The perivascular niche regulates breast tumour dormancy

Cyrus M. Ghajar1,11, Héctor Peinado2,3, Hidetoshi Mori1, Irina R. Matei2,3, Kimberley J. Evason4,6, Hélène Brazier2,3, Dena Almeida3, Antonius Koller5, Katherine A. Hajjar2,3, Didier Y. R. Stainier6,10, Emily I. Chen5,7, David Lyden2,3,8,9 and Mina J. Bissell1,11

In a significant fraction of breast cancer patients, distant metastases emerge after years or even decades of latency. How disseminated tumour cells (DTCs) are kept dormant, and what wakes them up, are fundamental problems in tumour biology. To address these questions, we used metastasis assays in mice and showed that dormant DTCs reside on microvasculature of lung, bone marrow and brain. We then engineered organotypic microvascular niches to determine whether endothelial cells directly influence breast cancer cell (BCC) growth. These models demonstrated that endothelial-derived thrombospondin-1 induces sustained BCC quiescence. This suppressive cue was lost in sprouting neovascularature; time-lapse analysis showed that sprouting vessels not only permit, but accelerate BCC outgrowth. We confirmed this surprising result in dormancy models and in zebrafish, and identified active TGF-β1 and peristin as tumour-promoting factors derived from endothelial tip cells. Our work reveals that stable microvasculature constitutes a dormant niche, whereas sprouting neovascularature sparks micrometastatic outgrowth.

We have long argued and provided evidence that basement membrane, in particular laminin-111, provides a hospitable microenvironment that allows mammary epithelial cell survival, quiescence and resistance to cytotoxic agents14-17, three properties commonly associated also with dormant DTCs (ref. 18). Thus, we suspected that basement membrane was a major component of the dormant niche in distant organs. Given that BCCs must take a haematogenous route to arrive at sites where breast tumours metastasize most often (that is, lung, bone marrow (BoMa), brain and liver)19, the microvascular basement membrane would be the first of its kind encountered by tumour cells as they disseminate to these tissues. Therefore, we reasoned that endothelial cells (ECs)—and factors deposited within their surrounding basement membrane—may be a prime player within the dormant niche.

To test this hypothesis, we used two mouse models of human breast cancer metastasis and discovered that dormant DTCs reside on the microvasculature of lung, BoMa and brain. By creating organotypic models of lung- and BoMa-microvascular niches, we demonstrated that ECs induce and sustain BCC quiescence.

It has been difficult, if not impossible, to predict whether and when metastases will occur1. The reason is that although the metastatic cascade is depicted typically as a linear process, in reality it is anything but. Some patients may experience metastatic relapse within months whereas others go several years or even decades without distant recurrence1-4. The recent discovery of tumour-promoting milieus (referred to as metastatic niches5-7) established at distant sites before, or on, the arrival of DTCs could explain the population that relapses early. However, in late-relapsing populations, what tumour cells do from the time of dissemination to the time they become clinically detectable is an outstanding question. Studies in mice and analysis of human clinical specimens revealed that single or small clusters of DTCs may persist long-term in a state of quiescence8-10. Precisely where these cells reside, how they are induced into a dormant state and what eventually causes them to awaken remain perplexing mysteries in tumour biology. Solving these problems is key to designing therapies that prevent relapse by either sustaining tumour dormancy or by selectively killing off dormant cells with minimal damage to normal tissues11.

© 2013 Macmillan Publishers Limited. All rights reserved.

1Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA. 2Department of Pediatrics, Weill Cornell Medical College, New York 10021, USA. 3Department of Cell and Developmental Biology, Weill Cornell Medical College, New York 10065, USA. 4Department of Pathology, University of California, San Francisco, California 94143, USA. 5Stony Brook University Proteomics Center, School of Medicine, Stony Brook, New York 11794, USA. 6Department of Biochemistry and Biophysics, University of California, San Francisco, California 94158, USA. 7Department of Pharmacological Sciences, Stony Brook University, Stony Brook, New York 11794, USA. 8Department of Pediatrics, Memorial Sloan-Kettering Cancer Center, New York 10065, USA. 9Champalimaud Metastasis Programme, Lisbon 1400-038, Portugal. 10Present address: Department of Developmental Genetics, Max Planck Institute for Heart and Lung Research, Bad Nauheim D-61231, Germany.

11Correspondence should be addressed to C.M.G. or M.J.B. (e-mail: cghajar@lbl.gov or mjbissell@lbl.gov)

Received 10 August 2012; accepted 22 April 2013; published online 2 June 2013; DOI: 10.1038/ncb2767
Proteomic and functional analyses of proteins deposited in organotypic microvascular niches identified thrombospondin-1 (TSP-1) as an endothelium-derived tumour suppressor. Importantly, TSP-1 was diminished near sprouting neovasculature, suggesting that tumours may escape growth regulation in this sub-niche. Time-lapse analysis confirmed that tumour growth was not just permitted, but in fact accelerated around neovascular tips, which we show are rich in tumour-promoting factors such as active TGF-β1 and periostin (POSTN). These findings establish a paradigm of differential regulation of DTC dormancy and relapse by distinct endothelial sub-niches, and suggest that preserving vascular homeostasis is critical to maintaining dormancy of DTCs.

