SUPPLEMENTAL MATERIALS AND METHODS

Cell culture

Human breast cancer cell line MDA-MB-231 cells (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Sigma Aldrich) with 10% fetal bovine serum (FBS) (Sigma Aldrich), 10 units/ml of penicillin and 0.1 mg/ml of streptomycin (Invitrogen). Human breast cancer cell line Hs578T cells (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich) with 10% FBS (Sigma Aldrich), 10 units/ml of penicillin and 0.1 mg/ml of streptomycin (Invitrogen). 4T1 cells (A kind gift from Dr. Meenakshi Upreti) were maintained in DMEM/F12 (Sigma Aldrich) with 10% FBS (Sigma Aldrich), 1 mM Sodium Pyruvate (Gibco), 10 units/ml of penicillin and 0.1 mg/ml of streptomycin (Invitrogen). HEK293 FT cells (A kind gift from Dr. Mina J Bissell [Lawrence Berkeley Natl Laboratory]) were maintained in DMEM (Sigma Aldrich) with 10% FBS (Sigma Aldrich), 0.1 mM Non-Essential Amino Acids (Hyclone), 6 mM L-glutamine (Sigma Aldrich), 1 mM Sodium Pyruvate (Gibco), 10 units/ml of penicillin and 0.1 mg/ml of streptomycin (Invitrogen). MCF10A cells and MCF10A-Twist cells were maintained in DMEM/F12 (Sigma Aldrich) with 5% horse serum, 20ng/ml EGF, 0.5 mg/ml Hydrocortisone, 100 ng/ml Cholera Toxin, 10μg/ml Insulin, 10 units/ml of penicillin and 0.1 mg/ml of streptomycin (Invitrogen). Human microvascular endothelial cells isolated from lung (HMVEC-L cells, Lonza, CC-2527) were maintained in EBMTM-2 Basal Medium (CC-3156) with EGMTM-2 MV Microvascular Endothelial Cell Growth Medium SingleQuots^{TM} supplements. Human umbilical vein endothelial (HUVEC) cells (American Type Culture Collection) were maintained in Vascular Cell Basal Medium (ATCC PCS100030) with Endothelial Cell Growth Kit-BBE (ATCC PCS100040). HMLE cells (American Type Culture Collection), HMLE-Snail cells and HMLE-Twist cells were maintained in MEGM™ Mammary Epithelial Cell Growth Medium (Lonza, CC3150). All cells

were treated with Plasmocin™ (Invivo Gen) to eliminate and prevent mycoplasma contamination. All the cells were cultured at 5% CO2, 95% O2 at 37°C.

Plasmids and virus preparation

SERPINH1 cDNA clones were purchased from Thermo Fisher Scientific. SERPINH1 cDNA was cloned into pCDH1 plasmid and generated expression vector pCDH1- SERPINH1-Flag. Knockout plasmid Crispr-SERPINH1 were constructed with gDNA primers: 5'-CACCGCTTTGGCCATCGCCTGATAT-3' and 5'-AAACATATCAGGCGATGGCCAAAGC-3'. SERPINH1 Knockdown plasmids shHSP47 were purchased from Sigma. SERPINH1 Knockdown plasmids shHSP47 were cloned into doxycycline-inducible knockdown vector pLKO-Tet-On (Addgene). HEK293 FT cells were transfected with pCDH1 vectors, or Crispr vectors or shRNA vectors (Sigma) plus lentivirus packaging vectors using FuGENE® HD Transfection Reagent (Promega). Culture supernatants containing viral particles were collected 48 hours after transfection. Cancer cells were infected with lentivirus and 48 hours after infection, cells were selected by puromycin (Gibco) for at least 3 days.

***In Vivo* Xenograft Experiments**

Six-week-old female SCID mice were randomly grouped and injected with 1×10^6 shcontrol/shHSP47 MDA-MB-231/Luc cells, 1×10^6 shcontrol/shHsp47 MDA-MB-231/GFP cells; 1×10^6 control/Hsp47-KO 4T1/Luc cells, or 1×10^6 control/SERPINH1-expression MCF10A/GFP cells via tail vein. All of the procedures during our study were performed within the guidelines of the Division of Laboratory Animal Resources at the University of Kentucky. For short term study, lungs were harvested at 4 hours after tail vein injection and embedded in OCT for IF staining analysis at 4 hours after injection. For long term study, mice with MDA-MB-231/Luc cells

injection were imaged using IVIS to detect lung metastasis. Mice developed the expected lung metastasis at week 5 and lung samples were harvested for H&E staining.

