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

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SUMMARY

Cells released from primary tumors seed metastases to specific organs by a nonrandom process, implying the involvement of biologically selective mechanisms. Based on clinical, functional, and molecular evidence, we show that the cytokine TGFβ in the breast tumor microenvironment primes cancer cells for metastasis to the lungs. Central to this process is the induction of *angiopoietin-like 4* (*ANGPTL4*) by TGFβ via the Smad signaling pathway. TGFβ induction of Angptl4 in cancer cells that are about to enter the circulation enhances their subsequent retention in the lungs, but not in the bone. Tumor cell-derived Angptl4 disrupts vascular endothelial cell-cell junctions, increases the permeability of lung capillaries, and facilitates the *trans*-endothelial passage of tumor cells. These results suggest a mechanism for metastasis whereby a cytokine in the primary tumor microenvironment induces the expression of another cytokine in departing tumor cells, empowering these cells to disrupt lung capillary walls and seed pulmonary metastases.

INTRODUCTION

The identification of metastasis genes and mechanisms is essential for understanding the basic biology of this lethal condition and its implications for clinical practice (Fidler, 2003; Gupta and Massagué, 2006). The predisposition of primary tumors to selectively invade different organs has been long recognized (Paget, 1889). Recent work has functionally identified and clinically validated sets of genes whose overexpression in breast cancer cells confers a selective advantage for the colonization of bones (Kang et al., 2003b; Lynch et al., 2005) or lungs (Minn et al., 2005). There is also the possibility that the microenvironment of a primary tumor may influence the fate of cancer cells that escape from this tumor. Among the factors in the tumor microenvironment that might play such a role, we chose to focus on the cytokine TGFβ, which modulates tumor progression in various experimental systems (Bierie and Moses, 2006; Dumont and Arteaga, 2003; Siegel and Massagué, 2003).

TGFβ is a multifunctional cytokine with diverse effects on virtually all cell types and with key roles during embryo development and tissue homeostasis (Massagué et al., 2000). It regulates the production of microenvironment sensors and modulators, including cytokines, extracellular matrix components, and cell-surface receptors. Additionally, TGFβ has potent inhibitory effects on cell proliferation, and, as such, it can deter tumor growth (Bierie and Moses, 2006; Dumont and Arteaga, 2003; Siegel and Massagué, 2003). Within the tumor microenvironment, TGFβ is produced by myeloid cells, mesenchymal cells, and the cancer cells themselves, as a natural response to the hypoxic and inflammatory conditions that occur during tumor progression. The TGFβ receptors, which are membrane serine/threonine protein kinases, and their substrates, the Smad transcription factors, are tumor suppressors that frequently suffer inactivation in gastrointestinal, pancreatic, ovarian, and hepatocellular cancinomas and subsets of gliomas and lung adenocarcinomas (Bierie and Moses, 2006; Levy and Hill, 2006). However, in breast carcinoma, glioblastoma, melanoma, and other types of cancer, selective losses of growth inhibitory responses often accrue through alterations downstream of Smad, leaving the rest of the TGFβ pathway operational and open to co-option for tumor-progression advantage (Massagué and Gomis, 2006). Low-level expression of TGFβ receptors in the ER-negative (ER−) breast tumors is associated with better overall outcome (Buck et al., 2004), whereas overexpression of TGFβ1 is associated with a high incidence of distant metastasis (Dalal et al., 1993). Studies in mouse models of breast cancer have implicated TGFβ in the suppression of tumor emergence (Bierie and Moses, 2006; Siegel and Massagué, 2003), but also in the induction of epithelial-mesenchymal transitions and tumor invasion (Thiery, 2002; Welch et al., 1990), the production of

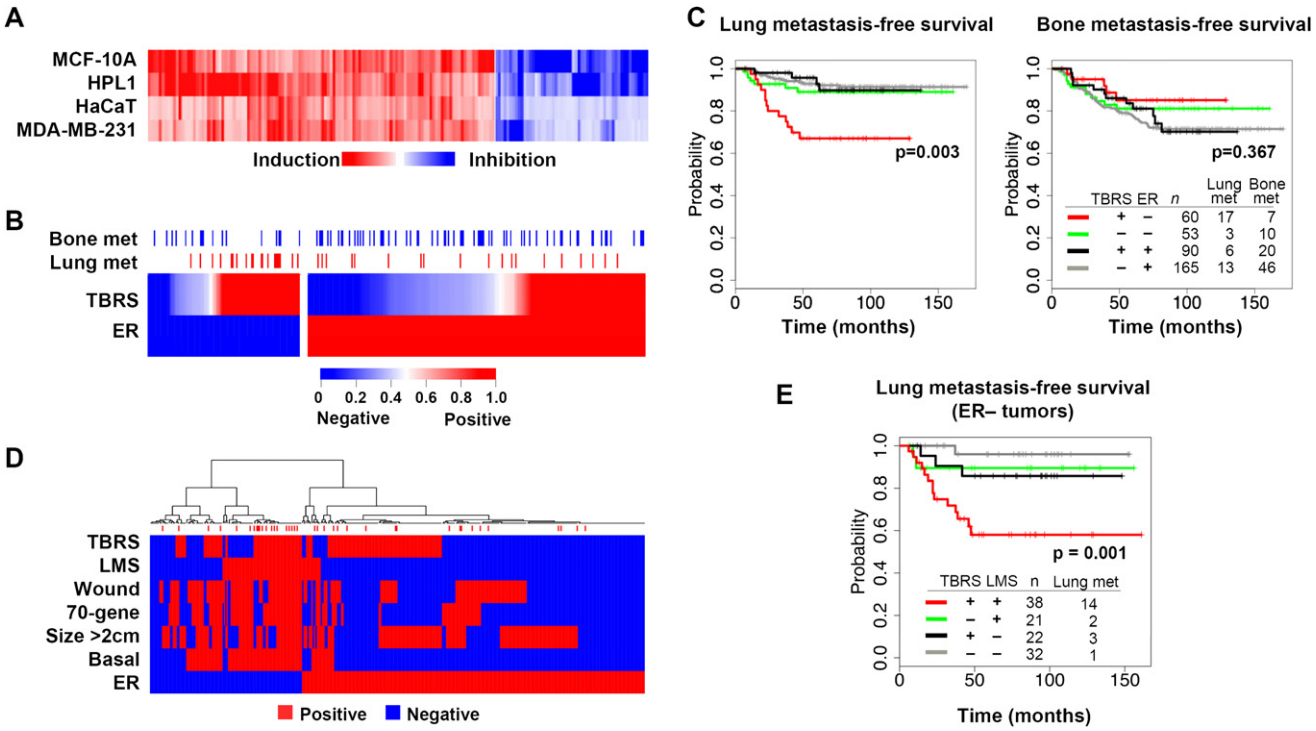

Figure 1. The TBRS Associates with Breast Cancer Metastasis in Humans

(A) The indicated epithelial cell lines were incubated for 3 hr with TGFβ, and then total RNA was subjected to microarray analysis. The heat map represents the change in expression levels of the 153 genes within the TBRS.

(B) TBRS status was assessed in a MSK/EMC cohort of 368 primary breast cancer tumors with known lung or bone metastatic outcomes. Red denotes a strong correlation between individual tumor gene-expression profiles and the TBRS, while blue indicates no correlation. Estrogen receptor α (ER) expression status is also indicated. Blue and red marks above the heat map indicate tumors that at any time developed bone or lung metastases, respectively.

(C) Kaplan-Meier curves representing the probability of cumulative lung (left panel) or bone (right panel) metastasis-free survival for this cohort. Tumors are categorized according to their TBRS and ER status. The P values for the ER-negative tumor comparisons are shown.

