Supporting Information

Duda et al. 10.1073/pnas.1016234107

SI Materials and Methods

Cell Lines and PCR Array. Lewis lung carcinoma (LLC-1) cells were purchased from ATCC. Another metastatic lung cancer cell line (LA-P0297) was established from spontaneous tumors that arose in FVB mice in our laboratory (1). The cancer cells have a mean diameter of 20 μm. We isolated carcinoma-associated fibroblasts (CAFs) from human breast cancer tissue obtained from the Department of Surgery at Massachusetts General Hospital and maintained cells in DMEM (ATCC) supplemented with 10% calf serum. Total RNA samples were isolated from cultured CAFs using TRIzol (Invitrogen). Expression profile of 84 key genes was determined using Human Angiogenesis PCR array plate (SABiosciences).

Tissue Preparation and Fluorescence Immunohistochemistry. Mice were perfusion fixed by infusion of 4% paraformaldehyde through the left ventricle, and lungs were postfixed for 2 h in 4% paraformaldehyde, followed by overnight incubation in 30% sucrose in PBS at 4 °C and subsequent embedding in freezing media. For immunohistochemical characterization of GFP-positive stromal cells, tissue sections were stained using anti-F4/80, anti-αSMA (α-smooth muscle actin), and anti-FSP1 (fibroblast-specific protein 1) (S100A4) antibodies. Tissue sections were incubated (*i*) 24 h at 4 °C with biotin-conjugated anti-F4/80 antibodies (Serotec), followed by 1-h incubation with a 1:200 dilution of Cy3-conjugated streptavidin (Molecular Probes); (*ii*) 2 h at room temperature using a primary Cy3-conjugated anti-mouse αSMA antibody (Sigma); or (*iii*) 24 h at 4 °C using a rabbit polyclonal anti-FSP1 (Abcam), followed by 1-h incubation at room temperature with a Cy3-conjugated anti-rabbit antibody (Jackson Immunoresearch). For human CAF identification, tissues were incubated for 30 min at room temperature using the monoclonal mouse, anti-human vimentin clone 3B4 antibody (#M7020; DAKO), or incubated 1 h at 37 °C using a monoclonal mouse anti-human HLA-ABC antigen clone W6/32 (#R7000; DAKO), both followed by 1-h incubation at room temperature with an FITC-labeled anti-mouse antibody.

Immunohistochemical Analysis of Brain Metastases from Carcinoma and Glioblastoma Patients. H&E sections of each clinical case were reviewed by a trained neuropathologist (M.S.). The following morphological criteria were applied in identifying benign fibroblasts on H&E sections: spindle shape, low nuclear/cytoplasmic ration, even chromatin pattern, and absence of atypical mitotic figures. For CD31/αSMA double staining, the sections were incubated with prediluted CD31 antibody (#N1596; DAKO) for 1 h at room temperature, and antigen detection was performed using Mouse EnVision Polymer. Next, sections were incubated with anti-αSMA 1:5,000 (catalog no. M0850; Sigma) in 5% NHS/PBS overnight at 4 °C, and double stain alkaline phosphatase polymer (DAKO) was used to retrieve the antigen for 30 min at room temperature. Tissues were counterstained using hematoxylin and coverslipped with Faramount fixation. Immunostaining for CD10 was performed using anti-CD10 antibody (IgG_1 isotype; clone 56c6, prediluted; Ventana Medical Systems) on BenchMark XT automated tissue staining systems (Ventana Medical Systems), followed by incubation with *UltraView* HRP-conjugated multimer antibody reagent (Igs; Ventana Medical Systems). Antigen detection was performed using *UltraView* diamino-benzidine chromogen (Ventana Medical Systems).

1. Huang P, Duda DG, Jain RK, Fukumura D (2008) Histopathologic findings and establishment of novel tumor lines from spontaneous tumors in FVB/N mice. *Comp Med* 58:253–263.

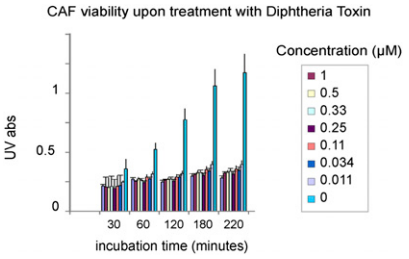

Fig. S1. In vitro effect of diphteria toxin (DT) on human CAFs. 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) cell survival/proliferation assay for DT treatment of human CAFs. The histogram plot shows that DT depletes human cells even at very low doses (0.011 μM).