For platelet depletion or collagen blocking experiments, mice were injected intraperitoneally with 100 μl anti-mouse GPIb monoclonal antibody (0.5 mg/ml, emfret #R300) or anti-mouse GPIb monoclonal antibody (0.5 mg/ml, emfret #M011-0, clone: JAQ1). Mice were injected with same amount of isotype-matched rat IgG (emfret #C301) as control (1). Four hours after injection with platelet depletion antibody, six-week-old female SCID mice were randomly grouped and injected with 1×10^6 shcontrol/shHsp47 MDA-MB-231/GFP cells, or 1×10^6 control or Hsp47-expressing MCF10A/GFP cells via tail vein. Lungs were harvested at 4 hours after tail vein injection and embedded in optimal cutting temperature (OCT) for immunofluorescence (IF) staining analysis.

For experiments in the orthotropic mammary tumor model, 0.5×10^6 control/Hsp47-knockout 4T1/Luc cells were injected into fourth mammary gland fat pad of six-week-old female BalB/C mice. Primary tumors were measured every two days. Primary tumors were removed by surgery about one month after implantation. Mice developed the lung metastasis at two-three weeks after surgery and lung samples were harvested for H&E staining.

Tumorsphere assay

Cells cultured on 2D plastic culture dish were trypsinized according to standard protocol. Cells suspensions were filtered using Cell Strainer (40μm, Corning®) and viable cells was calculated after trypan blue staining. 1 x 10^4 single viable cell in 1 ml of tumorsphere media [DMEM/F12 medium supplemented with B27 (1:50), EGF (20 ng/ml), bFGF (20 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), Gentamycin (100 μg/ml)] was added into each well of non-adherent 12-well culture plate (coated with poly-2-hydroxyethyl-methacrylate). After 5 days incubating

without moving or disturbing the plates, the numbers of tumorspheres greater than 50 μm diameter were counted using a microscope. Tumorsphere forming efficiency (%) is calculated as number of tumorspheres per well / number of cells seeded per well ×100.

Methylcellulose colony formation assay

Cells cultured on 2D plastic culture dish were trypsinized according to standard protocol. Cells suspensions were filtered using Cell Strainer (40μm, Corning®) and viable cells was calculated after trypan blue staining. 5000 single viable cell in 1 ml of tumorsphere media [DMEM/F12 medium supplemented with B27 (1:50), EGF (20 ng/ml), bFGF (20 ng/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), Gentamycin (100 μg/ml)] was added into each well of non-adherent 6-well culture plate (pre-treated with poly-2-hydroxyethyl-methacrylate). The medium was made semi-solid by the addition of 0.5% Methylcellulose (HSC011, R&D Systems™) to prevent cell aggregation. After 7 days incubating without moving or disturbing the plates, the phase images of colonies were taken by using Nikon microscope and the numbers of colonies for each well were counted.

Soft agar colony formation assay

Mixed 42 °C pre-warmed 2x cell culture medium (supplemented with 20% FBS and 2% penicillin/streptomycin) with 1% agar solution. For the bottom layer of agar, 1.5 ml of a mix of 1.0% agar and 2x cell culture medium per well of a 6-well plate. Allowed agar mixture to solidify at room temperature for 30 minutes in cell culture hood. Cells cultured on 2D plastic culture dish were trypsinized according to standard protocol. 5000 cells were suspended in 1.5 ml of a mix of 0.7% agar and 2x cell culture medium per well of a 6-well plate. Allow cell/agar mixture to solidify at room temperature, in cell culture hood, for 30 minutes before placing into a 37 °C humidified cell culture incubator. After 3 weeks, the colonies were stained with 0.1% crystal violet for 30 min.

Images of colonies were taken by using Nikon microscope and the numbers of colonies for each well were counted.

