Supplemental Data

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TGFβ Primes Breast Tumors for Lung Metastasis Seeding through Angiopoietin-like 4

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Experimental Procedures

Cell culture and reagents

HaCaT were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin, and fungizone. MCF-10A cells were maintained in a 1:1 mixture of DMEM and Ham's F12 supplemented with 5% horse serum, 10 μg/ml insulin (Sigma), 0.5 μg/ml hydrocortisone (Sigma), 0.02 μg/ml epidermal growth factor (Sigma), and antibiotics. HPL1 cells were maintained in Ham's F12 supplemented with 1% FBS, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 5 μg/ml transferrin (Sigma), 2×10^{-10}M triiode thyronine, and antibiotics. All tumor cell lines were cultured in DMEM supplemented with 10% FBS, glutamine, penicillin, streptomycin and fungizone. The pleural effusion samples were centrifuged at 1,000 r.p.m. for 10 min, cell pellets were re-suspended in PBS and treated with ACK lysis buffer to lyse blood cells. A fraction of these cells underwent negative selection to remove leukocytes ($CD45^+$ and $CD15^+$ cells), and EpCam-positive cells were sorted from the population upon recovery in tissue culture for 24 h. Human vascular endothelial cells (HUVECs) (ScienCell) were cultured in complete ECM media (ScienCell) and used between passages 3–6. The retroviral packaging cell line GPG29 was maintained in DMEM containing 10% FBS supplemented with puromycin, G418, doxycycline, penicillin, streptomycin and fungizone. Transfections were done using standard protocols with Lipofectamine 2000 (Invitrogen). After transfection, GPG29 cells were cultured in DMEM containing 10% FBS.

RNA isolation, labeling, and microarray hybridization

Methods for RNA extraction, labeling and hybridization for DNA microarray analysis of the cell lines have been described previously (Kang et al., 2003b; Minn et al., 2005). The EMC and MSK tumor cohorts and their gene expression data have been previously described (Minn et al., 2007; Minn et al., 2005; Wang et al., 2005). Bone or lung recurrence at any time is indicated.

The tissues for microarry analysis were obtained from therapeutic procedures performed as part of routine clinical management. Samples were snap-frozen in liquid nitrogen and stored at -80° C. Each sample was examined histologically in cryostat sections stained with hematoxylin and eosin. Regions were dissected manually from the frozen block to provide a consistent tumor cell content of greater than 70% in tissues used for analysis. RNA was extracted from frozen tissues by homogenization in TRIzol reagent (Gibco/BRL) and evaluated for integrity. Complementary DNA was synthesized from total RNA by using a dT primer tagged with a T7 promoter. The RNA target was synthesized by transcription *in vitro* and labelled with biotinylated nucleotides (Enzo Biochem). The labeled target was assessed by hybridization to Test3 arrays (Affymetrix). All gene expression analysis was performed with an HG-U133A GeneChip (Affymetrix). Gene expression was quantified with MAS 5.0 or GCOS (Affymetrix). All studies involving patient materials or data were conducted under protocols approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center, and that of the Hospital Clínic de Barcelona.

For the cell culture experiments, total RNA was prepared from 5×10^6 cultured cells that were untreated or treated with TGFβ. Twenty-five micrograms of total RNA was used to prepare cRNA probe using a Custom Superscript Kit (Invitrogen) and the BioArray HighYield RNA Transcript Labeling Kit (Enzo). Each sample was hybridized with an Affymetrix Human Genome U133A microarray for 16 hr at 45°C.

TGFβ response gene-expression signature and TBRS classifier

To generate a TBRS classifier, we carried out a “meta-gene” analysis based on this gene set and using the cell lines as references (Bild et al., 2006) and references therein. In short, expression values of the 153 TGFβ responsive genes in cell lines were linearly transformed and encapsulated into one or two “Meta genes”. A Bayesian Probit model was then trained based the cell line data and applied to the Meta genes of the tumor samples. For each tumor, a number between 0 and 1 was derived, indicating the likelihood that the TGFβ signaling is active in that tumor.

Generation of retrovirus and knockdown cells

Viruses were collected 48 and 72 h after transfection, filtered, and concentrated by ultracentrifugation. Concentrated retrovirus was used to infect cells in the presence of 8 μg ml^{-1} polybrene, typically resulting in a transduction rate of over 80%. Infected cells were selected

with puromycin or hygromycin. To generate knockdown-rescue cell lines, we used a similar method to produce virus encoding complementary DNAs for overexpression of the RNAi-targeted genes, along with a hygromycin or puromycin selectable marker. The overexpressing retrovirus vector, pBabe, was used to super-infect previously generated knockdown cells that were subsequently selected with either hygromycin or puromycin.

Analysis of mRNA and protein expression

Total RNA from subconfluent MDA-MB-231 cells was collected and purified using the RNeasy kit (Qiagen). Four-hundred nanograms of total purified RNA was subjected to a reverse transcriptase reaction according to the Hi-Capacity Archive kit (Applied Biosystems). cDNA corresponding to approximately 4 ng of starting RNA was used in three replicates for quantitative PCR. Indicated Taqman gene expression assays (Applied Biosystems) and the Taqman universal PCR master mix (Applied Biosystems) were used to quantify expression. Quantitative expression data were acquired and analyzed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). For immunoblotting, we used previously described methods (Calonge and Massagué, 1999). Briefly, proteins were separated by SDS–PAGE and transferred to nitrocellulose membranes (Bio-Rad) that were immunoblotted with mouse monoclonal antibodies that recognize Smad4 (Cell Signaling) and α-tubulin (Sigma). For analysis of secreted protein expression, cells were plated in triplicate at 90% confluency in 12-well plates, incubated in DMEM 0.2% FBS, and conditioned media was collected 72 h later. Media was cleared of cells by centrifuging at 2,000 r.p.m. for 5 min. Angptl4 concentrations were analyzed in conditioned media using an ELISA kits (BioVendor).

