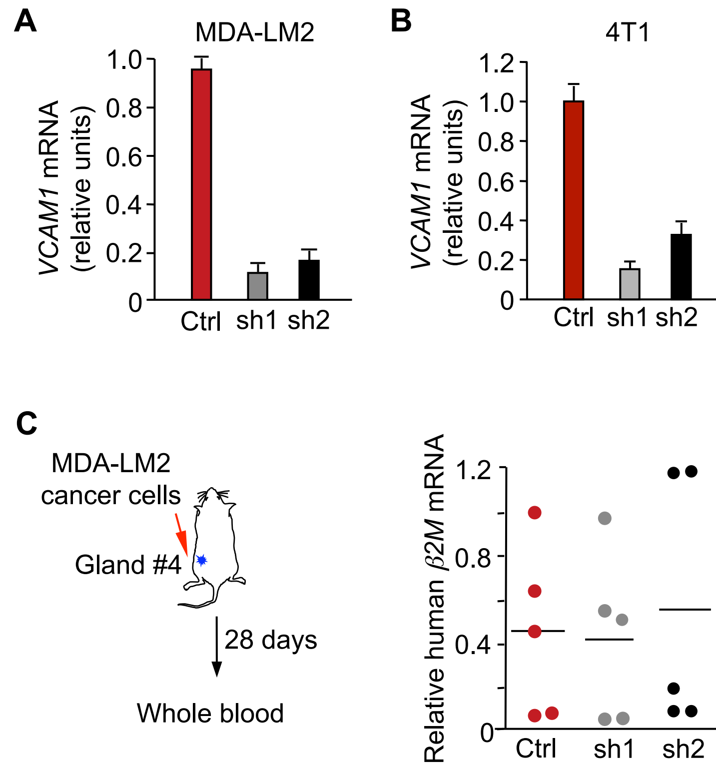

Figure S1, related to Figure 1.
(A, B) Generation of *VCAM1* knockdown cells by two independent short hairpins (sh1 and sh2) in human MDA-231-LM2 cells (A) and mouse 4T1 cells (B). *VCAM1* mRNA was detected by RT-PCR. Data are from one representative experiment of two times.

(C) Control (C) and *VCAM1*-depleted MDA231-LM2 cells (2 individual shRNAs) were injected in the fourth mammary fat pad of mice. Circulating cancer cells were assessed by normalizing human *β2-Microglobulim* expression by mouse *β2M* expression in whole blood from tumor-bearing mice when tumor sizes reached 300 mm^3.