Assessment of Endothelial Barrier Function

Endothelial barrier function was assessed by measurement real-time changes in electrical resistance across endothelial monolayers using electric cell-substrate impedance sensor (Applied Biophysics, Troy, NY, USA) (2, 3). Briefly, cultured HUVECs and HMVEC-L cells were digested and 400 µl volume of 3 x 10^5 cells/ml cell suspension were plated onto 8-chamber gold microelectrode arrays (8W10E+ PC, Applied Biophysics) and cultured to confluence. Resistance across the endothelial monolayer was monitored for 2 hours to ensure a stable baseline recording. Thereafter, the cells were stimulated with thrombin (1 nM), and subsequent change in TER was recorded in real time. Resistance data were normalized to the initial voltage and plotted as normalized resistance. The experiments were performed in duplicates.

Immunohistochemical staining

Protein levels of HSP47 expression in human breast cancer tissues were determined using tissue microarray (TMA, Us Biomax). TMA sections were deparaffinized and rehydrated through 100% alcohol, 95% alcohol, 70% alcohol to PBS solution. Endogenous peroxidase was blocked by incubation with 3% H_2O_2 for 20 min. At the antigen retrieval step, slides were steamed in citrate sodium buffer for 30 min. Slides were blocking with Avidin/Biotin Blocking Kit (Vector Laboratories, SP-2001) incubated with primary antibodies (Anti-HSP47, Santa Cruz sc-5293, 1:100) at 4°C overnight, and then the sections were incubated with Biotinylated Goat Anti-Mouse IgG Antibody (Vector Laboratories, BA-9200) at room temperature for 60 min. After incubated with Streptavidin, Horseradish Peroxidase (Vector Laboratories, SA-5704) at room temperature

for 60 min, slides were added substrate diaminobenzidine (DAB, Vector Laboratories, SK-4100), images were taken by Nikon Eclipse 80i microscope.

Primary mammary epithelial cell isolation and culture

Primary mammary epithelial cells were isolated from 8-week-old female MMTV-Cre:Hsp47$^{+/lox}$ mice and MMTV-Cre:Hsp47$^{lox/lox}$ mice. In general, the forth mammary glands were collected from 8-week-old female mice and minced with blades. Minced mammary gland tissue was gently shaken for 30 minutes at 37 °C in a 50-ml with collagenase-trypsin solution (0.2% trypsin, 0.2% collagenase type IV, 5% fetal calf serum, 5 μg/ml of insulin, and 50 μg/ml of gentamicin in 50 ml of DMEM/F12). The collagenase solution was discarded after centrifugation and the pellet was incubated for 5 min in 4 ml of DMEM/F12+40 μl of DNase (2 U/μl). The primary mammary gland epithelial organoids pellets were plated in two-dimensional plastic culture dish with growth medium (5% fetal calf serum, 5 ng/ml of EGF, 5 μg/ml of insulin, and 50 μg/ml of gentamicin in DMEM/F12). The primary mammary gland epithelial cells isolated were cultured at 5% CO2, 95% O2 at 37°C for several days until confluence. Primary mammary epithelial cells were fixed by 100% Methanol before IF staining.

Western Blot Assays

Cells were lysed in 2% SDS in PBS buffer containing phosphatase and protease inhibitor cocktails (EMD Millipore, 539131). Protein concentration was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, 23227). Conditioned medium was collected at 24h after replaced with plan medium and precipitated by Acetone. Equal amounts of protein lysates or conditioned medium (normalized to cell numbers) were subjected to SDS gel electrophoresis, immunoblotted with primary antibodies and horseradish peroxidase (HRP) conjugated-secondary antibodies or DyLight 680/800-conjugated secondary antibodies. Western blot results were

quantified by using AlphaInnotech analysis software. The secondary antibodies were used in the study were: HRP conjugated goat anti-rabbit IgG secondary antibody (Thermo Fisher Scientific, 31460), HRP conjugated goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific, 31440), HRP conjugated rabbit anti-goat IgG secondary antibody (Thermo Fisher Scientific, 31402); DyLight 680 conjugated goat anti-rabbit IgG secondary antibody (Thermo Fisher Scientific, 35569), DyLight 800 conjugated goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific, SA5-35521), DyLight 680 conjugated donkey anti-goat IgG secondary antibody (Thermo Fisher Scientific, SA5-10090). The primary antibodies used in this study were listed in Table S1.

Table S1. Primary antibodies.