Generation of retrovirus and knockdown cells

Knockdown of *SMAD4* and *ANGPTL4* was achieved using pRetroSuper technology (Brummelkamp et al., 2002) targeting the following 19-nucleotide sequences: 5'-GGTGTGCAGTTGGAATGTA -3' (*SMAD4*) and 5'-GAGGCAGAGTGGACTATTT-3' (*ANGPTL4*). To produce retrovirus for knockdown, the hairpin vector was transfected into the GPG29 amphotropic packaging cell line (Ory et al., 1996).

Immunostaining

For visualizing lung metastases, mice were killed and perfused with PBS and 4% paraformaldehyde through the left ventricle. Lungs were fixed and paraffin-embedded.

Immunohistochemical staining for vimentin (Novocastra) was performed on paraffin-embedded lung sections by the MSKCC Molecular Cytology Core Facility. Brightfield microscopic images were collected using an AxioImager Z1 microscopy system (Zeiss).

Immunofluorescence

HUVECs were grown to confluence on fibronectin coated chamber slides (BD Biosciences). The cells were fixed for 10 min in 4% paraformaldehyde in PBS, and incubated for 5 min on ice in 0.5% Triton X-100 in PBS. After blocking with 2% BSA, the monolayers were processed for staining with anti-ZO1 (Zymed), anti-beta-catenin (Santa Cruz), rhodamine phalloidin (Molecular Probes) for F-actin staining and DAPI (Vector Labs) for nuclear staining. Fluorescence images were obtained using an AxioImager Z1 microscopy system (Zeiss).

Animal studies

For primary tumor analysis, $5x10^5$ viable single cells were re-suspended in a 1:1 mixture of PBS and growth-factor-reduced Matrigel (BD Biosciences) and injected orthotopically into both mammary gland number four in a total volume of 100µL as previously described (Minn et al., 2005). Primary tumor growth rates were analyzed by measuring tumour length (*L*) and width (*W*), and calculating tumor volume based on the formula $\pi LW^2/6$. For lung colonization assays, 2×10^5 cells were re-suspended in 0.1 ml PBS and injected into the lateral tail vein. Lung metastatic progression was again monitored and quantified using bioluminescence. For bone metastasis, 30,000 cells in PBS were injected into the left ventricle of anaesthetized mice (100mg kg^{-1} ketamine, 10 mg kg^{-1} xylazine). For priming assays, cells were switched to low media (0.2% FBS) for 12 hours and then treated with 100pM of TGFβ for 6 hours. Mice were inoculated with 200,000 and 30,000 cells for lung and bone assays, respectively. Mice were imaged for luciferase activity immediately after injection to exclude any that were not successfully xenografted. Lymph node analysis was performed by ex vivo bioluminescent imaging of the peri-aortic lymph nodes.

Intravasation assays

Mice were perfused with 5 ml PBS through the left ventricle. Three millilitres of blood perfusate was collected from the atrium and lysed two times using ACK lysis buffer (Cambrex). Total RNA was extracted from the remaining cells and used for qRT–PCR as described above. The

presence of human circulating tumour cells was determined by the relative expression of human *GAPDH* normalized to murine β2-microglobulin.

Trans-endothelial migration assays

HUVECs were seeded into gelatin-coated Fluoro-Blok trans-well inserts (3 μm pore size, BD Falcon) at 100,000 cells per well, and allowed to grow to confluence for 4 days. Tumor cells were pulsed with 5 μM cell tracker green (Invitrogen) for 30 min before being conditioned overnight in 0.2% FBS ECM media without growth factors. The next day, 50,000 tumour cells were seeded into trans-well inserts with confluent endothelial monolayer, and the wells were fixed in 4% paraformaldehyde after 8 h. Cells on the apical side of each insert were scraped off and the trans-well membrane mounted onto slides. Migration to the basolateral side of the membrane was visualized with a Zeiss AxioObserver inverted immunofluorescent microscope at 20x magnification. Pictures of 5 random fields across three replicate wells were captured for quantification using Volocity software. In general, 8-10 cells per field were seen to migrate through an endothelial barrier. Migration of the indicated lines was plotted as a percentage of migrating LM2 control cells.

Supplemental Figures

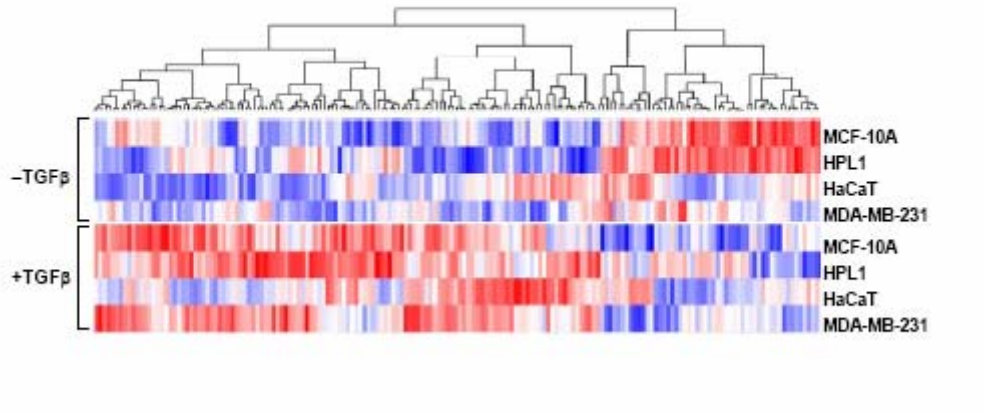

Figure S1 Heat map representing the raw expression values of the 153 gene TBRS in the four epithelial cell lines with or without TGFβ treatment.

