-9 secretion in S100A7 overexpressing MDA-MB-231 cells, while its activity was decreased in S100A7 overexpressing MCF7 cells (Fig. 3c, d). However, the MMP-2 activation was not affected in both vector and S100A7 overexpressing MDA-MB-231 and MCF-7 cells. Therefore, diverse MMP-9 activation in S100A7 overexpressing MDA-MB-231 and

MCF7 cells suggests the significance of MMP-9 in S100A7 associated invasiveness.

Role of NF- κ B in S100A7-mediated MMP-9 secretion in ER α^- cells

It is known that NF- κ B binding on MMP-9 gene regulates TNF- α -mediated MMP-9 secretion [27]. It has also been reported that S100A7 promotes pro-survival pathways in ER α^- cells through Akt-mediated NF- κ B activation [14]. Since, S100A7 regulates NF- κ B activation in ER α^- cells, we sought to see the effect of NF- κ B knockdown on MMP-9 secretion in S100A7 overexpressing MDA-MB-231 cells. We observed a significant NF- κ B downregulation in S100A7 overexpressing MDA-MB-231 cells (Fig. 3e, f). Active MMP-9 secretion was significantly reduced in NF- κ B-knocked-down cells compared to scramble siRNA

Fig. 3 Role of NF- κ B in S100A7-mediated MMP-9 secretion in ER α^- and ER α^+ cells. The effect of EGF on relative invasiveness of MDA-MB-231 (a) and MCF-7 cells (b) on S100A7 overexpression was analyzed by matrigel invasion assay and represented as percent invasion with p value <0.05 as * and <0.005 as **. Gelatin zymography revealing the level of MMP-9 secretion in MDA-MB-231 cells (c) and MCF-7 cells (d) upon S100A7 overexpression without any effect on MMP-2 levels. Images represent one of the three independently performed experiments. Knockdown of p65-NF- κ B subunit (e, f) markedly inhibited the MMP-9 secretion of MDA-MB-231 cells analyzed by gelatin zymography (g). Images represent one of the three experiments and their quantitative analyses were statistically significant with p value *<0.05 and **<0.005. Where, EGF used was 100ng/mL and *Scr* stands for scramble siRNA



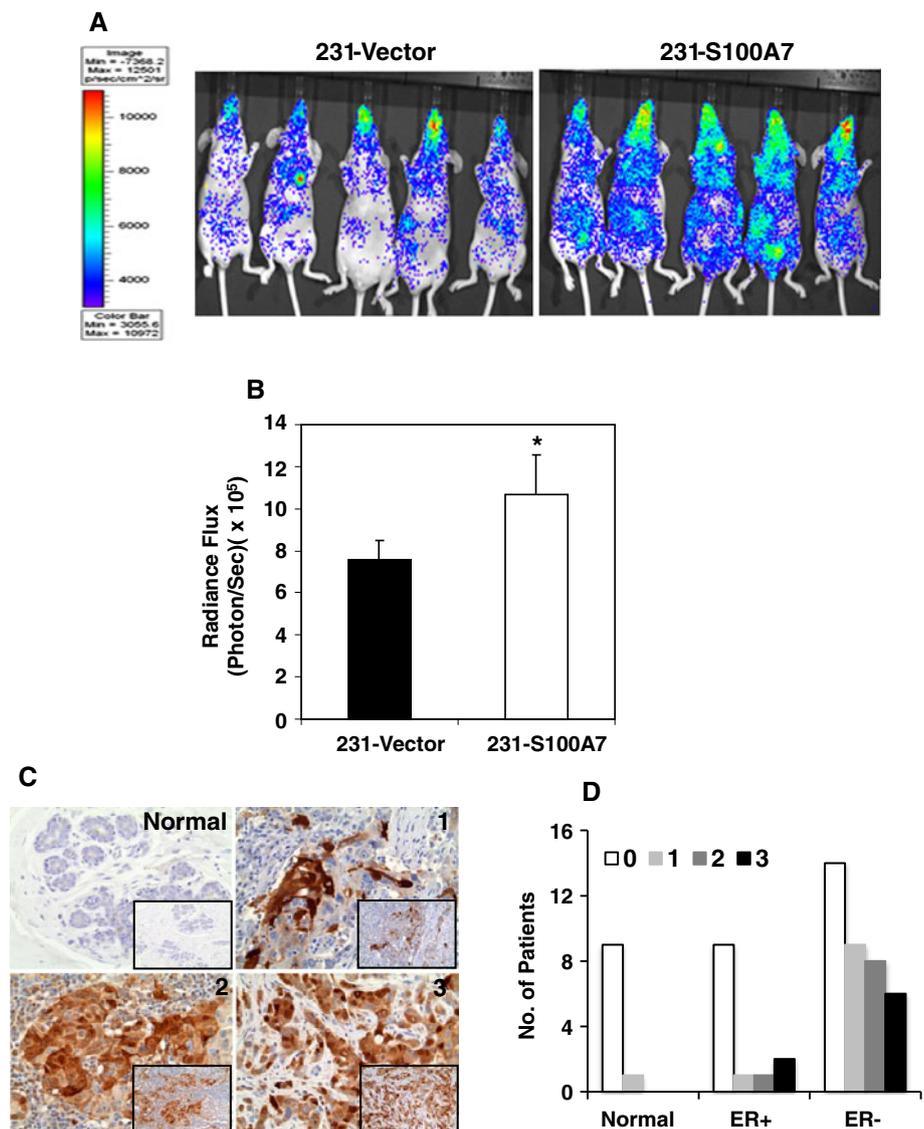
control indicating the direct role of NF- κ B in S100A7-mediated MMP-9 secretion (Fig. 3g).

In vivo metastatic potential of S100A7 overexpressing MDA-MB-231 cells and clinicopathological S100A7 expression analysis in breast carcinomas

Since, S100A7 overexpression in breast cancer cell lines has been shown to enhance tumor growth [12], we investigated its significance in metastasis *in vivo*. In this study, we have used IVIS imaging system to analyze the metastatic potential of S100A7 overexpressing MDA-MB-231 with luciferase reporter gene (Fig. 4a). Higher metastatic progression with elevated radiation flux was observed in intracardially injected nude mice with S100A7 overexpressing MDA-MB-231 cells compared to vector control (Fig. 4b). This demonstrates that S100A7 plays an

important role in promoting metastatic phenotype of ER α^- breast cancer cells. Furthermore, we have analyzed the S100A7 expression in a cohort of breast tissue specimens using tissue microarray ($n = 59$). The TMA and IHC data has been summarized as in Supplementary Table 1. Our study revealed that S100A7 expression was highly prevalent in metastatic tumors, especially at lymph node region (Fig. 4c). Ninety percent of metastatic tumors showed good expression of S100A7, while normal breast tissues ($n = 9$) were devoid of S100A7 protein. Interestingly, S100A7 expressing lymph node metastatic group were mostly ER α negative. In addition, we observed predominant S100A7 expression in ER α^- (~54%) and PR $^-$ (~50%) compared to ER α^+ type (~36%) and PR $^+$ (~44%) among 35 infiltrating ductal carcinoma carcinomas (Fig. 4d). Hence, the considerable role of S100A7 as a regulator of breast cancer metastasis seems to be directly linked to ER α status.

Fig. 4 In vivo metastatic potential of S100A7 in ER α ⁻ cells and its association with ER α status. Representative BLI images show comparative metastases of control and S100A7 overexpressing MDA-MB231-luc-D3H2LN cells in nude mouse model (a), while the statistically significant high radiance flux was observed in the presence of S100A7 with *p* value <0.05 (b). Representative immunohistochemistry images show different levels of S100A7 staining pattern in lymph node metastatic tissue specimens compared to normal tissue, as a negative control (c). Based on clinico-pathologist analysis, the overall S100A7 expression levels were quantitatively represented in ER α ⁻ and ER α ⁺ cells compared to normal breast tissues (d). Where 0, 1, 2, and 3 represent different S100A7 expression levels



Role of S100A7 overexpression on actin polymerization

Actin polymerization is a well-known process that drives cell migration with the most evident feature of lamellipodia formation at leading edges of motile cells. Our immunofluorescence studies showed increased actin accumulation at the leading edges of S100A7 overexpressing MDA-MB-231 cells compared to vector upon EGF stimulation (Fig. 5a). However, there was comparatively lesser actin accumulation at the leading edges of S100A7 overexpressing MCF7 cells compared to vector control. Instead, actin seems to be distributed as small actin-filament structures that became more prominent in vector compared to S100A7 overexpressing MCF-7 cells on EGF treatment (Fig. 5b). Actin accumulation at leading edges might be responsible for differential migratory

response of S100A7 in ER α ⁻ and ER α ⁺ breast cancer cells. Hence, a direct relationship between activity of actin polymerization and formation of migratory structures could be possible.

S100A7 overexpression affect EGF-induced Rac1 activation and associated signaling

In order to investigate the role of actin polymerization pathway in S100A7-mediated differential effect in ER α ⁻ and ER α ⁺ cells, we have analyzed the activity status of Rac1, which is a key molecule in actin polymerization. Rac1 pathway is downstream of LIMK1/2 and mediates its activation through intermediate kinases like PAK1 [28]. We observed that Rac1 activity was enhanced along with LIMK1/2 expression in S100A7 overexpressing MDA-MB-231 cells compared to vector control cells at

Fig. 5 Effect of EGF on actin polymerization of S100A7 overexpressing ER α^- and ER α^+ cells. Immunofluorescence images showing EGF-induced consequences on actin-based cell protrusions of MDA-MB-231 (**a**) and MCF-7 cells (**b**) on S100A7 expression. Images represent one of three independent experiments with phalloidin-568 and DAPI staining as indicated at 100ng/mL of EGF treatment

