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VEGF-D Promotes Tumor Metastasis by Regulating Prostaglandins Produced by the Collecting Lymphatic Endothelium

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SUMMARY

Lymphatic metastasis is facilitated by lymphangiogenic growth factors VEGF-C and VEGF-D that are secreted by some primary tumors. We identified regulation of PGDH, the key enzyme in prostaglandin catabolism, in endothelial cells of collecting lymphatics, as a key molecular change during VEGF-D-driven tumor spread. The VEGF-D-dependent regulation of the prostaglandin pathway was supported by the finding that collecting lymphatic vessel dilation and subsequent metastasis were affected by nonsteroidal anti-inflammatory drugs (NSAIDs), known inhibitors of prostaglandin synthesis. Our data suggest a control point for cancer metastasis within the collecting lymphatic endothelium, which links VEGF-D/VEGFR-2/VEGFR-3 and the prostaglandin pathways. Collecting lymphatics therefore play an active and important role in metastasis and may provide a therapeutic target to restrict tumor spread.

INTRODUCTION

The lethality of cancer is primarily associated with metastasis, the spread of cancer cells from a primary site to distant organs (Liotta, 1992). The spread of tumor cells to lymph nodes (LNs) is an important prognostic indicator for disease staging, and thus the lymphatic vasculature is considered a route of metastatic spread (Achen and Stacker, 2008). However, it is noteworthy that systemic metastasis can occur independently of LN spread highlighting the complex nature of the process of systemic disease (Sleeman et al., 2011).

Some tumors secrete lymphangiogenic growth factors that act on the lymphatic vasculature to facilitate metastasis. These factors can induce lymphangiogenesis, that is, the formation of

Significance

The lymphangiogenic growth factor VEGF-D promotes cancer spread via the lymphatics, a crucial step in metastasis. Elevated VEGF-D levels in human tumors correlate with lymph node metastasis and poor patient prognosis, yet mechanisms underlying lymphogenous spread to lymph nodes remain elusive. Transcriptional profiling of collecting lymphatics draining primary tumors to sentinel lymph nodes identified a link between VEGF-D-signaling and prostaglandin pathways. VEGF-D modulates prostaglandin levels to regulate collecting lymphatic vessel dilation, an effect blocked by NSAIDs. This key interaction between lymphangiogenic factors and prostaglandins reveals a mechanism for preparing collecting vessels for tumor cell dissemination, and a mechanism by which NSAIDs reduce lymphogenous metastasis. Collecting lymphatic vessels may therefore constitute a therapeutic target for prevention and treatment of metastatic disease.

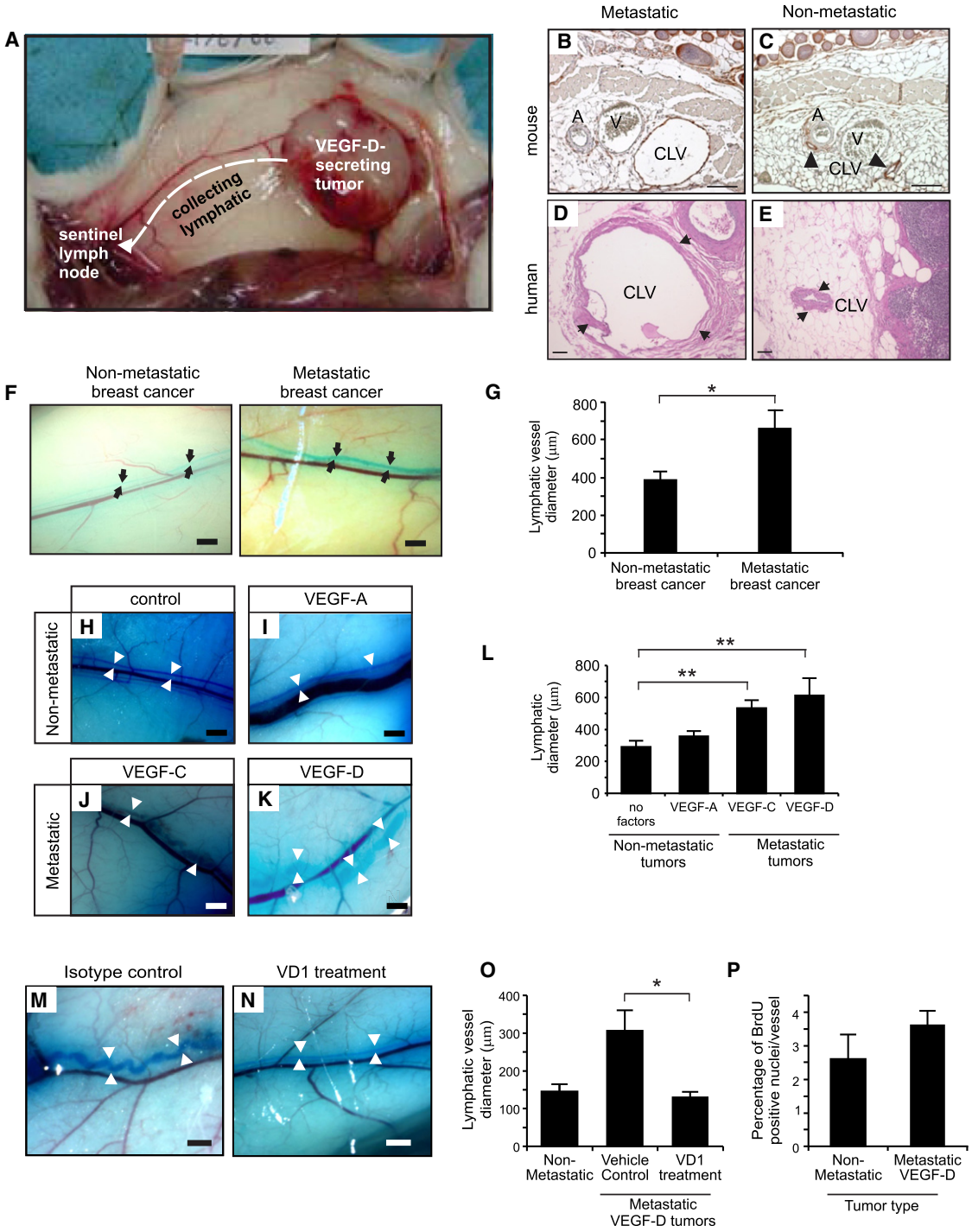


Figure 1. Lymphangiogenic Growth Factors Induce Collecting Lymphatic Vessel Dilation

(A) Representative image of the skin-flank 293EBNA tumor model, indicating primary tumor and the CLV (white dashed arrow) draining to the SLN.

(B and C) Representative images of flank skin containing the tumor-draining CLV stained for the lymphatic marker podoplanin, from mice bearing metastatic VEGF-D-293EBNA tumors (B) and nonmetastatic 293EBNA tumors (C). A, artery; CLV, collecting lymphatic vessel (arrowheads in C); V, vein. Scale bars: 200 μm.

(D and E) Hematoxylin and eosin (H&E) staining of LNs containing afferent CLVs. A dilated afferent CLV (arrowhead) can be seen in a patient with VEGF-D-positive metastatic breast carcinoma, (D) compared to a nonmetastatic control LN, which has a nondilated afferent collecting lymphatic (arrowhead) (E). CLV, collecting lymphatic vessel. Scale bars: 100 μm.

(F) Representative images of flank skin highlighting CLVs (black arrows) from mice bearing nonmetastatic and metastatic 66cl4 mammary adenocarcinoma. Scale bars: 1 mm.

(G) Quantitative analysis of CLV diameter in mice bearing nonmetastatic and metastatic 66cl4 mammary adenocarcinoma. Data are mean ± SEM; $n \geq 5$. *$p < 0.05$ by t test.

new lymphatics from preexisting vessels, in regions within or immediately adjacent to, a primary tumor (Tammela and Alitalo, 2010). They can affect vessels beyond the tumor environment, such as those within the sentinel lymph node (SLN) (Farnsworth et al., 2011) and can modulate immune responses to the tumor (Tammela and Alitalo, 2010). Despite its clinical implications, the mechanisms underpinning metastasis via the lymphatic network are not well understood.

Lymphaniogenic growth factors include two vascular endothelial growth factor (VEGF) family members, VEGF-C and VEGF-D, which act through the cell surface-localized receptor tyrosine kinases VEGFR-2 and VEGFR-3 (Joukov et al., 1996; Achen et al., 1998; Mäkinen et al., 2001). There is a strong association between elevated tumor expression of VEGF-C or VEGF-D, increased tumor lymphatic vessel density and enhanced rates of metastasis to LNs (Achen and Stacker, 2008).

The VEGF-C- or VEGF-D-signaling axes are pivotal in controlling lymphangiogenesis during cancer. Tumor models demonstrate that inhibiting this signaling may block lymphogenous cancer spread by restricting lymphatic vessel formation (Stacker et al., 2001; He et al., 2005; Hoshida et al., 2006). In addition, other VEGF-independent signaling pathways may operate in lymphatic endothelial cells (LECs) or associated cells such as mural cells or pericytes (Cao et al., 2004; Achen and Stacker, 2006).