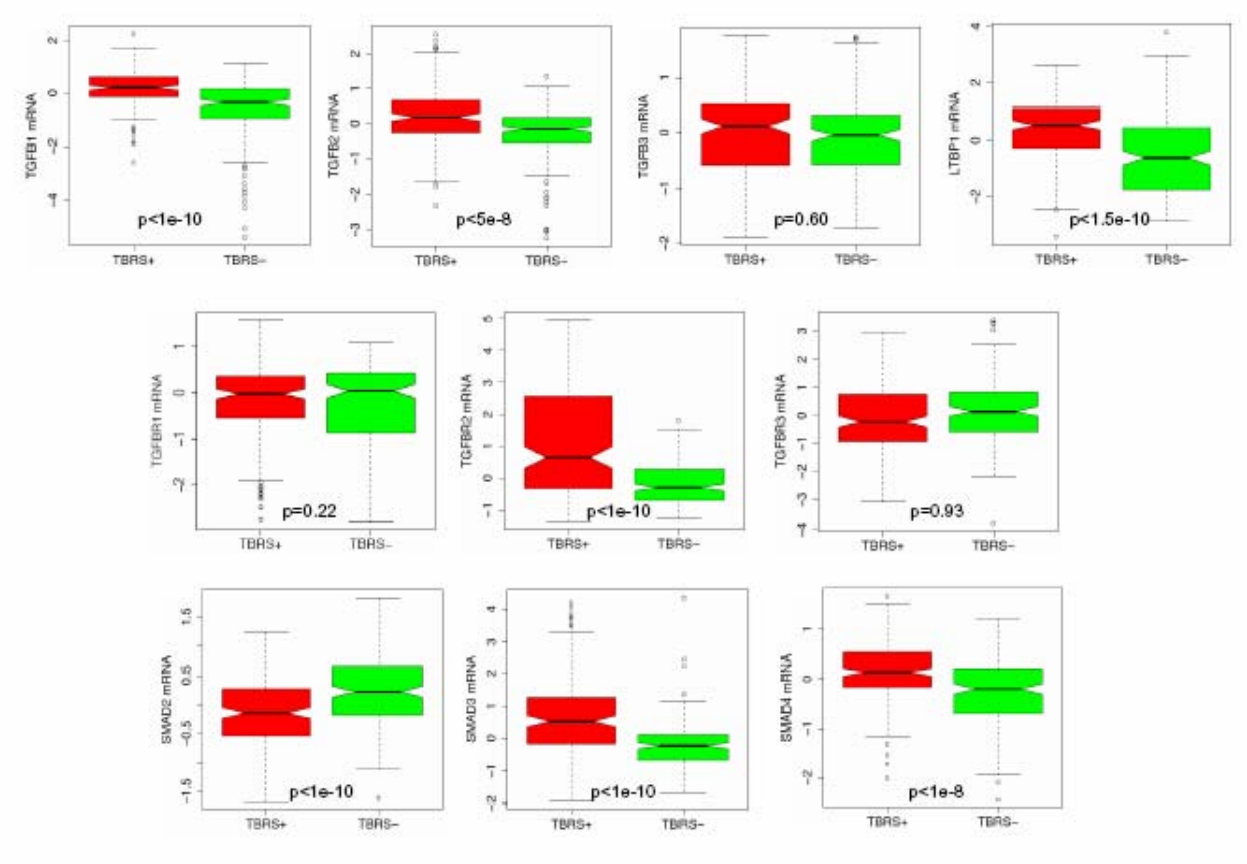

Figure S2 Box-and-whisker plots comparing the RNA expression of TGFβ1, TGFβ2, TGFβ3, and LTBP1 in TBRS positive or negative primary breast tumors.