(D) Hierarchical clustering was performed on the MSK/EMC cohort with the indicated pathological and genomic markers including the TBRS, the lung metastasis signature (LMS), the wound response signature (Wound), the 70-gene prognosis signature (70-gene), size (Size > 2cm), the basal molecular subtype (Basal), and the ER status. Red marks above the map indicate tumors that developed lung metastasis.

(E) Lung metastasis-free survival restricted to patients with ER-negative tumors. Patients were categorized according to their TBRS and LMS status. P values are shown for the LMS+ tumor comparisons.

osteoclast-activating factors in the bone metastasis microenvironment (Kang et al., 2003b; Mundy, 2002), and the context-dependent induction of metastasis (Dumont and Arteaga, 2003; Siegel and Massagué, 2003). Thus, the effects of TGFβ on breast cancer progression in mouse models are as profound as they are disparate, making it difficult to discern from these models the role that TGFβ may be playing in human breast cancer.

To investigate the contextual role of the TGFβ pathway in human cancer and the mechanism by which TGFβ may instigate metastasis, we based our present work on the weight of clinical evidence and the use of a bioinformatic tool that classifies tumors based on the status of their TGFβ transcriptional readout. Applying this tool to a wealth of clinically annotated samples and gene-expression data sets, we made the surprising observation that TGFβ activity in primary breast tumors is associated with an increased propensity of these patients to develop lung metastasis but not bone metastasis. This phenomenon implies a biologically selective TGFβ-dependent mechanism that favors tumor targeting of the lungs. We identify this mechanism based on *ANGPTL4* as a critical TGFβ target gene, whose induction in cancer cells in the primary tumor primes these cells for disruption of lung capillary endothelial junctions to selectively seed lung metastasis.

RESULTS

Development of a TGFβ Response Bioinformatics Classifier

In order to investigate the role of TGFβ in cancer progression, we set out to develop a bioinformatic classifier that would identify human tumors containing a high level of TGFβ activity. A gene-expression signature typifying the TGFβ response in human epithelial cells was obtained from transcriptomic analysis of four human cell lines (Figures 1A and S1). These cell lines include HaCaT keratinocytes, HPL1 immortalized lung epithelial cells, MCF10A breast epithelial cells, and MDA-MB-231 breast carcinoma cells. The cells were treated with TGFβ1 for 3 hr in order to

capture direct TGFβ gene responses (Kang et al., 2003a) and resulted in a 153-gene TGFβ response signature (TBRS) (174 probe sets; Table S1). When applied to metastatic lesions extracted from bones, lungs and other sites representing the natural metastatic spectrum of human breast cancer, the TBRS classifier identified TGFβ activity in a 38/67 of these samples (Table S2), which is in agreement with previous observations of activated Smad in a majority of human bone metastasis samples (Kang et al., 2005).

TGFβ Activity in Primary Breast Tumors Is Selectively Linked to Lung Metastasis

We applied the TBRS classifier to a series of primary breast carcinomas that were analyzed on the same microarray platform (Minn et al., 2005, 2007; Wang et al., 2005). This series includes 82 tumors collected at Memorial Sloan-Kettering Cancer Center (MSK cohort) and 286 tumors from the Erasmus Medical Center (EMC cohort). Both cohorts comprised a mix of breast cancer subtypes, with tumors in the MSK cohort being more locally advanced than those in the EMC cohort (Minn et al., 2007). Out of a combined total of 368 patients, 39 patients developed lung metastases and 83 developed bone metastasis after a median follow-up of 10 years, with some patients developing metastases in both sites (Figure 1B). TBRS+ tumors were similarly distributed between estrogen receptor-positive (ER+) and ER− tumors (Figure 1B). Microarray analysis revealed that the TBRS+ tumors expressed significantly higher mRNA levels for TGFβ1, TGFβ2, and the latent TGFβ-activating factor, LTBP1. TBRS− tumors had lower mRNA levels for type II TGFβ receptor, Smad3, and Smad4. The expression level of other TGFβ pathway components was the independent of TBRS status (Figure S2).

TBRS status in ER+ tumors did not correlate with distant metastasis. However, in ER− tumors there was a striking association between TBRS+ status and relapse to the lungs (Figure 1C). This association was observed regardless of whether the tumor ER status was assigned using the clinical pathology reports, which are based on immunohistochemical analysis (Figures 1B and 1C), or using a microarray probe level designation (Figures S3A and S3B). No link was observed between TBRS status and bone metastasis (Figures 1D and S3C) or liver metastasis; a brain metastasis association did not attain statistical significance (Figure S4). In univariate as well as multivariate analyses, the expression level of TGFβ pathway components was much inferior to the TBRS at linking these tumors with metastasis outcome (Table S3). These results indicate that TGFβ activity in ER− breast tumors is selectively associated with lung metastasis.

Cooperation between TGFβ and the Lung Metastasis Signature

The association of TBRS with lung relapse prompted us to search for links between the TBRS and a previously described lung metastasis signature (LMS) (Minn et al., 2005). The LMS is a set of 18 genes whose expression in ER− tumors indicates a high risk of pulmonary relapse in patients (Minn et al., 2007). Several of these genes have been validated as mediators of lung metastasis (Gupta et al., 2007a, 2007b; Minn et al., 2005). The TBRS+ subset of ER− tumors partially overlapped the LMS+ subset (Figure 1D). Remarkably, tumors that were positive for both the TBRS and LMS were associated with a high risk of pulmonary relapse, whereas single-positive tumors were not (Figure 1E). Within poor-prognosis tumor subsets defined by other features, such as size >2 cm, basal subtype gene-expression signature (Sorlie et al., 2003), 70-gene poor-prognosis signature (van de Vijver et al., 2002), or wound signature (Chang et al., 2005), TBRS status was associated with risk of lung metastasis in nearly every case (Figure 1D). The TBRS performed independently of these other prognostic features (Figure S5), as did the LMS (Figure S6) (Minn et al., 2007).

TGFβ Signaling in Mammary Tumors Enhances Lung Metastatic Dissemination

The MDA-MB-231 cell line derived from the pleural fluid of a patient with ER− metastatic breast cancer evades TGFβ growth-inhibitory responses through alterations downstream of Smads (Gomis et al., 2006). The lung metastatic subpopulation LM2-4175 (henceforth LM2) was isolated by in vivo selection of MDA-MB-231 cells in mice (Minn et al., 2005). We perturbed the TGFβ pathway in LM2 cells by overexpressing a kinase-defective, dominant-negative mutant form of the TGFβ type I receptor (Weis-Garcia and Massagué, 1996) or by reducing the expression of Smad4, which is an essential partner of Smad2 and Smad3 in the formation of transcriptional complexes (Massagué et al., 2005). Using a *SMAD4* short-hairpin RNA (shRNA) (Kang et al., 2005), we reduced Smad4 levels by 80%–90% in LM2 cells (Figure 2B).

Neither the dominant-negative TGFβ receptor nor the Smad4 knockdown decreased mammary tumor growth as determined by tumor volume measurements, or the extent of tumor cell passage into the circulation, as determined by quantitative (q)RT-PCR analysis of human *GAPDH* mRNA in blood cellular fractions (Figures 2C and 2D). Tumors inoculated into the mammary glands of immunocompromised mice and allowed to grow to 300 mm^3 were surgically removed and the emergence of disseminated cells to the lungs after the mastectomy was determined (Figure 2A). Inactivation of TGFβ signaling markedly inhibited the lung metastatic seeding of the tumors as determined by quantitative bioluminescence imaging (Figures 2E and 2F, insets) (Ponomarev et al., 2004) and histological examination (Figure 2F). These results suggest that the canonical TGFβ pathway enhances mammary tumor dissemination to the lungs.