Antibody	**Company**	**Catalog Number**	**Dilution**	**Species**
Anti-HSP47	Santa Cruz	sc-5293	WB: 1:500 IHC: 1:100	Mouse
Anti-Collagen I	Abcam	ab34710	WB: 1:1000 IF: 1:300	Rabbit
Anti-Collagen IV	Thermo Fisher	PA1-28534	WB: 1:1000 IF: 1:100	Rabbit
Anti-FN	Abcam	Ab6328	WB: 1:1000	Mouse
Anti-Snail	Cell signaling	4719S	WB: 1:1000	Rat
Anti-Flag	Sigma-Aldrich	F1804	WB: 1:1000	Mouse
Anti-Lamin A/C	Santa Cruz	sc-6215	WB: 1:500	Goat
Anti-Tubulin	Millipore Sigma	05-661	WB: 1:5000	Mouse
PE Anti-CD41	BD Biosciences	558040	IF: 1:100	Rat
FITC Anti-CD41	BD Biosciences	553848	FACS 1:20	Rat
Anti-Ecad	BD Biosciences	610181	WB 1:2000 IF: 1:100	Mouse
Anti-Ncad	BD Biosciences	610920	WB: 1:500	Mouse
Anti-Vimentin	Thermo Fisher	MS129P	WB 1:2000 IF: 1:100	Mouse

SUPPLEMENTAL REFERENCES

1. Xiang B*, et al.* (2013) Platelets protect from septic shock by inhibiting macrophage-dependent inflammation via the cyclooxygenase 1 signalling pathway. *Nat Commun* 4:2657.
2. Han J, Liu G, Profirovic J, Niu J, & Voyno-Yasenetskaya T (2009) Zyxin is involved in thrombin signaling via interaction with PAR-1 receptor. *FASEB J* 23(12):4193-4206.
3. Han J*, et al.* (2013) A critical role for Lyn kinase in strengthening endothelial integrity and barrier function. *Blood* 122(25):4140-4149.