**RESULTS**

**Dormant DTCs reside on microvascular endothelium**

To determine whether dormant DTCs occupy a specific niche, we searched first for DTCs lacking expression of the cell cycle marker Ki67 in a spontaneous metastasis model of breast cancer20. Tumours resulting from orthotopic injection of MDA-MB-231, a *bona fide* metastatic BCC line expressing GFP–luciferase, were resected after 3 weeks ($V_{avg} = 0.5 \text{ cm}^3$; Fig. 1a). Surviving mice that did not experience relapse at the primary site were euthanized 6 weeks later. Bioluminescence of dissected visceral organs confirmed that BCCs disseminated to the canonical target organs—lung, bone, liver and brain21 (Fig. 1a). In contrast to the resected primary tumours, in which BCCs proliferated...
Figure 2  Microvascular endothelium induces sustained quiescence of breast tumour cells in engineered cultures. (a) Lung and BoMa stroma (LFs and MSCs, respectively) were seeded alone or with mCherry E4-ECs. In co-culture, mCherry E4-ECs self-assembled into 3D microvascular networks over 7 d. YFP-expressing BCCs (T4-2) were then seeded sparsely (240 cm\(^{-2}\)) in SFM onto stroma or microvascular niche cultures and overlaid with a drip of laminin-rich ECM (LrECM) diluted in medium to provide BCCs with a 3D microenvironment\(^{58}\). Entire wells were imaged 10 days later. (b) Representative images of T4-2 cell growth within lung-like or BoMa-like niches containing stroma alone, or stroma + ECs, 10 days post-seeding. Scale bars, 500 μm. (c,d) Tumour cell area fraction of YFP T4-2 at day 10 (normalized by the value measured immediately post-seeding to correct for any minor variations in initial seeding density) in lung-like (c) or BoMa-like niches (d) (n = 5 sets of co-cultures analysed per condition). Error bars denote s.e.m. \(\ast\ast P = 0.001\) and \(\ast\ast\ast P < 0.0001\) by two-tailed t-test. Day 10 co-cultures were fixed and stained for CD31 to label ECs and Ki67 to identify actively cycling tumour cells (b, inset; scale bar, 50 μm). (e,f) The percentage of Ki67-negative clusters (white asterisk in b, inset) was quantified for T4-2 cells seeded on lung- (e) and BoMa-like (f) niches (n = 5 sets of co-cultures analysed per condition). Error bars denote s.e.m. \(\ast\ast\ast P < 0.0001\) by two-tailed t-test. Day 17 normalized by day 10) in lung- (g) and BoMa-like (h) niches to determine whether quiescent tumour clusters at day 10 remained quiescent (n = 5 sets of co-cultures analysed per condition). Error bars denote s.e.m. \(\ast\ast\ast P < 0.0001\) by two-tailed t-test. (i) Live images of representative T4-2 cells on lung-like stroma and microvascular niche for day 10 and day 17, with immunofluorescence staining to confirm Ki67 status. (j) The same as in i, for representative T4-2 cells on BoMa-like stroma and microvascular niche cultures. Note that stroma culture scale bars, 100 μm and microvascular niche culture scale bars, 50 μm.

This observation was confirmed also with a weakly metastatic BCC line (mCherry HMT-3522-T4-2; ref. 22), which was injected intra-cardially to facilitate dissemination to all target organs (Fig. 1c).

actively whether nearby tumour vasculature or not (Fig. 1b), we found small clusters of GFP-positive/Ki67-negative BCCs residing directly on microvascular endothelium of both lung (Fig. 1c) and BoMa (Fig. 1d).
Eight weeks after injection into the left ventricle of NOD-SCID mice, small clusters of mCherry-positive (false-coloured green)/Ki-67-negative T4-2 cells were found residing perivascularly in murine lung (Fig. 1f), BoMa (Fig. 1g) and brain (Fig. 1h). The consistent discovery of quiescent DTCs residing perivascularly—6 weeks after resection of the primary tumour in the first model, and 8 weeks post-injection in the second model—suggested that endothelium might play an active role in regulating tumour dormancy.

**Organotypic microvascular niches demonstrate that endothelial cells induce sustained quiescence of BCCs**

Determining whether microvascular endothelium could directly influence tumour cell quiescence necessitated lung- and BoMa-like designer microenvironmentsthat would allow quantitative assessment of human BCC growth in the presence or absence of a microvascular network. There are considerable hurdles to engineering such models. For example, whereas ECs do not survive in serum- and cytokine-free medium (SFM), the addition of exogenous factors could mask the effects of EC-derived angiocrine factors on tumour growth23-25.

To overcome this limitation, primary human umbilical vein endothelial cells (HUVECs) were transduced with a lentiviral construct containing the human adenoviral E4ORF1 gene24, which enables HUVECs to survive24 and form sustainable microvascular networks in SFM (Supplementary Fig. S1). E4ORF1-HUVECs (E4-ECs) expressing mCherry self-assembled into robust three-dimensional (3D) microvascular networks26 over 7 days when cultured with fibroblasts from lung (LFs) or with BoMa mesenchymal stem cells (MSCs). We then compared the growth of yellow fluorescent protein (YFP)-expressing T4-2 cells seeded sparsely in SFM onto lung- and BoMa-like microvascular niches or onto only the corresponding stroma (that is, LFs or MSCs) after a further 10 days (Fig. 2a). Whereas T4-2 cells grew extensively on lung and BoMa stroma, growth of T4-2 cells on organotypic microvascular niches was reduced markedly (threefold in lung-like- and fivefold in BoMa-like-microenvironments; Fig. 2b–d). Similar results were obtained also with a luminal, oestrogen-receptor-positive BCC line (MCF-7; Supplementary Fig. S2a,b). Highly metastatic MDA-MB-231 cells exhibited the same trend; in particular, cells adherent to microvasculature were Ki67-negative (Supplementary Fig. S2c-e). Ki67 immunofluorescence (Fig. 2b, inset) revealed further that most T4-2 cells seeded in organotypic microvascular niches became quiescent (77.4% Ki67-negative clusters in lung-like niche and 88.1% in BoMa-like niche; Fig. 2e,f). Importantly, this was not a transient phenotype, as microvascular-associate tumour clusters generally remained dormant, as opposed to BCCs cultured only on lung or BoMa stroma (Fig. 2g–j). Thus, our organotypic models recapitulated our in vivo findings and allowed us to pinpoint ECs as a prime regulator of DTC quiescence in lung and BoMa. We next sought to identify endothelium-derived factor(s) underlying this effect.

**TSP-1 is deposited around mature endothelium and is an angiocrine tumour suppressor**

We noted consistently that whereas the bulk of quiescent tumour clusters remained on or near microvascular endothelium in our culture models, those that had seeded—or strayed—to the edge of a well and off of microvasculature typically underwent marked expansion (Supplementary Fig. 3a; inset shows that all clusters originated from single cells). This observation hinted that the putative angiocrine tumour suppressor(s) was not freely diffusible. Indeed, medium conditioned daily by microvascular niche cultures did not reduce T4-2 cell growth on lung stroma when compared to control conditions (Supplementary Fig. S3b,c).

Accordingly, to identify factors deposited locally by ECs that could suppress tumour cell growth, we performed comparative proteomics on decellularized extracellular matrix (ECM) from lung- and BoMa-like microvascular niches (versus their respective stroma). A number of extracellular factors were upregulated in organotypic microvascular niches (Fig. 3a). Among these potential angiocrine tumour suppressors, TSP-1 caught our attention because: TSP-1 was expressed at higher levels in both organotypic lung- and BoMa-microvascular niches when compared with stroma alone (Fig. 3a), and TSP-1 overexpression in BCCs was shown previously to suppress metastatic outgrowth in lung27. However, these anti-tumour effects were attributed to the anti-angiogenic activity of TSP-1 (ref. 27). The possibility that TSP-1 could function to directly suppress tumour cell growth (particularly from a non-tumour source within the DTC microenvironment) had not been considered28.