TGFβ Primes Tumor Cells to Seed Lung Metastases

We wondered whether TGFβ within the breast tumor microenvironment could endow tumor cells with the ability to seed the lungs as these cells enter the circulation. To test this possibility, we mimicked the exposure of tumor cells to TGFβ by incubating LM2 cells with TGFβ for 6 hr prior to inoculation of these cells into the tail veins of mice. Interestingly, this pretreatment with TGFβ significantly increased the lung colonizing activity of LM2 cells, as determined by a higher retention of these cells in the lungs 24 hr after inoculation (Figure 3A). In this time frame LM2 cells extravasate into the lung parenchyma (Gupta et al., 2007a). A similar effect was observed when we carried out this experiment with malignant cells (CN34.2A) obtained from the pleural fluid of a breast cancer patient treated at MSKCC. The pretreatment

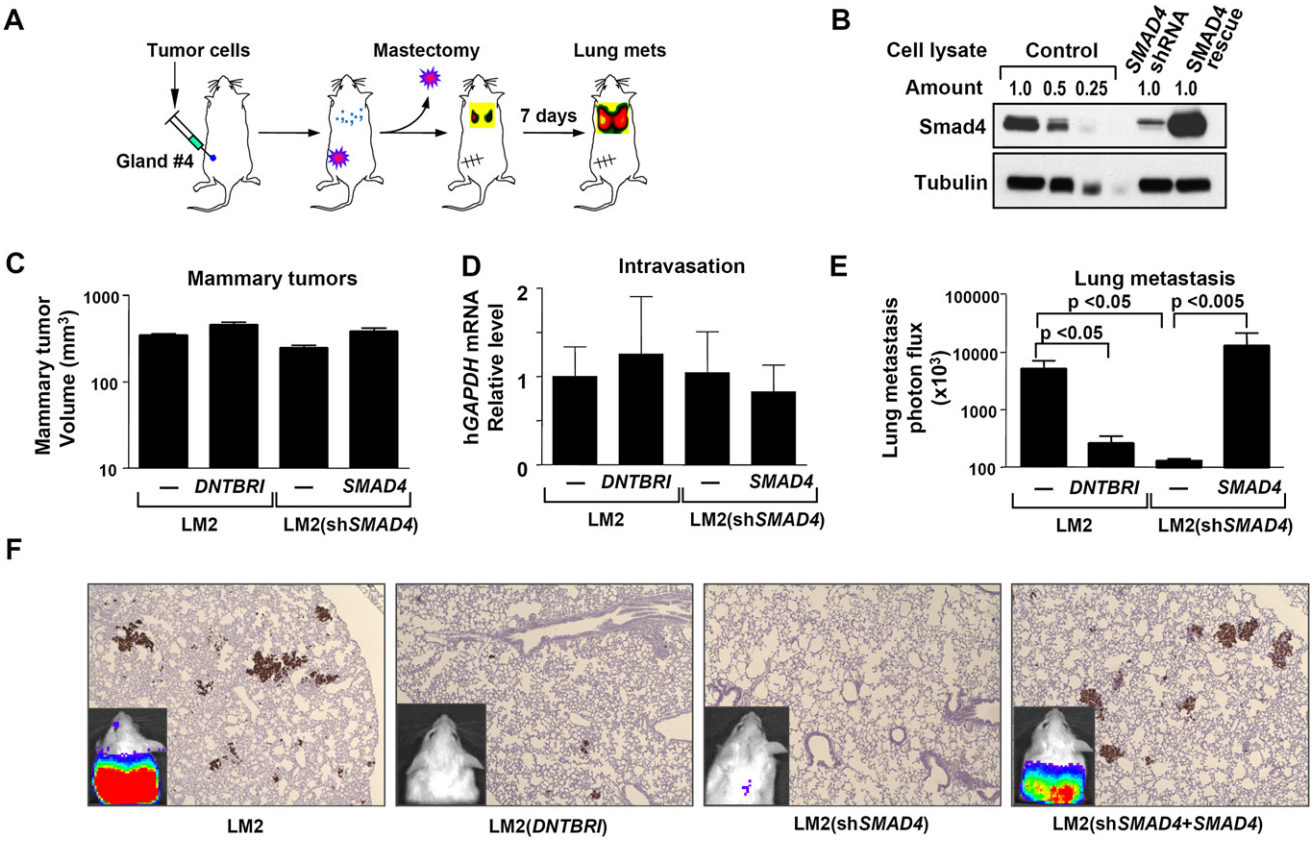

Figure 2. TGFβ Signaling Enhances Mammary Tumor Dissemination to the Lungs

(A) Schematic of lung metastasis assay from an orthotopic breast cancer inoculation.

(B) Immunoblots using indicated antibodies were performed on whole-cell extracts from control, Smad4 knockdown, and Smad4-rescue LM2 cells.

(C) Mice injected with 5×10^5 cells into the fourth mammary fat pads were measured for tumor size at day 28. n = 14; error bars indicated standard error of the mean (SEM).

(D) Blood from tumor-bearing mice was isolated and red blood cells lysed. RNA from the remaining cells was extracted for qRT-PCR. The presence of circulating tumor cells was assessed as a function of human-specific *GAPDH* expression relative to murine β2-microglobulin, in 3 ml of mouse blood perfusate. n = 8; error bars indicate SEM.

(E) Bioluminescent quantification of lung seeding elicited from orthotopically implanted breast tumors. Orthotopic tumors were grown to approximately 300 mm^3, mastectomies were performed, and lung seeding was quantified using bioluminescence imaging 7 days later. n = 7–15; error bars indicated SEM; p values calculated using the one-tailed unpaired t test.

(F) Representative bioluminescent images (inset) and lung histology of mice with the median value of bioluminescence in each experimental group in (D). Breast cancer cells were stained with human vimentin.

with TGFβ increased the lung seeding activity of LM2 and CN34.2A cells 3- and 5-fold, respectively (Figure 3B). The initial advantage provided by a transient exposure to TGFβ was sustained but not expanded during the ensuing outgrowth of metastatic colonies (Figure 3A and data not shown).

To investigate the selectivity of this lung metastasis-priming effect, we tested the effect of TGFβ preincubation on the establishment of bone metastases. LM2 cells have limited bone metastatic activity in addition to their high lung metastatic activity (Minn et al., 2005). The pretreatment of LM2 cells with TGFβ prior to their inoculation into the arterial circulation did not increase the ability of these cells to colonize the bone (Figure 3C). We also tested the effect of TGFβ on the metastatic seeding of an MDA-MB-231 subpopulation (BoM-1833) that is highly metastatic to bone (Kang et al., 2003b) and responsive to TGFβ (Kang et al., 2005). Preincubation of BoM-1833 cells with TGFβ did not increase their bone colonizing ability (Figure 3C) and had no discernible effect on the early seeding of the bones (Figure 3D). Thus, TGFβ stimulation primes tumor cells for an early step in lung metastasis but not bone metastasis, which is concordant with the selective association of TBRS+ status in primary tumors with risk of lung metastasis in clinical cohorts (refer to Figure 1C).