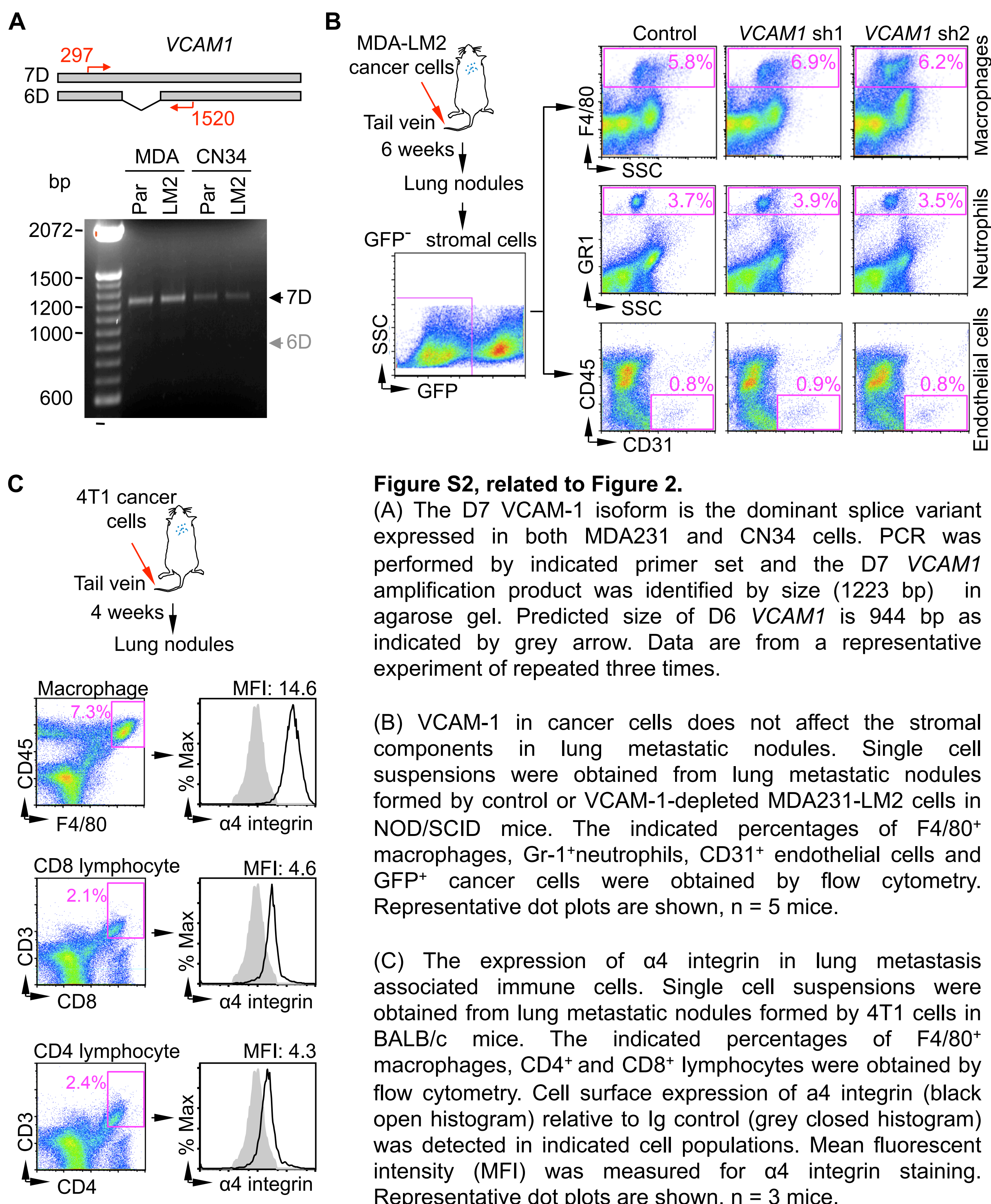

Figure S2, related to Figure 2.

(A) The D7 VCAM-1 isoform is the dominant splice variant expressed in both MDA231 and CN34 cells. PCR was performed by indicated primer set and the D7 *VCAM1* amplification product was identified by size (1223 bp) in agarose gel. Predicted size of D6 *VCAM1* is 944 bp as indicated by grey arrow. Data are from a representative experiment of repeated three times.

(B) VCAM-1 in cancer cells does not affect the stromal components in lung metastatic nodules. Single cell suspensions were obtained from lung metastatic nodules formed by control or VCAM-1-depleted MDA231-LM2 cells in NOD/SCID mice. The indicated percentages of F4/80$^+$ macrophages, Gr-1$^+$neutrophils, CD31$^+$ endothelial cells and GFP$^+$ cancer cells were obtained by flow cytometry. Representative dot plots are shown, n = 5 mice.

(C) The expression of α4 integrin in lung metastasis associated immune cells. Single cell suspensions were obtained from lung metastatic nodules formed by 4T1 cells in BALB/c mice. The indicated percentages of F4/80$^+$ macrophages, CD4$^+$ and CD8$^+$ lymphocytes were obtained by flow cytometry. Cell surface expression of a4 integrin (black open histogram) relative to Ig control (grey closed histogram) was detected in indicated cell populations. Mean fluorescent intensity (MFI) was measured for α4 integrin staining. Representative dot plots are shown, n = 3 mice.