We verified first that TSP-1 was present on lung microvessels associated with dormant DTCs in both spontaneous and experimental metastasis models (Fig. 3b,c). We confirmed also that TSP-1 is expressed in non-tumour-bearing mice in the microvascular basement membrane of murine lung (Fig. 3d), bone (Fig. 3e) and brain (Fig. 3f).

Similar peri-endothelial localization was observed also in organotypic microvascular niches. To determine whether perivascular TSP-1 is derived primarily from ECs, we used a 3D co-culture model consisting of EC-coated microcarrier beads embedded within a fibrin ECM several millimetres away from overlaid LFs. ECs form robust microvascular networks within 7 days under these conditions29, and TSP-1 was concentrated within the basement membrane of established microvessels (Fig. 3g,h). Gain-of-function studies confirmed that TSP-1 was sufficient to suppress BCC growth on lung stroma in the absence of endothelium (Fig. 3i). Furthermore, pre-treatment with a TSP-1-blocking antibody to interfere with T4-2 cell adhesion to TSP-1 within lung-like microvascular niches resulted in significantly increased tumour cell outgrowth when compared with IgG control-treated cultures (Fig. 3j). These gain- and loss-of-function experiments, combined with its presence in microvascular basement membrane and its association with dormant DTCs, identified TSP-1 as an angiocrine tumour suppressor.

**Neovascular tips accelerate breast tumour cell outgrowth**

As TSP-1 stabilizes microvascular endothelium by inhibiting EC motility and growth28, it was not surprising to find it expressed surrounding established microvasculature (Fig. 3d–g). However, loss of TSP-1 expression at neovascular tips (Fig. 3g,h) suggested that this physiological knockdown could result in a concomitant loss of tumour suppression within neovascular sub-niches. In support of this idea, we found that quiescent tumour clusters were often associated with stable endothelial stalks (Fig. 4a(i); asterisk, and also a(ii)–(iv), all from the same culture), whereas actively growing tumour clusters were often surrounded by sprouting neovascular tips (Fig. 4a(i); T). Therefore, we reasoned that these two sub-niches exert differential growth control over BCCs.
TSP-1 co-localized with type IV collagen in the basement membrane of established microvessel stalks (white arrowheads), but TSP-1 seemed to be downregulated at neovascular tips (white asterisks). Scale bar, 50 μm.

(b) This was confirmed by quantification of TSP-1 intensity at stalks versus tips (n = 16 microvessels pooled from 3 different experiments). Error bars denote s.e.m. *P < 0.005 by two-tailed paired t-test. (i) Add-back of TSP-1 to T4-2 cells plated on lung stroma effectively substituted for the presence of ECs by causing a significant reduction in tumour cell growth (normalized to vehicle condition; n = 3 sets of co-cultures analysed per condition). Error bars denote s.e.m. **P < 0.05 when compared with vehicle by one-way analysis of variance and Dunnett's multiple comparisons test. (j) Treating lung-like microvascular niches with a TSP-1-blocking antibody resulted in significantly enhanced tumour cell growth (versus IgG control; n = 5 sets of co-cultures analysed per condition). Error bars denote s.e.m. ***P = 0.0054 by two-tailed unpaired t-test.
Malignant T4-2 cells expressing histone H2B–GFP were seeded on top of microvascular niches and tracked for 72 h. Qualitative analysis of time-lapse videos revealed that tumour cells remaining near established vessel stalks divided more slowly than those that encountered neo-vascular tips (Fig. 4b; Supplementary Videos S1 and S2). To perform quantitative analysis, we defined 3 sub-niches: neovascular tip, for tumour cells within 50 μm of a sprouting endothelial tip; stable endothelium, for tumour cells within 50 μm of established, non-invasive endothelium; and stroma, for tumour cells >50 μm away from either type of endothelium. We quantified the aggregate time (that is, dwell time, t_dwell) that 229 tumour cells spent in each of these sub-niches before undergoing a single division (division time: t_div).

Thus, in Fig. 4c, the scatter plot represents the fraction of each T4-2 cell’s t_div spent near stable (red) or neo-vascular (green) endothelium, or on stroma (black). Pearson correlation analysis revealed that dwell time around stable endothelium (t_dwell, stable) correlated significantly
Figure 5 Notch1-mediated reduction in neovascular tips suppresses breast tumour cell outgrowth. Microvascular niches were created with stromal cells mixed with control shRNA E4-EC and/or Notch1 shRNA E4-EC. YFP T4-2 cells were then seeded in SFM and growth was analysed 10 days later. (a) Microvascular niches composed of control shRNA E4-EC, Notch1 shRNA E4-EC, or a 1:1 mix of the two were fixed and stained for CD31 at day 7. Scale bars, 200 μm. (b,c) Neovascular tip number per field (b; large white dots in a) and branch point density (c; small yellow dots in a) were quantified (n = 15 fields of microvascular networks pooled from 5 separate co-cultures). Error bars denote s.e.m

with \( t_{\text{div}} \) (two-tailed \( P = 0.014 \)); that is, BCCs with longer division times tended to reside longer near established endothelium (Fig. 4c, red trend line). Conversely, tumour cell dwell time around neovascular tips (\( t_{\text{dwell,neo}} \)) anti-correlated significantly with \( t_{\text{div}} \) (two-tailed \( P = 0.001 \); green trend line in Fig. 4c). Importantly, stromal dwell time (\( t_{\text{dwell,stroma}} \)) did not correlate with \( t_{\text{div}} \) (Fig. 4c, black trend line) at all. We extended this analysis further by examining the fastest- (\( t_{\text{div}} < t_{\text{avg}} - \text{s.d.} \)) and slowest- (\( t_{\text{div}} > t_{\text{avg}} + \text{s.d.} \)) dividing tumour cells and found that the fastest-dividing tumour cells resided 2.1 times longer within neovascular sub-niches than around stable endothelium, whereas the slowest-dividing tumour cells did the opposite (Fig. 4d).