The TBRS/LMS Gene *ANGPTL4* Is a TGFβ Target in Breast Cancer

Given the convergence of the TBRS and the LMS in linking human primary tumors to risk of lung metastasis, we wondered whether TGFβ may act by augmenting the activity of an LMS gene(s). The LMS includes 15 candidate mediators of lung metastasis and 3 suppressors (Minn et al., 2005) (see Figure 4C). Interestingly, the LMS genes *ANGPTL4*, which encodes the multifunctional

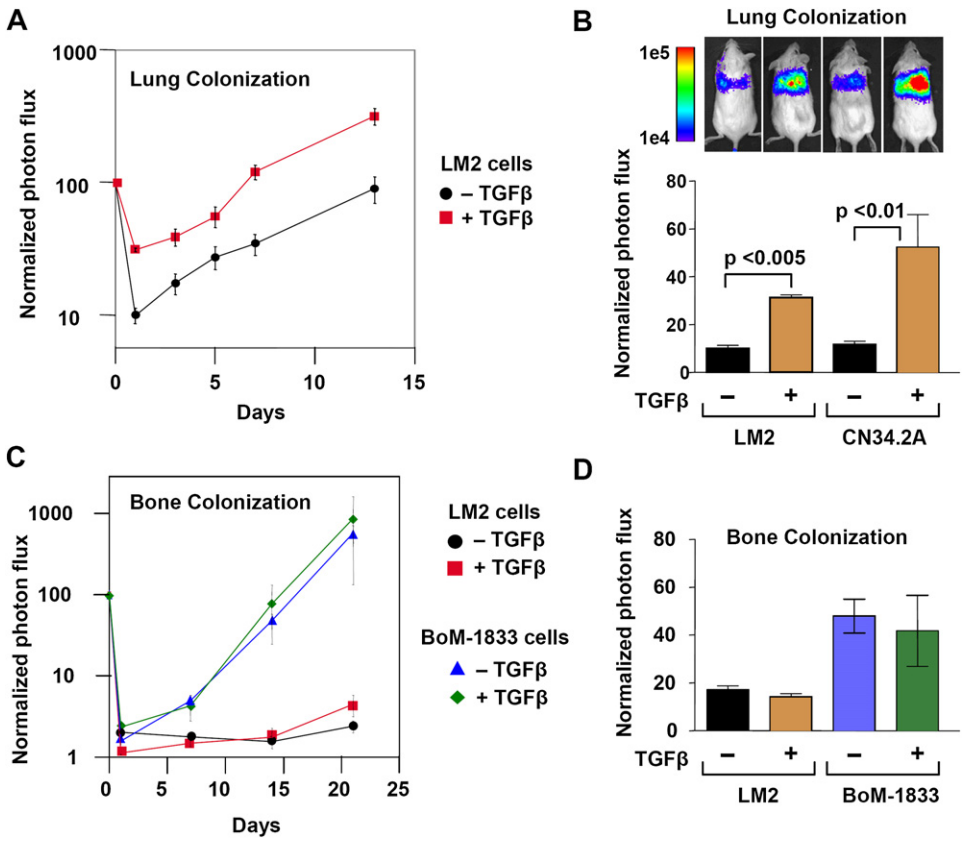

Figure 3. TGFβ Primes Tumor Cells for Metastatic Seeding of the Lungs

(A) LM2 cells and a clinically derived pleural effusion sample (CN34.2A) were pretreated with TGFβ for 6 hr. LM2 (2×10^5) and CN34.2A (4×10^5) cells were injected into the lateral tail vein, and lung colonization was analyzed by in vivo bioluminescence imaging.

(B) Bar graph represents 24 hr time-point measurements of the normalized photon flux from animals injected with either LM2 or CN34.2A cells. In vivo bioluminescent mouse images shown are from representative animals. n = 7 for the LM2 experiment, while n = 6 for the CN34.2A experiment; error bars indicate SEM; p values calculated using the one-tailed unpaired t test.

(C) Bone-colonization assays were performed by intracardiac injection of LM2 or BoM-1833 cells (3×10^4). Samples were pretreated with 100 pM of TGFβ for 6 hr and compared to an untreated control. Plot represents normalized photon flux from mouse hind limbs.

(D) Bar graphs represent 7-day time point analysis of the normalized photon flux from the mouse hind limbs. n = 8; error bars indicate SEM.

factor angiopoietin-like 4 (Oike et al., 2004), and *NEDD9*, which encodes an adaptor protein implicated in focal contact formation and cell motility (Kim et al., 2006), were present in the TBRS (Table S1). An induction of *ANGPTL4* by TGFβ was observed in four different epithelial cell types tested (Figure 4A). Moreover, among ER− tumors *ANGPTL4* expression was significantly higher in the TBRS+ tumors (median-centered intensity value = 1.07) than in TBRS− tumors (median value = 0.30). *NEDD9* expression was not different between these two groups (Figure 4B). TBRS+ and TBRS− tumors in the ER+ group showed a smaller difference in *ANGPTL4* expression (Figure S7).

To determine the effect of TGFβ on individual LMS genes, we used tumor cells isolated from pathological pleural fluids from patients with ER− and ER+ metastatic breast cancer. Lung metastasis was diagnosed in six out of seven of these cases. All samples were obtained from routine therapeutic procedures and were used under institutionally approved protocols and informed consent (Gomis et al., 2006). Carcinoma cells were isolated from these samples using the epithelial cell-surface marker EpCAM (Kielhorn et al., 2002). TGFβ addition increased *ANGPTL4* expression between 2- and 12-fold in all metastatic samples, and 16-fold in the LM2 cells, as determined by qRT-PCR (Figure 4C).

None of the other LMS genes, *NEDD9* included, was consistently regulated by TGFβ in this set of samples, with one exception: the transcriptional inhibitor of cell differentiation *ID1* was induced approximately 2-fold by TGFβ in most samples (Figure 4C). As a component of the LMS, *ID1* mediates tumor reinitiation after ER− cells enter the lung parenchyma (Gupta et al., 2007b). This induction of *ID1* by TGFβ is interesting given the fact that TGFβ represses *ID1* in untransformed breast epithelial cells (Kang et al., 2003a). This switched responsiveness of *ID1* is consistent with the pattern of loss of TGFβ growth-inhibitory responses in metastatic breast cancer cells (Gomis et al., 2006).

The induction of *ANGPTL4* expression by TGFβ was observed in all 13 malignant pleural cell samples tested, regardless of the ER, progesterone receptor, or ERBB2 receptor status (Table 1). Its induction by TGFβ was rapid and lasted for 8 hr (Figure 4D).