The lymphatic network is a well-characterized hierarchy of vessels, beginning as initial lymphatics in the superficial dermis that drain into deep dermal precollecting lymphatic vessels, which, in turn, drain into subcutaneous collecting lymphatic vessels (CLVs) (Oliver and Alitalo, 2005). Individual lymphatic vessel subtypes perform distinct, specialized functions; the smaller initial lymphatics perform an absorptive role, whereas precollector vessels guide lymph down to CLVs, a conduit to the draining LN basin (Shayan et al., 2006). These features are reflected in their respective morphologies. Unlike the initial lymphatics, the CVLs have circumferential smooth muscle cells (SMCs) and regular intraluminal valves to help propel a unidirectional flow of fluid (Shayan et al., 2006).

Determining the step(s) in metastatic spread regulated by lymphangiogenic growth factors is critical for developing optimal therapeutic strategies to control metastasis. Recently, the focus of much research has been to understand the mechanisms underlying the effects of tumor-derived VEGF-C or VEGF-D on initial lymphatics within and around the primary tumor (Mandriota et al., 2001; Skobe et al., 2001; Stacker et al., 2001; Björndahl et al., 2005; Hoshida et al., 2006; Roberts et al., 2006; Kopfstein et al., 2007). However, the influence of these factors on lymphatic vessels distal to the primary tumor, for example, the CLVs draining the primary tumor to the SLN, remains elusive. Historically, CLVs were considered passive conduits that drain defined tissue areas to LNs (Sappy, 1874). However, recent observations in tumor models of VEGF-C-driven lymphogenous spread indicate that CLVs may play an active role in metastasis by increasing lymph flow through vessel dilation (He et al., 2005; Hoshida et al., 2006).

In this study, we investigated how CLVs are altered during VEGF-D-driven metastasis and how the CLVs are prepared to facilitate tumor spread.

RESULTS

Lymphangiogenic Growth Factors Induce Dilation of Collecting Lymphatic Vessels

We examined the mechanism(s) by which VEGF-D could regulate CLVs that drain primary metastatic tumors using VEGF-D-expressing tumor models (Figure 1A). Our xenograft model of lymphogenous spread is based on the nonmetastatic 293EBNA cell line, which has negligible baseline expression of VEGF family members (Figures S1A–S1D available online). Lymphogenous spread occurs when lymphangiogenic growth factors are overexpressed (Stacker et al., 2001), allowing us to examine any changes to collecting lymphatic vessels exposed to tumor-secreted VEGF family members. Immunohistochemical staining of skin sections containing the CLVs draining primary VEGF-D-293EBNA metastatic tumors revealed them to be dilated compared to the same vessels from the nonmetastatic control animals (Figures 1B and 1C). The correlation between VEGF-D and dilated collecting lymphatics could also be observed in clinical specimens. The afferent CLVs were dilated in a patient with VEGF-D-positive metastatic breast cancer compared to the matched patient sample with nonmetastatic breast cancer (Figures 1D and 1E; Figures S1E and S1F).

To examine the breadth of these findings, we assessed additional tumor cell lines for endogenous levels of VEGF family members, in particular, VEGF-D. Expression analysis revealed both breast cancer cell lines 66c14 and MDA-MB-435 express endogenous levels of VEGF-D compared to other cell lines such as the prostate tumor cell line PC3 and the poorly metastatic breast cancer cell line MDA-MB-231 (Figures S1G–S1K). Therefore, in addition to our VEGF-D-overexpressing 293EBNA model, the 66c14 and MDA-MB-435 tumor cell lines were employed for subsequent manipulations.

We interrogated CLVs in our orthotopic model of breast cancer involving 66cl4 mammary adenocarcinoma cells (Sloan et al., 2010). This model of metastasis is associated with an upregulation of *vegf-A* and *vegf-D* but not *vegf-C* within the

(H–K) Representative images of flank skin containing CLVs draining 293EBNA tumors, highlighting collecting lymphatics (white arrowheads) from mice with nonmetastatic control (H) or VEGF-A-overexpressing tumors (I), or metastatic tumors overexpressing VEGF-C (J) or VEGF-D (K) . Scale bars: 1 mm.

(L) Quantitative analysis of CLV diameter in mice bearing 293EBNA nonmetastatic or overexpressing VEGF-A tumors, or metastatic overexpressing VEGF-C or VEGF-D tumors. Data are mean ± SEM; $n \geq 10$. **$p < 0.01$ by t test.

(M and N) Representative images of flank skin highlighting collecting lymphatics (white arrowheads) from mice bearing metastatic VEGF-D-293EBNA tumors treated with an isotype control antibody (M) or VD1, a neutralizing antibody against VEGF- D (N). Scale bars: 1 mm.

(O) Quantitative analysis of CLV diameter in mice bearing nonmetastatic 293EBNA or metastatic VEGF-D-293EBNA tumors treated with an isotype control and VD1. Data are mean ± SEM; $n \geq 4$. *$p < 0.05$ by t test.

(P) Quantification of the relative percentage of BrdU-positive nuclei per CLV in mice bearing nonmetastatic 293EBNA or metastatic VEGF-D-293EBNA tumors. Data are the mean of six sections/mouse ± SEM; $n \geq 3$.

See also Figure S1.

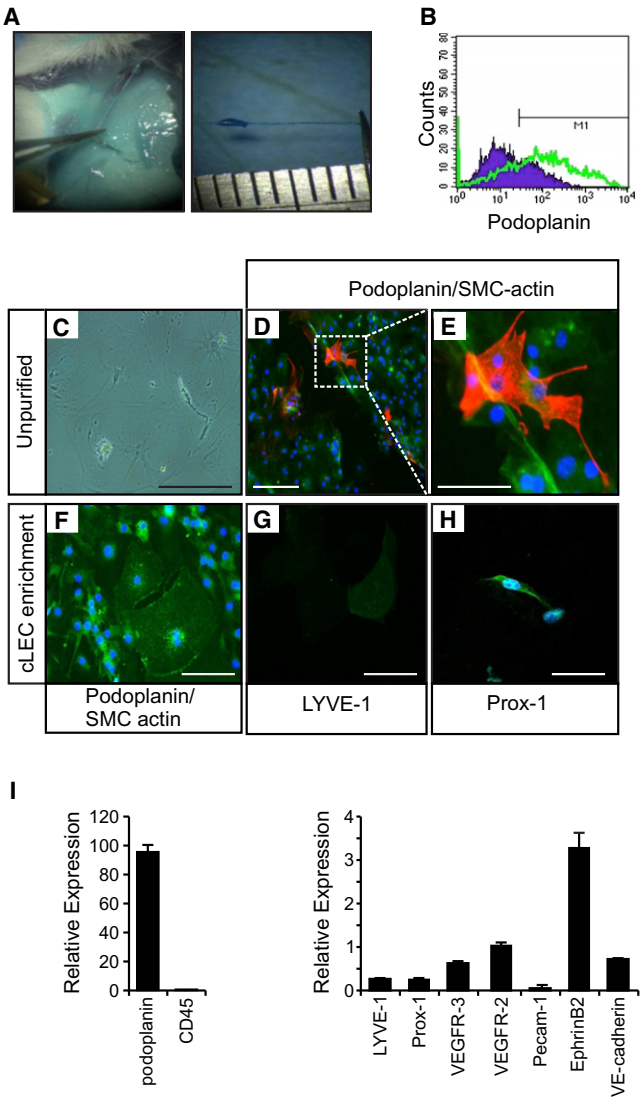


Figure 2. Purification and Characterization of Collecting Lymphatic Endothelial Cells

(A) Representative images of the microdissection procedure and a fully dissected CLV following identification with Patent Blue V. The ruler depicted indicates 1 mm graduation marks.

(B) Single-color flow cytometric analysis of unpurified single-cell suspension prepared from CLVs, stained with podoplanin (green) or an isotype-matched control (filled purple).

(C) Representative bright field image of unpurified cells following CLV harvest and culture. Scale bar: 10 μm.

(D and E) Immunofluorescence of single-cell suspension culture from CLVs stained for podoplanin (green) and SMC-actin (red). Scale bar: 50 μm. The boxed area in (D) is a magnified area from the original image to highlight the SMC-actin positive-staining cells and depicted as (E). Scale bar: 10 μm. Nuclei were counterstained with DAPI.

(F–H) Immunofluorescence of the enriched podoplanin-positive cLEC fraction from CLVs. Cells were stained with podoplanin (green) and SMC-actin (red) (F), LYVE-1 (green) (G) and Prox-1 (green) (H). Scale bars: 10 μm.

(I) Relative expression of lymphatic-specific genes following purification of cLECs from CLVs analyzed by qRT-PCR. Gene expression was normalized to β-actin. Data are mean ± SEM; $n \geq 5$.

primary tumor, increased tumor-associated lymphatic vessels, and subsequent LN and distant organ metastasis (Figures S1L–S1N). Tracking of collecting lymphatics in mice bearing 66c14 breast tumors revealed that CLVs were dilated in animals bearing metastatic tumors in which VEGF-D was upregulated, compared to nonmetastatic controls (Figures 1F and 1G).