Our analysis suggested that established endothelium steers BCCs towards a quiescent phenotype, whereas neovascular endothelium accelerates BCC growth. If this were indeed true, tumour growth should decrease if neovascular tips are depleted before tumour cell seeding, and increase if neovascular tip formation is promoted. Consistent with observations from the developing mouse retina, reduced expression of endothelial Notch1 through Notch1 targeting short hairpin RNA (shRNA; Supplementary Fig. S4) led to a hyperbranched network, but with a significant reduction in the number of endothelial tips (Fig. 5a–c). This was to be expected from a closed system (our case), as opposed to an open system (the developing retina). BCC growth followed suit, evidenced by the progressive reduction in growth of T4-2 cells seeded on either control shRNA (tip\(^{\text{high}}\))-EC, control shRNA:Notch1 shRNA chimaera (1:1)- or Notch1 shRNA (tip\(^{\text{low}}\))-EC cultures (Fig. 5d,e). The percentage of quiescent tumour clusters also increased modestly in cultures that contained fewer neovascular tips (Fig. 5f).

We investigated whether increased neovascular tip concentration would promote tumour cell growth in culture and \textit{in vivo}. To enrich for neovascular tips in culture, we allowed microvascular networks to develop for only 3 days before seeding T4-2 cells. The number of neovascular tips at day 3 of network formation was nearly double that of day 7 cultures (Supplementary Fig. S5a,b,e). Seeding tumour cells at each of these developmental time points and measuring growth 10 days later confirmed that BCC growth correlates positively with endothelial tip number; T4-2 cells grew nearly 6 times more when seeded on networks rich in neovascular tips, and significantly fewer of these tumour clusters became quiescent (Supplementary Fig. S5c,d,f,g).

To investigate whether a microenvironment rich in neovascular tips promotes tumour cell growth \textit{in vivo}, we used zebrafish with a mutation in the gene encoding microsomal triglyceride transfer protein (\textit{mtp}). These mutants, called \textit{stalactite}, have an ectopic microvascular sprouting phenotype that is especially pronounced in the perivitelline/subintestinal space at 3.5 days post-fertilization (dpf; ref. 31). Accordingly, we injected \( \sim 1-10 \) MDA-MB-231 cells expressing mCherry into the subintestinal space of wild-type (WT) and \textit{mtp}\(^{-/-}\) mutant zebrafish at this time point. Fish injected unsuccessfully, defined as those lacking red fluorescence in the subintestinal space, or those over-injected (an area fraction of red fluorescence over...
The above experiments confirmed that neovascular tips promote tumour cell outgrowth in organotypic culture and in vivo, implying production of distinct tumour-promoting factors by neovascular tips. To identify factors enriched around neovascular tips, we used tandem mass spectrometry and compared decellularized ECM from neovascular tip<sup>high</sup> (control shRNA) and tip<sup>low</sup> (Notch1 shRNA) cultures (Fig. 5a). Tip<sup>high</sup> cultures were characterized by enhanced expression of POSTN, tenascin, versican and fibronectin (Fig. 7a), all molecules involved in formation of the metastatic niche<sup>32–35</sup>. Further, tip<sup>high</sup> cultures exhibited reduced expression of molecules involved in sequestering another known mediator of metastatic outgrowth, TGF-β1 (biglycan and LTBP1, Fig. 7a)<sup>36</sup>, suggesting that active TGF-β1 itself would be expressed more highly at neovascular tips. Immunofluorescent staining of E4-ECs in 3D co-cultures confirmed that active TGF-β1 and POSTN were expressed highly at neovascular tips (Fig. 7b,c,e). In contrast, latent TGF-β1 was expressed prominently in endothelial stalks (Fig. 7d). These findings were confirmed in vivo in physiologic and pathologic settings. POSTN and active TGF-β1 were concentrated on/near endothelial tip cells in the developing mouse retina (Supplementary Fig. S6a,c,e), but were not detected consistently around established phalanx endothelium in the same tissue (Supplementary Fig. S6c,e; insets). Examining established
**Figure 7** Neovascular tips comprise micrometastatic niches enriched for POSTN and TGF-β1. (a) Heat map of ECM proteins (spectral counts) from (1) neovascular tiphigh cultures (LF + ctrl shRNA EC) normalized by lung stroma (LF); (2) neovascular tiphigh cultures (LF + Notch1 shRNA EC) normalized by lung stroma (LF); and (3) tiplow cultures normalized by tiplow cultures (sorted high to low with respect to this comparison, log2 scale). (b–d) Representative images of microvessels stained for POSTN (b), active TGF-β1 (c) and latent TGF-β1 (d). White asterisks in b,c denote enhanced intensity of POSTN and active TGF-β1 stain at endothelial tip. Scale bar, 20 μm. (e) Quantification of relative POSTN (left) and active TGF-β1 intensity (right) at the tip versus stalk of microvessels (n = 16 microvessels were pooled from 3 different experiments for analysis of POSTN intensity quantification; n = 16 microvessels were pooled and analysed for active TGF-β1 intensity quantification). Error bars represent s.e.m. ***P < 0.0001 by two-tailed t-test. (f,g) Representative images of microvascular niche cultures seeded with T4-2 cells and treated with vehicle (f) or a combination of POSTN (50 ng ml−1) and TGF-β1 (10 pg ml−1) (g) twice over the first 48 h, and imaged at day 10. Scale bar, 500 μm. (h) Normalized tumour cell area fraction of YFP T4-2 at day 10 treated by vehicle or by the combination of POSTN and TGF-β1 (combo; n = 5 sets of co-cultures analysed per condition). Error bars represent s.e.m. ***P < 0.0001 by two-tailed t-test. (i) A visual summary of our findings is as follows. In distant microenvironments, single or small clusters of DTCs reside in the perivascular niche and are maintained in a quiescent state by endothelial-derived factors. Here, we have identified TSP-1 as one such factor, and perlecan was identified by others as an EC-derived factor that suppresses tumour growth.1. Other ECM molecules such as laminins, type IV collagen and latent TGF-β1-binding proteins (LTBPs) may contribute directly or indirectly to the dormant niche. As vascular homeostasis is disrupted with induction of neovascular sprouting, endothelial architecture is perturbed. The result is not only loss of suppressive signals (for example, TSP-1), but deposition of ECM molecules and growth factors that promote micrometastatic outgrowth. Thus, maintaining vascular homeostasis could be the key to sustaining DTC dormancy long-term.
expressed highly at neovascular tips, promote BCC outgrowth within a tumour-suppressive microenvironment.

DISCUSSION

Using murine models, zebrafish and organotypic microvascular niches composed of human cells, we demonstrate here that: dormant DTCs from the breast reside on or near lung and BoMa microvasculature in vivo, stable microvasculature constitutes a dormant niche that induces sustained tumour cell quiescence through TSP-1, and the tumour-suppressive nature of microvascular endothelium is lost at sprouting endothelial tips, which are characterized by reduced TSP-1 expression and enhanced expression of pro-tumour factors POSTN and TGF-β1 (Fig. 7i). Studies of primary tumours have focused primarily on the tumour’s regulation of the endothelium. We believe this paradigm now shifts at secondary sites, where DTCs are the minority constituent of the tissue and subject to direct control by microvascular endothelium and perhaps other resident cell types.