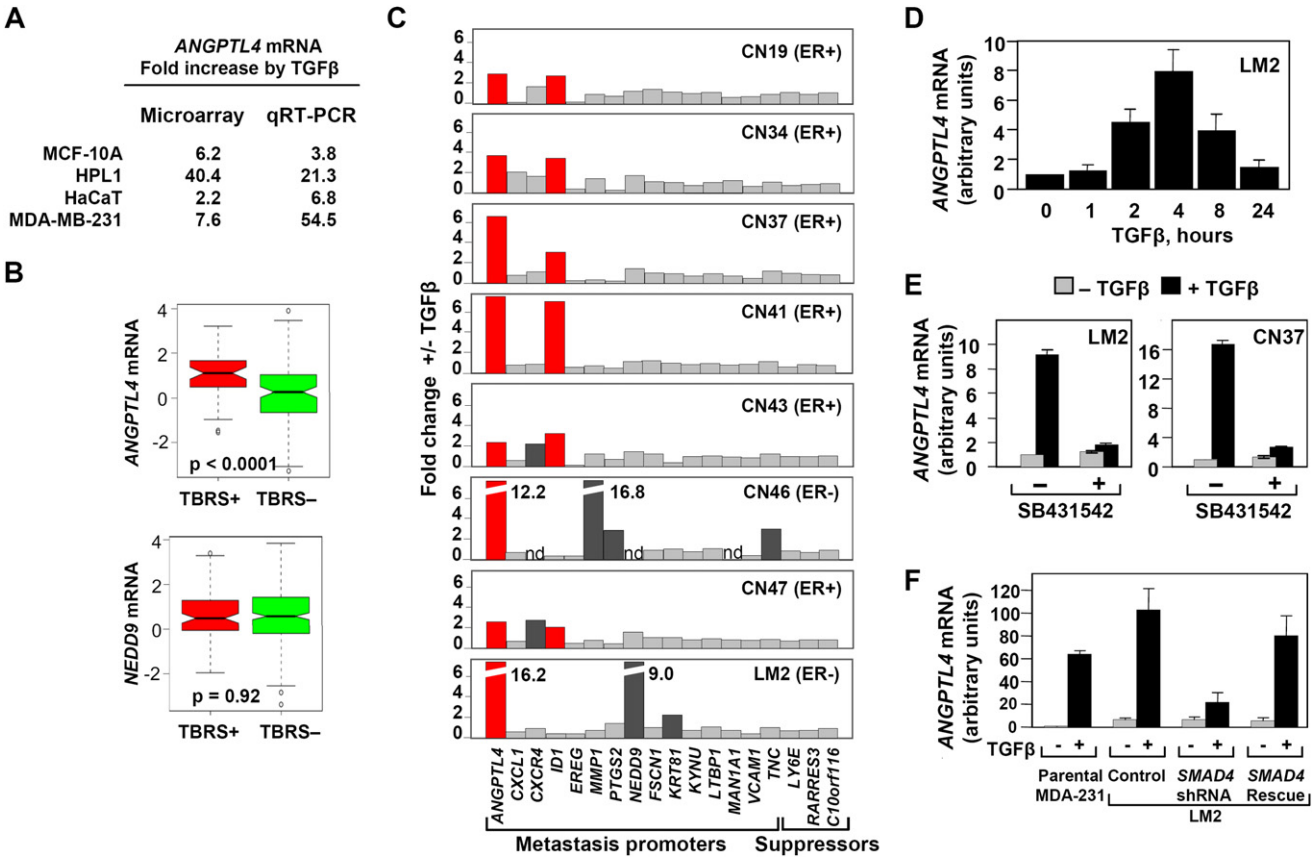

Figure 4. The TBRS/LMS Gene *ANGPTL4* Is a Smad-Dependent TGFβ Target

(A) Microarray and qRT-PCR analysis for the four epithelial cell lines treated with TGFβ. Fold change values for the TGFβ induction of *ANGPTL4* are indicated.

(B) Box-and-whisker plot comparing *ANGPTL4* and *NEDD9* TBRS-negative and -positive ER-negative tumors from the MSK/EMC cohorts. P value was calculated using the Wilcoxon rank sum test.

(C) TGFβ-induced changes in the mRNA expression of LMS genes in a panel of clinically derived pleural effusion samples and LM2 cells. Cells were treated with 100 pM of TGFβ for 3 hr and analyzed by qRT-PCR using primers for the indicated genes. ER status for each breast cancer patient is designated.

(D) LM2 breast cancer cells were treated with 100 pM of TGFβ for the indicated lengths of time, and *ANGPTL4* mRNA levels were analyzed using qRT-PCR. n = 3; error bars indicate standard deviation (SD).

(E) Treatment of LM2 (left panel) and pleural effusion-derived CN37 sample (right panel) with TGFβ and the TGFβ-receptor kinase inhibitor, SB431542. qRT-PCR expression levels are shown relative to the untreated control sample. n = 3; error bars indicate SD.

(F) MDA-231, LM2 control, LM2-Smad4-depleted, and LM2-Smad4-rescue cell lines were treated with 100 pM TGFβ for 3 hr. TGFβ-induced fold changes of *ANGPTL4* were analyzed by qRT-PCR analysis. n = 3; error bars indicate SD.

Addition of SB431542, an ATP analog inhibitor of the TGFβ type I receptor kinase (Laping et al., 2002), abolished the *ANGPTL4* response in LM2 and CN37 cells (Figure 4E). Smad4 knockdown markedly inhibited the *ANGPTL4* response to TGFβ, whereas a shRNA-resistant *SMAD4* cDNA containing two silent mutations in the shRNA-targeted sequence rescued this response (Figure 4F). Among various cytokines that are typical of the tumor microenvironment, TGFβ was the strongest inducer of *ANGPTL4* in the MDA-MB-231 cells (Figure S8). Thus, *ANGPTL4* induction in metastatic breast cancer cells is mediated by the canonical TGFβ-receptor-Smad pathway.

ANGPTL4 Participates in TGFβ Priming for Lung Metastasis

To investigate whether *ANGPTL4* participates in the prometastatic effects of TGFβ, we knocked down its expression in LM2 cells by means of a shRNA (Figure 5A). This knockdown did not decrease the ability of LM2 cells to grow as mammary tumors (Figure 5B) and to pass into the circulation (Figure 5C). The incidence of lymph-node metastases in LM2 tumor-bearing mice was also not affected by *ANGPTL4* knockdown (Figure 5D). However, the dissemination to the lungs from orthotopically implanted LM2 tumors was decreased more than 10-fold by the *ANGPTL4* knockdown, and this decrease could be prevented with the *ANGPTL4*-rescue construct (Figure 5E). *ANGPTL4* knockdown did not decrease the residual bone metastatic activity of LM2 cells (data not shown).

When orthotopically implanted, LM2 tumors accrue TGFβ activity that primes lung metastasis seeding (refer to Figure 2D). We subjected the *ANGPTL4* knockdown LM2 cells to the ex vivo TGFβ priming assay. Of note, the induction of *ANGPTL4* expression by TGFβ was blunted but not completely

Table 1. Clinical and Histological Staging of Malignant Pleural Effusion Samples, and Their *ANGPTL4* Response to TGFβ

	ER Status[a]	PR Status[a]	ERBB2 Status[a]	Tumor Type	Metastatis Sites	*ANGPTL4* mRNA +/− TGFβ
CN19	+	+	+	ductal	Pl, Lu, LN	2.9
CN34	+	−	++	ductal	Pl, Lu, Bo, LN	3.8
CN37	+	+	++	ductal	Pl, Lu, LN, Br, Li	6.7
CN41	+	+	++	ductal	Pl, Lu?, Bo, Br, CW	8.7
CN43	+	+	+	ductal	Pl, Lu, Bo, Li	2.3
CN46	−	−	+++	ductal	Pl, Bo, ST, LN	12.8
CN47	+	+	+	lobular	Pl, Lu, Li, LN, Ca, Co	2.8
CN90	−	−	−	ductal	Pl, Lu, Bo, Li	24.7
BCN5	+	+	+	ductal	Pl, Lu, Bo, LN, Sc	9.5
BCN6	+	+	+++	ductal	Pl, Lu, Bo, Li	10.9
BCN7	+	+	+	ductal	Pl, Lu, Bo, Li, LN, Ad	10.2
BCN8	+	−	−	lobular	Pl, Bo, As, ST	3.0
BCN9	−	−	−	ductal	Pl, LN	13.7

Pl, pleura; Lu, lung; Bo, bone; Br, brain; Sc, subcutaneous; LN, lymph nodes; Li, liver; Ad, adrenal; As, ascites; ST, soft tissue; Ca perinoteal carcinomatosis; Co, colon; CW, chest wall.
[a] Status in primary tumor.

eliminated in the knockdown cells (Figure 5F). This notwithstanding, the knockdown of *ANGPTL4* significantly blunted the priming effect of TGFβ on lung seeding by LM2 cells (Figure 5G). The constitutive overexpression of exogenous *ANGPTL4* in LM2 cells increased lung colonization by these cells (Figure 5H). These results provide evidence that *ANGPTL4* expression is necessary for the ability of TGFβ to prime LMS+ breast cancer cells and sufficient for increasing seeding of the lungs.

Angptl4 Mediates Endothelial Disruption and *Trans*-Endothelial Tumor Cell Passage

The ability of TGFβ to promote lung seeding through an induction of *ANGPTL4* suggested that this process may target an early step in pulmonary metastasis. Extravasation, or the passage of circulating tumor cells through the tight lung capillary endothelial junctions, is an important initial step. We, therefore, investigated whether Angptl4 might affect endothelial cell layers in a manner that would facilitate the passage of tumor cells across endothelia. HUVEC human vascular endothelial cells were allowed to grow to form tight monolayers on tissue-culture dishes, and at this point the monolayers were exposed to media containing human recombinant Angptl4 or no addition (Figure 6A), or media conditioned by control LM2 cells or by cells overexpressing Angptl4 (Figure 6B). In both cases Angptl4 caused an acute disruption of endothelial cell-cell junctions. Staining with antibodies against the tight junction component zonula occludens 1 (ZO-1), against the adherens junction component β-catenin, or staining of the actin cytoskeleton with phalloidin (Dejana, 2004) revealed that the monolayer integrity was dramatically perturbed by Angptl4 (Figures 6A and 6B).