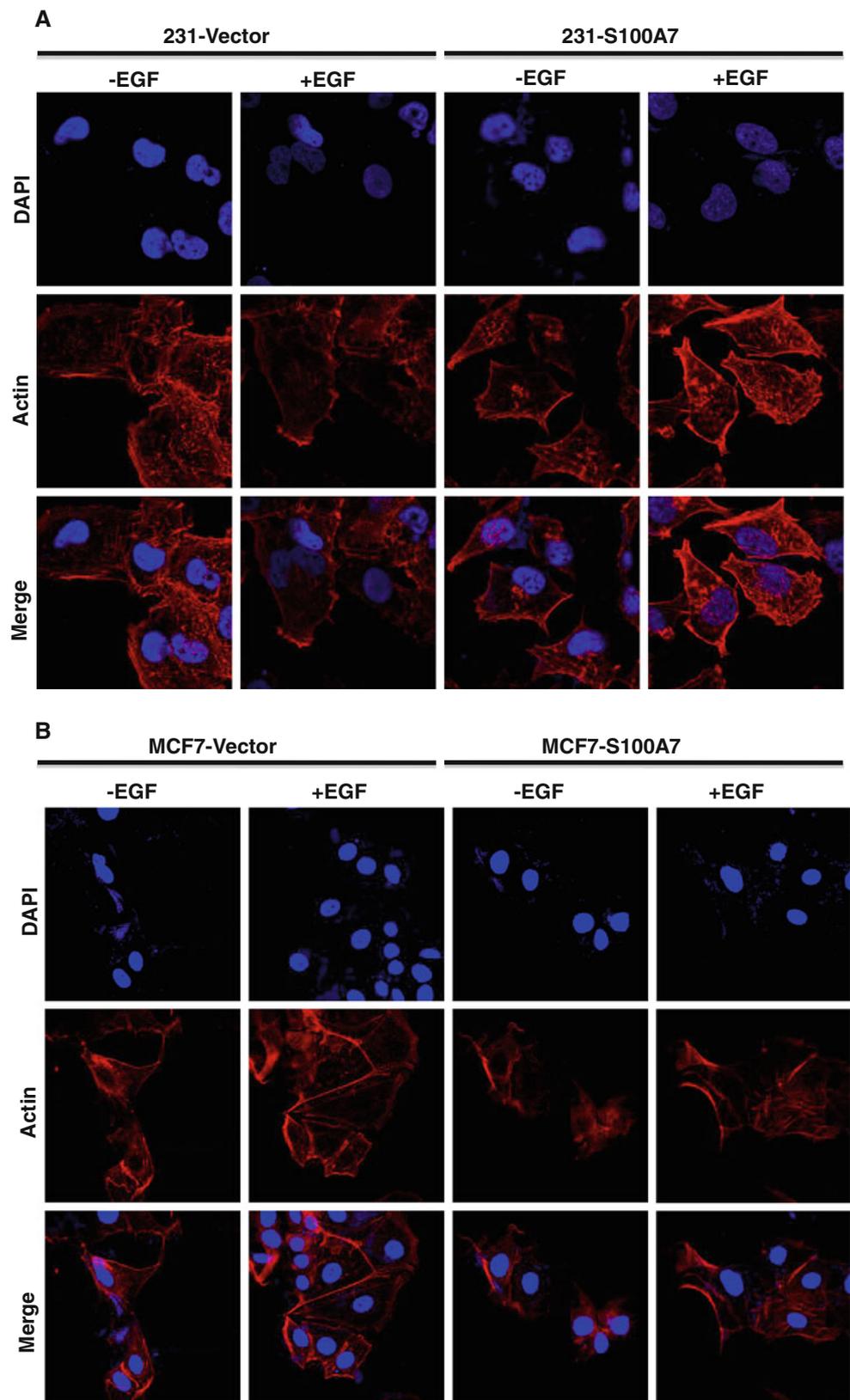
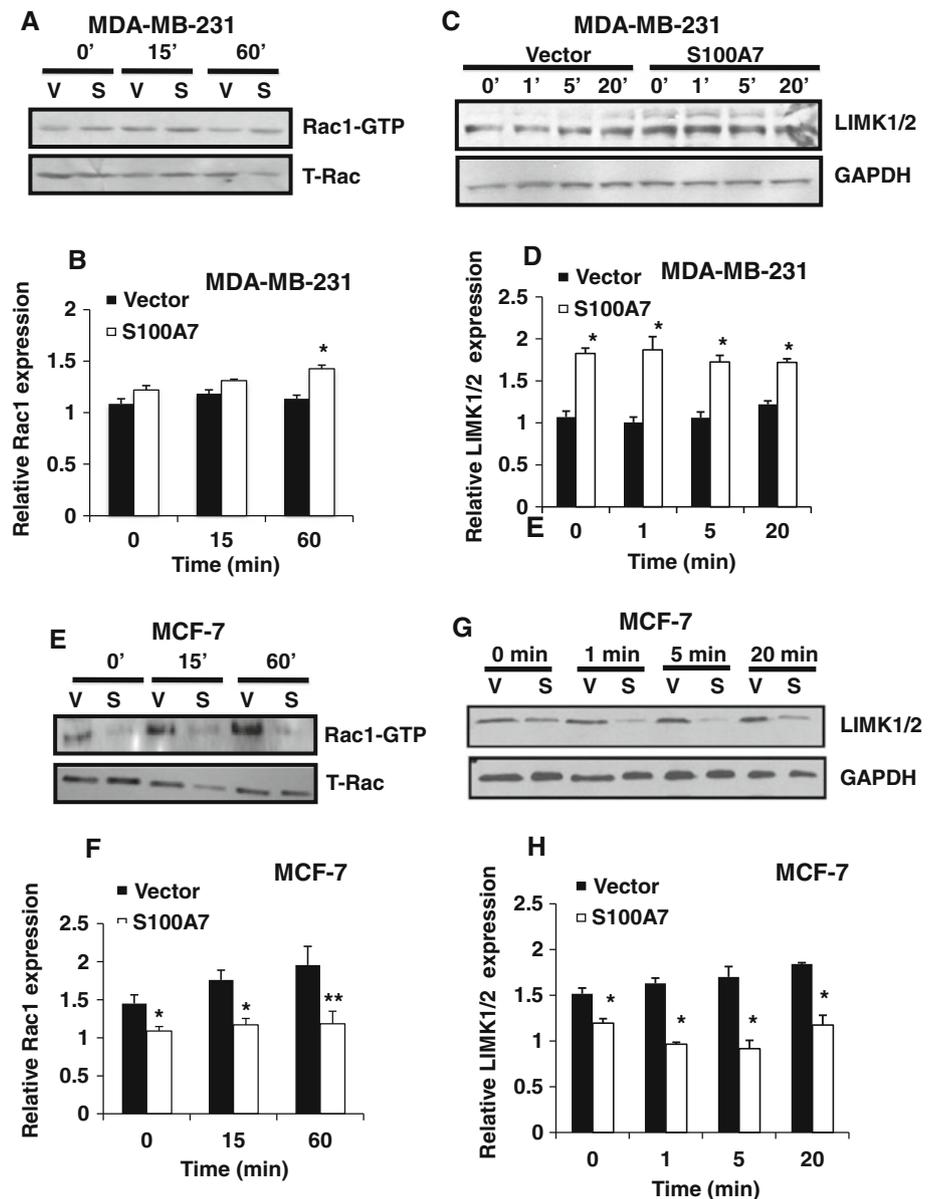


Fig. 6 Rac-1 signaling in EGF-induced cell motility of S100A7 overexpressing ER α ⁻ and ER α ⁺ cells. Actin regulators, Rac-1 and LIMK1/2 activation status were evaluated on EGF stimulation (100ng/mL) in S100A7 overexpressing MDA-MB-231 (a, c) and MCF-7 cells (e, g) by western blot. Their quantitative analysis corresponds to three independent repeats as indicated by *asterisks* * and ** with significant *p* value <0.05 and <0.005, respectively (b, d, f, h). Where, *V* stands for vector and *S* stands for S100A7



all time points (Fig. 6a, c). However, Rac1 activation was inhibited in MCF-7 cells on S100A7 overexpression and even EGF stimulation did not affect its activity suggesting the importance of Rac1 pathway (Fig. 6e, f). We also observed the downregulated expression of LIMK1/2 upon EGF treatment in S100A7 overexpressing MCF7 cells compared to vector control cells (Fig. 6g, h). Consistently enhanced Rac1 activity and LIMK1/2 expression reveal Rac-1 pathway as a modulator of increased migration and metastasis in S100A7 overexpressing MDA-MB-231 cells. However, Rac1 appears to act as a negative regulator in cell migration of S100A7 overexpressing MCF-7 cells.

S100A7 overexpression affects cofilin

Cofilin is one of the downstream molecules of actin polymerization that directly has impact on cell motility through activation of actin-filament dynamics. Cofilin is de-phosphorylated upon Rac1 activation and leads to the polymerization of the F-actin filaments and lamellipodium formation [29]. Our results showed time-dependent decrease in phosphorylation of cofilin in S100A7 overexpressing MDA-MB-231 cells on EGF stimulation compared to vector control cells (Fig. 7a, b). Since p-cofilin is an inactive form of cofilin, its declined level leads to increased actin polymerization–depolymerization and enhanced lamellipodium

formation as migratory structures in S100A7 overexpressing MDA-MB-231 cells seen in our immunofluorescence studies. This effect was not seen on cofilin phosphorylation in MCF7 cells upon S100A7 expression suggesting the effect of inactive Rac1 pathway and cofilin on slow mobility of S100A7 overexpressing ER α^+ cells (Fig. 7c, d). Hence, actin-associated regulatory pathway appears to play an important role in migratory potential of S100A7 in ER α^- and ER α^+ breast cancer cells.

S100A7 regulated EGF-induced actin turnover

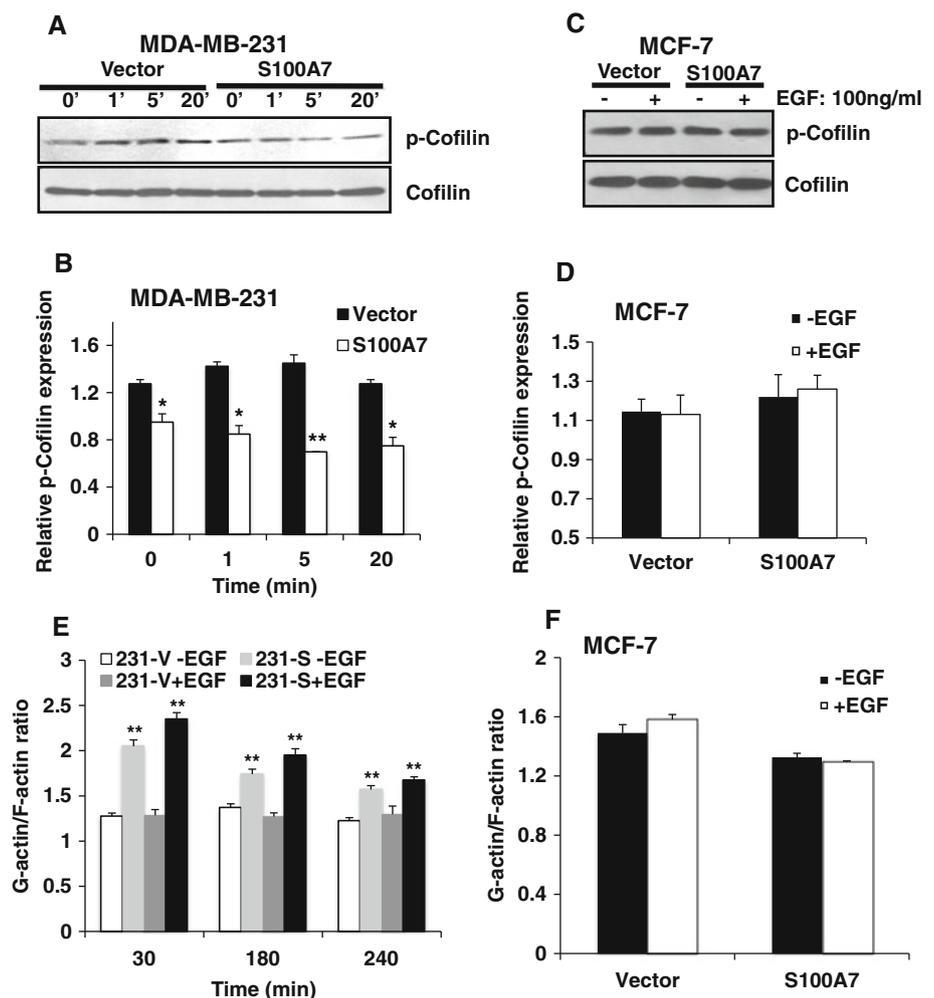
So far, our studies suggest that S100A7-mediated cell motility is controlled by actin polymerization and associated downstream signaling. However, actin-filament subunits (F-actin) need to be recycled back to monomeric forms (G-actin) to maintain further polymerization and motility [30]. Hence, the influence of S100A7 associated actin polymerization on G-actin/F-actin content upon EGF

