A ROLE FOR BONE MORPHOGENETIC PROTEIN-4 IN
LYMPH NODE VASCULAR REMODELING
AND PRIMARY TUMOR GROWTH

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Abstract

Lymph node metastasis, an early and prognostically important event in the progression of many human cancers, is associated with expression of vascular endothelial growth factor-D (VEGF-D). Changes to lymph node vasculature that occur during malignant progression may create a metastatic niche capable of attracting and supporting tumor cells. In this study, we sought to characterize molecules expressed in lymph node endothelium that could represent therapeutic or prognostic targets. Differential mRNA expression profiling of endothelial cells from lymph nodes that drained metastatic or non-metastatic primary tumors revealed genes associated with tumor progression, in particular bone morphogenetic protein-4 (BMP-4). Metastasis driven by VEGF-D was associated with reduced BMP-4 expression in high endothelial venules, where BMP-4 loss could remodel the typical high-walled phenotype to thin-walled vessels. VEGF-D expression was sufficient to suppress proliferation of the more typical BMP-4-expressing high endothelial venules in favor of remodeled vessels, and mechanistic studies indicated that VEGFR-2 contributed to high endothelial venule proliferation and remodeling. BMP-4 could regulate high endothelial venule phenotype and cellular function, thereby determining morphology and proliferation responses. Notably, therapeutic administration of BMP-4 suppressed primary tumor growth, acting both at the level of tumor cells and tumor stromal cells. Together, our results show that VEGF-D-driven metastasis induces vascular remodeling in lymph nodes. Further, they implicate BMP-4 as a negative regulator of this process, suggesting its potential utility as a prognostic marker or anti-tumor agent.
**Introduction**

Lymphatic dissemination is considered to be an early and crucial route of metastasis for many cancers (1, 2). Blind-ending lymphatic capillaries drain fluid, cells and macromolecules from tissue interstitium into a hierarchy of vessels punctuated by lymph nodes (LNs), which provide immunological surveillance for a particular lymphatic drainage basin (3). The presence of metastatic tumor cells in the “sentinel” LN draining a tumor site is a key factor in disease management: substantial clinical data indicates adverse prognostic significance of tumor-positive LNs for many tumor types (4, 5). However, a clear understanding of the mechanistic role of LNs in tumor progression is still lacking.

VEGF-D and VEGF-C are important inducers of the growth and differentiation of blood vessels and lymphatics. When overexpressed in experimental tumors these growth factors elicit angiogenesis and lymphangiogenesis, and are furthermore associated with increased metastasis to LNs and distant organs (1). VEGF-D and VEGF-C expression is also associated with metastasis to LNs in many human cancers, and is independently associated with poor prognosis (6). Recently, it has emerged that modulation of lymphatics and blood vessels - including high endothelial venules (HEVs); vessels specialized for leukocyte trafficking (7, 8) - also occurs in draining LNs of some tumors (9, 10). Such alterations can precede the arrival of metastatic cells (7, 11-13), and members of the VEGF family have been implicated in these changes (12-15). The importance of alterations to LN endothelium is highlighted by studies of human breast cancer: lymphangiogenesis or angiogenesis within metastatic tumor deposits in sentinel LNs was found to be associated with, and sometimes independently predictive of, distant metastasis or survival (9, 16, 17).

Here we sought to characterize changes to the vasculature within tumor-draining LNs, to identify molecules with prognostic or therapeutic potential. We compared the molecular
profiles of enriched endothelial cell (EC) populations from LNs draining non-metastatic tumors with those from LNs draining metastatic (VEGF-D-overexpressing) tumors. BMP-4 was downregulated in the HEVs of LNs draining metastatic tumors. This observation was linked with the remodeling of HEVs induced by VEGF-D-driven metastasis, thus implicating BMP-4 as a regulator of HEV morphology and cell function. Furthermore, therapeutically-applied BMP-4 protein inhibited primary tumor growth. This study indicates that VEGF-D’s pro-metastatic activity includes remodeling of specialized LN endothelium, and identifies new roles for BMP-4 in cancer and vascular biology.