SUPPLEMENTAL FIGURES

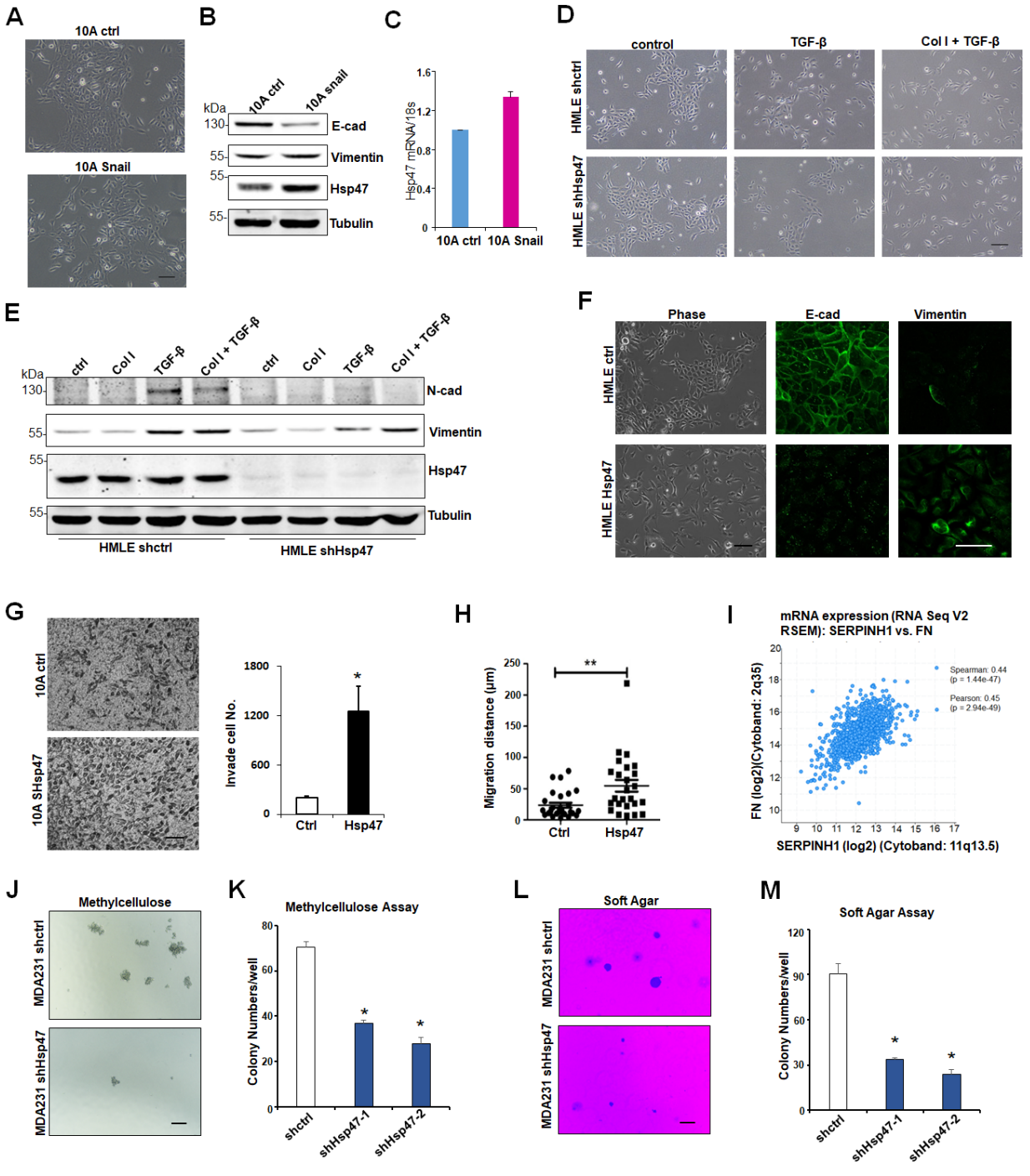

Fig. S1. Hsp47 expression is associated with EMT. (A-C) Phase images, EMT marker protein expression and SerpinH1 mRNA levels in MCF10A control cells and EMT cells. **(D-E)** Phase

images and EMT marker protein expression in TGF-β (5 ng/ml for 7 days) treated HMLE control and shHSP47 cells. **(F)** Represented phase images and IF staining images (E-cad and Vimentin) of control and Hsp47-expressing HMLE cells. Bar, 50 μm. **(G)** Phase images and quantification of cell invasion in control and Hsp47-expressing MCF10A cells. Bar, 50 μm. Results are presented as mean ± SEM; n= 3; *p < 0.05, independent Student's t test. **(H)** Quantification of single cell migration in control and Hsp47-expressing MCF10A cells. Results are presented as mean ± SEM; n= 25; **p < 0.01, independent Student's t test. **(I)** Co-expression of Hsp47 (SERPINH1) and EMT marker FN1 was assessed with spearman correlation analysis in human breast cancer tissue samples (TCGA, Provisional); n=960. **(J-K)** Represented phase images and quantification of colony numbers in control or shHsp47 MDA-MB-231/GFP cells by Methylcellulose colony formation assay. Bar, 250 μm. Results are presented as mean ± SEM; n= 3; *p < 0.05, independent Student's t test. **(L-M)** Represented phase images and quantification of colony numbers in control or shHsp47 MDA-MB-231/GFP cells by soft agar colony formation assay. Bar, 250 μm. Results are presented as mean ± SEM; n= 3; *p < 0.05, independent Student's t test.