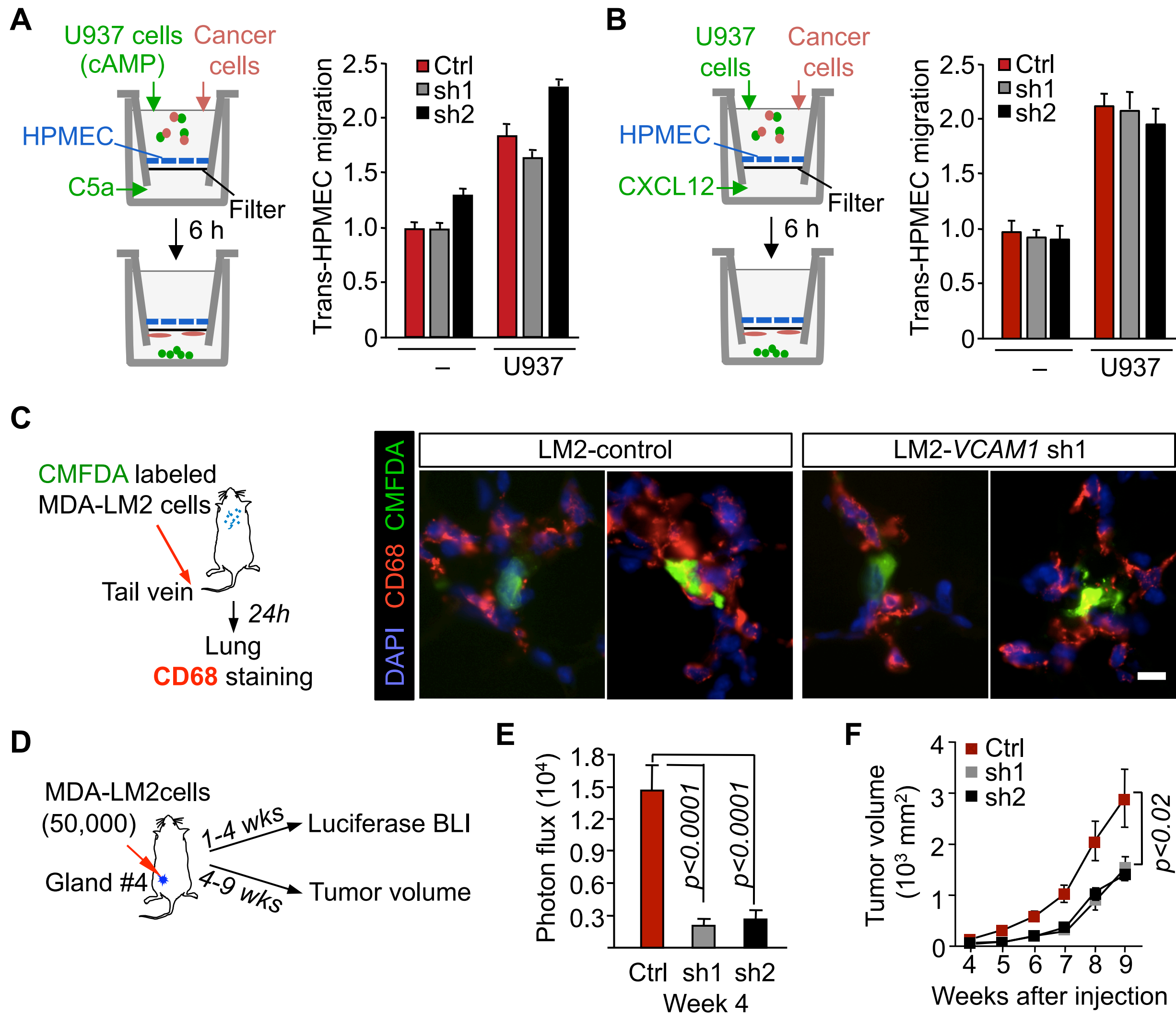

Figure S3, related to Figure 3.

(A,B) Trans-endothelial migration assay. (A) The indicated cancer cells were mixed with or without cAMP-stimulated U937 cells in the top chamber and allowed to migrate through a monolayer of human pulmonary microvascular endothelila cells (HPMEC) with U937 cell chemoattractant C5a in the bottom chamber. (B) The indicated cancer cells were mixed with or without U937 cells in the top chamber and allowed to migrate through a monolayer of HPMEC with tumor cell chemoattractant CXCL12 in the bottom chamber. Cancer cells that migrated through this layer were quantified. Data are average ± SEM; n = 10 unit areas.