**Materials and Methods**

Lists of antibodies, primers and detailed protocols are contained in the Supplementary Methods section linked to the online version of this manuscript.

**Metastatic and non-metastatic xenograft tumor models**

293 EBNA-1 tumor cell lines stably expressing full-length human VEGF-D (293-VEGF-D), human VEGF-C (293-VEGF-C), or vector alone (293-Apex) were established in SCID/NOD mice as described (18). 293 EBNA-1 cells were a gift from Kari Alitalo, University of Helsinki, Finland (1997). Regular growth and morphology of transfected cell lines was monitored routinely and growth factor expression verified by Western blot prior to each experiment. LNs were analyzed within the timeframe that metastasis typically occurs in this model; i.e. 2-4 weeks post-implantation. All animal experiments were performed with the approval of the institutional Animal Ethics Committee.

**Enrichment of LN EC populations**

Draining LNs of metastatic or non-metastatic tumors pooled from 1-5 mice were enzymatically digested, then tumor cells and leukocytes were depleted using immunomagnetic selection (Miltenyi Biotec) for class I HLA and CD16/CD32. The
remaining cells were cultured in EGM-2 MV media (Lonza) before enrichment for ECs by selection for podoplanin (19). See Supplementary Fig. S1 for detailed procedure.

**Microarray analysis**

Duplicate samples of LN EC total RNA (RNeasy Plus kit, Qiagen) were applied to Affymetrix® expression arrays (430 2.0; Australian Genome Research Facility). Raw intensity data were analyzed using GeneChip® Operating Software (Affymetrix®), and profiles compared via Robust Multiarray Analysis and linear modeling using AffylmGUI software (20). Microarray data are deposited in NCBI’s Gene Expression Omnibus; series accession number GSE31123 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31123).

**Human LNs**

Breast cancer-associated LNs with or without histologically-identifiable metastases (n=7 patients, 22 LNs), or control non-tumor-associated LNs collected during cardiac surgery (n=3 patients), were obtained as a pilot study. Access to de-identified tissue (formalin-fixed, paraffin-embedded) was provided by the Pathology Department, Royal Melbourne Hospital, with permission from the Melbourne Health Human Research Ethics Committee.

**Immunostaining and image quantitation**

For BMP-4/MECA-79 quantitation, 2-3 sections of each tumor-draining LN (~6 per group) were immunostained (18). For HEV morphometry, the luminal and basal edges of HEVs were traced using Metamorph Premier (Molecular Devices), to determine lumen area, average vessel wall width and endothelial area using Integrated Morphometry Analysis parameters (journal available on request). HEVs with ≥50% of their circumference staining for BMP-4 were designated BMP-4\textsuperscript{high}, or otherwise BMP-4\textsuperscript{low}. Data were analyzed according to a linear mixed model (Supplementary Methods).
Treatment of ear-draining LNs with recombinant VEGF-D

One μg of purified VEGF-D dimers (0.05 μg/μL; Vegenics Ltd.) in PBS, or PBS alone as control, was injected intradermally into the ears of SCID/NOD mice for three consecutive days. On Day 4, BrdU (Invitrogen) was injected intraperitoneally, and ear-draining (superficial parotid) LNs were harvested two days later.

Treatment of tumors with neutralizing antibodies

Mice bearing metastatic (VEGF-D-overexpressing) tumors received thrice weekly intraperitoneal injections of neutralizing antibodies (800 μg) to VEGF receptor-2 (DC101; Imclone) or VEGF-D (VD1; ref. 21), or PBS. For analysis of HEVs, sections of LNs draining non-metastatic and antibody-treated metastatic tumors were used from one experiment. LNs of PBS-treated metastatic tumors where HEVs were not obscured by tumor infiltration were included from an identically-performed experiment as a control.

BMP-4 therapeutic model

Tumor-bearing mice were injected intraperitoneally from day 1, thrice weekly, with 1.4 μg of human BMP-4 (R&D Systems) in 200 μl PBS with 0.652 mg/ml BSA, or a vehicle control of PBS with 0.32 mM HCl and 1 mg/ml BSA, until day 12 or experiment termination. Serum was sampled 60 minutes post-treatment and BMP-4 quantified by ELISA (R&D).