To ascertain the specific contribution of VEGF-D to collecting lymphatic dilation, we utilized variants of the 293EBNA model that overexpress VEGF-A, VEGF-C, or VEGF-D. When compared to nonmetastatic controls, mice bearing metastatic VEGF-D-expressing tumors had enlarged CLV diameters (Figures 1H, 1K, and 1L) consistent with observations made during VEGF-C-driven tumor spread (Figures 1J and 1L; He et al., 2005). This dilation was reversed when mice bearing VEGF-D tumors were treated with VD1, a neutralizing VEGF-D antibody (Figures 1M–1O). Collecting lymphatic dilation was not observed in mice bearing VEGF-A-expressing tumors (Figures 1I and 1L), suggesting that VEGF-A has little effect on collecting lymphatic dilation. Previous studies showed that lymphatic vessels have the capacity to functionally adapt by dilating in response to VEGF-C, and this was attributed to hyperplasia of the endothelium (Jeltsch et al., 1997; He et al., 2005). In contrast, we found no significant increase in the number of BrdU-positive endothelial cells in dilated collecting lymphatics from mice bearing LN metastasis in VEGF-D-secreting tumors, compared with nonmetastatic controls (Figure 1P; $p = 0.327$). These data suggest that VEGF-D drives morphological changes in CLVs that correlate with metastasis, and are not due to endothelial cell proliferation.

Isolation and Characterization of Collecting Lymphatic Endothelial Cells during Cancer Spread

To define the molecular mechanisms driving the morphological changes in collecting lymphatics during metastasis, we developed a method to isolate CLVs draining primary tumors. Large subcutaneous CLVs were visualized using Patent Blue V and separated from blood vessels and surrounding skin tissue by microdissection (Figure 2A).

LYVE-1 is strongly expressed on smaller caliber lymphatic vessels, namely, the initial and precollector lymphatics, whereas it is weakly expressed on collecting vessels (Mäkinen et al., 2005). In contrast, podoplanin is expressed strongly on all lymphatic vessel subtypes (Mäkinen et al., 2005), and podoplanin-positive selection was therefore used to isolate collecting lymphatic endothelial cells (cLECs). Flow cytometry of cell suspensions prepared from microdissected CLVs prior to purification showed that approximately 75% of cells were positive for podoplanin (Figures 2B and 2C) and immunofluorescence revealed the presence of podoplanin-positive cells interspersed with smooth muscle actin (SMA)-positive cells, a marker for mural cells (Figures 2D and 2E). The presence of SMA is consistent with in vivo observations of the association of mural cells with CLVs (Mäkinen et al., 2005). To allow molecular characterization of cLECs, podoplanin-positive cells were isolated. Immunofluoresence of the cLEC-enriched fraction revealed cells that stained weakly for the lymphatic markers, LYVE-1 and Prox-1 (Kriehuber et al., 2001), and contaminating SMC were not detected (Figures 2F–2H). The cLEC-enriched fraction was not contaminated by immune cells, as indicated by negligible levels

of the pan-immune marker CD45 (Figure 2I). Quantitative real-time (qRT)-PCR revealed expression of other lymphatic markers, such as VEGFR-3 (Podgrabinska et al., 2002). In addition, VEGFR-2, recently shown to be expressed on dermal microvascular LECs (Podgrabinska et al., 2002), was expressed by cLECs (Figure 2I). Other endothelial and LEC markers such as Pecam-1, VE-cadherin, and EphrinB2 (Kriehuber et al., 2001; Mäkinen et al., 2005; Baluk et al., 2007) were also detected (Figure 2I). Collectively, these data confirm the endothelial nature of the purified cLEC population that can be used to investigate the molecular pathways in collecting lymphatics necessary for tumor spread.

Collecting Lymphatic Vessels Alter Their Gene Signature during VEGF-D-Driven Metastasis

To understand the molecular mechanisms underlying morphological changes in CLVs, we compared the molecular signature of cLECs harvested from CLVs from animals with VEGF-D-driven metastasis to that from animals with nonmetastatic disease (Figure 3A). Whole-genome profiling revealed reproducible gene expression patterns observed among replicates, and distinct and unique molecular signatures that differentiated between cLECs from animals with metastatic disease to those with nonmetastatic tumors (Figure 3B).

Several key genes that were differentially expressed between these groups could be arranged according to their cellular roles: cell surface receptors, secreted factors, transcription factors and cytoskeletal or extracellular matrix remodeling genes (Figure 3C). The cell surface genes were those important in adhesion and inflammation; many of the secreted factors were also modulators of inflammatory responses such as IL-11. Interestingly, the majority of the differentially expressed genes fell into the cytoskeletal or extracellular matrix modulatory group, which is consistent with observation of dilated collecting lymphatics. Within this gene group was *pgdh,* encoding 15-hydroxyprostaglandin dehydrogenase (PGDH), an enzyme whose key function is degradation of prostaglandins (PGs), which are small lipid-based molecules that can act as potent vasodilators. PGDH was of interest as it has been identified as a tumor suppressor in colorectal cancers (Myung et al., 2006).

The Tumor Suppressor, PGDH, Is Downregulated in Collecting Lymphatics during VEGF-D-Driven Metastasis

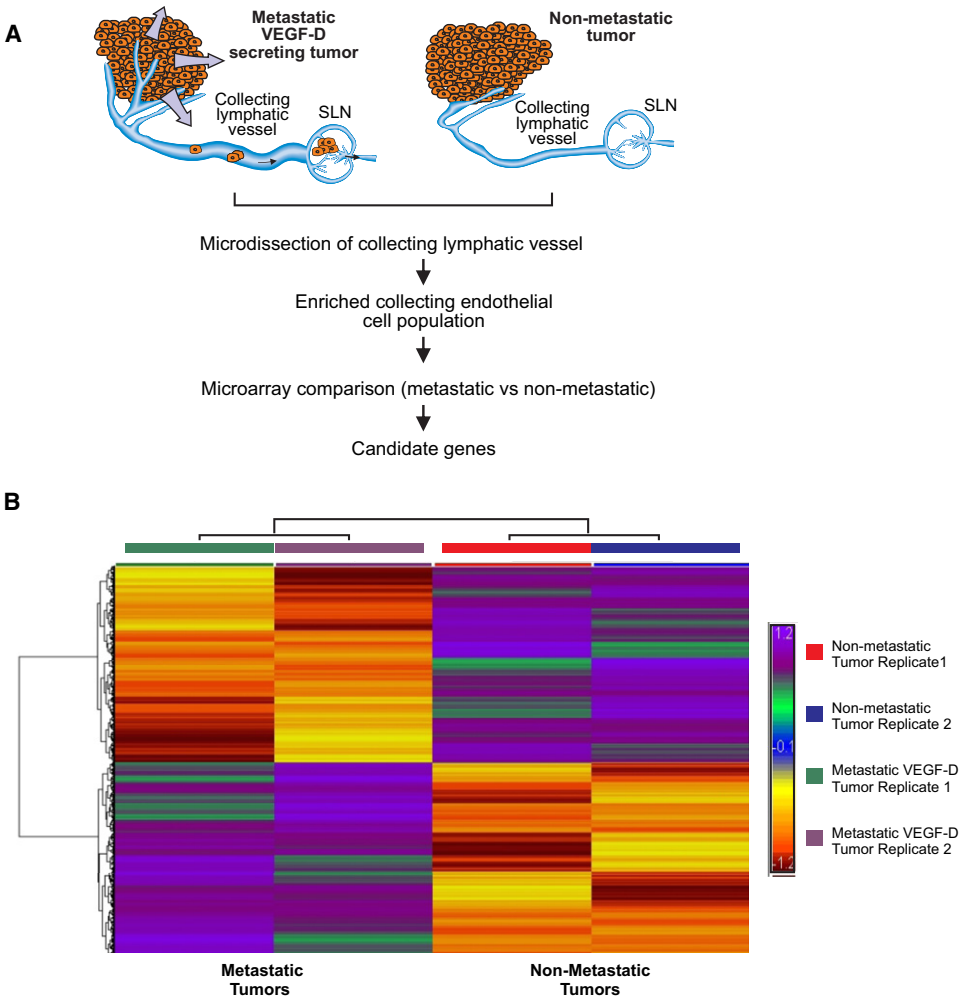
To validate differential *pgdh* expression levels, qRT-PCR was employed, revealing a 20-fold reduction in the level of *pgdh* gene expression in CLVs from metastatic VEGF-D-293EBNA tumors compared to those draining nonmetastatic tumors (Figure 4A), consistent with the whole-genome profiling. To test for the specificity of VEGF-D regulation of PGDH expression, we evaluated the influence of the related lymphangiogenic factor, VEGF-C, during metastasis. Collecting lymphatics from mice bearing metastatic VEGF-C-293EBNA tumors did not reveal a statistically significant change in *pgdh* expression (Figure 4A). PGDH is a component of the catabolic arm of the PG pathway whereas the COX proteins are key biosynthetic enzymes (Gupta and Dubois, 2001). Studies have shown a correlation between VEGF-C and COX-2 expression in metastatic cancers (Di et al., 2009; Liu et al., 2010). To determine if VEGF-D could also regulate the biosynthetic arm of the PG pathway, we assessed VEGF-D regulation of *cox-2* expression (Figure 4A). In contrast to the VEGF-C control, VEGF-D had no effect on *cox-2* levels in collecting lymphatics (Figure 4A). To confirm the specific effect of VEGF-D and to eliminate any possible effects of tumor-secreted VEGF family members or other tumor factors on *pgdh* expression levels, cLECs harvested from nontumor bearing mice were stimulated in vitro with VEGF-A, VEGF-C, or VEGF-D (Figure 4B). qRT-PCR revealed that *pgdh* expression was not altered by VEGF-A, in keeping with the lack of collecting lymphatic dilation observed in our nonmetastatic VEGF-A-overexpressing xenograft tumors (Figure 1L). Likewise, VEGF-C stimulation in vitro had little effect on *pgdh* expression in cLECs. Conversely, *pgdh* was significantly downregulated in cells stimulated with VEGF-D compared with unstimulated control cells (Figure 4B), suggesting that VEGF-D has a specific effect on *pgdh* expression in cLECs.