(C) Association of tail vein inoculated CMFDA-labeled MDA-LM2 cells with endogenous $CD68^+$ cells in the lungs of NOD.SCID mice. Two representative micrograph per group show no effect of VCAM1 depletion on the abundance of $CD68^+$ cells surrounding the cancer cells. Scale bar, 10 μm. Quantification of the percentage of cancer cells interacting with $CD68^+$ cells and numbers of $CD68^+$ cells surrounding the individual tumor cell are shown in Figure 3D,E.

(D-F) Schematic of the experimental protocol to test the ability of a low number of MDA-LM2 cells (50,000 cells) to form tumors in mammary fat pads (A). Mammary tumor growth was detected by bioluminescent quantification at week 4 (B), and by direct tumor size measurements thereafter (C). Data are average ± SEM; n = 5 mice.

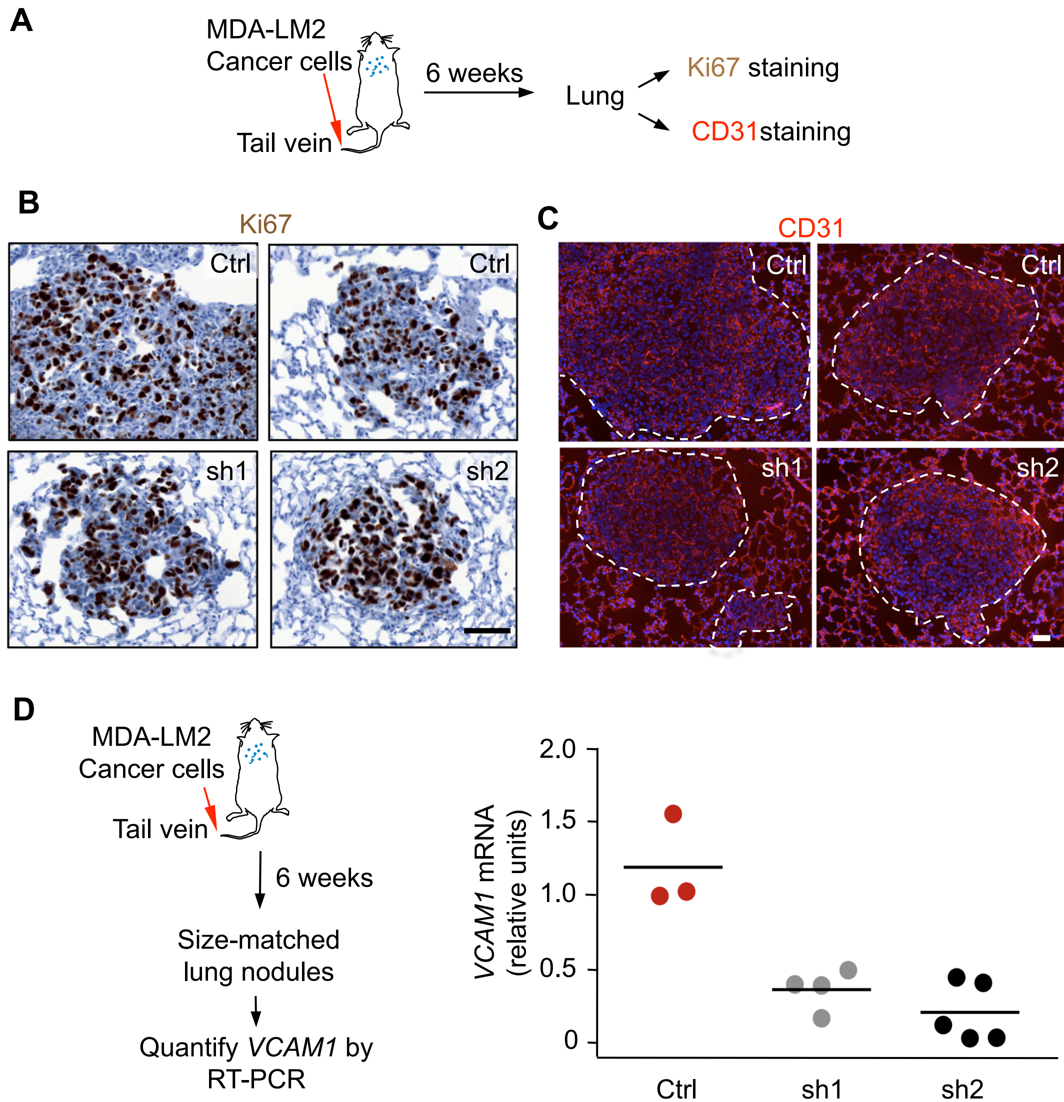

Figure S4, related to Figure 4.
(A-C) VCAM-1 does not affect the proliferation of cancer cells or angiogenesis in lung metastatic nodules. (A) Control and VCAM-1-depleted MDA231-LM2 cells (by 2 individual short hairpins: sh1 and sh2) were intravenously injected into mice. Ki67 (B) and CD31 staining (C) were performed in the lungs harvested 6 weeks after tumor cell injection. Representative photomicrographs are shown. Scale bar, 100 µm.

(D) Size-matched lung metastatic nodules were harvested and *VCAM1* expression was determined by qRT-PCR.

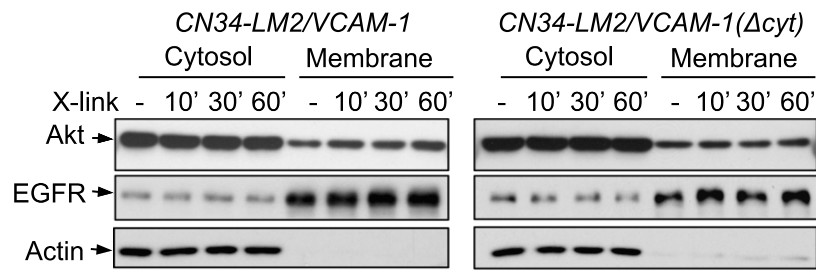

Figure S5, related to Figure 7.
Time course of Akt membrane localization after addition of VCAM-1 clustering antibodies to the indicated serum starved CD34-LM2 cells. Akt was detected in membrane and cytosolic fractions.