Statistical analysis

Data were compared using a two-tailed Student’s t-test, or Fisher’s exact test for comparison of proportions. Graphed data represent mean ± standard error (s.e.) unless specified otherwise.
Results

Enrichment of endothelial cells from tumor-draining LNs

A model of VEGF-D-driven tumor metastasis to regional LNs was used to examine molecular changes in LN endothelium during metastasis (Fig. 1A). Overexpression of VEGF-D in 293-EBNA-1 tumor cells drives metastasis to local LNs within 2-4 weeks of implantation in ~80% of cases. Vector-transfected tumor cells (no VEGF-D) served as a non-metastatic control (18). Podoplanin (19) was used as a highly-expressed, protease-resistant selection marker to derive cell populations enriched for lymphatic ECs and related EC types, which may respond to VEGF-D (Fig. 1B). Microarray analysis revealed expression of EC-characteristic genes, including VEGF receptor-2 (VEGFR-2), neuropilin-1 and neuropilin-2, endothelial nitric oxide synthase, CD34 and TIE-2; while desmin and calponin-1, found in fibroblastic lineages, and chondroitin sulfate proteoglycan 4 (NG-2 antigen), characteristic of pericytes, were absent. These findings confirmed that the podoplanin+ve cells were enriched for ECs. The LN ECs heterogeneously expressed ICAM-1 and endoglin, markers of endothelial activation in inflammation and angiogenesis, respectively (Fig. 1C; Supplementary Methods).

Identification of endothelial-expressed genes modulated during metastasis to LNs

LN ECs from metastatic and non-metastatic tumor models were compared by microarray (Fig. 2A). Of the top ten differentially-expressed genes (ranked by adjusted p-value), nine were downregulated in LNs draining metastatic tumors compared to their non-metastatic counterparts, and all ten showed >2-fold difference in expression (Table 1; Fig. 2B). Candidates were subsequently selected for further analysis based on relevance to endothelial and cancer biology. qRT-PCR validated the downregulation of Bmp4, Unc5c, Cfh, Emcn and Gpr39 in ECs from LNs draining metastatic tumors, as well as the upregulation of Nova1
(Fig. 2C). Bmp4 showed the greatest abundance and a >5-fold difference in expression, and was thus selected for further investigation.

**Localization of BMP-4 protein in HEVs and differential expression in metastasis**

Immunohistochemistry showed that BMP-4 protein was localized to HEVs (Fig. 3A), confirmed by co-staining for the specific MECA-79 epitope (22). BMP-4 protein was present in a subset of HEVs in LNs draining both non-metastatic and metastatic tumors (Fig. 3A), and in LNs from non-tumor-bearing SCID/NOD and immunocompetent mice (Fig. 3A, data not shown). HEVs did not endogenously express podoplanin (Supplementary Fig. S2), suggesting podoplanin probably became upregulated in HEV ECs during the brief culturing between extraction from LN and purification for microarray analysis (23, 24). While MECA-79 stained the surface of HEV ECs, BMP-4 appeared primarily in the cytoplasm (Fig. 3A inset), implying that HEV ECs express BMP-4 protein. No other sites of BMP-4 localization were observed in the LN or primary tumor. This supported the conclusion that HEV ECs are the main source of BMP-4 mRNA and protein in LNs.

Quantitation of staining revealed that HEV-expressed BMP-4 was significantly reduced (by ~50%), in LNs draining metastatic versus non-metastatic tumors (p<0.001; Fig. 3B, C). This illustrated a shift from predominately BMP-4^{high} to predominately BMP-4^{low} HEVs in LNs draining non-metastatic versus metastatic tumors respectively; however, both LN types contained some BMP-4^{high} and some BMP-4^{low} HEVs (Fig. 3B, C). Therefore, the downregulation of BMP-4 mRNA was reflected at the protein level *in vivo*.