We previously showed that cLECs express both VEGFR-2 and VEGFR-3 (Figure 2I). To determine which of these receptors mediates the downregulation of *pgdh*, selective blockade of VEGFR-2 or VEGFR-3 was performed. VEGF-D stimulation of cLECs following pretreatment with neutralizing antibodies against VEGFR-2 or VEGFR-3 (Figures S2A and S2B) prevented VEGF-D-induced downregulation of *pgdh* (Figure 4C).

Immunoblot analysis of cellular lysates prepared from CLVs from mice bearing metastatic VEGF-D-293EBNA tumors (Figure 4D) demonstrated that PGDH protein levels were downregulated. To assess the distribution and expression pattern of PGDH with respect to lymphatic vessel subtypes, skin sections containing major longitudinal CLVs from non-tumor-bearing mice were stained with antibodies against PGDH. These showed cytosolic protein expression in nondilated CLVs, the identity of which was confirmed by positive staining with the lymphatic marker, podoplanin (Figures 4E and 4H). In animals with VEGF-D-driven metastatic disease, however, PGDH expression in the dilated CLVs was reduced (Figures 4G and 4J) compared to the CLVs in mice bearing nonmetastatic 293EBNA tumors (Figures 4F and 4I). In combination with the microarray data, these results suggest that VEGF-D mediates the downregulation of *pgdh* expression in dilated CLVs during VEGF-D-driven lymphogenous spread, and that this process requires the actions of both VEGFR-2 and VEGFR-3.

VEGF-D/VEGFR-2/VEGFR-3-Signaling Axes Modulate Prostaglandins Produced by Collecting Lymphatic Vessels during Metastasis

To maintain homeostasis of PGs in tissues, there is a balance between PG synthesis and degradation. We rationalized that the downregulation of the PG degrading enzyme, PGDH, may alter PG levels secreted by the collecting lymphatics, in particular PGE2, which is the main target for PGDH activity (Cha and DuBois, 2007). Since the lymphatic vasculature eventually drains into the circulatory system, we reasoned that local, continual secretion of PGs by endothelial cells may be detected in the plasma (Challis et al., 1976; Albuquerque et al., 2009). We tested the plasma of mice exposed to VEGF-D for the PGs previously shown to have vasodilatory effects (Olsson and Carlson, 1976; Whorton et al., 1978) and found that PG levels, specifically PGE2, in mice with VEGF-D-driven metastasis were elevated



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Biofunctions		Gene name	Genebank accession number	Log_2 fold change	P value
Cell surface markers	up	IL-1rl1	NM_008362	-3.6	0.013
		ICAM-1	NM_010493	-3.2	0.040
	down	Ramp2	NM_019511	3.6	0.038
Secreted factors	up	C1qtnf3	NM_030888	-4.2	0.010
		Sema3c	NM_013657	-3.3	0.022
		CxCl14	NM_019568	-3.2	0.022
		IL-11	NM_008350	-3.2	0.034
	down	Dkk2	NM_020265	5.3	0.010
Cytoskeletal and ECM modulation	up	Tnc	NM_011607	-3.8	0.038
		Serpina3c	NM_008458	-3.3	0.046
	down	Pgdh	NM_008278	4.2	0.014
		Col6a3	AF034136	11.6	0.019
		Lmo7	NM_201529	3.7	0.020
		Aspn	NM_025711	3.8	0.040
		Dsp	NM_023842	3.5	0.040
		Adam12	NM_007400	3.1	0.046
Transcription factors	down	HoxA9	NM_010456	4.1	0.013

Figure 3. Collecting Lymphatic Vessels from Metastatic VEGF-D Tumors Have Distinct Gene Signature

(A) Schematic representation of experimental paradigm used to identify gene expression signatures in CLVs during VEGF-D-driven metastatic disease. This involved microdissection of the CLVs draining primary tumors to the SLN, purification of cLECs and microarray analysis (tumor cells: orange; collecting lymphatic vessels and SLN: blue).

(B) Microarray analysis of cLECs isolated from mice bearing metastatic VEGF-D-293EBNA or nonmetastatic 293EBNA tumors from four independent experiments. Data were visualized using Partek Genomics Suite software, by displaying a hierarchical cluster with average linkage analysis of normalized gene

compared to those with nonmetastatic control or nonmetastatic VEGF-A-secreting tumors (Figures S3A and S3B). A similar trend regarding PGE2 was also observed in collecting lymphatic tissues harvested from the same animals (Figure S3C). Next, we assessed the ability of cLECs as an isolated cell type to produce PGs in vitro and to determine whether there is a causal relationship between VEGF-D signaling and modulation of PG production by cLECs. Upon VEGF-D stimulation of cLECs, there was an increase in PG levels (Figure 5A), further supporting the notion that VEGF-D modulates tissue-specific PG production by downregulating the PG degrading enzyme, *pgdh* (Figures 4A and 4B).

We endeavored to understand the VEGFR-2/VEGFR-3-signaling mechanism necessary for VEGF-D-dependent PG modulation by CLVs. Stimulation with VEGF-D led to phosphorylation of VEGFR-2 in cLECs, which could be blocked by pretreatment with neutralizing antibodies against either VEGFR-2 or VEGFR-3 (Figure 5B). Likewise, phosphorylation of VEGFR-3 was also blocked by both neutralizing antibodies (Figure 5B), which is consistent with our observation that either anti-VEGFR-2 or anti-VEGFR-3 antibody blocked the reduction in PGDH expression induced by VEGF-D (Figure 4C). Next, we assessed whether attenuation of VEGF-D signaling by blocking VEGFR-2/VEGFR-3 activation would affect PG production by cLECs. We found a significant reduction in PGs secreted by cLECs that had been pretreated with either neutralizing anti-VEGFR-2 or anti-VEGFR-3 antibodies (Figure 5C). Finally, we investigated the effects of attenuating VEGF-D signaling in vivo by blocking VEGFR-2 and VEGFR-3 activity, and hence PG production by cLECs, on dilation of CLVs draining tumors. CLVs in animals bearing metastatic VEGF-D-293EBNA tumors treated with either VEGFR-2 or VEGFR-3 neutralizing antibodies were not dilated (Figures 5D, 5E, and 5H) in contrast to dilated collectors found in the isotype-treated and nonmetastatic controls (Figures 5F–5H). Collectively, these data suggest that VEGF-D—via the activities of both VEGFR-2 and VEGFR-3—is able to regulate the levels of vasodilatory PGs produced by collecting lymphatic vessel endothelium.

Anti-inflammatory Drugs Reduce VEGF-D-Driven Metastasis by Reversing the Morphological Changes in Collecting Lymphatic Vessels

The discovery that the lymphangiogenic molecule VEGF-D modulates the morphology of collecting lymphatics via a tissue-specific regulation of PG activity led us to investigate whether pharmacologically manipulating this pathway would reverse the effect of VEGF-D on metastasis. NSAIDs are a class of commonly used analgesic and anti-inflammatory drugs and are prototypical inhibitors of COX enzymes. To address whether PGs contribute to "preparing" the collecting lymphatics that drain the primary tumor for spread to the SLN, we sought to shut down COX-2 and thereby ablate PG production. We reasoned that a VEGF-D-mediated increase in PG production by cLECs could be reduced concomitantly by inhibiting the biosynthetic arm of the PG pathway by treatment with a COX-2 inhibitor, Etodolac (Glaser et al., 1995). After testing the efficacy of this drug in vitro (Figure S4A), we monitored its effects on metastasis in the VEGF-D-293EBNA model. It has been shown that NSAID treatment can reduce the levels of VEGFs in some tumor models (Iwata et al., 2007), yet we found no difference in plasma VEGF-D levels during NSAID treatment (Figure S4B).