The notion that ECs directly regulate cells in the perivascular microenvironment is rooted in a number of biological studies on normal tissues (reviewed in ref. 25). ECs with phenotypic characteristics of neovascular tip cells spark growth and morphogenesis of the liver and regeneration of lung alveoli. On the other hand, established endothelium promotes pancreatic differentiation, inhibits smooth muscle cell proliferation and maintains pluripotency of neural, haematopoietic and mesenchymal stem cells. Our study demonstrates that this scenario—that mature microvasculature confers tissue quiescence and sprouting endothelium promotes tissue growth—is at work also in the DTC microenvironment. These findings may apply generally to primary tumours also, thus shedding light on the apparent dichotomy of EC function at the primary site.

The therapeutic implications of our results are multi-fold. Foremost is that identification of tumour-suppressive factors derived from stable endothelium may guide therapies designed to enforce DTC dormancy. This raises the question of whether other molecules in the microvascular basement membrane function as tumour suppressors, and whether these can be used in combination with TSP-1 to stave off metastatic relapse. Second is that factors enriched in neovascular sub-niches may be targeted early in tumour progression to prevent establishment of micro-metastatic niches that disrupt DTC quiescence. In this regard, our study complements previous work pinpointing POSTN, TGF-β1 and other molecules as potential therapeutic targets, and reveals further that these molecules arise from an unexpected source, namely neovascular endothelium.

Surprisingly, many of the factors upregulated in neovascular tip-enriched cultures are documented components of pre-metastatic and metastatic niches. Given the nature of our results, this commonality provides further evidence for the in vivo relevance of our model systems. It also raises a question about whether metastatic niche formation is dependent on the induction of neovascularisation. In this respect, it is interesting to note that nascent endothelium was shown recently to initiate a Th2-mediated inflammatory response in asthma, a response that is also associated with accelerated metastatic outgrowth in tumour models. Thus, by direct deposition of tumour-promoting factors, as well as by secreting cytokines that stimulate macrophage polarization to a pro-tumour phenotype, neovascular tips may function as a nexus that directly and indirectly catalyses formation of a micrometastatic niche. Accordingly, long-term administration of drugs aimed at preventing neovascular formation through inhibition of VEGFR2- or integrin αvβ3- (ref. 56) driven signalling, or by targeting more recently discovered pro-angiogenic signalling mechanisms, may prove effective in delaying relapse of early-stage breast cancer patients. We believe that it will be crucial to deliver these drugs in a manner that prevents cultivation of the pro-tumour neovascular niche while preserving the dormant niche fostered by stable microvasculature.

It remains to be determined whether the mechanisms we have identified here apply also to other tumour types and in other secondary tissues. We propose that a systematic understanding of interactions between DTCs and their microenvironment will provide a vehicle by which we can design more effective therapies to keep DTCs dormant—or eradicate them—in early-stage cancer patients.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

ACKNOWLEDGEMENTS

We thank S. Rafii and B. Weinstein for generously providing the E4ORF1 lentiviral plasmid and staurosporine mutant zebrashark, respectively. We are grateful to N. Boudreau, S. Rafii, R. Schwartz, R. Xu, A. Bruni-Cardoso and L. Correia for critical insight, and to other present members of the Bessell laboratory for helpful discussions. We thank A. Lo and C. Williams for technical assistance and T. Varimezova for performing blinded quantitative analysis. C.M.G. was financially supported by a Glenn T. Seaborg Postdoctoral Fellowship from LBNL and by a NCI-U54CA143836 training grant. K.I.E. is a Robert Black Fellow supported by the Damon Runyon Cancer Research Foundation (DRG-109-10). K.A.H.’s laboratory is supported by grants from the NIH (R01HL042493 and R01HL090895) and from the March of Dimes Foundation (6-FY12-356). The work in D.Y.R.S.’s laboratory was financially supported in part by grants from the NIH (HL54737) and the Packard Foundation. E.I.C.’s laboratory is supported by a Carol Baldwin Breast Cancer Award, a shared instrument grant (NIH/NCRR 1 S10 RR023680-1) and a DOE Subcontract (DE-AC02-05CH1123) from LBNL. D.L. is supported by The Hartwell Foundation. The work from M.J.B.’s laboratory is supported by grants from the US Department of Energy, Office of Biological and Environmental Research and Low Dose Scientific Focus Area (contract no. DE-AC02-05CH1123); by the National Cancer Institute (awards U51CA169538 to D.L. and M.I.B.); U54CA126552, R37CA064786 and U54CA143836—Bay Area Physical Sciences-Oncology Center, University of California, Berkeley, California); by a grant from the Breast Cancer Research Foundation; and by the US Department of Defense (W81XWH-081075).

AUTHOR CONTRIBUTIONS

C.M.G., D.L. and M.I.B. conceived of the study. C.M.G., H.P., I.R.M. and H.B. conducted animal studies and analysed resulting data. C.M.G., K.I.E. and D.Y.R.S. planned and executed zebrafish experiments. D.A. and K.A.H. lent expertise in the zebrafish model. C.M.G. and H.M. collected and analysed data, and H.M. also provided conceptual advice. A.K. and E.I.C. conducted LC-MS/MS analysis and analysed data. C.M.G. and M.I.B. wrote the manuscript. All authors read and critiqued the manuscript extensively.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at www.nature.com/doifinder/10.1038/ncb2767

Reprints and permissions information is available online at www.nature.com/reprints

1. Aguirre-Ghiso, J. A. Models, mechanisms and clinical evidence for cancer dormancy. Nat. Rev. Cancer 7, 834–846 (2007).
2. Goss, P. E. & Chambers, A. F. Does tumour dormancy offer a therapeutic target? Nat. Rev. Cancer 10, 871–877 (2010).
3. Klein, C. A. Parallel progression of primary tumours and metastases. Nat. Rev. Cancer 9, 302–312 (2009).
METHODS