Table S1, related to Figure 7. Screening of signaling pathways regulated by clustering VCAM-1 in LM2 cancer cells (MDA231-LM2-sh1 and CN34-LM2) expressing wild-type VCAM-1. Indicated cancer cells were treated with VCAM-1 clustering antibody for 5 minutes or comparable Ig control. Activation of different signaling pathways was detected by Human Phopho-Kinase Array Kit (R&D system) or by western immunoblotting (indicated by *). Fold change between VCAM-1 clustering and control was determined using the Image J software. Phosphorylation changes > 30% that were concordant in both cell lines are highlighted in red. N.D., non-detectable.

	MDA231	**CN34**
Akt (S473)	**1.45**	**1.73**
MEK1/2 (S218/S222, S222/S226)	1.09	1.49
STAT4 (Y693)	1.05	1.39
eNOS (S1177)	1.03	1.33
FAK (Y397)	1.08	1.32
RSK1/2/3 (S380)	0.89	1.23
Chk-2 (T68)	1.20	1.21
ERK1/2 (T202/Y204, T185/Y187)	0.98	1.20
Akt (S308)	0.97	1.19
MSK1/2 (S376/S360)	0.96	1.19
p53 (S392)	0.98	1.18
STAT3 (Y705)	0.95	1.16
p53 (S46)	0.94	1.15
PLCγ-1 (Y783)	1.09	1.13
p53 (S15)	0.93	1.11
AMPKα1 (T172)	0.98	1.09
TOR (S2448)	0.65	1.06
Src (Y419)	0.65	1.06
Smad3 (S423/S425)*	0.90	1.05
p38α (T180/Y182)	1.20	1.04
STAT5b (Y699)	0.99	1.04
STAT6 (Y641)	1.04	1.02
STAT5a/b (Y699)	1.00	1.02
AMPKα2 (T172)	0.80	1.01
STAT2 (Y689)	0.80	1.01
p27 (T157)	1.26	1.00
RSK1/2 (S221)	1.02	0.98
STAT1 (Y701)	0.97	0.98
Hck (Y411)	0.98	0.96
Pyk2 (Y402)	1.09	0.90
c-Jun (S63)	1.15	0.88
p70 S6 Kinase (T421/S424)	1.18	0.87
Yes (Y426)	0.93	0.84
p70 S6 Kinase (T389)	0.95	0.84
Paxilin (Y118)	1.06	0.82
HSP27 (S68/S82)	0.89	0.82
Lck (Y394)	0.89	0.82
Fyn (Y420)	0.82	0.81
IκBα*	1.13	0.80
Fgr (Y412)	0.94	0.79
JNK pan (T183/Y185,T221/Y223)	0.60	0.77
p70 S6 Kinase (T229)	1.07	0.76
p27 (T198)	0.79	0.74
β-Catenin	1.11	0.73
STAT5a (Y699)	1.11	0.73
GSK-3α/β (S21/S9)	**0.59**	**0.70**
CREB (S133)	1.30	0.54
Lyn (Y397)	1.30	0.54
Smad2 (S465/S467)*	N.D.	N.D.
NK-κB (S468)*	N.D.	N.D.