In addition, we employed the metastatic MDA-MB-435 breast cancer model, which was shown to express endogenous VEGF-D (Figures S1H–S1K). Primary tumor growth was not significantly affected by NSAIDs in either VEGF-D-293EBNA or MDA-MB-435 models (Figure 6A), in contrast to previous studies (Greenhough et al., 2009).

The COX biosynthetic pathway has been shown to promote metastasis by stimulating tumor-associated angiogenesis and lymphangiogenesis (Iwata et al., 2007; Williams et al., 2000; Tsujii et al., 1998). We found no significant difference in the density of tumor-associated lymphatic or blood vessels between the NSAID-treated and vehicle control groups in either VEGF-D-293EBNA or MDA-MB-435 metastatic models (Figures 6B–6G; Figures S4C–S4H). When we assessed dilation of collecting lymphatics that drain the primary tumors in these models, we found a compelling reduction in vessel diameter in NSAID-treated mice in both tumor models (Figures 6I, 6L, and 6M) compared with the nonmetastatic and vehicle control groups (Figures 6H, 6J, 6K, and 6M). Cox-2-derived PGE2 promotes tumor progression and metastasis and is significantly increased in malignant tissue (Jaffe et al., 1971, Rigas et al., 1993). The chemoprotective effects of NSAIDs are mediated by reducing PGE2 levels (Hansen-Petrik et al., 2002). Treatment with Etodolac significantly reduced PGE2 levels in both the VEGF-D-293EBNA and the MDA-MB-435 breast cancer model, suggesting that the dilation observed during metastasis may, in part, be attributed to PGE2 (Figure 6N). PGs exert their biological actions, such as vasodilation, by engaging specific receptors (Amano et al., 2003). Protein expression of PGE receptors on CLVs was found at low levels (Figure S4I), with a differential expression pattern during metastasis and upregulation of EP3, the receptor commonly engaged by PGE2 (Figure S4J).

To ascertain whether NSAID-mediated reversal of CLV dilation affects metastasis, we examined the SLN (Kerjaschki et al., 2011). In both the VEGF-D-EBNA293 and MDA-MB-435 models, we found a reduction of tumor cells in LNs from NSAID-treated animals compared to the vehicle control (Figures 6O–6U). Further, in the MDA-MB-435 model which displays systemic metastasis to the lung, micrometastatic deposits in both the pleural and subpleural area of lung sections could not be detected in NSAID treated animals compared to those readily observed in the vehicle control animals (Figures 6V and 6W; Figure S4K). NSAIDs have been shown to have antiproliferative, proapoptotic, and antiangiogenesis effects (Cha and DuBois, 2007), yet we found no statistically significant differences in the proliferation/apoptosis index or blood vessel density in LNs

expression (>1.5-fold change, adjusted p values < 0.05). These data illustrate differentially upregulated (red/orange) or downregulated genes (purple/blue) in cLECs during metastatic disease compared to those from nonmetastatic tumor bearing mice. The gene expression patterns are consistent among replicates. (C) Selected genes whose expression was up- or downregulated in cLECs during VEGF-D-driven metastatic disease in the 293EBNA model. Statistical significance corresponds to $p < 0.05$ by HOLMS test.

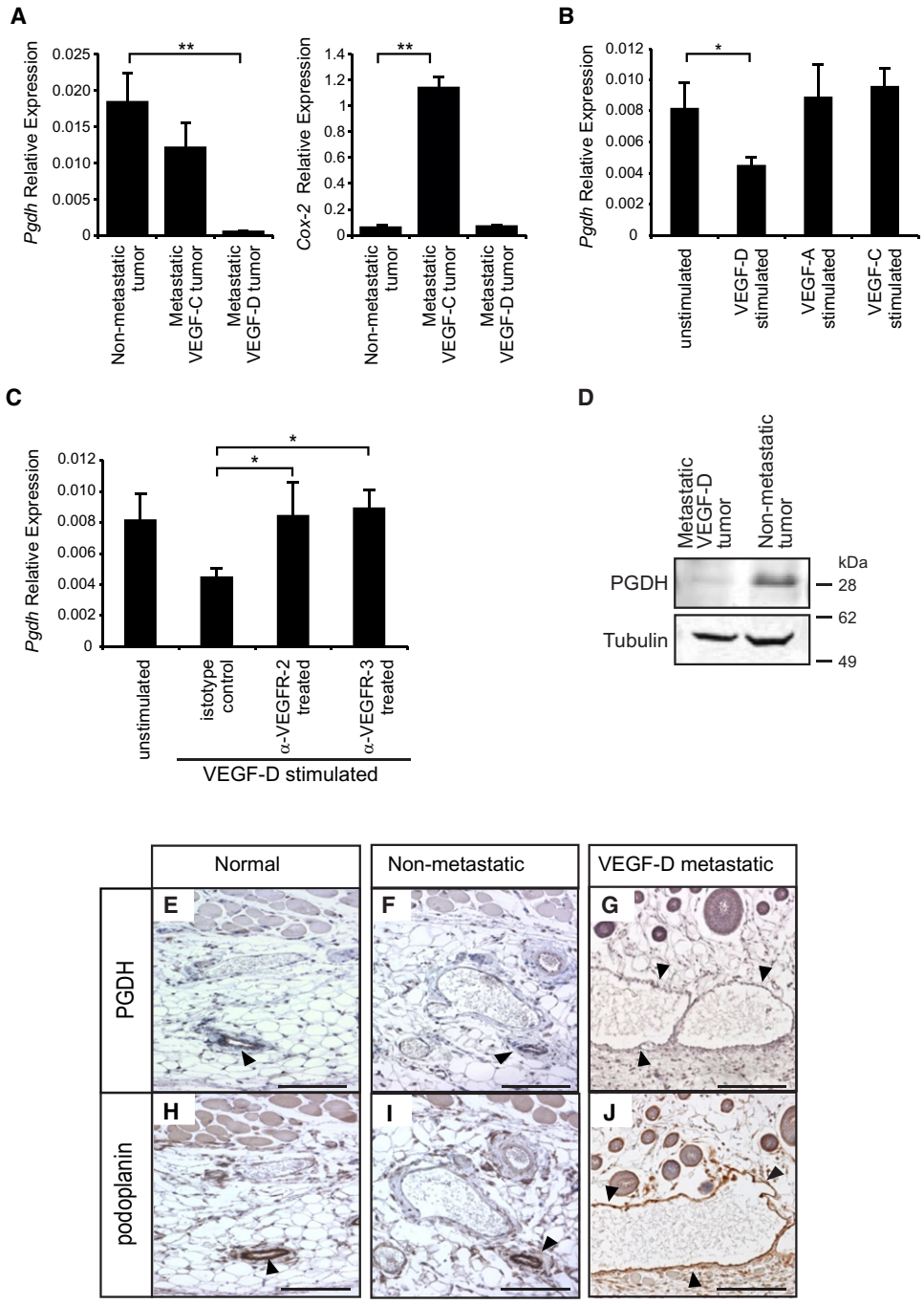

Figure 4. VEGF-D Downregulates PGDH Expression in Collecting Lymphatic Vessels during Metastasis

(A) Real-time qPCR analysis of *Pgdh* and *Cox-2* expression in cLECs harvested from mice bearing nonmetastatic 293EBNA or metastatic VEGF-C- or VEGF-D-293EBNA tumors. Gene expression was normalized to β-actin. Data are mean ± SEM; $n \geq 5$. **$p < 0.01$ by t test.

(B) Real-time qPCR analysis of *Pgdh* expression in cLECs stimulated for 24 hr with VEGF-A (10 ng/ml), VEGF-C (100 ng/ml), and VEGF-D (100 ng/ml). Gene expression was normalized to β-actin. Data are mean ± SEM; *$p < 0.05$ by t test.

(C) Real-time qPCR analysis of *Pgdh* expression in cLECs pretreated with neutralizing VEGFR-2 (DC101) or VEGFR-3 (mF4-31C1) antibodies before stimulation with VEGF-D (100 ng/ml) for 24 hr. Data are mean ± SEM; *$p < 0.05$ by t test.

(D) Immunoblots of CLV lysates harvested from pools of mice bearing VEGF-D metastatic and nonmetastatic 293EBNA tumors, probed for PGDH. $n \geq 5$.

(E–J) Immunohistochemical staining of serial sections of flank skin containing collecting lymphatics (arrowheads) from non-tumor-bearing mice (E and H), or mice bearing nonmetastatic 293EBNA (F and I), or metastatic VEGF-D-293EBNA tumors (G and J), stained for the cytosolic protein PGDH, or the lymphatic marker podoplanin. Representative images are shown. Scale bars: 200 μm.

See also Figure S2.

from either NSAID or vehicle-treated groups, suggesting that the reduction of tumor cells in LNs of NSAID-treated animals was not due to changes in proliferation, apoptosis, or angiogenesis within the LN (Figures S4L and S4M) or primary tumor (data not shown). Collectively, these data suggest that NSAIDs can affect VEGF-D-regulated PG production by the collecting lymphatic endothelium. This in turn may provide an antimetastatic effect by preventing critical morphological alterations to CLVs that are necessary to facilitate tumor cell spread to the SLN (Figure 7).