Animal studies. All mouse work was performed in accordance with institutional, IACUC and AAALAS guidelines. For spontaneous metastasis assays, GFP–luc MDA-MB-231 (1 × 106 cells) were injected into the inguinal mammary gland of 7-week-old female NOD-SCID mice (Jackson Laboratory, Bar Harbor, Maine). For cancer cell colonization studies, mice were deburred, rehydrated in PBS and incubated in a 1:1 solution of LeEGM (Growth-factor reduced Cultrex; Trevigen). Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen/Gibco). Tumours 0.5 cm3 were resected 3 weeks later. Mice were monitored weekly for relapse by bioluminescence imaging and those that did not experience gross metastatic relapse early on were euthanized and dissected at 6 weeks. Lungs were collected after saline perfusion. Primary tumours and lungs were fixed overnight in 1:60% paraformaldehyde (PFA)/PBS solution and then dehydrated to 100% cold cutting temperature (CCT) compound (Tenckhoff). Femurs and tibia were fixed in an identical fashion and then decalciﬁed by gentle shaking in decalciﬁcation solution (0.1 M Tris–HCl, 0.2 M EDTA, at pH 7.4) for 1 week protected from light and with intermittent changes of decalciﬁcation solution before overnight incubation in 30% sucrose, incubation in 1:1 sucrose/CCT (1 h overnight), and ﬁnally embedding in OCT compound.

For experimental metastasis assays, mCherry T-4-2 cells (1 × 106 cells in 100 µl PBS) were injected into the left cardiac ventricle of 6–8-week-old nude NOD-SCID mice with a 26 1/2 gauge needle. Successful injection was characterized by the pumping of arterial blood into the syringe. Mice that did show any signs of tumour burden were euthanized and dissected 8 weeks post-injection. Tissues were processed as described above.

For the retinal angiogenesis assay, retinas were dissected from 3.5 dpf zebrafish and whole-mounted and stained as described elsewhere. Sections were thawed, rehydrated in PBS and incubated in whole-mounted retina, endothelial cells were labelled with a rat monoclonal antibody targeting CD31/PECAM-1 (BD Pharmingen 553373, clone: MEC 13.3, 1:250), TSP-1 was stained with a rabbit polyclonal antibody (Abcam ab85762, 1:500), all at 1:500. Tissues were imaged on a Zeiss LSM 710 confocal microscope using either a 1.1 NA 63–water-immersion objective or a 1.4 NA 63–oil-immersion objective. Three-dimensional cultures (see below) were stained after ﬁxation with Alexa Fluor 568 phalloidin (Invitrogen A12380, 1:200) to detect F-actin or with the following antibodies: mouse monoclonal antibody targeting human CD31/PECAM-1 (Millipore C2608, clone: HCL/6 1:200), rabbit polyclonal antibody against Ki67 (see above), rabbit polyclonal antibody against peristin (Abcam ab14041, 1:100), chicken polyclonal antibody against active TGF-β1 (see above), goat polyclonal antibody against LAC TGF-β1 (R&D Systems AB-246-B-A, 10 µg ml−1), and mouse monoclonal antibody against type IV collagen (University of Iowa Developmental Studies Hybridoma Bank, clone: M537, 1:100).

Cell culture and reagents. HUVECs isolated freshly from human umbilical cord veins were propagated in EGM-2 growth medium (Lonza). Human MSCs and LFs were obtained commercially (Lonza) and propagated in low-glucose (MSCs) or high-glucose (LFS) DMEM supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals) and 1% penicillin/streptomycin (P/S, UCSF Cell Culture Facility). All primary human cells were used in experiments after passage 10.

Malignant T4-2 cells were grown in H14 medium on collagen-coated tissue culture ﬂasks. MCF-7 and MDA-MB-231 cells were grown in high-glucose DMEM supplemented with 10% FBS and 1% P/S.

mCherry E4-ECs were generated by retroviral infection of E4-ECs with a pBBM/mCherry plasmid19, YFP-T4-2, -MCF-7 and -MDA-MB-231 were generated by infection of tumour cells with pLentiCMV/YFP lentivirus followed by selection for 1 in 10 µg ml−1 puromycin. Histone H2B–GFP T-4-2 have been described previously20.

Generation of E4ORF1 lentivirus and E4ORF1-HUVECs. pCCL-PGK lentiviral vector containing the human adenosoviral E4ORF1 gene (serotype 5) was a gift from S. Rabin (Weill Cornell Medical College, HHMI, USA; ref. 21). Lentivirus was generated by co-transfection of sub-confluent 293FT cells with 2 µg each of PLP1, PLP2, VSVG and E4ORF1 plasmid DNA in DMEM containing a 3:1 (ulug) ratio of FuGene6 (Roche) and L-HEPES (Gibco). Lentiviral RNA was harvested at 5–7 days post-transfection and transfected into 293FT cells. After incubating 10 µl of viral suspension per well on a 96-well plate, cells were left undisturbed on a flat surface for 20 min to allow even seeding before incubation.

After 7 days, YFP tumour cells were suspended in unsupplemented DMEM/F12 (800 cells ml−1). YFP tumour cells were seeded (100 µl well−1) after washing cultures three times with PBS. Cells were allowed to settle for 15 min at room temperature, and then a drip of LeEGM (ref. 58) in DMEM/F12 was slowly added to each well (ﬁnal concentration = 20%). The drips condensed for 10 min at room temperature before polymerizing fully at 37°C before imaging. Cultures were imaged immediately after seeding on a Zeiss LSM 710 confocal microscope using a 0.3 NA x10 air objective. The objective was centred to each well before acquisition of 6 x 6 tiles that captured the near-entirety of each well. Cultures were maintained with media changes every 72 h and imaged at day 10.

For TSP-1-blocking antibody experiments, cultures were treated at day 5 and again at day 7 (on tumour cell seeding) with 20 µg ml−1 of a mouse monoclonal antibody that blocks binding of CD47 to TSP-1 (Thromboscience MS-420-PIABX, clone: C5, 1:200), or with 20 µg ml−1 of IgG control (Acros Antibodies AM3095AF-N).

Time-lapse acquisition. Time-lapse sequences were acquired with a Zeiss LSM 710 confocal microscope with an environmental chamber to maintain temperature (37°C), humidity and CO2(5%). H2B–GFP T-4-2 cells were starved for 24 h in unsupplemented DMEM/F12 before seeding on microvascular niches (see above). Images (6 x 6 tiles, 512 x 512 resolution, 8-bit) were acquired every 20 min for 72 h. Medium was replenished at 24 h.