stimulation was examined by an in vivo assay kit. The assay showed more G-actin content with decreased F-actin quantity in S100A7 overexpressing MDA-MB-231 cells on EGF stimulation (Fig. 7e). The decrease in F-actin and simultaneous increase in G-actin content might be responsible for enhancing ATP based actin recycling during actin polymerization, thus significantly enhancing actin turnover with time. Moreover, there was no effect of EGF on F-actin and G-actin content on S100A7 overexpression in MCF7 cells (Fig. 7f). The diverse influence of EGF on G-actin/F-actin content and actin turnover in S100A7 overexpressing ER α^- and ER α^+ cells significantly correlated with the activation of actin polymerization pathway.

Discussion

In this study, we report for the first time that S100A7 differentially regulates EGF-induced EGFR phosphorylation

Fig. 7 Role of Cofilin signaling on actin remodeling of S100A7 overexpressing ER α^- and ER α^+ cells. Phosphorylation status of cofilin as a negative actin regulator was assessed to show effect of EGF on S100A7 overexpressing MDA-MB-231 (a) and MCF-7 cells (c). The relative phosphorylation levels appear to be significant with p value $* < 0.05$ and $** < 0.005$ (b, d). Effect of EGF-induced actin regulation in S100A7 overexpressing MDA-MB-231 (e) and MCF-7 (f) cells were quantitatively evaluated by in vivo actin turnover assay as a change in G-actin/F-actin ratio over the indicated time. Where EGF used was 100 ng/mL



and migration of $ER\alpha^-$ and $ER\alpha^+$ breast cancer cells. We observed an increase in EGFR phosphorylation in S100A7 overexpressing $ER\alpha^-$, whereas reduced EGFR phosphorylation was seen in $ER\alpha^+$ cells. The estrogen receptor pathway is known to crosstalk with EGFR pathway and since S100A7 negatively regulates ER, it could be possible that S100A7 may likely inhibit EGF activity in $ER\alpha^+$ cells [13, 16]. However, EGF-induced downregulation of EGFR phosphorylation in S100A7 overexpressing $ER\alpha^+$ cells could also be due to the downregulation of β -catenin/TCF4 pathway shown in previous studies [16, 31]. Therefore, it is reasonable to mention that S100A7-mediated differential EGF receptor activation appears to be regulated through different pathways in $ER\alpha^-$ and $ER\alpha^+$ cells.

Increased invasive and migratory properties are important characteristics of metastatic breast cancer cells. Our previous studies on MVT-1 orthotopic syngeneic bi-transgenic mS100a7a15 mouse model showed enhanced metastasis through M2-macrophage recruitment [23]. EGF/EGFR-axis is known to regulate cell spreading, motility and invasion through extracellular matrix (ECM). In this study, we demonstrate EGF-induced increase in migration and invasion of S100A7 overexpressing MDA-MB-231 cells. In addition, our in vivo nude mouse model study revealed that S100A7 is associated with increased metastatic capacity of $ER\alpha^-$ cells. Furthermore, our studies revealed that S100A7 enhanced NF- κ B-mediated MMP-9 secretion in MDA-MB-231 cells. MMP-9 has been shown to play an important role in breast cancer invasion and metastasis [32]. Consistent with our studies, other S100 gene family proteins also promote tumor metastasis through MMP's activation [33, 34]. Our patient sample data also suggest that S100A7 is widely expressed in metastatic carcinoma, especially in lymph node regions. Interestingly, all S100A7 expressing metastatic samples were $ER\alpha$ negative providing further evidence of S100A7 involvement in $ER\alpha^-$ tumor metastasis. However, S100A7-mediated effects on cell migration and invasion were inhibited in $ER\alpha^+$ breast cancer cells with decreased MMP-9 activity.

Metastasis is a multi-step process which can be driven by several ways such as actin polymerization, cell adhesion, and acto-myosin contraction [35]. Hence, we have analyzed the influence of S100A7 in $ER\alpha^-$ and $ER\alpha^+$ cells on actin polymerization pathway, which has been extensively studied in cancer metastasis. We revealed an increase in actin polymerization in MDA-MB-231 cells and decrease in MCF7 cells on S100A7 overexpression compared to vector control. Furthermore, we have shown that increased actin polymerization in S100A7 overexpressing $ER\alpha^-$ cells is due to more lamellipodia formation at leading edges that is regulated by Rac1 pathway. Rac1 has been shown to control cofilin phosphorylation

through the activity of class II PAKs that is regulated through LIM kinases and other downstream effectors of the Rho family of GTPases, Cdc42, Rac, and Rho [36]. Our results suggest that S100A7 overexpression in $ER\alpha^-$ cells downregulates cofilin phosphorylation with increased LIMK1/2 expression. This can increase the number of barbed ends available during directional cell movement [28, 36]. Therefore, increased LIMK1/2 and cofilin dephosphorylation mediates enhanced directional migration of S100A7 overexpressing $ER\alpha$ cells. However, downregulated LIMK1/2 expression and presence of inactive phosphorylated form of cofilin appears to inhibit local actin polymerization at leading edges and reduced EGF-induced migration in S100A7-overexpressing $ER\alpha^+$ cells. Interestingly, prominent cytoplasmic staining of actin filaments in S100A7 overexpressing MDA-MB-231 cells on EGF stimulation could be explained by increased actin turnover. Our in vivo G-actin/F-actin assay demonstrates that G-actin/F-actin ratio regulates actin turnover, which is maintained by S100A7-mediated activation of Rac1 pathway on EGF stimulation in MDA-MB-231 cells. However, ineffective actin turnover in MCF-7 cells could be due to inhibited actin regulatory Rac1 pathway.

In summary, our proposed model describes the EGF-induced differential role of S100A7-mediated actin remodeling and MMP-9 in $ER\alpha^+$ and $ER\alpha^-$ breast cancer cells (Supplementary Fig. 3). S100A7 was expressed predominantly in lymph node $ER\alpha^-$ metastatic tumors. Its overexpression enhanced in vivo metastasis of $ER\alpha^-$ cells. Furthermore, our studies suggest that S100A7 regulates breast cancer metastasis through a novel pathway by modulating actin cytoskeleton and MMP-9 activation. Since metastasis is the leading cause of cancer-related deaths, S100A7 could be a novel therapeutic target in $ER\alpha^-$ metastatic breast cancer.

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Conflict of interest None.

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S100A7 Enhances Mammary Tumorigenesis through Upregulation of Inflammatory Pathways

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Abstract

S100A7/psoriasin, a member of the epidermal differentiation complex, is widely overexpressed in invasive estrogen receptor (ER) α -negative breast cancers. However, it has not been established whether S100A7 contributes to breast cancer growth or metastasis. Here, we report the consequences of its expression on inflammatory pathways that impact breast cancer growth. Overexpression of human S100A7 or its murine homologue mS100a7a15 enhanced cell proliferation and upregulated various proinflammatory molecules in ER α -negative breast cancer cells. To examine *in vivo* effects, we generated mice with an inducible form of mS100a7a15 (MMTV-mS100a7a15 mice). Orthotopic implantation of MVT-1 breast tumor cells into the mammary glands of these mice enhanced tumor growth and metastasis. Compared with uninduced transgenic control mice, the mammary glands of mice where mS100a7a15 was induced exhibited increased ductal hyperplasia and expression of molecules involved in proliferation, signaling, tissue remodeling, and macrophage recruitment. Furthermore, tumors and lung tissues obtained from these mice showed further increases in prometastatic gene expression and recruitment of tumor-associated macrophages (TAM). Notably, *in vivo* depletion of TAM inhibited the effects of mS100a7a15 induction on tumor growth and angiogenesis. Furthermore, introduction of soluble hS100A7 or mS100a7a15 enhanced chemotaxis of macrophages via activation of RAGE receptors. In summary, our work used a powerful new model system to show that S100A7 enhances breast tumor growth and metastasis by activating proinflammatory and metastatic pathways. *Cancer Res*; 72(3); 604–15. ©2011 AACR.

Introduction

Human S100A7 (hS100A7) is present within the epidermal differentiation complex on 1q21 chromosome (1) and is predominantly expressed in high-grade ductal carcinoma *in situ* (DCIS; refs. 2–6). In addition, its expression is significantly associated with estrogen receptor (ER) α -negative and nodal metastasis in invasive ductal tumors (2, 4–6). Furthermore, hS100A7 expression is associated with increased angiogenesis (7). hS100A7 has been shown to modulate tumor growth by activating several signaling pathways (5, 8–10).

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hS100A7 has also been associated with increased inflammatory cell infiltrates in invasive breast tumors (2) and various inflammatory disorders (2). Cytokines, including oncostatin M (OSM), interleukin (IL)-6, and IL-1, have been shown to induce hS100A7 (10). These cytokines directly or indirectly signal through STAT3 pathways (11, 12). STAT3 has been shown to be constitutively activated in 35% to 60% of human breast cancers (13). Activated STAT3 has also been shown to be associated with increased expression of cytokines, growth factors, matrix metalloproteinases (MMP), and angiogenic factors (12). In addition, STAT3 signaling modulates tumor growth and metastasis by recruitment of tumor-associated macrophages (TAM) to tumors (14, 15). TAMs, which often constitute a major part of leukocyte infiltrates present in the tumor microenvironment, have been shown to enhance the tumor growth and metastasis of various cancers (16, 17). In addition, collaborative interactions of tumors with TAMs have been associated with poor prognosis in breast cancer (16, 18). Studies with mouse models have shown that ablation of macrophages leads to inhibition of tumor progression and metastasis (19–21). Factors produced by tumor cells, especially cytokines/chemokines, activate TAMs, which in turn release factors that stimulate tumor cell proliferation, angiogenesis, and metastasis (17, 20).