GFP-labeled MDA-MB-231 cells either expressing a control vector or expressing Angptl4 were inoculated into NOD/SCID mice. One day post inoculation, the animals were injected with a rhodamine-conjugated dextran, in order to measure vessel permeability. The lungs were then extracted and analyzed for retained rhodamine using fluorescent microscopy. No rhodamine signal was present in the lungs of mice that were not inoculated with cancer cells (data not shown). In inoculated animals, however, diffuse areas of rhodamine signal surrounded the cancer cells that lodged in the lungs (Figure 6C). Cells overexpressing Angptl4 showed a 3-fold increase in surrounding rhodamine signal, as determined by quantitative analysis of the fluorescent area (Figures 6D and S9). To test the effect of Angptl4 on cell migration across an endothelial layer, endothelial monolayers were set on *trans*-well tissue culture inserts. LM2 cells overexpressing Angptl4 passed twice as efficiently through these layers into the lower chamber of the *trans* well compared to control LM2 cells (Figure 6E). Collectively, these data demonstrate that Angptl4 disrupts the integrity of vascular endothelial cell layers both in vitro and in the lungs, facilitating the passage of breast cancer cells.

DISCUSSION

Primary tumor microenvironments may promote metastasis by selecting for highly invasive and resistant cancer cell phenotypes (Bernards and Weinberg, 2002) and systemically fostering the mobilization of marrow-derived progenitor cells (Kaplan et al., 2005). The ability to subsequently colonize distant organs depends on the organ-colonizing faculties of disseminated tumor cells as well as on certain permissive conditions that may be present in the otherwise restrictive microenvironment of target organs (Gupta and Massagué, 2006). The present results suggest a distinct mechanism for the colonization of a distant organ, one that relies on a stimulus in the primary tumor microenvironment to enhance the ability of departing tumor cells to seed the lungs (Figure 6F).

Angptl4 as an Inhibitor on Endothelial Integrity that Mediates Lung Metastasis Seeding

Angptl4 is expressed in the liver, adipose tissue, and placenta, as well as in ischemic tissues. It was identified in a search for new

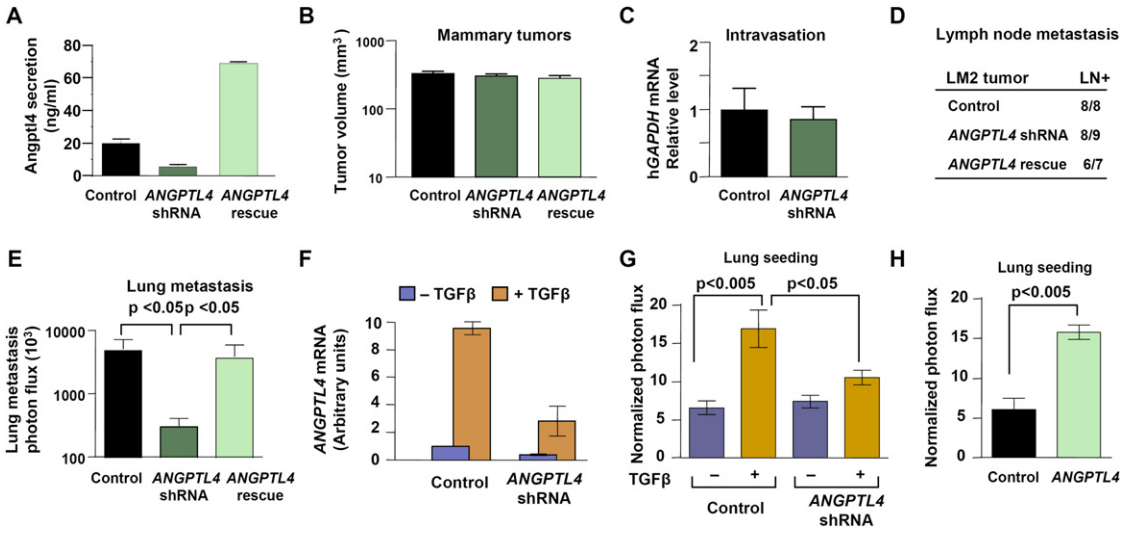

Figure 5. Angptl4 Mediates TGFβ Priming for Mammary Tumor Dissemination to the Lungs

(A) Secreted Angptl4 protein levels in the control, *ANGPTL4* knockdown, or *ANGPTL4*-rescue LM2 cells were analyzed by ELISA. n = 3; error bars indicate SD.

(B) Tumor size measurements were taken from mice inoculated into the fourth mammary fat pads with 5×10^5 control, *ANGPTL4* knockdown, or *ANGPTL4*-rescue LM2 cells. Tumor size was measured at day 28. n = 14; error bars indicated SEM.

(C) The presence of circulating tumor cells was assessed by qRT-PCR as a function of human-specific *GAPDH* expression relative to murine β2-microglobulin, in 3 ml of mouse blood perfusate. n = 10; error bars indicate SEM.

(D) Peri-aortic lymph node metastasis from indicated tumors was analyzed by ex vivo detection of luciferase activity. Positive bioluminescent signal in extracted lymph nodes indicated presence of metastasized tumor cells.

(E) Photon flux measurements of breast cancer cells seeding the lung from orthotopically injected tumors as indicated. n = 13–15; error bars indicated SEM; p values calculated using the one-tailed unpaired t test.

(F) *ANGPTL4* mRNA levels were determined by qRT-PCR analysis in cells that were incubated for 6 hr with or without TGFβ. n = 3; error bars indicate SD.

(G) Lung-colonization analysis was performed by injecting 2×10^5 cells into the lateral tail vein. Prior to injection, cells were treated as indicated with 100 pM TGFβ for 6 hr. Bar graphs represent 24 hr time-point measurements of the normalized photon flux. n = 14–21; error bars indicate SEM; p values calculated using the one-tailed unpaired t test.

(H) Normalized photon flux measurements from tail vein injected animals. Lung-colonization measurements were taken from animals injected with control or Angptl4-overexpressing LM2 cells. n = 6; error bars indicate SEM; p values calculated using the one-tailed unpaired t test.

members of the angiopoietin family of vascular regulators, and independently in a search for targets of the PPAR family of metabolic response transcription factors (Oike et al., 2004). While Angptl4's role in lipid metabolism has been well characterized, little is known about its role in vascular biology. Indeed, the effects of angiopoietin-like proteins in experimental systems are complex, at times acting as general endothelial cell survival factors (Kim et al., 2000), modulating endothelial cell adhesion (Cazes et al., 2006), or paradoxically stimulating (Hermann et al., 2005; Le Jan et al., 2003) as well as inhibiting angiogenesis (Ito et al., 2003). Chronic systemic secretion of Angptl4 from a transgene expressed in muscle tissue in mice inhibited metastasis by xenografted melanoma cells (Galaup et al., 2006). These diverse responses are suggestive of a context, tissue-specific activity of this multifaceted molecule.