Three-dimensional sprouting angiogenesis assay. E4-ECs were coated on dextran microcarrier beads (Sigma), suspended within a 3 mg ml−1 solution of bovine ﬁbrinogen (Sigma), and gelated within a No. 1.5 thickness 8-well borosilicate chamber slide (Thermo Scientiﬁc/Nunc) using 50 U ml−1 (1:25 v/v) thrombin (Sigma). LFs (2 × 105) were overlaid in 250 µl of EGME2 well. Cultures were analysed at day 7.
subintestinal space at 3.5 dpf and 7.5 dpf using the macro described above. Tumour cell area fractions measured at 7.5 dpf were normalized by the corresponding values obtained post-injection to yield normalized tumour cell growth for each animal.

To quantify Ki67-negative, tumour clusters totally devoid of nuclear Ki67 were counted manually. The Ki67-negative fraction was obtained by dividing this number by the total number of YFP-positive clusters per well.

To analyse tumour cell division time as a function of microvascular sub-niche, a 50 μm x 50 μm grid was superimposed on image sequences loaded into Imaris software to facilitate measurement of the distance between H2B–GFP T4-2 cells and mCherry-positive E4 EC structures. When in question, distances were measured manually using the Measurement Points tool in Imaris. H2B–GFP T4-2 cells were tracked until first evidence of division, and the total time spent in an endothelial tip sub-niche (within 50 μm of a microvascular tip), in an endothelial stalk sub-niche (within 50 μm of microvasculature but not within 50 μm of a tip), or in the stromal sub-niche (>50 μm away from microvasculature) was tabulated for each of 229 cells that could be tracked accurately during the entire 72 h time period. Analysis was conducted in a blinded fashion.

Tip number and branch point density were counted manually using the cell counter application in ImageJ.

Intensity of protein immunofluorescence at endothelial tips and stalks was quantified using NIH ImageJ. A minimum of 15 vessels from 2 separate experiments were quantified to determine the relative intensities of TSP-1, POSTN and active TGF-β1 at the endothelial tip versus endothelial stalk. Images were contrast enhanced (saturated pixels = 0.5%) before analysis. Average intensity of a ~150 pixel-squared region of a tip cell and a stalk cell two cells away from that tip were measured. Background intensity was subtracted from the measured intensities. Tip and stalk intensities were each normalized by the average intensity obtained for all stalks and reported as normalized average intensities.

**Notch1 knockdown.** E4ORF1-HUVECs were infected at a multiplicity of infection of 5 with custom-made lentiviruses (Sigma) containing shRNA targeting human Notch1 in a pLKO.1–puro–CMV–TagRFP vector. Empty vector was used as a control (ctrl shRNA). Sequences for shRNA were as follows: sh6393: 5′-CCGGCCITTTGTTTCAGGTTCAGTATTCTCGAGAATACTGAACCTGAAAC-3′; sh1510: 5′-CCGGCGCTTGCGCAGACAAGTAACATCCTCGAGATGTATTTGCTCAGGCGCCTTTT3′; sh2304: 5′-CCGGCCAAAAGCATGACCCAAGTGGGACTCTCGAGTACCCACTTGTTTGTCTTTTT3′.

**Western blotting.** Notch1-shRNA-E4-ECs and control-shRNA-E4-ECs were lysed in 2% SDS/PBS. Twenty micrograms of each lysate was then separated on a Tris–glycine 4–20% gel. Notch1 was probed with a rabbit polyclonal antibody (Abcam ab27526, 1:500). The blot was stripped and re-probed with a rabbit polyclonal antibody against the nuclear membrane protein Lamin A/C, used here as a loading control (Santa Cruz Biotechnology sc-206811, 1:2,000).

**LC–MS/MS.** Samples were prepared for LC-MS/MS analysis as follows. Cultures were established for 7 days in EGM-2, washed extensively with PBS to remove medium, and incubated in 0.1% Triton X-100/PBS (with added protease inhibitor cocktail, EMD Biosciences) for 30 min at 4 °C to de-cellularize the cultures. After washing, cultures were incubated overnight at 4 °C in 0.5 M acetic acid solution. The following day, acetic acid was collected and protein was precipitated from the acetic acid solution through TCA/DOC precipitation. The precipitate was washed twice in acetone, dried at room temperature, and then dissolved overnight in 5× Invitrosl LC/MS protein solubilizer (Invitrogen) under constant agitation. Invitrosl was brought to 1× with 25 mM NH4 (HCO3) and final protein concentration was measured by A280nm using a NanoDrop spectrophotometer (Thermo Scientific). Precipitates were stored at −80 °C until analysis.

Immediately before analysis, 30 micrograms of protein from each experimental condition was proteolytically cleaved by modified, sequencing-grade trypsin (Promega) in 50 mM NH4 (HCO3) digestion buffer containing 1 μg trypsin and 2 mM CaCl2 for 16 h at 37 °C. Reactions were then acidified with 90% formic acid (2% final) to stop proteolysis. Samples were centrifuged for 30 min at 16,100 g to remove insoluble material, and then subjected to LC–MS/MS analysis.

**Methods for LC–MS/MS and tandem mass spectra analysis** were conducted essentially as described previously64. The resulting list of proteins was culled to ECM proteins and related growth factors/cytokines by referencing protein ontology in http://www.uniprot.org. Spectral counts for each condition were normalised by one another and log2 values of those products were plotted in heat-map format using TreeView open source software.

**Statistical analysis.** Statistical analyses were conducted with GraphPad Prism 5 software. See the figure legends for individual n and P values, and specific statistical test(s) employed. Unless noted otherwise, data are reported as mean±s.e.m.

59. Pitulescu, M. E., Schmidt, I., Benedito, R. & Adams, R. H. Inducible gene targeting in the neonatal vasculature and analysis of retinal angiogenesis in mice. Nat. Protoc. 5, 1518–1534 (2010).
60. Herbert, S. P., et al. Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. Science 326, 294–298 (2009).
61. Nicoli, S. & Presta, M. The zebrafish/tumor xenograft angiogenesis assay. Nat. Protoc. 2, 2918–2923 (2007).
62. Baluk, P., Morikawa, S., Haskell, A., Mancuso, M. & McDonald, D. M. Abnormalities of basement membrane on blood vessels and endothelial sprouts in tumors. Am. J. Pathol. 163, 1801–1815 (2003).
63. Tanner, K., Mori, H., Mroue, R., Bruni-Cardoso, A. & Bissell, M. J. Coherent angular motion in the establishment of multicellular architecture of glandular tissues. Proc. Natl Acad. Sci. USA 109, 1973–1978 (2012).
64. Beliveau, A., et al. Raf-induced MMP9 disrupts tissue architecture of human breast cells in three-dimensional culture and is necessary for tumor growth in vivo. Genes Dev. 24, 2808–2811 (2010).
E4ORF1 mediates survival and functional differentiation of endothelial cells in serum- and cytokine-free conditions. (a) Comparison of wild-type (wt)- and E4ORF1-HUVEC survival on tissue culture plastic after 48 h of culture within SFM. Scale bar = 100 µm. (b) wt- (left) and E4-ECs (right) after 24 h culture atop of LrECM in serum- and cytokine-free conditions. Scale bar = 100 µm. (c) wt- (left) and E4-ECs (right) in co-cultures with LFs were allowed to form microvascular networks for 7 d in growth medium prior to washout and addition of serum- and cytokine-free medium for an additional 10 d. Scale bar = 100 µm.