Transgenic mouse models of human breast cancer have provided important information about the initiation and progression of breast cancer and thus have emerged as powerful

tools for preclinical research. Phylogenetic analyses have shown the mouse ancestor mS100a7a15 to be most related to S100A7 and S100A15 among the human paralogs (22, 23). mS100a7a15 has been shown to be upregulated in carcinogen-induced mammary tumorigenesis (22). However, the direct functional role of mS100a7a15 in disease progression is not well characterized. In this study, we have generated a novel transgenic mouse model MMTV-rtTA; tetO-mS100a7a15 (MMTV-mS100a7a15) to study the functional significance of mS100a7a15 in breast tumorigenesis. We have used this model to analyze the role of mS100a7a15 in breast cancer growth/metastasis and have shown that mS100a7a15 may enhance tumorigenesis by inducing proinflammatory molecules and recruiting TAMs.

Materials and Methods

Cell culture and transfection

Human breast carcinoma cell line MDA-MB-231 (American Type Culture Collection) and MVT-1 cells derived from MMTV-c-Myc; MMTV-VEGF bitransgenic mice (obtained from Dr. Johnson) were cultured (24, 25). The identity of these cell lines was regularly verified on the basis of cell morphology. cDNA of hS100A7 (OriGene Technologies) and cDNA of mS100a7a15 were subcloned into pIRES2-EGFP (Invitrogen). Cells were transfected with pIRES2-EGFP-hS100A7 or pIRES2-EGFP-mS100a7a15 or pIRES-2-EGFP using Lipofectamine reagent according to the manufacturer's instructions and stable clones were generated using G418 (500 µg/mL).

Cell proliferation

Cell proliferation of hS100A7 or mS100a7a15 overexpressing or vector expressing MDA-MB-231 cells was determined as described (24).

Chemotaxis

The chemotactic assays were carried out using Transwell chambers (Costar 8 µm pore size; ref. 24). Briefly, phorbol-12-myristate 13-acetate (100 ng/mL) THP1-differentiated macrophages (TDM) or murine macrophage RAW264.7 cells (MMR) were serum starved. Top chambers were loaded with 150 µL of 1×10^6 cells/mL in serum-free medium (SFM) and bottom chambers had 600 µL of SFM containing 50 µg of concentrated supernatant obtained from hS100A7- or mS100a7a15-overexpressing or vector-expressing MDA-MB-231 cells. Migrated cells were fixed and documented as described (24).

Western blot analysis

Western blot analysis of lysates was done as described (24).

Microarray analysis

Total RNA was collected from hS100A7-overexpressing or vector expressing MDA-MB-231 cells using TRIzol reagent (Invitrogen). Microarray analysis was done at the Ohio State University (Columbus, OH) core facility using an Affymetrix Microarray gene U133 chip containing 40,000 human genes. The data were deposited in the GEO Expression Omnibus under accession no. GSE32052 (Supplementary Table S1).

Generation of transgenic mice

TetO-mS100a7a15 mice (26) were cross-bred with MMTV-rtTA mice (provided by Dr. Chodosh) to generate bitransgenic MMTV-mS100a7a15 mice. Transgenic littermates were genotyped by PCR using tetO-mS100a7a15 primers (Supplementary Table S1). Female mice were fed with Dox-chow 1 g/kg (Harlan laboratories) and mice fed with normal diet served as controls. All transgenic mice were kept in animal facility of Ohio State University in compliance with the guidelines and protocols approved by the IACUC.

Whole mount analysis of mammary glands

Right inguinal mammary gland #4 were spread on glass slides, fixed and stained overnight with 0.2% (w/v) carmine (Sigma) and 0.5% (w/v) aluminum sulfate (Sigma) as described (27).

Orthotopic injection assay

A total of 1×10^5 /100 µL of murine MVT-1 cells were injected into mammary gland (#4) of transgenic mice. Injected mice were either fed with Dox-chow 1 g/kg for 28 days or normal diet (control). Tumors were measured weekly with external calipers, and volume was calculated according to the formula $V = 0.52 \times a^2 \times b$, where a is the smallest superficial diameter and b is the largest superficial diameter. Orthotopically injected animals were sacrificed 28 days postinjection and tumors were excised and processed (28).

Depletion of macrophages using clodronate liposomes

Clodronate liposomes (clodrolip) were prepared as described (21). Briefly, clodrolip (1.5 mg/kg) was injected intraperitoneally 6 hours after tumor cell implantation and followed by 0.75 mg/kg treatments every 4 days. Control groups received PBS-liposomes at the same time points. The mice were sacrificed 25 days postinjection and tumors were excised and processed.

FACS analysis

For fluorescence-activated cell-sorting (FACS) analysis, freshly prepared single-cell suspension of tumor-infiltrating cells was incubated with anti-F4/80 PE, anti-Cd11b APC, and anti-CD206 Alexa Flour 488 (29). Receptor for advanced glycation end products (RAGE) expression was analyzed by staining with RAGE antibody (Abcam) followed by Alexa Flour 488 antibody. After staining, the cells were analyzed by FACS Caliber using CellQuest software (BD Biosciences).

Immunohistochemistry

Samples from mammary gland and tumors were dissected, fixed in formalin and embedded in paraffin for sections. Standard immunohistochemical techniques were used according to the manufacturer's recommendations (Vector Laboratories) using antibodies against Ki67 (Neomarkers, 1:100), CD31 (Santa Cruz 1:100), Keratin-8 (Troma-1 1:100), mS100a7a15 (custom, 1:250), F4/80 (AbD Serotec, 1:50), arginase1 (Santa Cruz, 1:200), and rabbit anti-mouse inducible nitric oxide synthase (iNOS; Abcam, 1:200) for 60 minutes at room temperature. Vectastain Elite ABC reagents (Vector

Laboratories), using avidin DH:biotinylated horseradish peroxidase H complex with 3,3'-diaminobenzidine (Polysciences) and Mayer's hematoxylin (Fisher Scientific), were used for detection of the bound antibodies.

Reverse transcriptase and real-time PCR

RNA was isolated from cells, mouse mammary gland, and tissues using TRIzol reagent (Invitrogen). Reverse transcriptase PCR (RT-PCR) reaction was carried out using RT-PCR kits (Applied Biosystem). Expression of genes analyzed by quantitative PCR (qPCR) was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18SRNA using the $2^{(-\Delta C_t)}$ method (30). Primers used for RT-PCR and qPCR are listed in Supplementary Table S1.

Statistical analysis

Student *t* test was used to compare different experimental groups. $P < 0.05$ was considered to be statistically significant. For all graphs, *, $P < 0.05$; **, $P < 0.01$.

Results

hS100A7 and mS100a7a15 overexpression induce proliferation and expression of inflammatory cytokines/chemokines

hS100A7 has been shown to be highly associated with ER α ⁻ breast cancers. Therefore, we first analyzed the effect of hS100A7 overexpression on proliferation of the ER α ⁻ MDA-MB-231 cell line using 2 different clones, S1 and S2. hS100A7 expression was confirmed by Western blot (Fig. 1A, left). hS100A7 overexpression significantly enhanced growth in both the clones, compared with vector control (V; Fig. 1A, right). To determine the mechanism by which hS100A7 may enhance tumorigenesis, we carried out microarray analysis and found that hS100A7 overexpression induced high levels of proinflammatory cytokines/chemokines CXCL1, CXCL8, IL-1 α , IL-11, and CSF2 as compared with control (Fig. 1B). The expression of these hS100A7-induced target proteins was further confirmed using qPCR in 2 different clones, S1 and S2 (Fig. 1C).

Phylogenetic analyses have shown that mS100a7a15 is most related to hS100A7 and hS100A15 (22, 23). mS100a7a15 has also been shown to be associated with inflammation (31). Similar to hS100A7, mS100a7a15 overexpression in 2 different clones of MDA-MB-231 (M1 and M2) enhanced proliferation (Fig. 1D, bottom) and expression of inflammatory molecules CXCL1, CXCL8, IL-1 α , IL-11, and CSF2 as compared with vector (Fig. 1E). These results suggest that hS100A7 and mS100a7a15 overexpression enhance growth and upregulate proinflammatory cytokine/chemokine production in breast cancer cells.

mS100a7a15 induces mammary hyperplasia in bitransgenic mice

It has been reported that mS100a7a15 is upregulated during carcinogen-induced mammary tumorigenesis (22). However, to the best of our knowledge, there is no transgenic/knockout mouse model available to study the role of mS100a7a15 in breast tumorigenesis. Very recently, K5-tTA; tetO-mS100a7a15 mice were generated for studying the role of mS100a7a15 in

psoriasis (26). To determine the role of mS100a7a15 in tumorigenesis, we generated an inducible transgenic mouse model by crossing tetO-mS100a7a15 mice with tetracycline-responsive transactivator protein under the murine mammary tumor virus (MMTV-rtTA) promoter mice. In the presence of doxycycline, rtTA protein changes its conformation and binds to tet operator (tet-O) sequences that result in expression of mS100a7a15 in mammary epithelial cells (Fig. 2A). The mice were genotyped with mS100a7a15 and MMTV-rtTA-specific primers (data not shown). Mammary gland derived from MMTV-mS100a7a15 mice that were subjected to Dox-chow (1 g/kg) for 3 months showed mS100a7a15 expression at mRNA levels (Fig. 2B, left). We also observed enhanced mS100a7a15 expression in these mice by immunohistochemistry (IHC; Fig. 2B, right). We further identified the mS100a7a15-overexpressing cells to be of luminal epithelial origin as these cells also express CK8 (Fig. 2B, right). Further morphologic examination of whole mount virgin mammary gland by carmine (Fig. 2C, top) or hematoxylin and eosin (H&E; Fig. 2C, bottom) staining showed ductal hyperplasia in the doxycycline-induced MMTV-mS100a7a15 mice compared with uninduced mice. These findings indicate that overexpression of mS100a7a15 in mouse mammary gland induces hyperplasia.