ANGPTL4 is one of the top performing genes in the LMS with a highly significant association with lung relapse ($p < 0.000001$) (Minn et al., 2005). In the present work, we show that TGFβ stimulation sharply increased the expression of *ANGPTL4*, and we have functionally validated *ANGPTL4* as a mediator of breast cancer lung metastasis. *ANGPTL4* knockdown in LMS+ cells inhibits their ability to seed the lungs, and it does so without affecting the growth of these cells as mammary tumors, their passage into the circulation, or their invasion of lymph nodes. Angptl4 antagonizes vascular endothelial tight junctions and adherens junctions, and disrupts the integrity of capillary walls when secreted from metastatic breast cancer cells that have lodged in the lungs. These results strongly suggest that Angptl4 acts as an enhancer of breast cancer cell extravasation by transiently suppressing the integrity of capillaries. These observations fit with the role of Angptl4 as a vascular regulator in ischemia and tumor hypoxia conditions (Le Jan et al., 2003) and are in line with the role of the angiopoietin and angiopoietin-like factors in vascular remodeling (Camenisch et al., 2002; Gale et al., 2002; Parikh et al., 2006). Together with the presence of *ANGPTL4* in two distinct gene-expression signatures—the LMS and the TBRS—that are associated with lung metastasis in breast cancer patients, this evidence suggests that Angptl4 is a clinically relevant mediator of lung metastasis in breast cancer.

TGFβ Activity in Primary Breast Tumors Is Linked to Lung Metastasis

The role of TGFβ in breast cancer progression has remained baffling given the disparate results from various animal models. In transgenic mouse models, TGFβ action can enhance extravascular lung metastasis formation (Siegel et al., 2003), whereas a conditional knockout of TGFβ receptor in the mammary epithelium showed that TGFβ can suppress both primary tumor growth

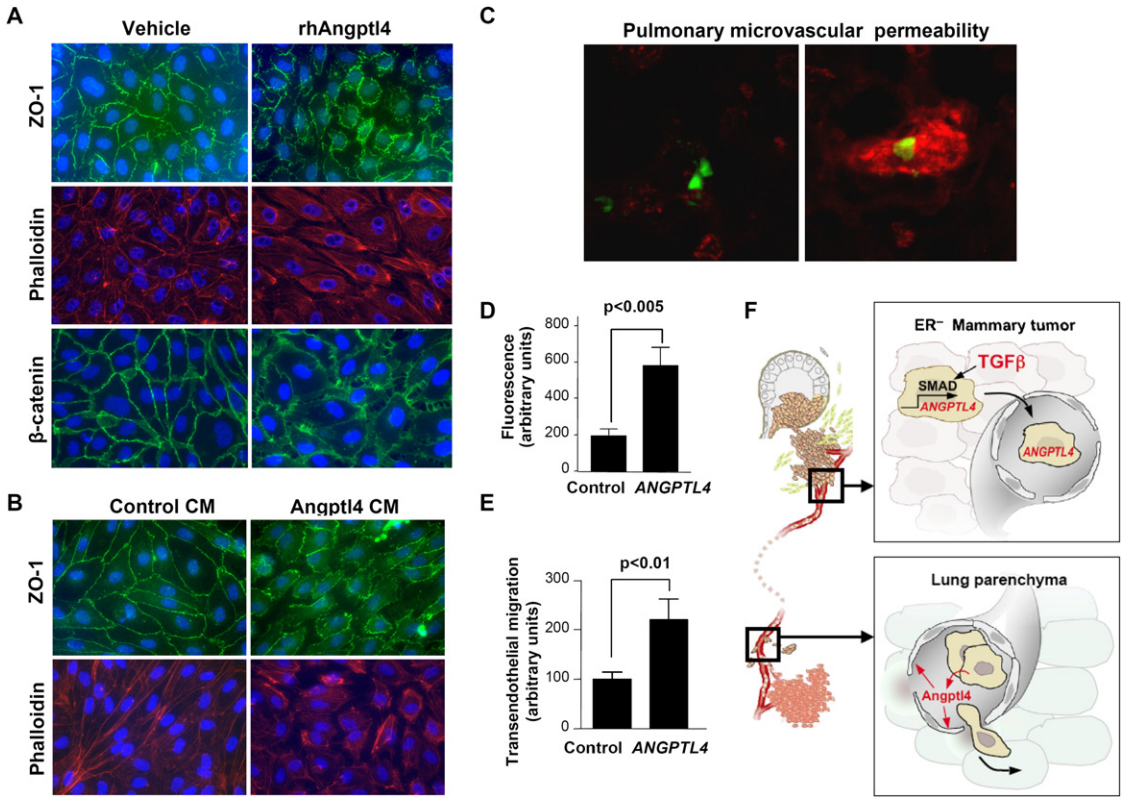

Figure 6. Angptl4 Mediates Endothelial Monolayer Disruption, Lung Capillary Permeability, and *Trans*-Endothelial Tumor Cell Migration

(A) HUVEC monolayers were grown to confluence on fibronectin-coated slides and then treated for 24 hr with rhAngptl4. Slides were subsequently fluorescently stained with anti-ZO-1 antibody, phalloidin, and anti-β-catenin antibody.

(B) HUVEC monolayers were treated for 24 hr with media conditioned by control LM2 cells or LM2 cells that overexpress Angptl4. Samples were stained for ZO-1 and phalloidin.

(C) GFP-labeled MDA-231 cells were injected via the tail vein and allowed to lodge in the lungs. One day post injection, a rhodamine-dextran dye was injected into circulation. Three hours after dye injection, lungs were extracted and frozen sections were obtained. Representative confocal images are shown here of cells with and without accumulation of dye in the lung parenchyma.

(D) Images were obtained as described in (C) with control or Angptl4-overexpressing MDA-MB-231 cells. A region of interest was drawn around the GFP-labeled cells and the amount of dextran dye was quantified based on rhodamine emissions. n = 40 cells; error bars indicate SEM; p values calculated using the one-tailed unpaired t test.

(E) Indicated cell lines were seeded into *trans*-well inserts that were previously covered with a HUVEC monolayer. Cells that migrated cross the endothelial layer into the bottom side of the *trans*-well membrane were quantified with Volocity software. n = 15; error bars indicate SEM. P values calculated using the one-tailed unpaired t test.

(F) Schematic model of the cytokine relay set up by TGFβ activity in the primary tumor. ER− primary tumor cells that are exposed to TGFβ respond with *ANGPTL4* induction via the Smad pathway. As they enter the circulation and reach the lung capillaries, these cells secrete Angptl4, which disrupts endothelial cell junctions thereby enabling the cancer cells to more efficiently enter the lung parenchyma.

and lung metastases (Forrester et al., 2005). Therefore, the causal relationship between TGFβ and breast cancer progression in humans, and the identity of downstream TGFβ targets that may be involved in this action, has remained unknown.

To address this problem, we have developed a bioinformatic classifier, the TBRS, based on the TGFβ gene-response signature of human epithelial cells. The TBRS can not only classify tumor tissue samples that have a gene-expression profile corresponding to active TGFβ signaling but can also help identify key downstream TGFβ mediators, as shown in this work. Using this tool to interrogate a wealth of existing clinical breast cancer data sets, we have found that the presence of TGFβ activity in primary tumors is selectively associated with risk of lung metastases. Surprisingly, this association is restricted to ER− tumors. Both ER+ and ER− cancer cells exhibit *ANGPTL4* induction by TGFβ, although the Angptl4 expression level is higher in TBRS+/ER− than in TBRS+/ER+ tumors. An explanation for the selective association with lung metastasis in the ER− group may lie with the fact that the contributions of TGFβ and *ANGPTL4* to lung metastasis occur in the context of the LMS+ phenotype. The TBRS+ status is not associated with metastasis in either ER−/LMS− tumors or in ER+ tumors, which are LMS− (refer to Figure 1D). ER− tumors that score positive for both TBRS and LMS are the ones with a high risk of lung metastasis (refer to Figure 1E).

We observed a high expression level of TGFβ1, TGFβ2, and LTBP1 in TBRS+ tumors, which is consistent with the TGFβ activity typified by the TBRS and is in line with a reported association of high TGFβ1 levels with lung metastasis (Dalal et al., 1993).