**Figure S1.**
**Figure S2** Microvasculature suppresses growth of luminal, ER+ BCCs as well as high metastatic, triple-negative BCCs. (a) Representative images of YFP-MCF-7 growth within lung-like niches containing only stroma or stroma + ECs ('microvascular niches') after 10d. Scale bar = 500 µm. (b) Quantification of YFP-MCF-7 area fraction in each of these conditions (n=5 sets of co-cultures analyzed per condition). Error bars represent s.e.m. ***p<0.0001 by two-tailed t test. (c) Representative images of YFP-MDA-MB-231 BCCs after 10d on lung-like niches containing only stroma or stroma + ECs. Scale bar = 500 µm. (d) Quantification of YFP-MDA-MB-231 area fraction in each of these conditions (n=5 sets of co-cultures analyzed per condition). Error bars represent s.e.m., p=0.09 by two-tailed t test. (e) Representative images of lung-like stroma and microvascular niche cultures seeded with YFP-MDA-MB-231, fixed 10d later and stained for CD31 (red) and Ki67 (green). Note on right that Ki67-negative MDA-MB-231s are located on microvasculature. Scale bar = 50 µm.
Figure S3 Medium conditioned by microvascular niche cultures does not substitute for presence of microvasculature. (a) Representative image of YFP-T4-2 cells cultured on lung-like microvascular niche for 10d, fixed and stained for CD31 (red) and Ki67 (green; yellow mark towards center of image is debris). Scale bar = 200 μm. Inset shows corresponding field at time of seeding. Note that all tumor cell clusters (white arrow heads) appear to be derived from single tumor cells (white dotted circles, inset). The presence of a large, proliferative tumor cluster at the culture’s edge (white arrow) hinted that the angiocrine tumor suppressor(s) was not a freely diffusible molecule. Conditioned media (CM) from established LF cultures or lung microvascular niches (LF+EC) were added daily to cultures consisting of LFs seeded with YFP-T4-2 cells. (b) Representative images of tumor growth after 7 days of CM treatment. Scale bar = 500 μm. (c) Quantification of normalized tumor area cell fraction at day 7 (n=5 sets of co-cultures analyzed per condition). Error bars represent s.e.m. ‘NS’ denotes no significance by two-tailed unpaired t test.
Figure S4 Validation of a shRNA clone that significantly reduced endothelial cell Notch1 expression at the protein level. Representative immunoblots for Notch1 (intracellular domain (ICD) detected, top) and the nuclear membrane protein Lamin A/C (bottom). Values correspond to knockdown achieved with each shRNA clone after normalizing to Lamin A/C using band densitometry. Clone sh8393 was used for all experiments presented in Figure 5.
**Supplementary Figure 5:** Enriching naturally for neovascular tips promotes outgrowth of breast tumor cells. Lung-like microvascular niche cultures were fixed at (a) day 3 and (b) day 7 of network development and stained for CD31 (red) and Hoechst 33342 (blue) to label DNA. Neovascular tips are labeled with white dots. Scale bars = 200 µm. Microvascular niche cultures were seeded with T4-2 BCCs at (c) day 3 or (d) day 7 and assessed after 10 days. Scale bar = 200 µm. (e) Quantification of neovascular tips at day 3 of network development vs. day 7 of network development (n=15 fields of microvascular networks pooled from 5 separate co-cultures). Error bars denote s.e.m. *** p<0.0001 when compared by two-tailed t test. (f) T4-2 tumor cell area fraction 10 days after seeding microvascular networks at either day 3 or day 7 of development (normalized to day 7 values; n=5 sets of co-cultures analyzed per condition). Error bars represent s.e.m. *** p<0.0001 when compared by two-tailed t test. (g) Percentage of Ki67-negative T4-2 clusters 10 days after seeding microvascular networks that developed for 3 or 7 days (n=5 sets of co-cultures analyzed per condition). Error bars represent s.e.m. * p=0.016 when compared by two-tailed t test.
Figure S6  *In vivo* validation of POSTN and TGF-β1 expression around endothelial tip cells in physiologic and pathologic contexts. Schematics of (a) whole-mounted neonatal (postnatal day 5, P5) retina and (b) brain metastasis sections used to analyze expression of identified tip cell-derived tumor promoters in physiologic and pathologic contexts. POSTN is deposited by endothelial tip cells in (c) the developing retina and (d) within brain metastases (*white arrows*). POSTN is expressed sporadically on established phalanx endothelium within the retina (c, *inset*), and is absent from endothelium on the contralateral side of the brain (*normal tissue; d, inset*). Active TGF-β1 is expressed in the immediate vicinity of endothelial tip cells in (e) the developing retina and (f) within brain metastases (*white arrows*). Conversely, active TGF-β1 is absent around retinal phalanx endothelium (e, *inset*) and is expressed randomly around microvasculature on the contralateral side of the brain (f, *inset*). Scale bars = 20 μm.
Supplemental Video Legends

**Supplemental Video 1** Opposite regulation of tumor quiescence and growth by endothelial sub-niches: stable endothelium inhibits breast tumor cell growth. H2B-GFP T4-2 cell residing predominantly around stable endothelium does not undergo division over a 72h period. Intervals: 20min. Scale bar = 50 µm.

**Supplemental Video 2** Opposite regulation of tumor quiescence and growth by endothelial sub-niches: neovascular tips promote breast tumor cell growth. H2B-GFP T4-2 cells are observed dividing soon after encountering neovascular tips. Highlighted cell divides after encountering an endothelial ‘probe.’ At right of screen, another cell divides at −1d, 16:00 as it encounters an approaching neovascular tip. One of the daughter cells divides again as the couplet resumes interaction with the tip. At bottom, another cell ‘surfs’ past a neovascular tip and eventually divides. Intervals: 20min. Scale bar = 100 µm.