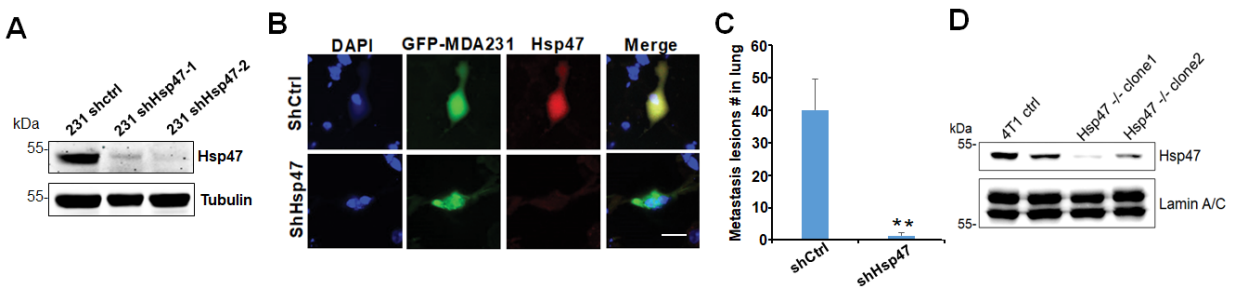

Fig. S2. Knockdown Hsp47 expression in breast cancer cells. (A) Knockdown efficiency of shHSP47 was assessed in MDA-MB-231 cells. (**B)** Knockdown efficiency of shHSP47 was assessed in MDA-MB-231 cells adhesion in lung tissue. Bar, 20 µm. **(C)** Quantification of lung metastasis lesions (4 weeks after tail vein injection with 1 x 10^6 control and Hsp47-silenced MDA-MB-231/luc cells) of control and MDA-MB-231 cells in H&E staining images. Results are presented as mean ± SEM; n= 5; independent Student's t test. **(D)** Knock down efficiency of Crispr-Hsp47 was assessed in 4T1 cells.

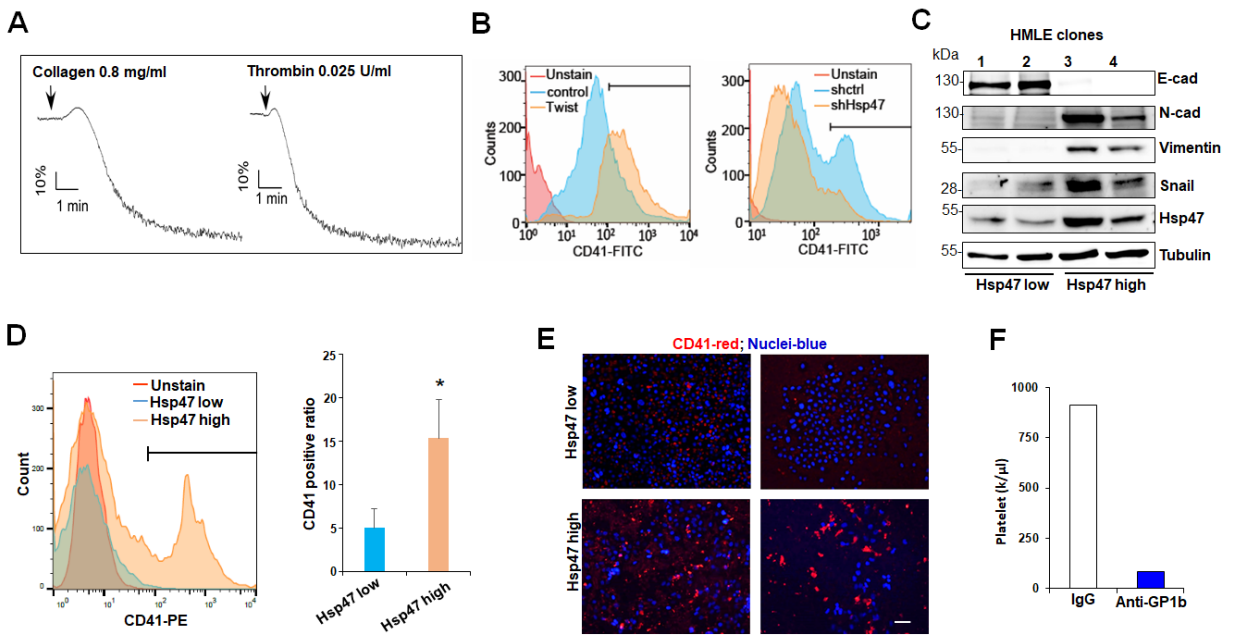

Fig. S3. Hsp47 enhanced mammary epithelial cell-platelet interaction. (A) Platelet aggregation was measured in a lumi-Aggregometer with 0.8 mg/ml Collagen or 0.025 U/ml Thrombin stimulation. **(B)** FACS analysis images of platelet binding in control and MCF10A-Twist cells (left); and in control and shHsp47 MDA-MB-231 cells (right). (**C)** Hsp47 protein level and EMT markers protein levels were assessed in HMLE clones. **(D)** FACS analysis of platelet binding with Hsp47-low and Hsp47-high HMLE clones. Results are presented as mean ± SEM; n= 4; *$p < 0.05$, independent Student's t test. **(E)** IF staining images of platelets binding with Hsp47-low and Hsp47-high HMLE clones cultured on plastic. Bar, 50 μm. **(F)** Platelets depletion efficiency was assessed in mice at 4 hours after injection with IgG or Anti-GPIb antibody injection.