DISCUSSION

Entry of tumor cells into the lymphatic system and subsequent dissemination to LN and distant organ sites is an important event in the metastasis of many solid tumors. In contrast to our knowledge of the influence of lymphangiogenic factors on initial lymphatics within and surrounding the primary tumor (Koukourakis et al., 2000; Pepper, 2001; Stacker et al., 2004; Sleeman and Thiele, 2009), the effect of these factors on lymphatics beyond the tumor environment is only beginning to emerge.

Isolation of endothelial cells from normal and tumor samples has identified genes that are important during tumor-associated angiogenesis and lymphangiogenesis (St Croix et al., 2000; Clasper et al., 2008). We have isolated LECs from vessels beyond the tumor microenvironment and have investigated the molecular regulation that occurs in collecting lymphatics during the metastatic process. Transcriptional profiling of cLECs from CLVs draining metastatic VEGF-D-secreting tumors identified gene signatures that are implicated in tissue remodeling and inflammation. These studies found that VEGF-D regulates the structure of the tumor-draining CLVs through a PG-dependent mechanism, consistent with the elevated levels of inflammatory mediators, such as PGs (Mantovani et al., 2008). In contrast, tumor-associated initial lymphatics differentially expressed genes encoding components of endothelial junctions, subendothelial matrix, and vessel growth/patterning (Clasper et al., 2008). Such variation between gene signatures may reflect the different responses that lymphatic vessel subtypes exhibit to lymphangiogenic growth factors and the roles they perform during the course of lymphogenous spread.

As an initial step toward evaluating mechanisms of metastasis and potential therapeutic targets by which to restrict metastatic disease, we focused on *pgdh*, which catalyzes the rate-limiting step in the catabolism of PGs (Pichaud et al., 1997) and has been identified as a tumor suppressor (Myung et al., 2006). Endothelial-derived PGs are potent regulators of vasodilation, attenuating or amplifying the response of blood vessels to modulate vascular tone during normal and pathological states (Messina et al., 1974; Gupta and Dubois, 2001). We extend this finding to the CLVs, with ''tissue-specific'' regulation of PGs by these vessels when exposed to VEGF-D. The importance of PGDH during blood vessel dilation was initially demonstrated in PGDH null mice, which have increased tissue PGE2 levels, and as a consequence, a patent blood vessel shunt between the lungs and heart, causing them to die soon after birth (Coggins et al., 2002). It will be interesting to assess the lymphatic vasculature in PGDH heterozygous mice during normal and pathological states.

PGs are produced in tissues by COX enzymes and levels are balanced by the degrading activities of PGDH (Gupta and Dubois, 2001). Clinically, high COX-2 expression in some tumors is associated with poor patient prognosis and survival (Ristimäki et al., 1997). The protumorigenic effects of COX-2 are believed to be largely attributed to its role in synthesizing PGE2 (Pugh and Thomas, 1994). Similarly, reduced PGDH expression and a consequential rise in PGE2 levels enhance tumor growth in colon, gastrointestinal, and breast cancers (Backlund et al., 2005a; Backlund et al., 2005b; Wolf et al., 2006). Our data extend this knowledge by demonstrating that reduced PGDH expression and elevated levels of PGE2 are important in lymphatic endothelial cells beyond the tumor microenvironment, possibly ''preparing'' the CLVs to promote tumor spread.

It is not apparent at this stage whether lymphatic endothelium-derived PGs act in an autocrine or paracrine manner to modulate the overall tone of the CLVs during metastasis. The actions of prostaglandins such as PGE2 are mediated by engagement with their cognate receptors, EP1-4 (Greenhough et al., 2009), with EP3 signaling shown to contribute to tumor lymphangiogenesis (Kubo et al., 2010). In collecting lymphatics, cLEC-derived PGs may activate SMCs associated with the vessel walls; PG ligation of receptors on SMCs may induce vessel wall relaxation to accommodate expansions in vessel size (Tang and Vanhoutte, 2008). This effect is akin to that of nitric oxide- and PG-dependent modulation of blood vessel tone (Fukumura et al., 2006).

Lymphangiogenic growth factor modulation of PGs in CLVs is mediated by both VEGFR-2 and VEGFR-3 signaling, possibly via heterodimers. Recently published data showed that VEGF-D stimulation of endothelial cells promotes the formation of VEGFR-2/VEGFR-3 heterodimers (Nilsson et al., 2010). Consistent with these findings, anti-VEGFR-2 or anti-VEGFR-3 therapeutic agents were shown to block CLV dilation, which correlated with decreased lymph flow rates and consequently, a reduction in the number of tumor cells reaching the SLN (He et al., 2005; Hoshida et al., 2006). Based on data presented in this study, it is feasible that the mechanism of metastatic suppression by VEGFR-2 and VEGFR-3 blockade is, in part, due to attenuation of PG levels in the collecting endothelium. Since lymphatics are a conduit for immune cells, it cannot be excluded that VEGF-D-induced dilation of collecting lymphatics may contribute to metastasis by altering the traffic and behavior of immune cells in the SLN, consequently modulating metastatic burden.

NSAIDs are commonly used for the treatment of inflammatory disease and can restrict the development of colon cancer (Thun and Heath, 1995; Mantovani et al., 2008; Hirsch et al., 2010) and tumor spread in breast and prostate cancer patients (Holmes et al., 2010; Leitzmann et al., 2002), yet the precise antimetastatic mechanism is unclear. Emerging evidence suggests that COX-2 overexpression and high PGE2 levels are associated with tumor angiogenesis and lymphangiogenesis, processes crucial for metastasis (Iwata et al., 2007; Amano et al., 2003; Tsujii et al., 1998). Recently, the effect of NSAIDs on the lymphatic vasculature has been assessed within tumors, where it was shown that treatment with a COX-2 inhibitor reduced tumoral lymphangiogenesis, in turn, leading to decreased metastasis to the SLN (Iwata et al., 2007). Evidence

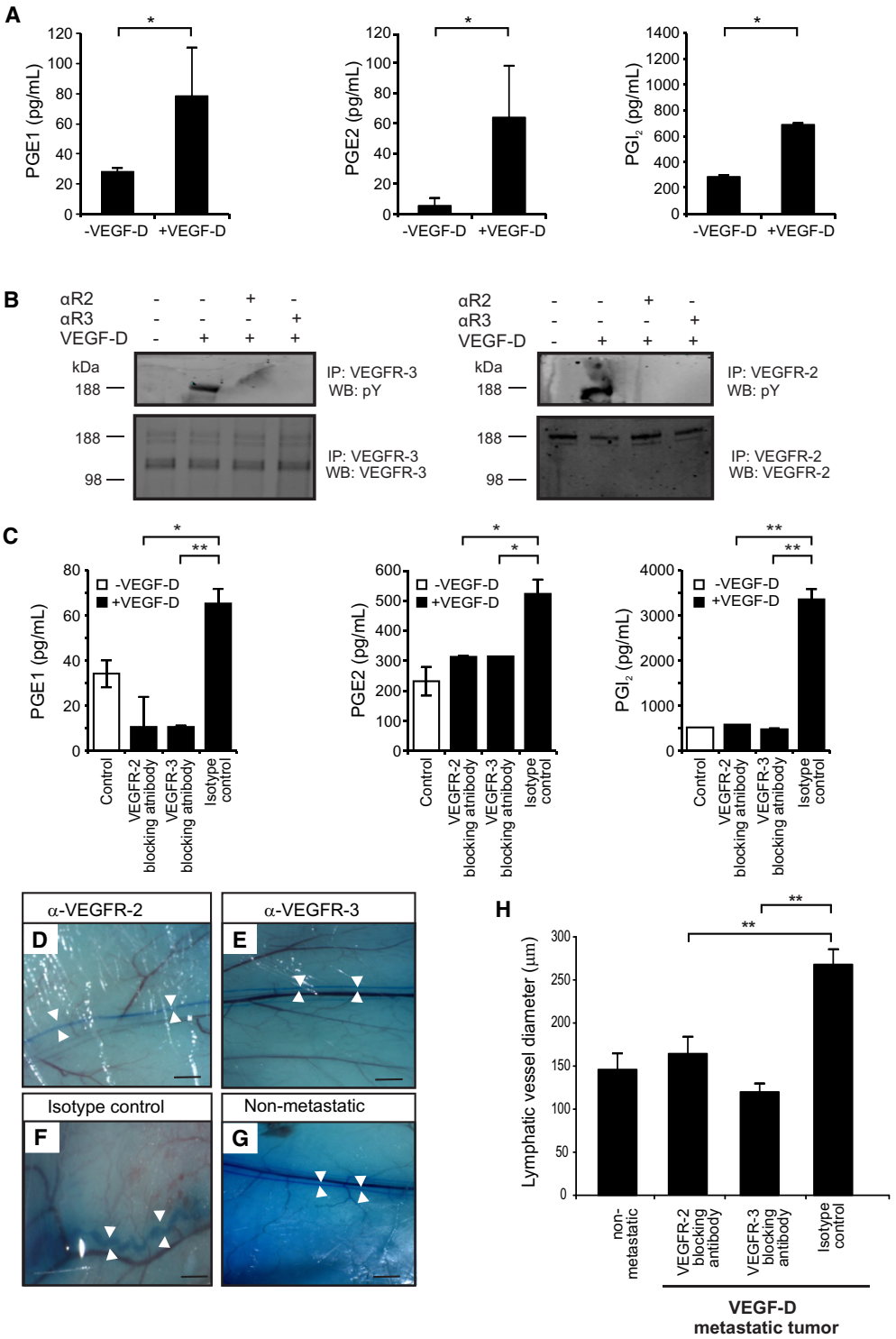

Figure 5. VEGF-D/VEGFR-2/VEGFR-3 Signaling Regulates Prostaglandins Produced by cLECs during Metastasis

(A) PG levels in supernatants of cLECs stimulated with VEGF-D (100 ng/ml) for up to 7 days. Assays were performed in triplicate. Data are mean ± SEM; *$p < 0.05$ by t test.