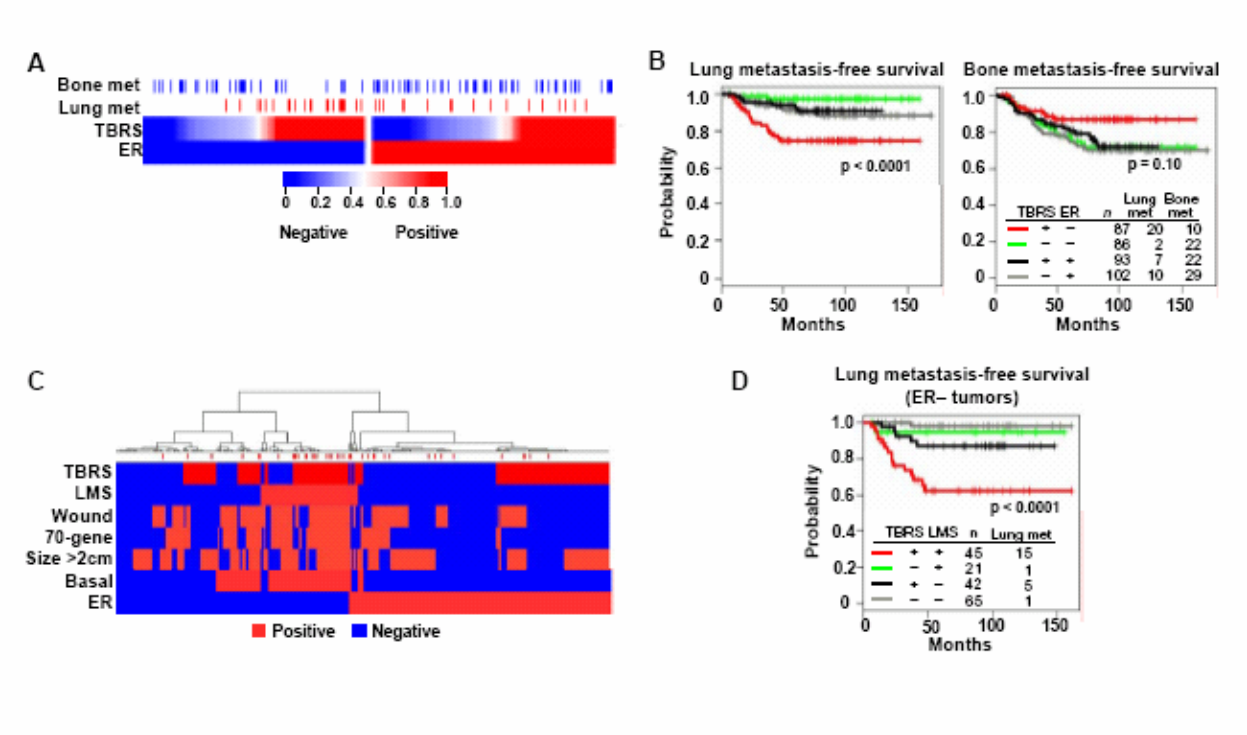

Figure S3 Analysis of Figure 1 data using an ER status designated by microarray probe levels rather than the clinical designations

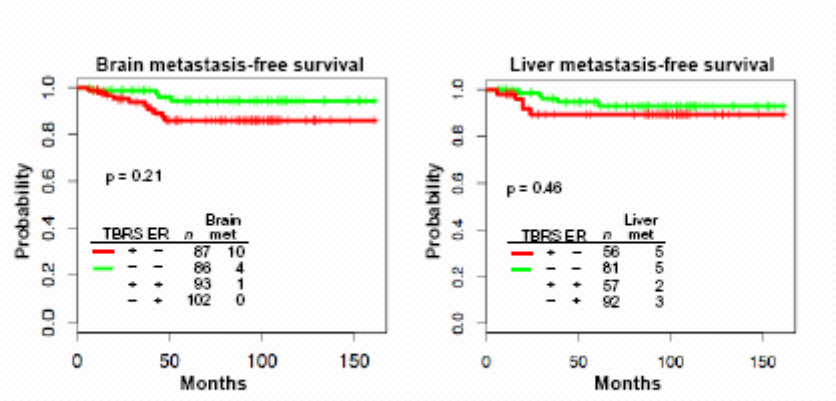

Figure S4 Kaplan-Meier curves of brain and liver metastasis-free survival from ER negative breast cancer patients either TBRS positive or negative.

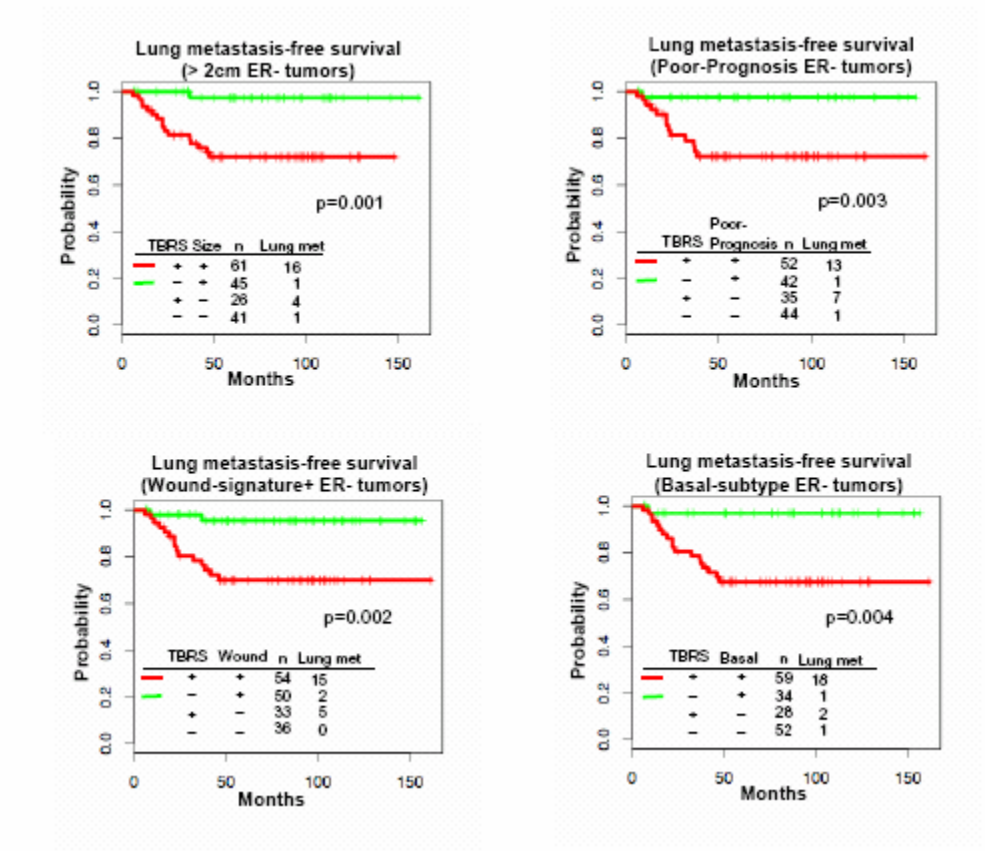

Figure S5 Kaplan-Meier curves of ER negative breast cancer patients comparing TBRS status and other markers of poor clinical outcome such as large size, poor-prognosis signature positive, wound signature positive, and basal molecular subtype tumor designation.