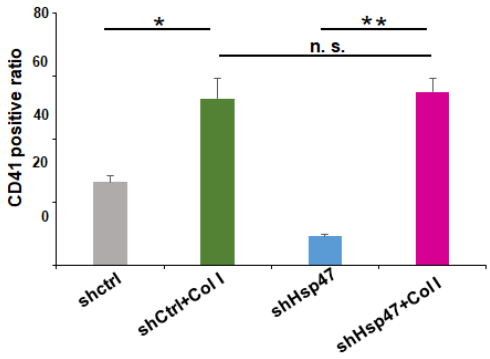

Fig. S4. Hsp47 enhanced mammary epithelial cell-platelet interaction via type I collagen. FACS analysis of platelet binding in vector control and shHsp47 MDA231 cells pre-treated with/without collagen I. Results are presented as mean ± SEM; n= 6; independent Student's t test. n.s., no significance; *, $p < 0.05$; **, $p < 0.01$.

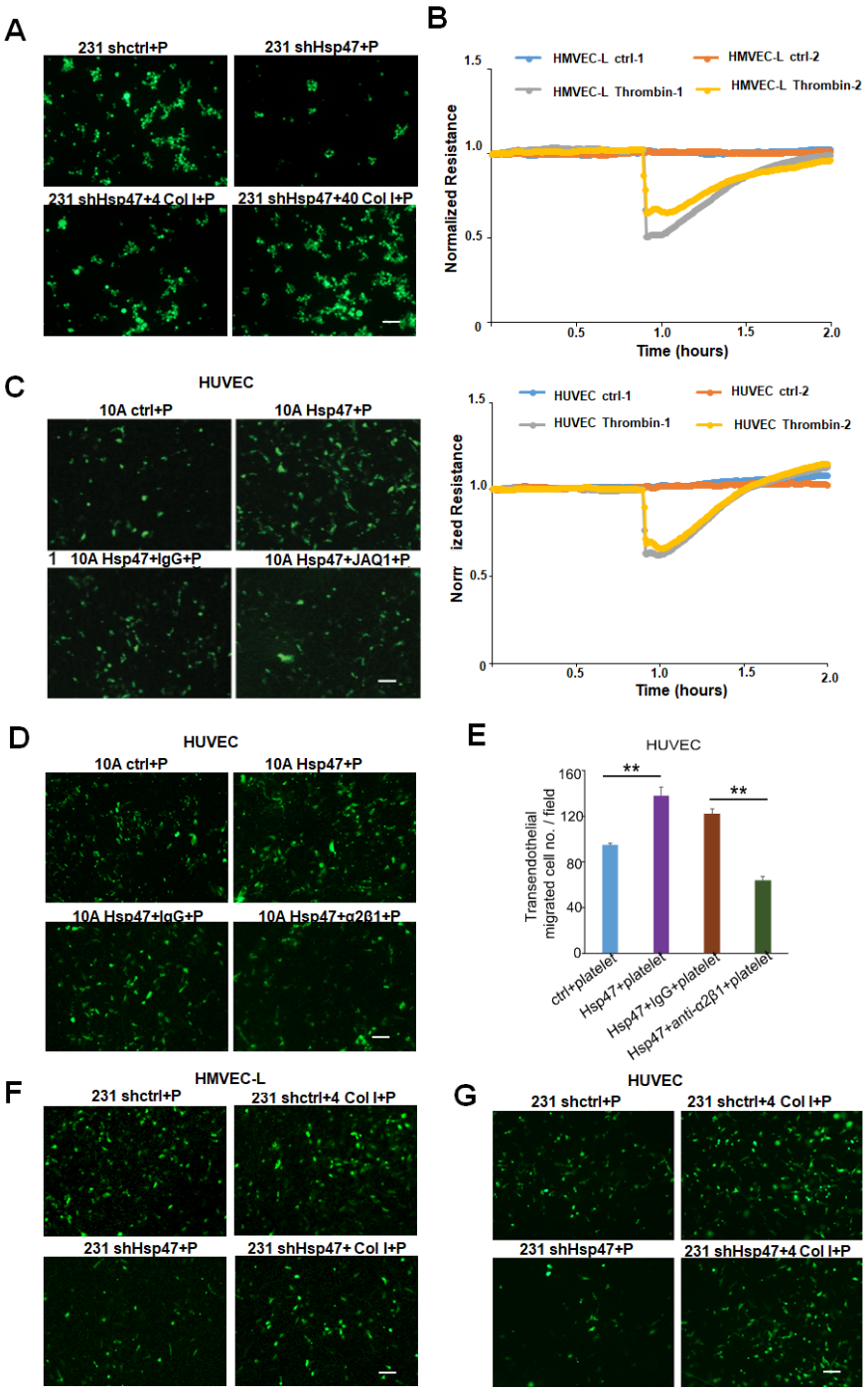

Fig. S5. Hsp47-dependent collagen I deposition and platelet recruitment enhanced cancer cell clustering and extravasation. (A) Images of cell clustering in collagen I (0 μg/ml, 4 μg/ml or 40 μg/ml) treated control or shHsp47 MDA-MB-231/GFP cells incubated with mouse platelets. **(B)** Endothelial barrier function measurement in HMVEC-L cells and HVUEC cells with/without

1 nM Thrombin treatment. **(C)** Images of extravasated control or Hsp47-expressing MCF10A/GFP cells in HUVEC transendothelial migration assay; cells were incubated