(B) VEGF-D-mediated activation of VEGFR-2 and VEGFR-3 in cLECs. cLECs were pretreated for 1 hr with neutralizing VEGFR-2 (DC101) or VEGFR-3 (mF4-31C1) antibodies before stimulation with VEGF-D (100 ng/ml) for 10 min. VEGFR-2 and VEGFR-3 were immunoprecipitated from cLEC lysates and immunoblots were probed for phosphorylation of VEGFR-2 and VEGFR-3.

(C) PG levels in supernatants of cLECs were measured by ELISA. cLECs were pretreated for 1 hr with neutralizing VEGFR-2 (DC101) or VEGFR-3 (mF4-31C1) antibodies before stimulation with VEGF-D (100 ng/ml) for up to 7 days. Assays were performed in triplicate. Data are mean ± SEM; *$p < 0.05$ and **$p < 0.01$ by t test.

presented in the current study indicates an additional mechanism for the antimetastatic effects of NSAID treatment beyond the tumor environment, one that involves normalizing the diameter of CLVs that facilitate tumor cell trafficking to the SLN (Figure 7B).

An emerging "subtypes-based" model for lymphogenous spread suggests that lymphangiogenic growth factors have two modalities. The first consists of proliferation or alteration of tumor-associated initial lymphatics that enables tumor cells to access to the lymphatic network (Figure 7A); the second involves dilation of the CLVs beyond the tumor, which facilitates trafficking to the SLN (Figure 7B). Understanding the functionally important effects that VEGF-C and VEGF-D have on lymphatic vessel subtypes may provide one of the missing links in the metastatic process, and further refine our knowledge of the complex nature of lymphogenous spread. These insights may assist with the design of additional therapeutic avenues for cancer patients and/or enhance current approaches to antilymphangiogenic therapies, such as blocking or neutralizing antibodies in combination with other treatments such as NSAIDs.

EXPERIMENTAL PROCEDURES

Mice

Female SCID/NOD (IMVS, Adelaide, Australia) or Balb/c (ARC, Perth) mice 6–8 weeks of age were used for tumor studies and/or isolation of cLECs. Ethics for approval for research using animals was obtained from the Ludwig Institute for Cancer Research, Peter MacCallum Cancer Centre and Monash University Animal Ethics Committees, in accordance with National Health and Medical Research Council of Australia guidelines.

Human Tissue Specimens

Tissues were collected following surgical resection at the Peter MacCallum Cancer Center. Ethics approval for research using human tissue was obtained from the Peter MacCallum Cancer Centre (approval number 10/16) and includes a waiver for consent. A tissue microarray from basal-like breast carcinoma was constructed from a series of breast tumors screened for ER, PR, HER2, EGFR, and cytokeratin 5 (ck5). Those tumors that were triple negative (ER, PR, and her2) and ck5-positive and EGFR-positive were considered basal-type. 1 mm cores of tissue were punched from donor blocks. Tissue microarrays were immunostained with anti-human VEGF-D antibody (R&D Systems).

Metastatic and Nonmetastatic Xenograft Models

Stably transfected 293EBNA-1 cell lines expressing full-length human VEGF-D (VEGF-D-293EBNA), human VEGF-C (VEGF-C-293EBNA) and mouse VEGF-A (VEGF-A-293EBNA), vector alone (293EBNA), or MDA-MB-453-expressing endogenous VEGF-D were established in SCID/NOD mice as described (Stacker et al., 2001).

Orthotopic Metastasis Model

Six-week-old female Balb/c mice (ARC, Perth) were housed under PC2 barrier conditions. 66cl4 mammary adenocarcinoma cells were transduced with the FUhlucW lentiviral vector containing firefly luciferase under control of the ubiquitin-C promoter and were inoculated as previously described (Sloan et al., 2010). See Supplemental Experimental Procedures for full methods.

Treatment of Tumors with Neutralizing Antibodies and NSAIDs

Mice bearing VEGF-D-expressing tumors received thrice weekly, beginning 5 days post-tumor inoculation, intraperitoneal injections of 800 μg of neutralizing anti-VEGFR-2 antibody (DC101; ImClone) or VD1 (Achen et al., 2000); 1 mg of neutralizing anti-VEGFR-3 antibody (mF4-31C1; ImClone); or isotype matched antibody/PBS as vehicle control. For NSAID treatment, mice were treated daily by oral gavage with 5 mg/kg of Etodolac (Sigma-Aldrich) (Iwata et al., 2007) dissolved in 5% (w/v) carboxymethylcellulose, beginning 5 days post-tumor inoculation and continued daily until tumors reached a size of 1,500–2,000 mm^3 (typically 3–4 weeks).

Collection of tissue and isolation of cLECs used for various analyses including immunohistochemistry, flow cytometry, BrdU analysis, prostaglandin assays, RNA purification, microarray analysis, and qRT-PCR are described in detail in the Supplemental Experimental Procedures.

Harvest of Collecting Lymphatic Vessels

0.25% (w/v) Patent Blue V dye (Sigma-Aldrich) was injected postmortem into hindlimb footpads of normal SCID/NOD mice, or intratumorally in mice bearing tumors, and massaged to promote uptake by the collecting lymphatics. Collecting lymphatics thus identified were microdissected using a Zeiss OPMI MDI dissecting microscope.

Isolation of Endothelial Cells from Collecting Lymphatic Vessels

Single-cell suspensions of cLECs were prepared by digestion of freshly dissected CLVs with a cocktail of Blendzyme III (Roche) and DNase (Sigma-Aldrich) for 1.5–2 hr at 37°C. Cells were either seeded on fibronectin (5 μg/ml; Sigma-Aldrich)-coated dishes for in vitro expansion, or subjected to immunoisolation by MACS (Miltenyi Biotec) using rabbit anti-mouse podoplanin antibody (30 μg/ml, Sigma-Aldrich). See Supplemental Experimental Procedures for full method.

Flow Cytometry and Immunofluorescence

See Supplemental Experimental Procedures for full method.

Immunoprecipitation and Western Blotting

See Supplemental Experimental Procedures for full methods.

Immunohistochemistry and BrdU Incorporation

Mouse tissues were fixed in 4% paraformaldehyde before paraffin embedding for analysis. See Supplemental Experimental Procedures for full methods.

RNA Isolation and Microarray Analysis

RNA was isolated using the RNeasy kit as per manufacturer's instructions (QIAGEN). See Supplemental Experimental Procedures for full methods.

Quantitative PCR

See Supplemental Experimental Procedures for a list of primers.

Quantification and Statistical Analysis

Images were quantified using Metamorph software. Student's t test (Minitab for Windows, MiniTab Inc) and HOLMS test were used for statistical analyses where indicated.

ACCESSION NUMBERS

Microarray data were deposited in NCBI's Gene Expression Omnibus; series accession number GSE34135. (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34135).

(D–G) Representative images of CLVs filled with Patent Blue V from mice bearing metastatic VEGF-D-293EBNA tumors treated with neutralizing VEGFR-2 (DC101) (D), VEGFR-3 (mF4-31C1) (E), or isotype control antibody (F) and nonmetastatic tumor control (G). Scale bars: 1 mm.
(H) Quantification of CLV diameter in mice bearing nonmetastatic and metastatic VEGF-D-293EBNA tumors treated with neutralizing VEGFR-2 (DC101), VEGFR-3 (mF4-31C1), or isotype control antibodies. Data are mean ± SEM; $n \geq 4$. $^{**}p < 0.01$ by t test.
See also Figure S3.