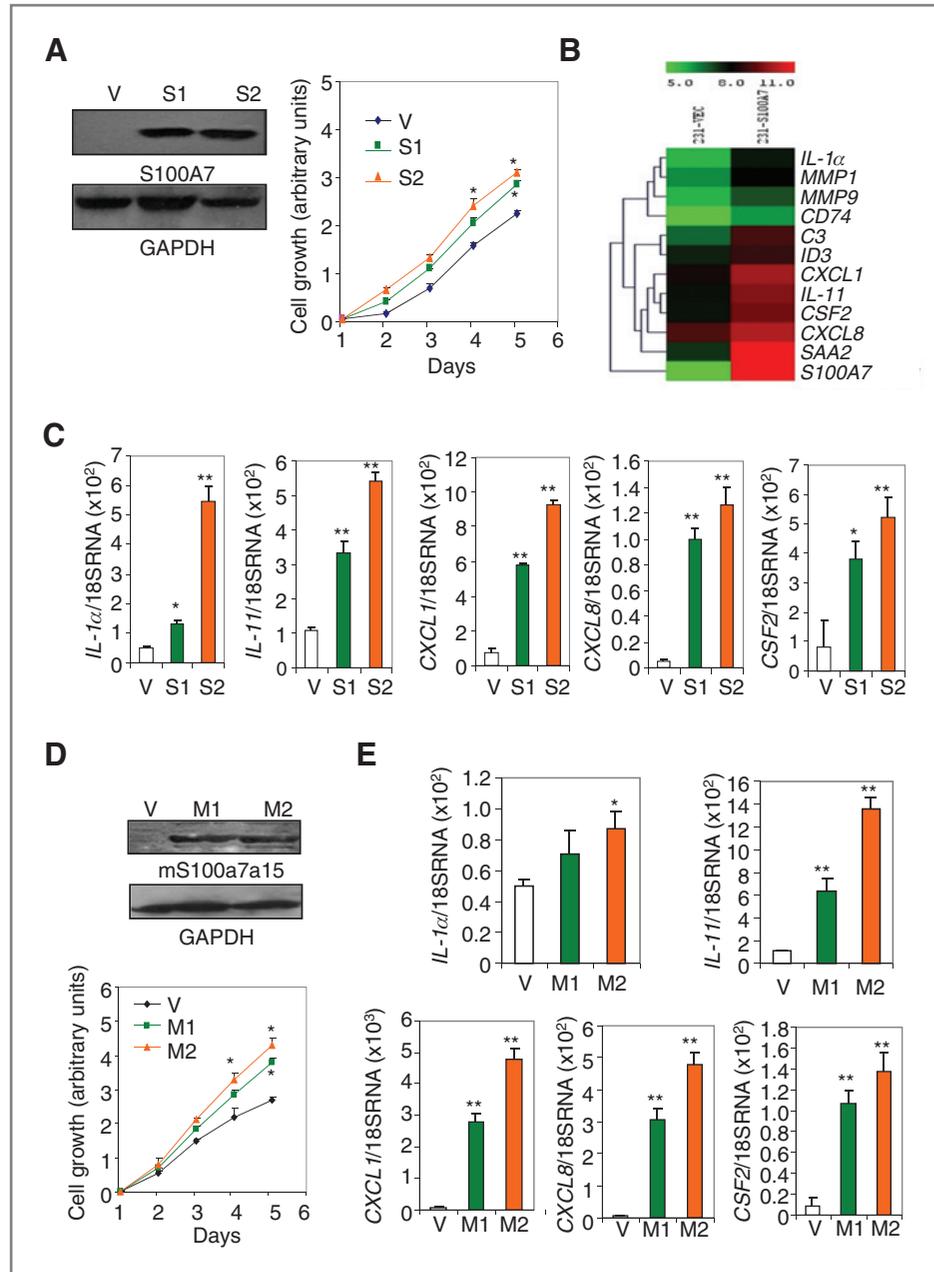
mS100a7a15 overexpression in mammary glands enhances proliferative, inflammatory, and signaling pathways

We analyzed the expression of phospho-STAT3, phospho-AKT, phospho-ERK, and cyclin D1 in mammary gland as these molecules have been shown to be associated with proinflammatory and proliferative responses and are activated in breast cancer tissue (12, 13, 32). We observed enhanced phosphorylation of STAT3, ERK, and AKT in doxycycline-treated MMTV-mS100a7a15 mice (Fig. 2D). We also observed enhanced expression of cyclin D1 by Western blot (Fig. 2D) and expression of Ki67 and cyclin D1 by IHC (Fig. 2E) in doxycycline-induced MMTV-mS100a7a15 mice. Because STAT3 has been shown to enhance macrophage infiltrations to the tumors (12), we further analyzed the recruitment of macrophages in the mammary gland of these mice. We found an increase in macrophages in doxycycline-induced MMTV-mS100a7a15 compared with uninduced mice (Fig. 2E). MMPs are known to degrade extracellular matrix (ECM) proteins in the cellular microenvironment and significant correlation between TAM count and MMP expression has been observed in tumor (33–35). We observed enhanced MMP2 expression in the mammary gland of doxycycline-induced MMTV-mS100a7a15 compared with uninduced mice (Fig. 2D). These data indicate that mS100a7a15 overexpression induces hyperplasia, activates STAT3/AKT/ERK pathways, and enhances the macrophage recruitment.

mS100a7a15 enhances tumor growth in an orthotopic syngeneic breast cancer model

hS100A7 has been shown to increase tumor growth in nude mice (5, 7). We further analyzed the role of mS100a7a15 in tumor progression, by implanting highly aggressive MVT-1 cells (25) into the mammary gland of MMTV-mS100a7a15 mice. Five days prior to injection, mice ($n = 5$) were fed with

Figure 1. Effect of hS100A7 and mS100a7a15 overexpression on proliferation and proinflammatory gene expression. **A**, left, the expression of hS100A7 in 2 different clones of MDA-MB-231 cells (S1 and S2) was analyzed by Western blot using hS100A7-specific antibody. GAPDH was used as the loading control. **A**, right, proliferation of hS100A7-expressing MDA-MB-231 (S1 and S2) and vector control cells (V) was analyzed using MTT assay. **B**, heatmap of differentially expressed genes in MDA-MB-231-overexpressing hS100A7 (231-S100A7) compared with control (231-Vec). **C**, expression of transcripts for indicated inflammatory markers relative to 18SRNA in vector or hS100A7-overexpressing cells (S1 and S2) by qPCR. **D**, top, the expression of mS100a7a15 in 2 different clones of MDA-MB-231 cells (M1 and M2) was analyzed by Western blot using mS100a7a15 antibody. **D**, bottom, proliferation of mS100a7a15-expressing MDA-MB-231 (M1 and M2) and vector cells was analyzed using the MTT assay. **E**, expression of transcripts of inflammatory markers as analyzed by qPCR in mS100a7a15 overexpressing clones M1 and M2. All the experiments were repeated 3 times and representative ones are shown. Graphs represent the mean \pm SD for each experimental group. *, $P < 0.05$; **, $P < 0.01$.



1 g/kg Dox-chow to induce mS100a7a15 and mice maintained on normal diet served as control. These mice were observed for tumor growth (Fig. 3A, left). Interestingly, MVT-1-derived tumor growth was enhanced 2-fold in doxycycline-treated MMTV-mS100a7a15 compared with the uninduced mice (Fig. 3A, middle and right). These studies show that mS100a7a15 expression in mammary gland enhanced growth of breast cancer cells in syngeneic mouse models.

mS100a7a15 overexpression enhances TAM recruitment in a syngeneic mouse model

TAMs have been shown to be a major component of inflammatory infiltrates seen in tumors (18, 20). Initially,

MVT-1-derived primary tumors were evaluated by IHC with macrophage marker F4/80. F4/80⁺ macrophages were enhanced in tumor tissues of doxycycline-induced MMTV-mS100a7a15 compared with uninduced mice (Fig. 3B). We further analyzed macrophage infiltration in the tumors by flow cytometry. As shown in Fig. 3C, the CD11b⁺/F4/80⁺ macrophage infiltration was increased by approximately 42% in doxycycline-induced MMTV-mS100a7a15 compared with uninduced mice. We also analyzed other cell types such as Gr-1, T, and B cells but did not notice any significant increase in the doxycycline-induced MMTV-mS100a7a15 compared with uninduced mice (data not shown).

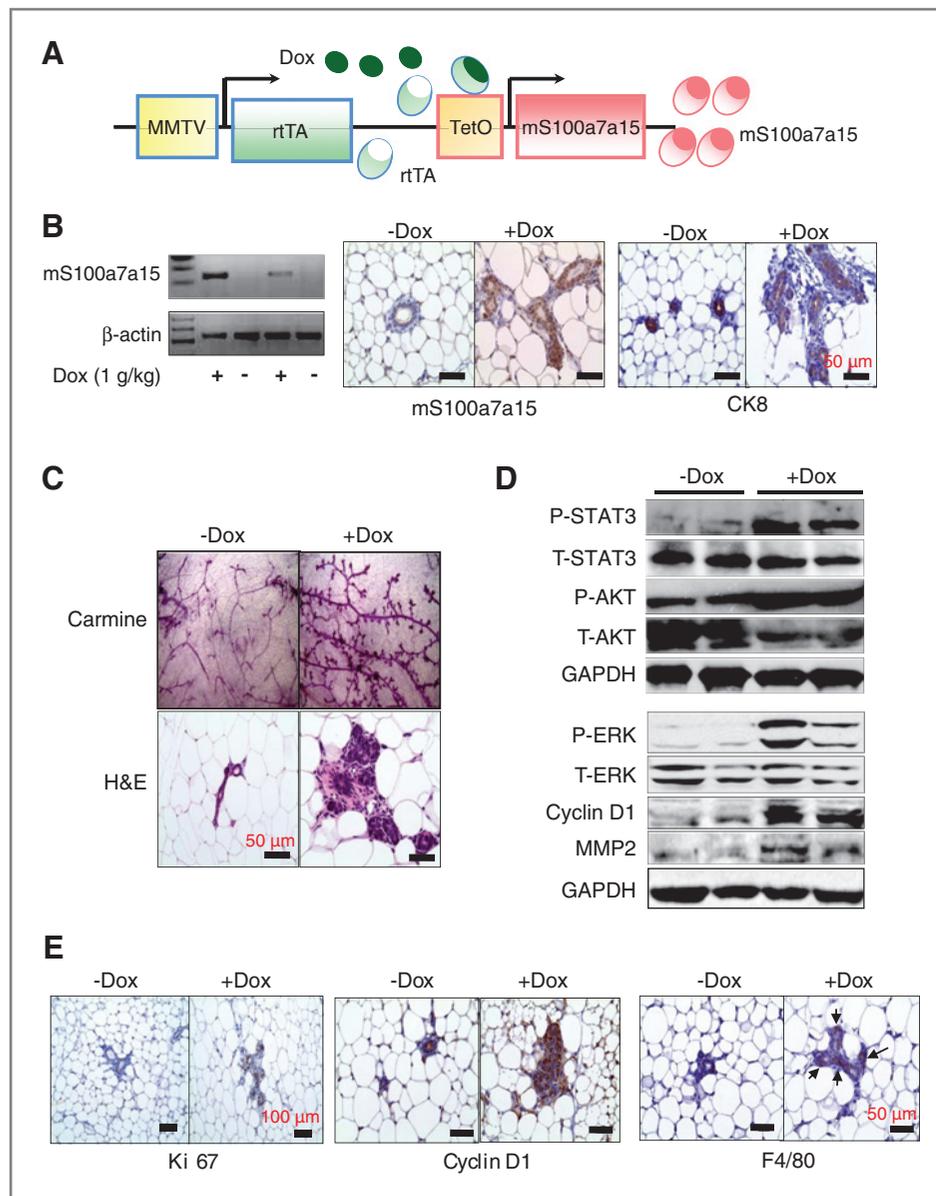


Figure 2. Characterization of the inducible, mammary-specific mS100a7a15 transgenic mouse model. **A**, schematic representation of the inducible, MMTV-mS100a7a15 (Tet-O, tet operator) mouse model system. **B**, left, RT-PCR analysis of mS100a7a15 expression in mammary gland of doxycycline-induced and uninduced mice ($n = 5$). **B**, right, immunohistochemical analysis of mS100a7a15 and CK8 of mammary gland from doxycycline-treated (+Dox) and untreated (-Dox) mice. **C**, top, mammary gland from doxycycline-treated (+Dox) and untreated (-Dox) mice were subjected to whole mount carmine staining (Original magnification of 40 \times) or (**C**, bottom) H&E staining. **D**, mammary gland lysates (50 μ g) from MMTV-mS100a7a15 mice treated with doxycycline or untreated were subjected to Western blot using phospho-STAT3, phospho-ERK, phospho-AKT (P-STAT3, P-ERK, P-AKT), cyclin D1, and MMP2 antibodies. Blot showing anti-GAPDH indicates equal loading of lysates. **E**, mammary gland isolated from doxycycline-treated and untreated ($n = 5$) mice were subjected to IHC of Ki67, cyclin D1, and F4/80. Representative photomicrographs of 5 mammary tissues per experimental group.