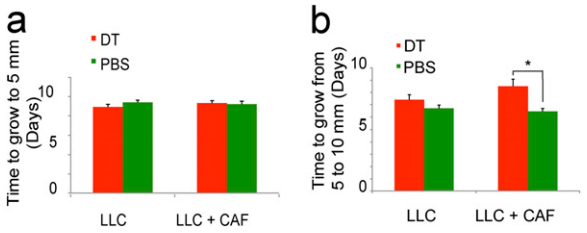

Fig. S2. In vivo growth of LLC1 tumors after implantation of LLC1 cells alone or coimplantation of LLC1 cells with CAFs: effect of diphtheria toxin (DT) treatment. (*A*) Tumor growth after coimplantation of CAFs with LLC1 cells was not significantly different from after implantation of LLC1 alone. (*B*) Depletion of CAFs with DT treatment significantly delayed the established tumor growth only in the coimplantation group.

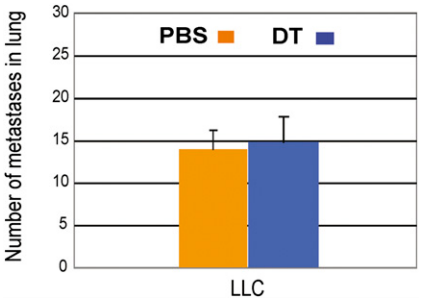

Fig. S3. Effect of diphtheria toxin (DT) treatment on metastasis in LLC1 alone implantation model. DT treatment 1 d after primary LLC1 tumor resection does not affect metastasis.

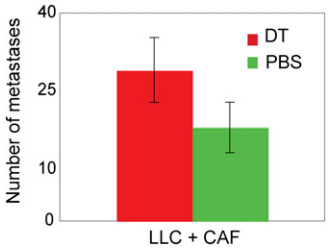

Fig. S4. Metastasis formation after i.v. infusion of LLC1 cells with CAFs: effect of diphtheria toxin (DT) treatment. Depletion of CAFs with DT after i.v. infusion of LLC1 and CAFs does not affect metastasis formation.

Table S1. Size distribution of heterotypic cell fragments

Diameter (μm)	No. of clumps with GFP$^+$ cells per h per tumor (n = 5 mice), mean ± SEM (% of total)
<50	152 ± 52 (77)
50–99	23 ± 15 (12)
100–149	18 ± 9 (9)
150–200	5 ± 5 (3)
Total	198 ± 116

Table S2. Gene expression profile in CAFs using a 96-gene PCR array

Gene (symbol)	Ct value	Comments
Angiopoietin-like 4 (*ANGL4*)	22.19	Identified as a player in lung metastatic colonization
Alanyl aminopeptidase (*ANPEP*)	22.32	Proangiogenic factor
Monocyte-chemoattractant protein 1 (MCP-1) (*CCL2*)	24.24	Proangiogenic and prometastasis chemokine
Sphingosine-1-phosphate receptor 1 (*S1PR1*)	24.58	Receptor involved in angiogenesis, cell–cell adhesion and migration
Basic fibroblast growth factor (*FGF2*)	21.39	Proangiogenic growth factor
Hepatocyte growth factor (*HGF*)	24.39	Proangiogenic and migration factor
Hypoxia-inducible factor 1 alpha (*HIF1A*)	20.95	Proangiogenic signaling factor
Inhibitor of differentiation gene 3 (*ID3*)	21.21	Proangiogenic signaling factor
Interleukin 6 (*IL6*)	24.69	Proangiogenic inflammatory cytokine
Interleukin 8 (*IL8*)	23.3	Proangiogenic inflammatory cytokine
Integrin alpha v (*ITGAV*)	22.86	Receptor involved in angiogenesis, cell–cell adhesion and migration
Gellatinase A (*MMP2*)	20.82	Proangiogenic enzyme
Neuropilin 1 (*NRP1*)	21.45	Receptor involved in angiogenesis and migration
Sphingosine kinase 1 (*SPHK1*)	23.53	Lipid kinase that catalyzes formation of S1P and induces MMP1, Erk, and Ets1
Thrombospondin 1 (*THBS1*)	17.58	Endogenous antiangiogenic factor
Tissue inhibitor of metalloproteinase 1 (*TIMP1*)	17.22	Negative regulator of MMPs
Tissue inhibitor of metalloproteinase 2 (*TIMP2*)	20.33	Negative regulator of MMPs
Vascular endothelial growth factor A (*VEGFA*)	22.93	Proangiogenic growth factor
Vascular endothelial growth factor C (*VEGFC*)	22.95	Proangiogenic growth factor
B2M (control)	25.35	Housekeeping gene
HPRT1 (control)	26.08	Housekeeping gene
RPL13A (control)	21.87	Housekeeping gene
GAPDH (control)	18.99	Housekeeping gene
ACTB (control)	19.32	Housekeeping gene

Data are shown for the genes with a Ct value of <25 and for housekeeping genes.