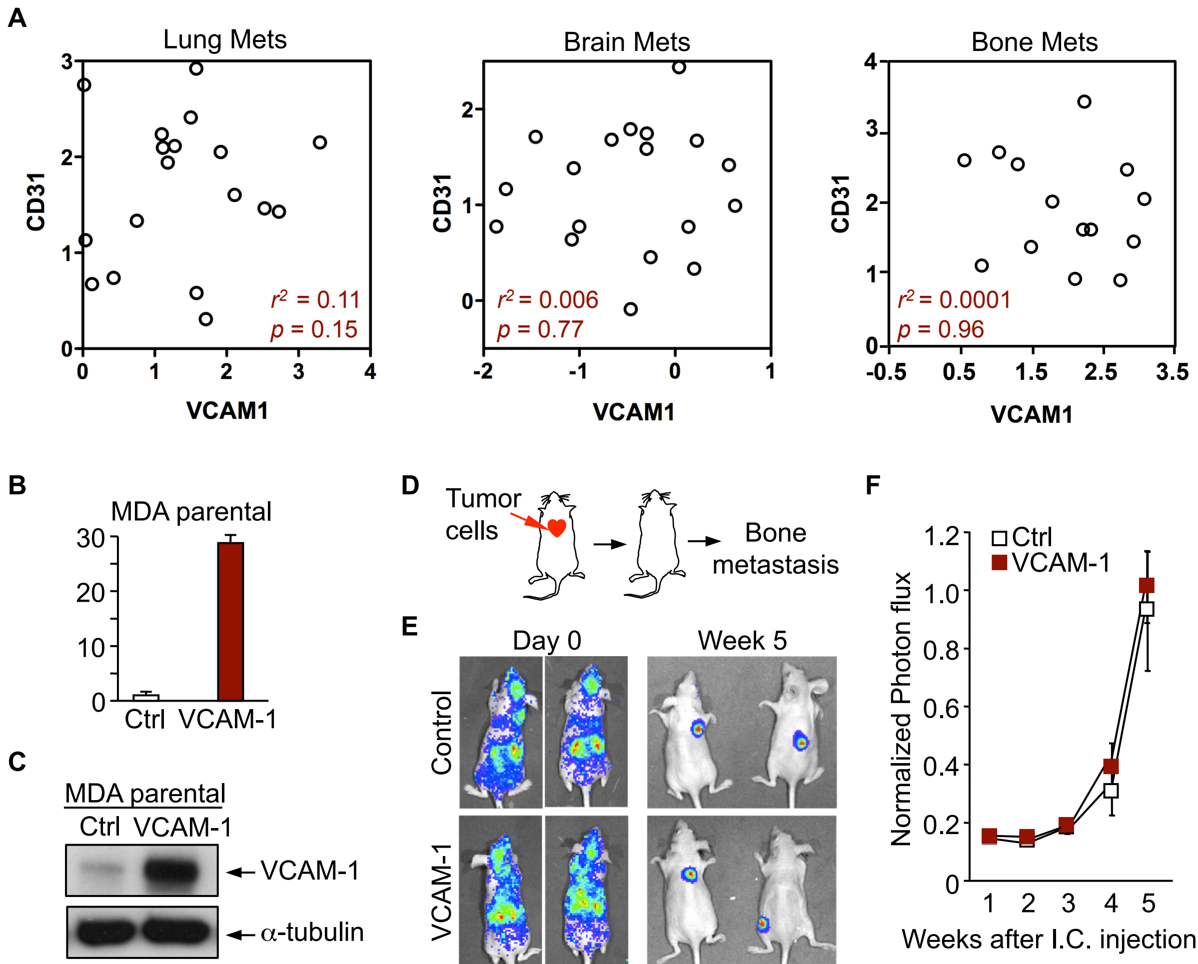

Figure S6, related to Figure 8.

(A) No correlation between *VCAM1* and *CD31* expression in metastatic lesions from cancer patients. *VCAM1* and *CD31* transcript levels were obtained from microarray dataset of lung (n = 18), brain (n = 19) and bone (n = 17) metastasis from breast cancer patients. Correlation coefficient was calculated in each dataset.

(B-F) VCAM-1 does not affect bone metastasis at early stage. (B,C) VCAM-1 was overexpressed in MDA231 parental cells. *VCAM1* mRNA and protein levels were detected by RT-PCR (B) and western immunoblotting (C), respectively. (D-F) Bone metastasis assay. (D) Schematic of experimental procedure. Control (Ctrl) or VCAM-1 overexpressed (VCAM-1) MDA231 parental cells were intracardiacly injected in NOD/SCID mice. (E) Representative BLI at the indicated time points. (F) Normalized BLI of bone metastasis in mice inoculated with indicated cancer cells. Data are average ± SEM; n = 6 mice.

SUPPLEMENTAL EXPERIMENTAL PROCEDURE

Cell culture

MDA-231 parental and metastatic derivatives cells were cultured in DME media with 10% fetal bovine serum (FBS). CN34 parental and metastatic derivative cell lines cells were cultured in M199 media with 2.5% FBS, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 20 ng/mL EGF, 100 ng/mL cholera toxin. 4T1 cells were cultured in DME media with 10% fetal bovine serum (FBS). Retroviral packaging cell line GPG29 was maintained in DME media with 10% FBS, 20 ng/mL doxycycline, 2 μg/mL puromycin and 0.3 mg/mL G418. Lentivirus packaging cell line 293T was cultured in DME media