with human platelets pretreated with IgG/JAQ1 antibody. **(D-E)** Images and quantification of control or Hsp47-expressing MCF10A/GFP cell extravasation in HUVEC transendothelial migration assay. Cells were incubated with IgG/anti-integrin α2β1 antibody, then treated with platelets; n= 5; one-way ANOVA test. **(F-G)** Images of MDA-MB-231/GFP cell extravasation in HMVEC-L and HUVEC transendothelial migration assay. Control and shHsp47 MDA-MB-231 cells were treated with Collagen I (0 µg/ml, 4 µg/ml), then incubated with mouse platelets before the assay. Bar: 100 µm.

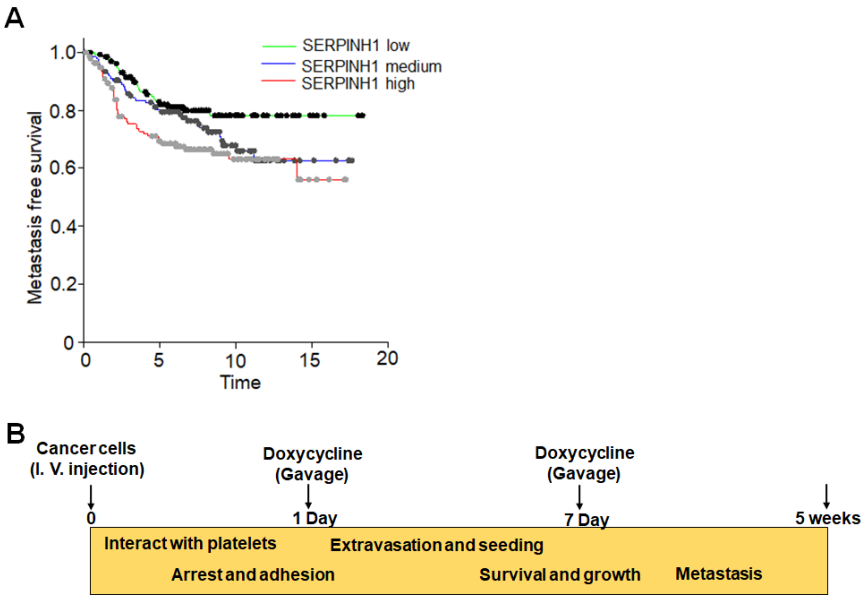

Fig. S6. Hsp47 is associated with in human breast cancer patients. **(A)** Kaplan-Meier analysis showed the association of Hsp47 expression with metastasis-free survival in breast cancer patients; n=1746. **(B)** A scheme of experiment design for in vivo metastasis model using Doxycycline inducible Hsp47 knockdown MDA-MB 231 cells.