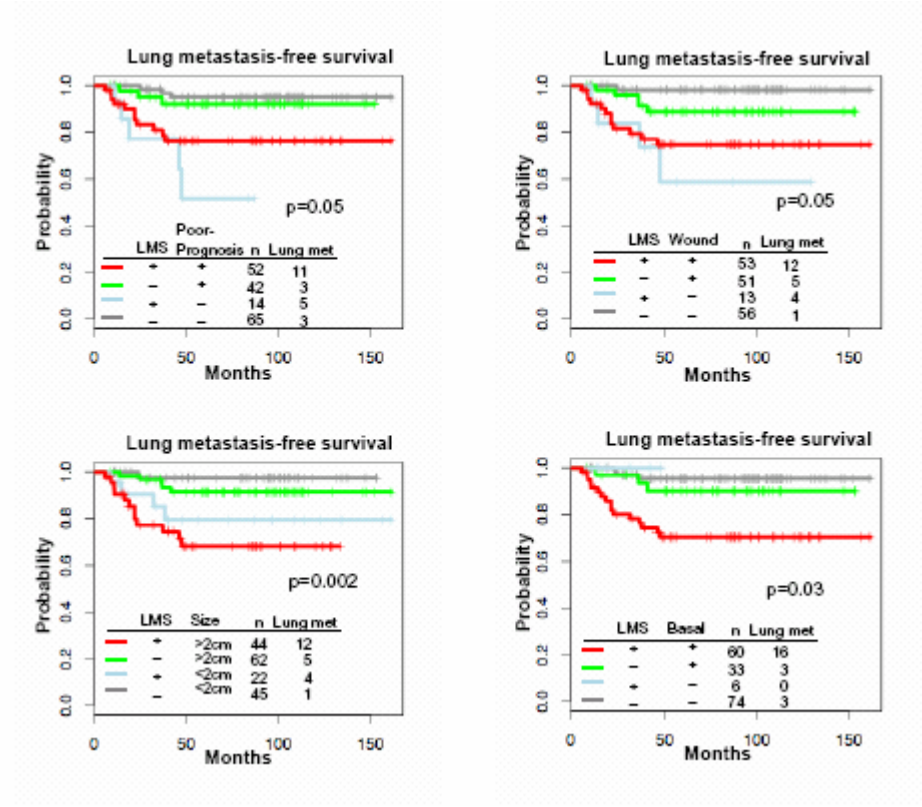

Figure S6 Kaplan-Meier curves of breast cancer patients comparing LMS status and other markers of poor clinical outcome such as large size, poor-prognosis signature positive, wound signature positive, and basal molecular subtype designation.

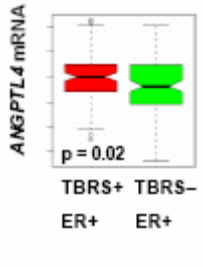

Figure S7 Box-and-whisker plot of ER positive primary breast cancer comparing the expression of *ANGPTL4* in primary tumors that are either TBRS positive or negative