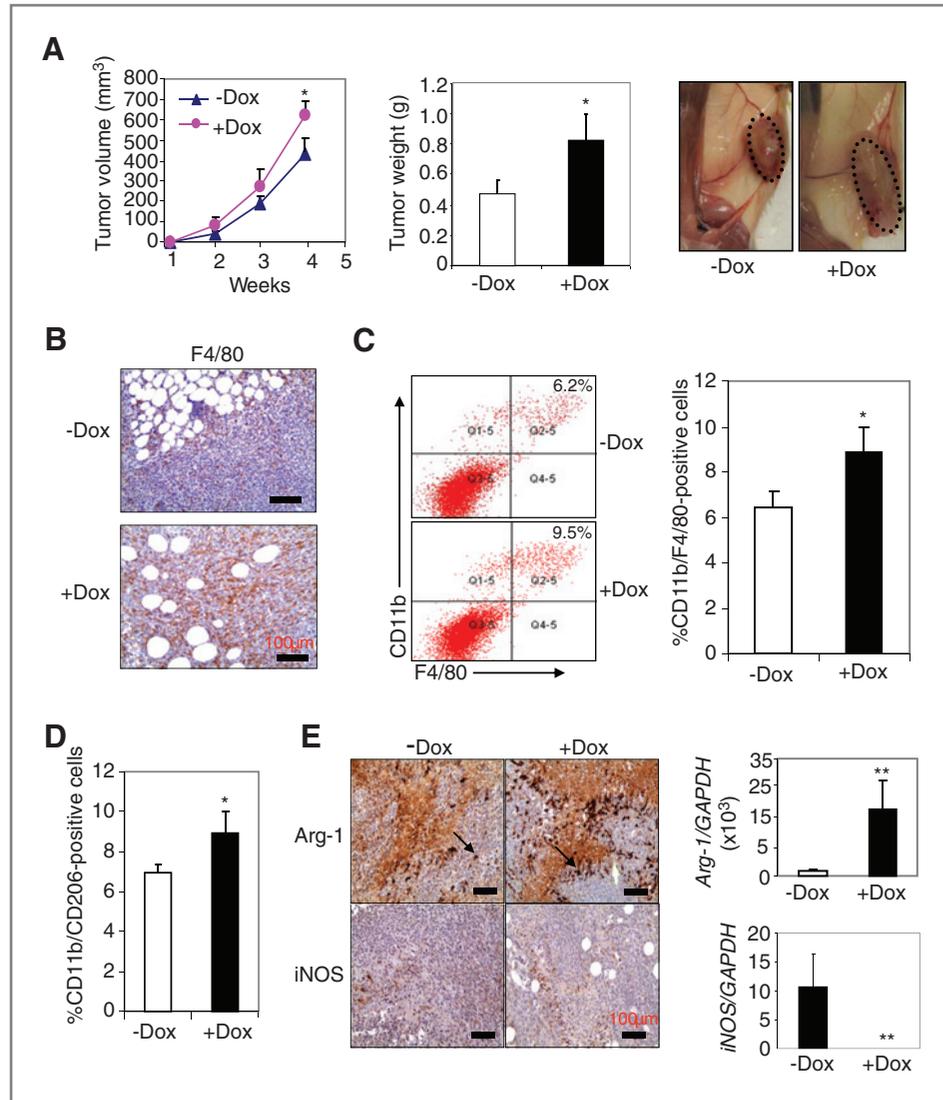
TAMs can be divided into 2 main classes, tumor-suppressive M1 (classically activated) and tumor-promoting M2 (alternative). M1 macrophages are characterized among other factors by expression of iNOS whereas M2 macrophages have a decreased level of iNOS and are identified by their signature expression of arginase-1 (Arg-1) and mannose receptor (CD206; ref. 36). An increase of 29% CD11b⁺/CD206 (M2 TAM) was observed in tumors derived from doxycycline-induced MMTV-mS100a7a15 compared with uninduced mice (Fig. 3D). We further confirmed increased M2 phenotype by IHC for enhanced expression of Arg-1 and decreased iNOS expression (Fig. 3E, left). Changes in expression of *Arg-1* or *iNOS* genes were also detected by qPCR (Fig. 3E, right). These results suggest that mS100a7a15 may

enhance tumor growth by recruiting M2 macrophages to the tumor site.

mS100a7a15 overexpression induces the expression of metastatic and angiogenic markers

We examined the expression of prometastatic and angiogenic genes, such as *CCL2*, *COX2*, *MMP9*, and *VEGF*, in the MVT-1-derived tumors. These genes were significantly upregulated in doxycycline-induced MMTV-mS100a7a15 compared with uninduced mice (Fig. 4A and B). We also observed an approximately 2.7-fold increase in CD31⁺ blood vessels as detected by IHC in doxycycline-induced MMTV-mS100a7a15 compared with uninduced mice (Fig. 4C and D). These studies suggest that mS100a7a15

Figure 3. Effect of mS100a7a15 on tumor growth in orthotopic syngeneic model. A, left, MVT-1 cells were injected into the mammary gland of the MMTV-mS100a7a15 mice ($n = 5$) and tumor volume was measured every week. A, middle, after 28 days, the tumors were excised from mice and weighed. A, right, representative photograph of mice showing tumors dissected from different experimental groups. B, MVT-1 cell line derived tumors from doxycycline-treated and untreated MMTV-mS100a7a15 mice were subjected to immunohistochemical staining for macrophage marker, F4/80. (C) CD11b⁺F4/80⁺ cells and (D) CD11b⁺CD206⁺ were quantified by flow cytometry in disaggregated MVT1 primary tumors harvested 28 days after implantation from doxycycline-treated and untreated MMTV-mS100a7a15 mice. E, right, IHC of Arginase-1 (Arg-1) and iNOS. E, left, expression of *Arg-1* and *iNOS* by qPCR. Data represent the mean \pm SD of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$.



may enhance expression of metastatic and angiogenic markers.

mS100a7a15 overexpression enhances metastasis in orthotopic breast cancer models

We further investigated the role of mS100a7a15 on spontaneous metastasis in MMTV-mS100a7a15 mice injected with MVT-1 cells. We observed a significant increase in surface lung metastases in the mice treated with doxycycline compared with untreated mice ($P < 0.049$; Fig. 5A and B). Because TAMs have been shown to enhance metastasis (17, 18, 20), we further analyzed the infiltrations of macrophages in the lung tissues and observed enhanced expression of F4/80⁺ macrophages (Fig. 5C) and Arg-1 expression but decreased iNOS expression (Fig. 5C) in doxycycline-induced MMTV-mS100a7a15 compared with untreated mice. We also observed a significant increase in prometastatic genes, such as *CCL2* and *VEGF*, in the metastatic lung tissue of doxycycline-induced MMTV-mS100a7a15 compared with uninduced mice (Fig. 5D). These

studies suggest that mS100a7a15 may enhance metastasis through enhancement of prometastatic genes in the metastatic lungs.

Macrophage depletion inhibits tumor growth and angiogenesis

To specifically analyze the role of mS100a7a15 overexpression in TAM recruitment, we selectively inhibited macrophages using clodrolip (liposome-encapsulated clodronate) as previously described (21). Clodrolip treatment significantly reduced tumor growth in MVT-1-derived doxycycline-induced MMTV-mS100a7a15 compared with control liposome-treated mice (Fig. 6A and B). Quantification of the number of F4/80⁺ TAMs and CD206⁺ M2 TAMs by FACS (Fig. 6C) and IHC (Fig. 6D and E left) revealed a significant decrease in TAMs and M2 TAMs in clodrolip treated compared with control liposome-treated mice fed with doxycycline diet. We also observed significant reduction in angiogenesis as detected by CD31⁺ immunohistochemical staining in clodrolip-treated MMTV-

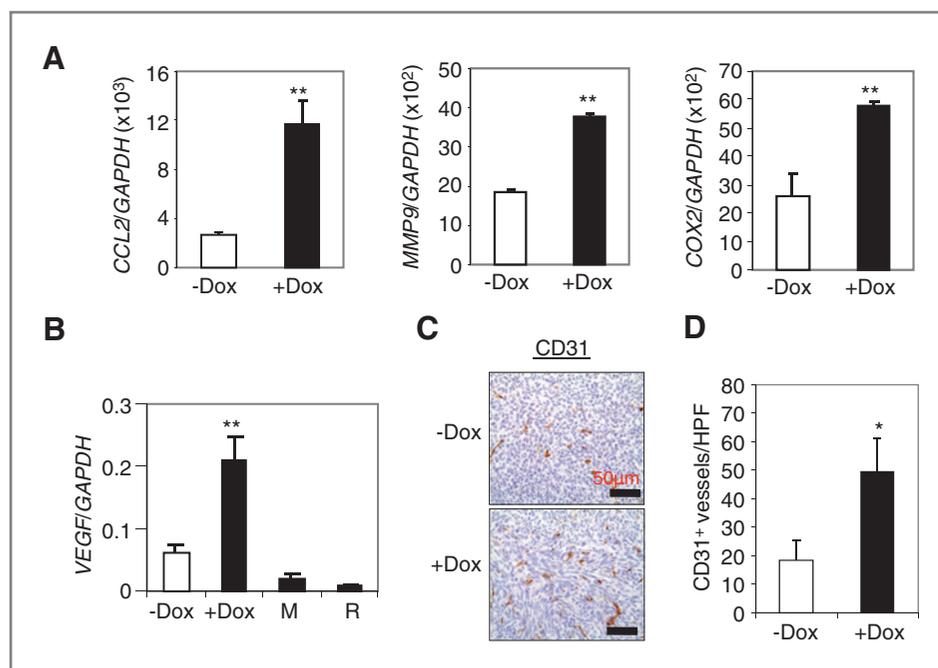


Figure 4. Effect of mS100a7a15 expression on prometastatic and angiogenic markers. A, gene expression was quantified by qPCR in mammary tumors from doxycycline-treated and untreated MMTV-mS100a7a15 mice ($n = 5$). B, VEGF expression in doxycycline-treated and untreated MMTV-mS100a7a15 mice and MVT-1 (M) or RAW264.7 (R) cell lines. C, representative IHC with endothelial marker CD31 antibody to assess the number of blood vessels in tumors from doxycycline-treated compared with untreated mice. D, bars represent the mean \pm SD of the number of CD31⁺ blood vessels shown in (C) counted in 5 random high-power fields (HPF, 20 \times) per tissue section ($n = 5$). *, $P < 0.05$; **, $P < 0.01$.

mS100a7a15 compared with control liposome-treated mice fed with doxycycline diet (Fig. 6D, bottom, and 6E, right). These studies further confirm that mS100a7a15 may enhance tumorigenesis and angiogenesis through recruitment of macrophages.