**BMP-4 loss marks HEV remodeling in cancer**

We examined LNs for evidence of tumor-induced HEV remodeling (7), and explored whether VEGF-D or BMP-4 was associated with this process (Fig. 4A). In LNs draining non-metastatic tumors, BMP-4^{high} HEVs had significantly smaller lumen areas than BMP-4^{low}...
HEVs (p=0.0017; Fig. 4B). In LNs draining metastatic tumors, however, the BMP-4\textsuperscript{high} HEVs were more dilated than in the non-metastatic context (p=0.028; Fig. 4B). Significantly, BMP-4\textsuperscript{high} vessels had thicker vessel walls than BMP-4\textsuperscript{low} HEVs in all LNs (p<0.001; Fig. 4B), suggesting that BMP-4 expression was closely linked with HEV morphology. While the remaining BMP-4\textsuperscript{high} vessels in LNs draining metastatic tumors largely retained their greater vessel wall width, there was a strong trend suggesting reduced width compared to those in LNs draining non-metastatic tumors, indicating that VEGF-D-driven metastasis could affect the endothelial width of BMP-4\textsuperscript{high} HEVs (p=0.064; Fig. 4B). We also observed remodeled HEVs in a pilot study of human breast cancer-associated LNs with or without histologically-identifiable metastasis (Fig. 4E), confirming its occurrence in human disease (7).

We next investigated whether HEV remodeling involved EC proliferation. Interestingly, BMP-4\textsuperscript{high} HEV ECs in LNs draining metastatic tumors had a significantly lower proliferation rate than those from the non-metastatic model (p=0.026; Fig. 4C). Furthermore, BMP-4\textsuperscript{low} HEV ECs in LNs draining metastatic tumors had a significantly higher proliferation rate than the BMP-4\textsuperscript{high} HEV ECs (p=0.015; Fig. 4C). These results indicated that the proliferation response of HEV ECs to tumor-secreted VEGF-D may be modulated by BMP-4; another way in which VEGF-D-driven metastasis may induce remodeling of HEV characteristics via reduction of BMP-4 expression.

The role of VEGF-D and VEGFR-2 in HEV remodeling

To determine whether HEVs could respond directly to tumor-secreted human VEGF-D we examined VEGFR-2 and VEGFR-3 expression in LNs. VEGFR-2 was expressed on most HEVs, blood vessel capillaries and lymphatics (Fig. 4D). VEGFR-3 was strongly expressed on lymphatics, but was essentially absent from HEVs. Thus HEVs are capable of responding to VEGFR-2 ligands.
In vivo approaches were utilized to investigate the specific pathways controlling HEV remodeling. Injection of VEGF-D into the mouse ear mimics tumor-secreted growth factor draining to regional LNs. After three days of VEGF-D treatment, proliferation of BMP-4\textsuperscript{high} HEV ECs was decreased (p=0.034; Fig. 5A), suggesting VEGF-D was responsible for the effect observed in tumor-draining LNs (Fig. 4C), and that suppression of proliferation in BMP-4\textsuperscript{high} HEVs by VEGF-D may occur early in the metastatic process. Alteration of lumen area, vessel wall width and BMP-4 expression may require a longer stimulation period as neither was affected in this experiment (Fig. 5A, B); however BMP-4\textsuperscript{high} HEVs again exhibited significantly thicker vessel walls (Fig. 5A).