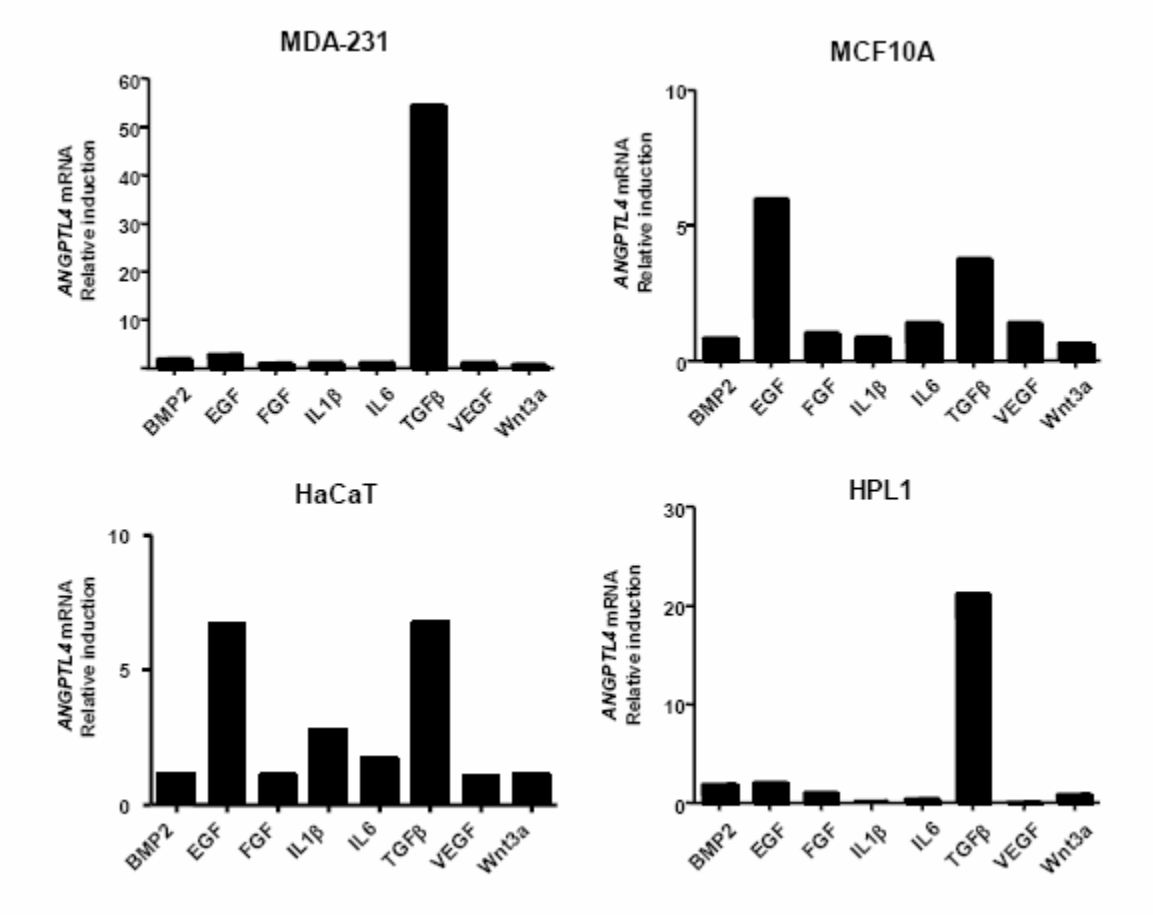

Figure S8 The four epithelial cell lines were treated with the indicated cytokines for 3h. ANGPTL4 mRNA fold induction are plotted relative to untreated samples.

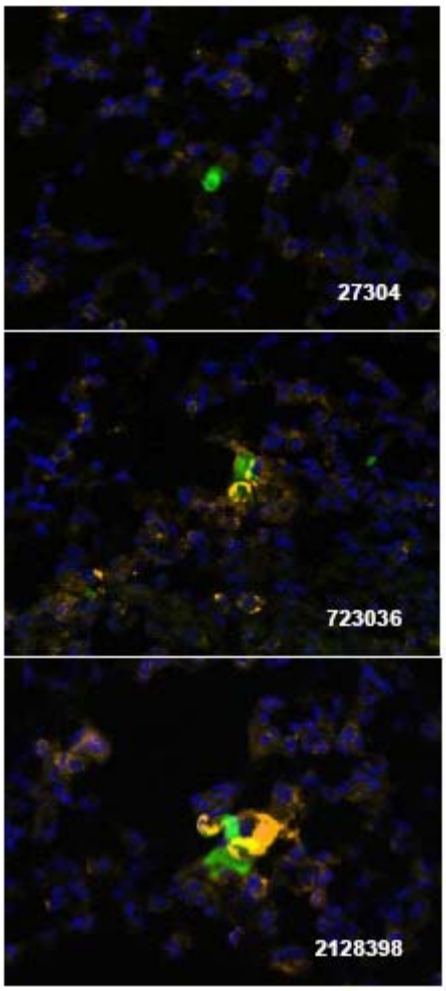

Figure S9 Representative images used in the quantification of in vivo permeability. Top image is representative of a low level of extravasated rhodamine-dextran, middle image corresponds to a mean level of extravasated dextran, while bottom image is that of cell inducing high levels of permeability and as a result dextran extravastation.

Table S1 The 153 genes in the TBRS. Gene symbols, gene description, and TGFβ induction levels are displayed.

Probes	MDA-MB-231	HaCaT	HPL1	MCF10A	Symbol
213497_at	1.88	2.14	2.24	1.53	ABTB2
206466_at	1.09	2.90	17.93	1.18	ACSBG1
222090_at	0.97	0.55	0.44	0.46	ADCK2
206170_at	0.37	0.40	0.73	0.52	ADRB2
207294_at	0.98	0.69	0.35	0.06	AGTR2
221008_s_at	1.01	0.76	0.15	0.34	AGXT2L1
220842_at	1.01	1.03	0.13	0.04	AHI1
204174_at	1.28	1.36	2.19	2.02	ALOX5AP
222108_at	4.26	1.81	5.54	1.51	AMIGO2
217630_at	1.02	0.53	0.16	0.47	ANGEL2
221009_s_at	7.58	2.18	40.35	6.26	ANGPTL4
207087_x_at	1.02	1.23	7.13	4.36	ANK1
217888_s_at	2.23	1.02	1.45	2.01	ARFGAP1
207606_s_at	0.40	1.10	0.50	0.54	ARHGAP12
212614_at	0.40	0.41	0.31	0.42	ARID5B
217579_x_at	1.04	1.69	9.19	2.77	ARL6IP2
216197_at	1.07	0.64	0.40	0.05	ATF7IP
218631_at	0.49	0.76	0.42	0.37	AVPI1
220470_at	1.13	1.05	5.04	4.41	BET1L
201169_s_at	3.67	2.21	9.39	2.27	BHLHB2
201170_s_at	2.99	3.37	7.51	2.45	BHLHB2
206119_at	1.15	1.66	2.13	4.44	BHMT
209920_at	1.72	2.35	2.18	1.29	BMPR2
210214_s_at	2.22	1.45	2.14	2.61	BMPR2
206787_at	0.98	0.72	0.12	0.06	BRDT
218723_s_at	0.50	0.56	0.05	0.24	C13orf15
217508_s_at	1.87	1.73	2.62	1.47	C18orf25
221533_at	1.18	1.18	2.04	3.22	C3orf28
219474_at	2.00	1.80	2.46	2.14	C3orf52
212923_s_at	2.12	2.21	1.61	1.52	C6orf145
220513_at	1.09	1.95	7.90	2.09	C6orf148
209688_s_at	1.63	1.36	2.07	2.30	CCDC93
219774_at	2.14	1.26	1.04	2.52	CCDC93
203645_s_at	0.96	0.76	0.36	0.15	CD163
208592_s_at	1.23	1.37	4.40	4.29	CD1E
211861_x_at	1.09	0.68	0.48	0.08	CD28
202284_s_at	3.18	2.70	2.44	1.11	CDKN1A
218929_at	0.59	0.96	0.37	0.47	CDKN2AIP
203973_s_at	0.21	0.57	0.44	0.53	CEBPD
207331_at	1.08	2.05	10.46	1.02	CENPF
207980_s_at	0.12	0.34	0.17	0.23	CITED2
209357_at	0.13	0.19	0.22	0.19	CITED2
205295_at	1.11	0.65	0.47	0.23	CKMT2
202310_s_at	4.75	1.33	1.90	5.05	COL1A1