Soluble hS100A7 and mS100a7a15 enhance chemotaxis in macrophages *in vitro*

Previously, soluble hS100A7 and mS100a7a15 were shown to induce chemotaxis in leukocytes by binding to RAGE (26, 37). However, not much is known about the role of these proteins in regulating monocyte/macrophage chemotaxis. We analyzed the effect of hS100A7 secreted into the conditioned media on chemotaxis of the differentiated monocytic cell line THP-1. hS100A7 expression was observed in the supernatant of hS100A7-overexpressing MDA-MB-231 cells (Fig. 7A, left). We also observed expression of RAGE in TDM (Fig. 7A, right). Furthermore, we observed a significant increase in the chemotaxis of TDM upon stimulation with conditioned media of hS100A7-MDA-MB-231 cells. These effects were significantly abrogated by blocking RAGE (Fig. 7B). We have also shown that RAGE is expressed on the surface of MMR (Fig. 7C). We also observed mS100a7a15 expression in the conditioned media of mS100a7a15-overexpressing MDA-MB-231 cells (Fig. 7C, right). In addition, conditioned media of mS100a7a15-expressing MDA-MB-231 cells enhanced migration of MMR and these effects were blocked by murine RAGE-neutralizing antibodies (Fig. 7D). These studies suggest that hS100A7/mS100a7a15 may enhance monocyte/macrophage chemotaxis through RAGE.

Discussion

hS100A7 has been shown to be associated with the ER α ⁻ phenotype and is predominantly expressed in high-grade DCIS.

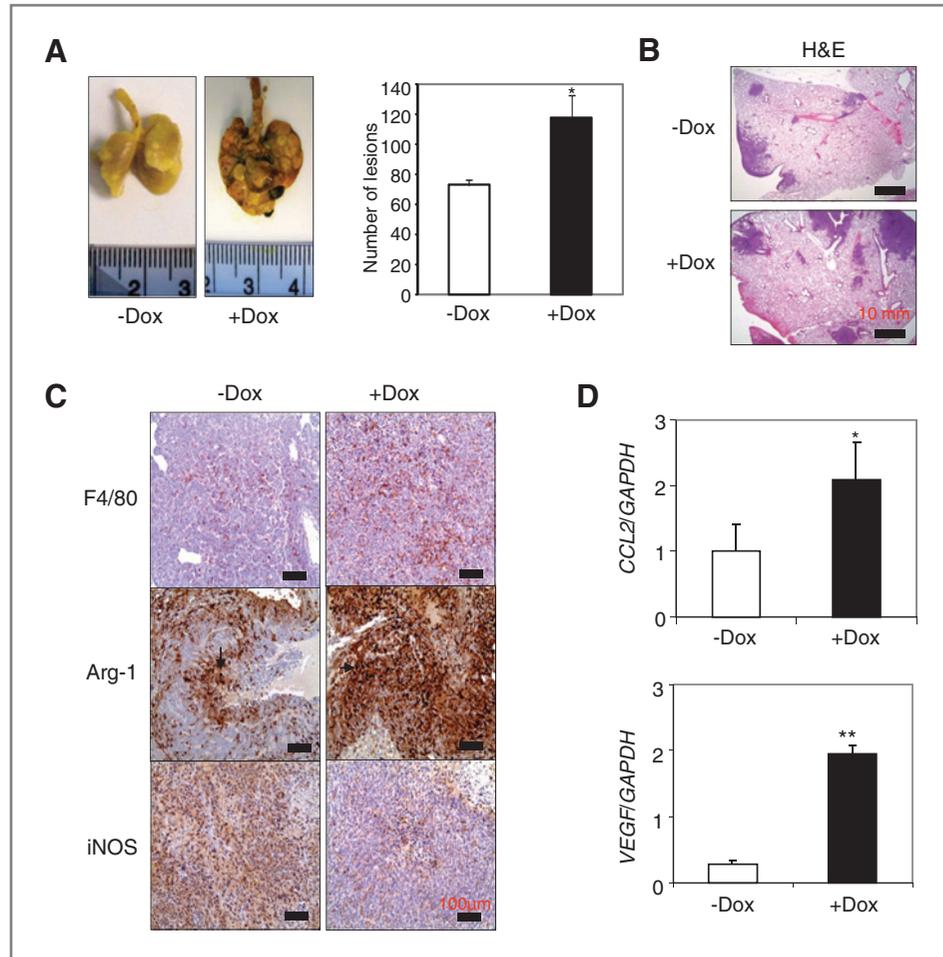
Furthermore, expression of hS100A7 in breast tumors represents a poor prognostic marker and correlates with lymphocyte infiltration and high-grade morphology (2, 6, 7). Although a number of putative functions have been proposed for hS100A7, its biologic role particularly in breast cancer remains to be defined.

In this study, we characterized the tumor-enhancing effects of hS100A7 and mS100a7a15 in MDA-MB-231 breast cancer cells and inducible MMTV-mS100a7a15 mouse model systems. We observed enhanced proliferation and production of proinflammatory molecules IL-1 α , IL-11, CSF2, CXCL1, and CXCL8 in hS100A7 and mS100a7a15-overexpressing cells compared with vector control. These molecules have been shown to play a major role in tumor progression and invasion (38, 39).

In an inducible transgenic mouse model system, we observed a significant increase in the number of primary ducts and side branches in mice expressing mS100a7a15 in mammary epithelial cells. This increase in mammary ductal epithelial hyperplasia was caused by enhanced proliferation as indicated by increased expression of Ki67 and cyclin D1 in the ductal epithelial cells of induced mice. We observed increased expression of STAT3 and MMP2 in mammary gland of inducible mice. Overexpression of cyclin D1 has been reported in up to 50% of primary breast tumors (40). In addition, STAT3 has been shown to be constitutively activated in 35% to 60% of breast cancers (12).

We also showed that mS100a7a15 overexpression significantly increased tumor growth in the syngeneic orthotopic model. Further elucidation of mechanisms revealed that mS100a7a15 may enhance growth and metastasis through recruitment of M2 TAMs. M2-polarized TAMs are known to drive tumor progression by stimulating angiogenesis and metastasis (17, 18, 20). We have shown that M2-specific markers are increased whereas expression of M1 markers is

Figure 5. Effect of mS100a7a15 on metastasis and TAM infiltrations. MVT-1 cells were injected into the mammary gland of the inducible MMTV-mS100a7a15 mice. A, left, representative photographs of metastatic nodules in the lung of doxycycline-treated ($n = 4$) and untreated ($n = 5$) mice. A, right, lungs were removed and inflated with Bouin's fixative, and the number of metastatic nodules on the lungs was counted with the aid of a dissecting microscope (29). B, H&E staining of metastatic nodules in the lung of doxycycline-treated MMTV-mS100a7a15 or untreated mice. C, IHC of F4/80, Arg-1, and iNOS in metastatic lung tissues obtained from doxycycline-treated and untreated MMTV-mS100a7a15 mice. D, expression of *CCL2* and *VEGF* by qPCR. Data represent the mean \pm SD per experimental group. *, $P < 0.05$; **, $P < 0.01$.



decreased in MVT-1-derived tumors and lung tissues of doxycycline-induced mS100a7a15 mice. We further determined whether selective depletion of macrophages would inhibit tumor growth. It has been shown previously that macrophages may be selectively depleted in mice using clodrolip (21). Therefore, we treated MVT-1 tumor-bearing mice with intraperitoneal inoculations of clodrolip or with an empty liposome control at various points throughout tumor progression. We observed approximately 80% depletion of macrophage content of the tumors compared with control liposome-treated tumors in doxycycline-induced MMTV-S100a7a15 mice. We observed that clodrolip-mediated reduction of TAMs also caused dramatic reduction in tumor growth in doxycycline-induced MMTV-mS100a7a15 mice. These results suggest that mS100a7a15 may enhance tumor growth through enhancing recruitment of macrophages to the tumors. Previous studies have reported that an intimate relationship between macrophages and tumor cells is required for tumor growth and metastasis (18, 41). We have shown that hS100A7 and mS100a7a15 enhanced chemotaxis of monocyte/macrophages through RAGE. RAGE expression has been detected in a variety of human tumors including breast (42). It has been shown that the blockade of RAGE in glioma-suppressed tumor growth (43).

Although mS100a7a15 has been shown to enhance CD4-positive T-cell populations in mS100a7a15-overexpressing keratinocytes from psoriasis mouse model (26), we did not observe a significant change in CD4-positive T cells as detected by FACS in tumors derived from our MVT-1 orthotopic syngeneic model. This difference may be attributed to the different model systems used in each study. Another possibility is that the recruitment of macrophages could result from enhanced production of chemokine CCL2 in tumors from doxycycline-induced MMTV-mS100a7a15 mice. CCL2 has been shown to recruit inflammatory monocytes/macrophages that in turn stimulate breast tumor growth and metastasis (44). In breast cancer, macrophage infiltration and CCL2 expression have been correlated with metastatic disease and poor prognosis (45–47).

We also observed significant increase in spontaneous metastasis and M2 TAMs in orthotopic syngeneic MMTV-mS100a7a15 mouse model. Previous studies have shown that TAMs promote metastasis by enhancing prometastatic and proangiogenic activities within the tumor microenvironment (17, 18, 20). We have shown enhanced expression of prometastatic and proangiogenic molecules such as CCL2 and VEGF in metastatic lung tissues. Also, we observed enhanced gene expression of *CCL2*, *VEGF*, *COX2*, and *MMP9* in primary tumors.