Additionally, mice bearing metastatic tumors were treated with neutralizing antibodies to VEGF-D or VEGFR-2. These antibodies can reduce rates of VEGF-D-driven metastasis to LNs (M. Matsumoto et al., unpublished). Anti-VEGFR-2 treatment of metastatic tumors significantly reduced the lumen area of BMP-4\textsuperscript{high} HEVs (versus metastatic + PBS p=0.039; versus non-metastatic tumors p=0.049; Fig. 5C). Vessel wall width of BMP-4\textsuperscript{high} HEVs was again reduced in LNs draining metastatic tumors (p=0.019; Fig 5C). Importantly, both anti-VEGFR-2 and anti-VEGF-D treatments effectively blocked this remodeling (p=0.049 for anti-VEGFR-2 and p=0.008 for anti-VEGF-D treatments), returning the vessel wall width to that of the non-metastatic control. VEGFR-2 blockade also increased the vessel wall width of BMP-4\textsuperscript{low} HEVs (versus metastatic + PBS p=0.007; versus non-metastatic p=0.053; Fig. 5C), while BMP-4\textsuperscript{high} HEVs still had significantly thicker walls than BMP-4\textsuperscript{low} HEVs in all conditions (p<0.001). Proliferation rates of both BMP-4\textsuperscript{high} (p=0.032) and BMP-4\textsuperscript{low} (p=0.026) HEV ECs were reduced by anti-VEGFR-2 treatment, while anti-VEGF-D treatment reduced proliferation of BMP-4\textsuperscript{low} HEVECs particularly (p=0.018; Fig. 5C). These analyses implicate VEGFR-2 in mediating HEV EC dilation and proliferation, and reiterate that BMP-4 expression modulates the proliferation response of HEV ECs to VEGF-D.
Interestingly, anti-VEGFR-2 treatment did not restore the reduced BMP-4 expression in LNs draining metastatic tumors; in contrast, BMP-4 expression under anti-VEGF-D treatment was very close to that of LNs draining non-metastatic tumors (Fig. 5D). This suggests that VEGF-D may induce BMP-4 downregulation, via a VEGFR-2-independent mechanism.

**Effects of exogenous BMP-4 on tumor progression**

As this study was designed to identify and analyze molecular targets with prognostic and/or therapeutic potential, we established a therapeutic model to determine the effects of exogenously-administered BMP-4. Activity and stability of recombinant human BMP-4 were verified by bioassay (Supplementary Fig. S3A; Supplementary Methods). As shown in Fig. 6A, BMP-4 inhibited the exponential growth of VEGF-D-overexpressing primary tumors by approximately 50% (day 20 p=0.056; day 22 p=0.036; day 24 p=0.080). Additionally, similar tumors overexpressing VEGF-C were reduced in size by approximately 56% by BMP-4 treatment (day 15 p=0.067; day 18 p=0.021; day 23 p=0.026). BMP-4 could thus inhibit tumor growth driven by two different lymphangiogenic/angiogenic growth factors. ELISA results confirmed that injected BMP-4 reached systemic circulation at approximately 1200 pg/ml in serum after 60 minutes (Fig. 6B). Interestingly, under the conditions and timecourse of these experiments the BMP-4 treatment did not appear to affect metastasis to LNs or HEV morphology (Fig. 6C and data not shown). Analysis of HEVs did reveal a trend suggesting that in metastasis-positive LNs draining VEGF-D-overexpressing tumors, more BMP-4\(^{\text{high}}\) HEVs were observed under BMP-4 treatment than for the control (mean ± s.e.: BMP-4, 40.9 ± 10.1; vehicle, 29.5 ± 10.0; n=5, p=0.16). Furthermore, BMP-4\(^{\text{high}}\) HEVs again exhibited thicker vessel walls than BMP-4\(^{\text{low}}\) HEVs in both treatment conditions (p<0.001; Supplementary Fig. S3B), confirming the importance of endogenous BMP-4 expression.
Mechanisms of BMP-4-induced tumor growth suppression

To clarify the mechanism by which BMP-4 suppressed primary tumor growth, we first examined the distribution of its receptors. BMPs bind a heterodimeric complex of type I and type II receptors (25). Immunohistochemistry for BMPR-II revealed broad expression on multiple cell types including tumor cells, stroma and endothelium of large blood vessels (Fig. 6D). Microarray analysis indicated that the VEGF-D-overexpressing tumor cells expressed \textit{BMPR2}, as well as \textit{BMPRIA} and \textit{ACTRIA}, but not \textit{BMPR1B} (Supplementary Table S2), while immunocytochemistry confirmed expression of BMPR-IA and BMPR-II protein on tumor cells and tumor-derived fibroblasts (Supplementary Fig. S4A; Supplementary Methods). Interestingly, Western blotting revealed that BMPR-II protein was more abundant in BMP-4-treated than control-treated VEGF-D-overexpressing tumors (p=0.048; Fig. 6E and Supplementary Methods), potentially representing a feedback loop that could contribute to tumor suppression.