211980_at	2.07	1.47	2.64	4.02	COL4A1
211981_at	1.84	1.40	2.99	2.27	COL4A1
211964_at	1.27	1.19	2.08	2.08	COL4A2
221900_at	1.21	1.10	2.01	3.43	COL8A2
209101_at	4.01	5.88	2.12	5.20	CTGF
206775_at	1.22	1.07	3.13	2.26	CUBN
203923_s_at	1.13	0.76	0.07	0.19	CYBB
202887_s_at	0.50	0.93	0.79	0.47	DDIT4
215252_at	1.12	1.56	2.08	3.67	DNAJC7
40612_at	0.94	0.55	0.15	0.30	DOPEY1
218995_s_at	3.76	2.13	1.50	1.03	EDN1
206127_at	2.01	1.56	2.11	1.35	ELK3
221773_at	2.09	1.90	3.03	1.62	ELK3
201328_at	1.72	3.35	2.86	6.83	ETS2
201329_s_at	1.75	2.65	1.72	3.95	ETS2
219427_at	0.61	0.70	0.50	0.39	FAT4
204988_at	1.01	0.55	0.18	0.36	FGB
218818_at	2.21	1.19	1.60	2.51	FHL3
204135_at	2.28	1.38	1.23	2.44	FILIP1L
220326_s_at	1.30	1.58	2.06	2.10	FLJ10357
58780_s_at	1.50	1.81	2.22	2.19	FLJ10357
210316_at	0.96	0.48	1.15	0.14	FLT4
219316_s_at	0.85	0.48	0.87	0.17	FLVCR2
218618_s_at	2.01	1.25	1.26	2.09	FNDC3B
203592_s_at	1.90	2.20	3.24	1.71	FSTL3
209414_at	1.45	1.17	2.08	2.73	FZR1
209416_s_at	1.47	1.31	2.00	2.02	FZR1
207574_s_at	3.88	3.79	2.80	1.45	GADD45B
209304_x_at	3.37	3.21	2.45	1.30	GADD45B
209305_s_at	3.38	3.06	2.58	1.11	GADD45B
215248_at	1.16	1.21	2.99	7.32	GRB10
214438_at	1.17	2.07	1.55	4.48	HLX
203665_at	3.05	1.29	6.19	1.70	HMOX1
204111_at	1.02	0.65	0.30	0.19	HNMT
205580_s_at	2.08	1.14	2.06	1.47	HRH1
208937_s_at	1.04	0.54	0.38	0.34	ID1
206924_at	9.37	2.46	4.91	2.70	IL11
207952_at	1.04	0.65	0.66	0.12	IL5
204686_at	0.41	0.80	0.67	0.50	IRS1
209098_s_at	2.26	2.06	1.76	2.45	JAG1
41387_r_at	1.22	1.64	2.02	2.06	JMJD3
201464_x_at	2.19	2.15	2.62	1.82	JUN
201466_s_at	2.81	2.58	5.10	2.65	JUN
201473_at	7.33	2.65	8.17	1.87	JUNB
218651_s_at	2.19	3.08	3.96	2.86	LARP6
221011_s_at	2.32	1.87	5.40	6.95	LBH
218604_at	1.62	2.08	2.67	1.23	LEMD3
218574_s_at	3.41	1.13	5.25	1.67	LMCD1
204089_x_at	1.40	1.56	2.18	2.04	MAP3K4
208210_at	1.00	0.69	0.21	0.41	MAS1