Among ER− tumors, a low expression of the TGFβ type II receptor is associated with favorable outcome (Buck et al., 2004). Our data are also in line with these findings. Additionally, we find that the Smad levels are differentially expressed with TBRS+ tumors expressing higher levels of Smad3 and Smad4 while expressing lower levels of Smad2. Indeed, Smad3, more than Smad2, is critical for the induction of TGFβ gene responses (Chen et al., 2001, 2002; Gomis et al., 2006; Seoane et al., 2004). Despite these interesting links, the TGFβ pathway components tested individually or as a group did not perform as strongly as did the TBRS at linking ER− primary tumors with lung metastasis.

A TGFβ-Angptl4 Relay System Primes Mammary Tumors for Seeding of Lung Metastases

Several activities have been ascribed to TGFβ that would favor tumor progression in general, including the maintenance of a mesenchymal phenotype (Shipitsin et al., 2007) or the dampening of immune functions (Gorelik and Flavell, 2002). However, it is not obvious how these effects of TGFβ would favor metastasis to one particular organ over another. Yet, our clinical and functional evidence selectively links TGFβ in the primary breast tumor microenvironment to lung metastasis and not bone metastasis. This observation implies a biologically selective mechanism, and our results point at *ANGPTL4* induction by TGFβ as a centerpiece of this mechanism. We provide evidence that TGFβ stimulation of mammary carcinoma cells before they enter the circulation primes these cells for seeding of the lungs through a transient induction of *ANGPTL4*. This effect is mediated by the canonical TGFβ receptor and Smad signaling pathway, which in normal breast epithelial cells would suppress cell proliferation, but in metastatic breast cancer cells fails to efficiently trigger cytostatic gene responses (Gomis et al., 2006). Given the disruptive effect of Angptl4 on endothelial cell junctions, we suggest that TGFβ-mediated induction of this factor increases the extravasation capabilities of breast cancer cells as they arrive in the lungs. Thus, a cytokine in the microenvironment of mammary tumors can endow departing cancer cells with increased expression of another cytokine to more efficiently seed a distant organ.

A vasculature disruptive mechanism may provide a selective invasive advantage in lung but not bone because of the inherent differences in the microvasculature of these two tissues. Lung vascular endothelial junctions act as a barrier that restricts the passage of cells. In contrast, the bone-marrow vasculature consists of capillary vascular channels, called sinusoids, which have a discontinuous endothelium to facilitate the passage of hematopoietic and other cells (Oghiso and Matsuoka, 1979). Therefore, lung metastasis may require robust extravasation functions such as those provided by Angptl4 and other factors (Gupta et al., 2007a), and additional lung-colonizing functions (Gupta et al., 2007b). In contrast, osteolytic metastasis by breast cancer cells may principally require their adaptation to the bone microenvironment and the recruitment and activation of osteoclasts (Mundy, 2002).

The TGFβ-Angptl4 cytokine relay system described here provides an example of how stimuli in the primary tumor can affect distant metastases. We envision that TGFβ and other factors in different tumor microenvironments may act in this manner to influence metastases from other tumor types, or to other organ sites. The ability of TGFβ to prime disseminating breast cancer cells for lung metastasis is clinically and mechanistically distinct from the advantage that metastatic colonies may later extract from locally produced TGFβ (Mundy, 2002). Indeed, of 67 samples of human breast cancer metastasis to bone, lung, brain liver, and other sites that we analyzed, more than half scored as TBRS+. This result is also consistent with our previous observation of activated Smad in a majority of bone metastases from breast cancer patients (Kang et al., 2005) and the involvement of several TGFβ target genes in the bone osteolytic process (Kang et al., 2003b; Mundy, 2002). However, TGFβ metastatic lesions might support subsequent rounds of metastatic dissemination by the mechanism outlined here, pointing at opportunities for therapeutic intervention based on the present findings.

EXPERIMENTAL PROCEDURES

Additional methods can be found in the Supplemental Data.

Cell Culture and Reagents

MDA-MB-231 and its metastatic derivatives LM2-4175 and BoM-1833 have been described previously (Kang et al., 2003b; Minn et al., 2005). Breast carcinoma cells were isolated from the pleural effusion of patients with metastatic breast cancer treated at our institution upon written consent obtained following IRB regulations as previously described (Gomis et al., 2006). BCN samples were obtained and treated as per Hospital Clinic de Barcelona guidelines (CEIC approved).

TGFβ and TGFβ-receptor inhibition used 100pM TGFβ1 (R&D Systems) for 3 or 6 hr as indicated and 10 μM SB431542 (Tocris) with 24 hr pretreatment. Epithelial cell lines were treated for 3 hr with BMP2 (25 ng/mL, R&D), Wnt3a (50 ng/mL, R&D), FGF (5 ng/mL, Sigma), EGF (100 ng/mL, Invitrogen), IL6 (20 ng/mL, R&D), VEGF-165 (100 ng/mL, R&D), and IL1β (100 ng/mL, R&D). Conditioned media experiments were performed by growing cells in serum-deprived media for 48 hr. Recombinant human Angptl4 (Biovendor) was used at 2.5 μg/mL for 24 hr.

TGFβ-Response Gene-Expression Signature and TBRS Classifier

Cell lines with and without TGFβ1 treatment (3 hr, 100 pM) were subject to expression profiling using Affymetrix U133A or U133 plus2 microchips. Microarray results were preprocessed using RMA algorithm (carried with *affy* package of R statistical program). The first comparison was conducted between all TGFβ-treated samples versus all untreated samples. Three hundred and fifty genes that yielded a p value of 0.05 or less (after Benjamini and Hochberg correction for multiple tests) were kept. Among these genes, we chose to focus on the genes that are significantly changed in at least two different cell lines when the cell lines are considered separately. This step resulted in 174 probe sets corresponding to 153 distinct human genes, which were collectively designated as the TGFβ gene-response signatures.

Animal Studies

All animal work was done in accordance with a protocol approved by the MSKCC Institutional Animal Care and Use Committee. NOD/SCID female mice (NCI) age-matched between 5–7 weeks were used for xenografting studies. For experimental metastasis assays from bilateral orthotopic inoculations, the tumors were extracted from both mammary glands when they each reached 300 mm^3, approximately 30 days. Seven days after mastectomies, lung metastases were monitored and quantified using noninvasive bioluminescence as previously described (Minn et al., 2005).

In Vivo Lung Permeability Assays

To observe in vivo permeability of lung blood vessels, tumor cells were labeled by incubating with 5 μM cell tracker green (Invitrogen) for 30 min and inoculated into the lateral tail vein. One day post inoculation, mice were injected

intravenously with rhodamine-conjugated dextran (70 kDa, Invitrogen) at 2 mg per 20 g body weight. After 3 hr, mice were sacrificed; lungs were extracted and fixed by intratracheal injection of 5 ml of 4% PFA. Lungs were fixed frozen, and 10 μm sections were taken to be examined by fluorescence microscopy for vascular leakage. Images were acquired on an AxioImager Z1 microscopy system (Zeiss). To analyze, a uniform ROI of approximately 3 nuclei in diameter was drawn around the tumor cells and applied to each image. A second larger ROI was also applied with similar results. Signal from the ROI was quantified using Volocity (Improvision).

Statistical Analysis

Results are reported as mean ± SEM unless otherwise noted. Comparisons between continuous variables were performed using an unpaired one-sided t test. Statistics for the orthotopic lung metastasis assays were performed using log transformation of raw photon flux.

ACCESSION NUMBERS

The normalized data has been deposited in the Array Express database (E-TABM-420).

SUPPLEMENTAL DATA

Supplemental data include nine figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/133/1/66/DC1/.

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