In vitro stimulation showed that proliferation of VEGF-D-overexpressing tumor cells was unaffected by BMP-4 (Supplementary Fig. S4B). Conversely, preliminary experiments suggested that 100 ng/ml BMP-4 could induce detectable death in flow cytometric and microscopy-based assays (6.9\% ± 0.3\% versus control 4.3\% ± 1.0\% 7-aminoactinomycin-D-positive cells, p=0.036; 396 ± 22 versus control 270 ± 23 propidium iodide-positive cells/field, p=0.002). Tumor-derived fibroblasts proliferated significantly when stimulated with BMP-4 (Supplementary Fig. S4C). Interestingly, BMP-4-stimulated human lymphatic ECs showed increased Ki-67 expression while being slightly reduced in number, suggesting a short-term apoptosis effect followed by a slower proliferation response (Supplementary Fig. S4D). These data collectively suggest that BMP-4-induced tumor growth suppression is enacted via multiple cell types.
Discussion

Changes to the blood or lymphatic vasculature in tumor-draining LNs have prognostic significance in cancer (9, 16, 17, 26), and may facilitate metastasis (11-13). Understanding the mechanisms and functional consequences of these alterations will be critical in determining the overall role of LN metastasis in tumor progression, and could advance prognostication and treatment for cancer patients. Here we have identified molecules involved in the remodeling of HEVs in tumor-draining LNs, and an additional role for BMP-4 in suppressing primary tumor growth.

Microarray analysis of enriched LN EC populations revealed differential expression of several genes with significance to endothelial/tumor biology. Analysis of isolated EC subtypes has enabled identification of important functional molecules (27, 28). While our isolation strategy utilized podoplanin, commonly used to distinguish lymphatic endothelium, immunohistochemical validation revealed BMP-4 to be differentially-expressed in HEVs, a specialized venous endothelial type that did not express podoplanin in vivo. Subsequent to observations that blood vascular ECs co-cultured with lymphatic ECs could spontaneously acquire expression of lymphatic-characteristic molecules including podoplanin (23), it has been shown that substantial plasticity exists between arterial, venous and lymphatic EC lineages, controlled by specific transcription factors and reflecting their common embryonic origin (24). Our observations provide further confirmation of this plasticity and relatedness. Another similar study used microarray analysis of isolated lymphatic ECs from primary tumors, which were briefly cultured, to identify novel markers with prognostic significance (29). Our study advances upon this by examining the endothelium of tumor-draining LNs.

The morphological changes we observed to be associated with VEGF-D-driven metastasis and BMP-4 reduction – i.e. remodeling of the normally “high”-walled HEVs into flat-walled,
more dilated vessels with altered proliferation responses – were consistent with those observed in mouse models and human breast cancer (7). Others observed suppression of the HEV-expressed lymphotactic chemokine CCL21 and reduced lymphocyte recruitment in tumor-draining LNs (30). Such physical and molecular features of HEVs endothelium are integral to their role in trafficking leukocytes into the LN to facilitate immune responses (8). While these investigators analyzed total HEVs, we identified HEV subtypes (BMP-4\textsuperscript{high} and BMP\textsuperscript{low}) which can respond differentially to tumor-associated stimuli. Although the functional significance of HEV height is poorly understood, flattening of HEV ECs appears to reduce leukocyte transmigration rates (31). Lower branching-order HEVs were observed to support lower rates of lymphocyte adhesion than higher-order HEVs (30); interestingly, in our studies lower-order HEVs tended to have flatter endothelium and lower BMP-4 expression than higher-order HEVs. It is possible that HEV remodeling may echo homeostatic differences in the morphological, molecular and functional characteristics of different branching-order HEVs. Ultimately, tumor-induced HEV remodeling could assist in generating a metastatic niche (32): proliferating, dilated blood vessels derived from remodeled HEVs could enrich the nutrient and oxygen supply to a LN, while impaired immune function would promote tumor cell survival. The proximity of remodeled HEVs and lymphatic vessels could provide a shortcut for metastatic cells into the blood vasculature and thus systemic circulation (32, 33).