with 10% FBS. Human monocytic cell line U937 was maintained in RPMI 1640 medium with 10% FBS. Additional 2 mM L-Glutamine, 100 IU/mL penicillin and 100 μg/mL and 0.5 μg/mL amphotericin B were added into all the media described above. Human pulmonary microvascular endothelila cells (HPMEC) (ScienCell) were cultured in complete ECM media (ScienCell) and used between passages 2-4.

Knockdown and overexpression constructs

Stable human VCAM-1 knockdown MDA231-LM2 cells were generated using two independent shRNAs in pGIPZ lentival vector (Open Biosystems) targeting the following sequences: GCCATAGCAAGATTGCTTA (shRNA1) and GGGAGTATATGAATGTGAA (shRNA2). Stable mouse VCAM-1 knockdown 4T1 cells was generated using two independent shRNAs in pLKO.1 lentivirus vector (Sigma) targeting the following sequences: AGATCCTTAATACTGTTTA (shRNA1) and AAATTGATTCTACACTCAA (shRNA2). Virus-infected cells were selected with 5 μg/ml puromycin. For inducible VCAM-1 knockdown system, shRNA1 were inserted into TRIPZ vector (Open Biosystems). 1μg/mL doxycycline hyclate (Sigma-Aldrich) were added to induce the expression of shRNA. Since shRNA1 targets the 3'-UTR region of the *VCAM1* transcript, *VCAM1*-depleted cells expressing shRNA1 (MDA231-LM2-sh1) were used for the generation of *VCAM1* rescue derivatives expressing the full-length VCAM-1 vector or *VCAM1-ΔCyt* vector. A pBabe-retro vector encoding human *VCAM1* was used(Gupta et al., 2007). The *VCAM1-ΔCyt* construct was generated by introducing a stop codon (TAA) at the start of the intracellular region (Lys722) using Site-Directed Mutagenesis Kit (Stragagene).