214696_at	2.02	1.46	2.26	1.51	MGC14376
202519_at	2.05	1.67	1.35	2.54	MLXIP
216303_s_at	1.02	0.58	0.52	0.34	MTMR1
213906_at	0.41	0.85	0.83	0.42	MYBL1
202431_s_at	1.02	0.40	0.39	0.37	MYC
201496_x_at	1.06	0.66	0.11	0.31	MYH11
218149_s_at	0.49	0.84	0.72	0.30	NA
207760_s_at	2.50	1.11	2.43	1.91	NCOR2
202607_at	1.35	1.11	4.21	2.02	NDST1
202150_s_at	2.74	3.08	3.05	3.56	NEDD9
201695_s_at	2.12	1.26	1.14	2.08	NP
206699_x_at	1.00	1.43	2.09	22.54	NPAS1
209120_at	0.19	0.64	0.49	0.34	NR2F2
215073_s_at	0.32	0.60	0.32	0.40	NR2F2
213824_at	0.91	0.71	0.50	0.23	OLIG2
221868_at	1.08	0.61	0.32	0.18	PAIP2B
206594_at	1.05	1.80	3.47	2.08	PASK
221918_at	2.17	2.27	1.34	1.61	PCTK2
205463_s_at	2.30	1.53	7.00	1.73	PDGFA
218691_s_at	2.43	1.50	18.06	1.15	PDLIM4
202464_s_at	2.02	2.15	1.83	1.65	PFKFB3
212134_at	2.06	1.36	2.85	1.96	PHLDB1
204612_at	1.31	2.09	1.15	8.96	PKIA
204958_at	1.20	1.48	6.24	2.08	PLK3
209740_s_at	0.87	0.83	0.49	0.24	PNPLA4
218849_s_at	2.22	1.41	2.83	1.57	PPP1R13L
202879_s_at	1.29	2.10	1.42	2.16	PSCD1
202880_s_at	1.22	2.09	2.08	2.09	PSCD1
206977_at	0.96	1.23	15.95	4.12	PTH
219812_at	1.18	1.19	2.43	2.52	PVRIG
216719_s_at	1.06	0.50	0.16	0.93	RAB11FIP4
219440_at	1.10	0.79	0.21	0.16	RAI2
203749_s_at	3.39	1.93	4.10	1.46	RARA
206850_at	1.23	1.34	4.49	2.30	RASL10A
203748_x_at	1.42	1.33	2.02	2.21	RBMS1
207266_x_at	1.42	1.36	2.18	2.09	RBMS1
209868_s_at	1.42	1.37	2.07	2.06	RBMS1
212099_at	3.92	2.05	4.73	3.16	RHOB
205158_at	0.71	0.78	0.48	0.26	RNASE4
202627_s_at	3.67	11.31	7.36	7.64	SERPINE1
202656_s_at	0.20	0.77	0.56	0.45	SERTAD2
202657_s_at	0.23	0.59	0.38	0.53	SERTAD2
201739_at	3.23	2.29	4.88	1.11	SGK
206675_s_at	1.26	4.80	8.33	16.58	SKIL
217591_at	2.02	3.25	3.13	2.52	SKIL
217685_at	0.96	0.76	0.44	0.11	SLC16A3
207298_at	1.23	1.19	3.02	2.01	SLC17A3
204790_at	4.17	5.95	10.80	6.39	SMAD7
210357_s_at	1.60	2.29	2.40	3.03	SMOX
207390_s_at	1.40	1.36	2.01	3.00	SMTN

212666_at	1.62	1.66	2.30	2.02	SMURF1
212668_at	1.32	2.10	1.24	4.46	SMURF1
215458_s_at	1.49	1.31	2.00	2.36	SMURF1
219480_at	2.01	1.38	6.11	0.90	SNAI1
219257_s_at	2.36	1.66	2.11	2.09	SPHK1
209875_s_at	1.00	0.90	0.15	0.23	SPP1
219677_at	2.15	1.26	1.27	2.11	SPSB1
217991_x_at	1.43	1.10	2.43	2.29	SSBP3
216917_s_at	1.05	0.50	0.72	0.20	SYCP1
212796_s_at	1.73	1.29	1.81	2.26	TBC1D2B
211462_s_at	1.04	1.24	8.78	2.08	TBL1Y
208398_s_at	2.09	1.40	1.33	2.17	TBPL1
221866_at	1.47	1.35	5.80	2.91	TFEB
50221_at	1.82	2.25	4.16	2.41	TFEB
211154_at	0.99	0.49	0.80	0.08	THPO
217875_s_at	5.44	2.96	31.67	1.62	TMEPAI
208296_x_at	2.11	1.33	1.24	2.01	TNFAIP8
218368_s_at	1.36	2.00	2.11	1.39	TNFRSF12A
210987_x_at	2.00	1.27	2.11	1.43	TPM1
212664_at	1.08	1.66	2.37	3.15	TUBB4
205807_s_at	2.92	1.85	2.23	2.75	TUFT1
221098_x_at	1.03	0.50	0.73	0.06	UTP14A
211527_x_at	2.56	1.45	8.61	21.20	VEGFA
221423_s_at	2.00	1.11	1.19	2.16	YIPF5
208078_s_at	3.17	2.49	2.47	2.05	ZEB1
211962_s_at	2.07	2.07	1.44	1.14	ZFP36L1
211965_at	1.56	2.28	1.43	3.34	ZFP36L1
203520_s_at	0.81	0.82	0.50	0.16	ZNF318
221123_x_at	0.42	0.96	0.93	0.50	ZNF395
206810_at	1.02	0.56	0.23	0.25	ZNF44

Table S2 TBRS status was assessed in each of the breast cacner metastasis samples submitted for microarray analysis. The percentage of TBRS positive samples in each metastasis site is shown.

Site	TBRS+ (%)
Lung	14/18 (78)
Bone	8/16 (50)
Brain	6/19 (32)
Liver	5/5 (100)
Other sites*	5/9 (56)
All sites	38/67 (58)

* Includes ovary, duodenum and chest wall

Table S3 Univariate and multivariate analyses correlating TGFβ pathway components and incidence of lung metastasis in the breast cancer patient cohorts. P-values for these associations are listed.

	All tumors	ER- tumors
TGFBR1	0.7740	0.2560
TGFBR2	0.1700	0.0110
TGFB1	0.5460	0.1800
TGFB2	0.8750	0.5120
TGFB3	0.001[a]	0.0271[a]
Smad2	0.1990	0.0425
Smad3	0.3090	0.0111
Smad4	0.0937	0.1780
TGFBR2+Smad3[b]	0.3380	0.0106
All (except *TGFB3*)[b]	0.6260	0.0533
TBRS	0.0133	< 0.0001

a: Negtive correlation
b: Multivairate analyses using Cox proportional hazards regression model