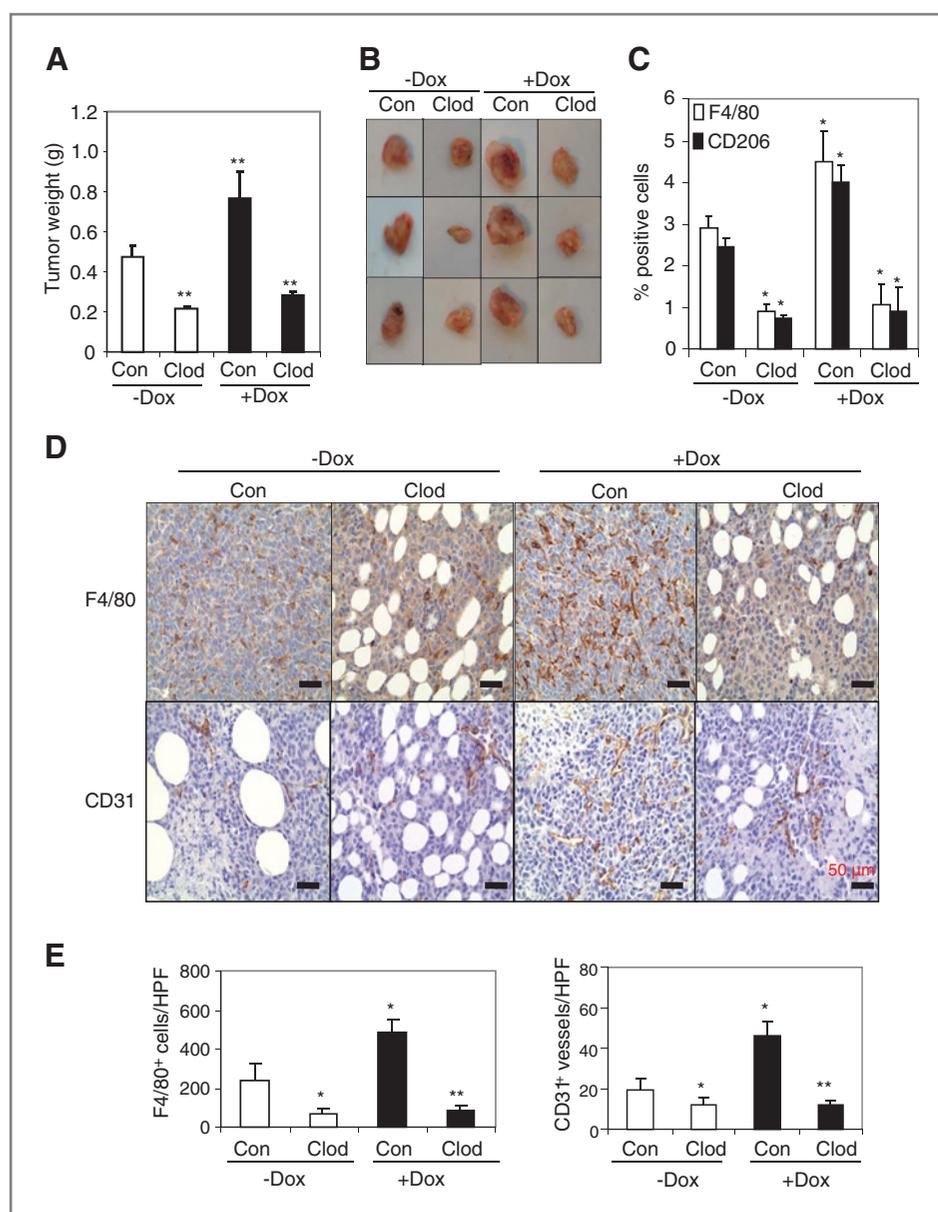


Figure 6. Effect of macrophage depletion on tumor growth and angiogenesis. **A**, growth of MMTV-1-derived tumors in doxycycline-induced or uninduced MMTV-mS100a7a15 mice treated with either clodrolip (Clod) or control liposomes (Con). **B**, representative photograph of tumors derived from different experimental groups. **C**, quantitative analyses of F4/80⁺ macrophages (white columns) or CD206⁺ M2 macrophages (black columns) by FACS. Graphs represent the mean \pm SD (control, $n = 4$; clodrolip, $n = 5$), * $P < 0.05$; ** $P < 0.01$. **D**, representative immunohistochemical staining of mammary tumor sections treated with clodrolip and control liposomes with macrophage marker F4/80 antibody (top) and with endothelial marker CD31 antibody (bottom) to assess the number of macrophages infiltrating into tumors and increase angiogenesis in tumors from doxycycline-treated compared with untreated mice. **E**, bars represent the mean \pm SD of the number of F4/80⁺ macrophages (left) and CD31⁺ blood vessels (right) as shown in (D) and counted in 5 random HPF (20 \times) per tissue section (control, $n = 4$; clodrolip, $n = 5$), * $P < 0.05$; ** $P < 0.01$.

These molecules have been shown to enhance metastasis of various cancers (33, 44, 48–50). Previously, it has been shown that hS100A7 modulates VEGF expression in MDA-MB-468 cells (7). These studies suggest hS100A7 which has been shown to be associated with highly invasive breast cancer subtypes (31) may enhance metastasis through enhancement of prometastatic and angiogenic molecules.

In summary, using novel mS100a7a15 transgenic and orthotopic syngeneic mouse models, we have shown that mS100a7a15 overexpression in mammary epithelial cells enhances hyperplasia, tumor growth, angiogenesis, and metastasis. As shown in model (Supplementary Fig. S1), our studies for the first time revealed that hS100A7/mS100a7a15 produced by epithelial cells may enhance proliferation and recruit TAMs to tumor site by endocrine mechanism through RAGE activa-

tion. Recruitment of TAMs into tumor microenvironment may in turn stimulate tumor growth and metastasis by enhancing expression of prometastatic and proinflammatory molecules such as CCL2, COX2, MMP9, and VEGF. Thus, these studies suggest that S100A7 may enhance tumor growth and metastasis especially in ER α ⁻ tumors through a novel mechanism by activating proinflammatory and metastatic pathways.

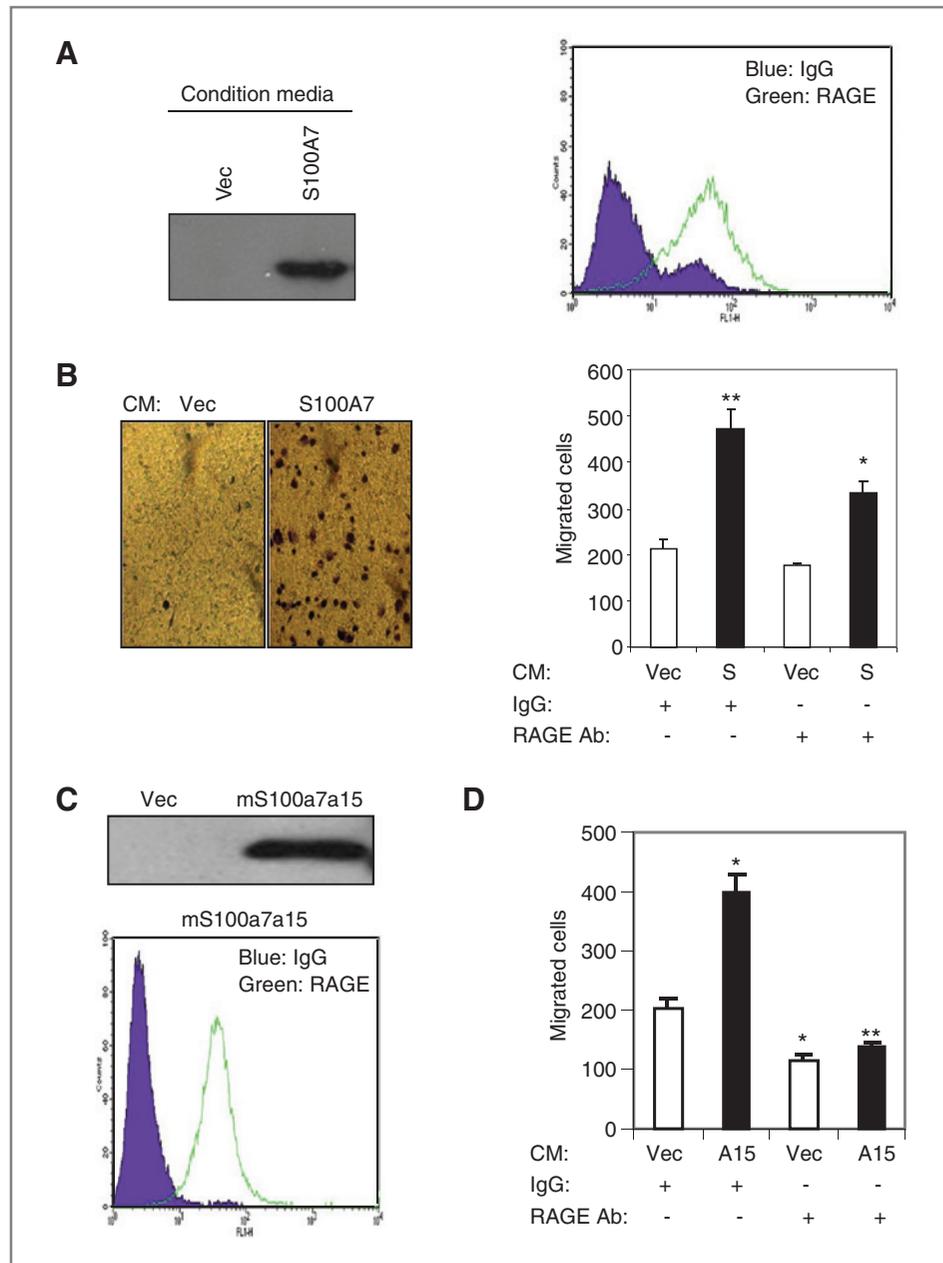
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 7. Role of RAGE in hS100A7 and mS100a7a15-induced chemotaxis of macrophages. A, Western blot of conditioned media (CM) obtained from vector (Vec) or hS100A7-overexpressing MDA-MB-231 cells. A, left, FACS analysis of RAGE expression in TDM. B, left, representative photographs of migrated TDM under phase contrast microscope. B, right, TDMs were subjected to hS100A7 or vector conditioned media-induced migration in presence of RAGE-neutralizing or control antibodies. C, top, Western blot of conditioned media of vector or mS100a7a15-overexpressing cells. C, bottom, FACS analysis of RAGE expression in MMR. D, MMR were subjected to vector or mS100a7a15 conditioned media-induced migration in presence of murine RAGE neutralizing or control antibodies. S represents hS100A7 and A15 represents mS100a7a15. Graphs represent the mean \pm SD for each experiment repeated 3 times with similar results. *, $P < 0.05$; **, $P < 0.01$.



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