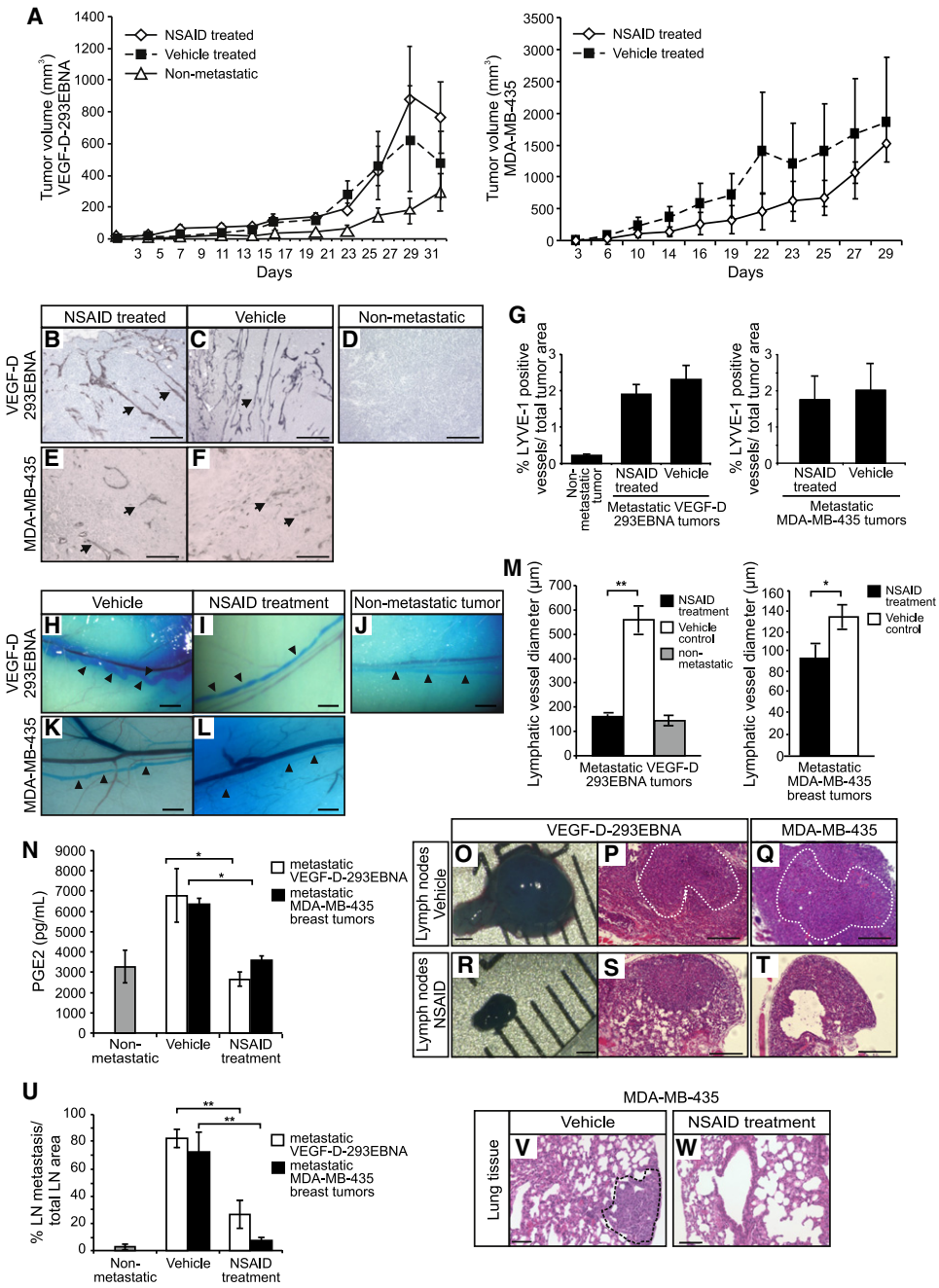

Figure 6. NSAIDs Reverse Collecting Lymphatic Vessel Dilation during Metastatic Disease and Suppress Tumor Spread

(A) Measurement of subcutaneous VEGF-D-293EBNA and MDA-MB-435 tumors in mice undergoing treatment with NSAID, (Etodolac). Data are presented as mean tumor volume (mm^3) ± SEM. Tumor volumes are shown as a function of time (in days); n ≥ 6.

(B–F) Immunohistochemical staining for the lymphatic marker LYVE-1 from mice bearing metastatic VEGF-D-293EBNA (B and C) and MDA-MB-435 (E and F) tumors treated with NSAID (B and E), vehicle (C and F), or nonmetastatic 293EBNA tumor control (D). Arrows indicate vessels. Representative images are depicted. Scale bars: 200 μm.

(G) Tumoral lymphatic vessel density (LYVE-1) in VEGF-D-293EBNA and MDA-MB-435 breast tumors from mice treated with either NSAID or the vehicle control. Data are the mean of three sections/mouse ± SEM; n ≥ 6.

(H–L) Macroscopic appearance of CLVs filled with Patent Blue V from mice bearing metastatic VEGF-D-293EBNA tumors (H and I) or MDA-MB-435 breast tumors (K and L) treated with either vehicle (H and K) or NSAID (I and L), compared to the nonmetastatic 293EBNA tumor control (J). Scale bars: 1 mm.

(M) Diameter of CLVs in mice bearing metastatic VEGF-D-293EBNA or MDA-MB-435 breast tumors treated with either NSAID or vehicle. Data are mean ± SEM; n ≥ 6. *p < 0.05 and **p < 0.01 by t test.

(N) PGE2 levels in plasma from mice bearing metastatic VEGF-D-293EBNA or MDA-MB-435 breast tumors treated with either NSAID or vehicle. Assay was performed in triplicate. Data are mean ± SEM; n ≥ 5. *p < 0.05 by t test.

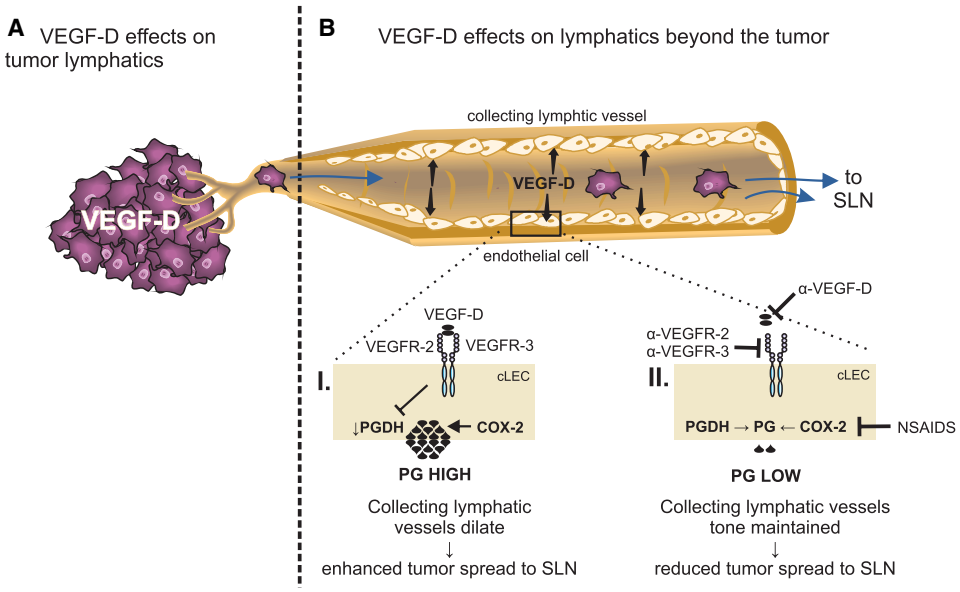

Figure 7. Schematic Overview of VEGF-D Effects in Tumor Metastasis

VEGF-D secreted by tumor cells promotes LN metastasis by (A) facilitating entry of tumor cells into initial lymphatics and (B) altering CLV dilation to facilitate transit of tumor cells to the SLN. This is modulated by downregulation of PGDH, resulting in high levels of PGs secreted by cLECs as depicted in (I). Inhibitors targeting the VEGF-D/VEGFR-2/VEGFR-3 or PG pathways in cLECs are likely to reduce PG levels and thereby restore vessel tone, suppressing metastasis as shown in (II).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.12.026.

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(O–T) Macroscopic appearance of LN from VEGF-D-293EBNA tumor bearing mice treated with vehicle (O) or NSAID (R) Scale bars: 1 mm. H&E staining (P, Q, S, and T) of LN sections from mice bearing VEGF-D-293EBNA (P and S) or MDA-MBA-435 tumors (Q and T), treated with either NSAID (S and T) or vehicle (P and Q). Dotted lines indicate micrometastatic foci within the LN. Scale bars: 200 μm.

(U) Quantification of metastatic burden in LNs from mice bearing metastatic VEGF-D-293EBNA or MDA-MBA-435 tumors and treated with the NSAID or vehicle, or the nonmetastatic 293EBNA tumor control. Data are mean ± SEM; $n \geq 6$. $^{**}p < 0.01$ by t test.

(V and W) H&E staining of lung tissue from mice bearing metastatic MDA-MB-435 breast tumors from animals treated with either vehicle (V) or NSAID (W). Dotted lines indicate micrometastatic foci. Scale bars: 100 μm.

See also Figure S4.

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