mRNA and protein levels

Total RNA from cancer cells was extracted using the PrepEase RNA spin kit (USB) and subjected to a reverse transcriptase reaction SuperScript III first-strand synthesis system (Invitrogen). cDNA was used in three replicates for quantitative PCR using pre-designed Taqman gene expression assay primers (Applied Biosystems). Expression data was acquired and analyzed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). For western immunoblotting, cell pellets were lysed with RIPA buffer and protein concentrations determined by BCA Protein Assay Kit (Pierce). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were immunoblotted with monoclonal antibodies against human VCAM-1 (Santa Cruz), α-tubulin (Sigma), mouse VCAM-1 (Santa Cruz) and b-actin (Cell Signaling).

Flow cytometry

Single cells suspensions were incubated with fluorochrome-conjugated monoclonal antibodies that recognize human VCAM-1 (BD Pharmingen) or mouse CD45, Gr-1, F4/80, CD31 or a4 integrin (eBioscience). To identify the cell populations that bind VCAM-1, lung metastatic nodules were isolated from mice and single cell suspensions were obtained by digesting tumor nodules with 0.5% collegenase type 3 (Worthington) and 1% dispase II (Roche) in PBS. The stromal cells were negatively selected with human EpCAM beads (Miltenyi Biotec). Cells were incubated with fluorochrome-conjugated monoclonal antibodies or Ig controls and recombinant human VCAM-1 or bovine serum albumin prelabeled with Alexa Fluor 647 Protein Labeling Kit (Invitrogen). The cell surface binding of VCAM-1 in different cell subpopulations was detected by flow cytometry.

Immunohistochemical and TUNEL staining

To visualize lung metastases, mice were perfused with PBS through the left ventricle. Lungs were either freshly frozen in O.C.T. Compound (VWR) or fixed with 4% paraformaldehyde and paraffin-embedded. Cryosections were used for mouse CD31 (BD Bioscience), CD4, CD8 (BD Biosciences) and CD68 (AbD Serotec) immunofluorescent staining; GFP (Invitrogen) immunoflurescent staining; and TUNEL staining (Roche). Immunohistochemical staining for human vimentin, Ki67 and activated caspase 3 was performed on paraffin-embedded lung sections using standard procedures (MSKCC Molecular Cytology Core Facility).

Trans-endothelial migration assays

HPMECs were plated in the upper chamber of Fluoro-Blok trans-well inserts (3 μm pore size, BD Falcon) in 24-well plates. In the first set of migration assays, HPMEC-coated inserts were placed upside-down and 1.5×10^5 U937 cells in 100 μl of culture medium were plated on the lower surface of filter. 20 nM PMA (Sigma) was added to the medium to induce adhesion of U937 cells to the trans-well membrane surface. The U937 cells were fed with PMA-containing culture medium every 15 to 20 min, for 5 h. The inserts were flipped and placed back into 24-well plates and 50,000 green CMFDA labeled cancer cells were placed in the upper chamber. In the second set of migration assays, U937 cells were activated 1mM dibutyryl cAMP (Sigma) for 24 h to induce the expression of C5a receptor. 5×10^4 green CMFDA labeled cancer cells were mixed with or without 2.5×10^5 activated U937 cells and placed in the upper trans-well chamber. 1 ml of culture medium with 10 nM recombinant C5a (R&D Systems) was placed in the lower chamber. In the third set of migration assays, 5×10^4 green CMFDA labeled cancer cells were mixed with or without 2.5×10^5 U937 cells and placed in the upper trans-well chamber. 1 ml of culture medium with 100 ng/ml recombinant human CXCL12 (R&D) was placed in the lower chamber. In all sets of migration assays, cancer cells were allowed to migrate for 6 h and the trans-well inserts were fixed with 1% paraformaldehyde (Electron Microscopy Sciences) for 10 min. The lower side of the filter of each insert were scraped off and scored under fluorescence microscope. At least 10 random fields per sample were scored. Volocity software was used to quantify the surface area covered by green fluorescence as a representation of migrating cancer cells. Values were plotted as a percentage of migrating LM2 control cells.