Our study provides an important contribution to understanding the molecular mechanisms driving tumor-induced HEV remodeling (Fig. 5D). The effects of BMP-4 and VEGF-D-driven metastasis on HEV vessel wall width were strongly evident, while differences in lumen area and proliferation were more dynamic and may be sensitive to other factors. The differing impacts of VEGFR-2 and VEGF-D blockade suggest involvement of another VEGFR-2 ligand. Several studies have implicated VEGF-A in stimulating HEV growth and
remodeling in immune responses (34, 35); thus endogenous VEGF-A could contribute to VEGFR-2-mediated HEV dilation in tumor-draining LNs. Additionally, VEGF-A might be involved in the differential proliferative response of BMP-4\textsuperscript{high} and BMP-4\textsuperscript{low} HEVs to VEGF-D. BMP-4 can increase expression and phosphorylation of VEGFR-2 in ECs, thus enhancing responsiveness to autocrine or paracrine VEGF-A (36). BMP-4 itself could signal to ECs in an autocrine manner (37), and might upregulate VEGF-A expression by LN stromal cells (35, 38), thus potentiating a VEGF-A/VEGFR-2 signaling loop. VEGF-D may then inhibit proliferation of BMP-4\textsuperscript{high} HEV ECs in the tumor context by competing with VEGF-A for binding to VEGFR-2. In contrast, HEVs expressing less BMP-4 may have less dependency on endogenous VEGF-A/VEGFR-2 signaling, and be sensitive to pro-angiogenic stimulation by tumor-derived VEGF-D (Supplementary Fig. S6). VEGF-D might induce this sensitization via downregulation of BMP-4 over longer exposure periods. Preferential proliferation of BMP-4\textsuperscript{low} HEVs earlier during VEGF-D-driven metastasis could also contribute to the preponderance of these vessels over time.

Signaling through HEV-expressed VEGFR-2 by VEGF-D (potentially also VEGF-A) likely contributes partially to altering HEV vessel wall width, and the physical presence of tumor cells in LNs could also affect HEV remodeling. Notably, the difference in vessel wall width corresponding to BMP-4 expression is larger than that attributable to VEGF-D and VEGFR-2 blockade in the metastatic tumor context. BMP-4 may thus act as a key regulator of HEVs, controlling EC morphology and responsiveness to angiogenic factors. BMP-4 induces a columnar epithelial phenotype in premalignant Barrett’s oesophagus (39), and could play a similar role in maintaining high endothelium. Loss of BMP-4 may enable completion of the remodeling process induced by VEGF-D-driven metastasis (Fig. 5D). Whilst the mechanism controlling BMP-4 expression remains unclear, the restorative effect of VEGF-D blockade suggests a VEGF-D-driven mechanism independent of VEGFR-2. Lymphatics and HEVs are
regulated in a coordinated manner during immunization responses, via cross-talk involving lymphotoxin β receptor (33); similarly, modulation of lymphatics in tumor-draining LNs (e.g. via VEGF-D/VEGFR-3 signaling) may influence HEV characteristics.

As a member of the TGF-β superfamily of multipotent cytokines, the role of BMP-4 in tumor progression can be complex and highly context-specific (25, 40). We showed that while endogenously-expressed BMP-4 regulates HEVs, exogenous BMP-4 can restrict primary tumor growth. BMP-4 is also known to induce apoptosis of other tumor cell lines (41, 42) and microvascular ECs (43), although in other studies pro-angiogenic responses were observed, possibly due to potentiation of VEGF-A/VEGFR-2 signaling (36). Our data suggests that lymphatic ECs may respond to BMP-4 in a similar way. An increase in proliferation of tumor-derived fibroblasts stimulated with BMP-4 in vitro is intriguing considering that cancer-associated fibroblasts are commonly implicated in promoting tumorigenesis (44). The upregulation of BMPR-II expression in BMP-4-treated tumors recapitulates a similar observation in *Xenopus* embryos indicating that *Bmpr2* is a target gene of BMP-4 signaling (45). Expression of several other regulators of BMP-4 signaling is also induced by BMP-4, raising the possibility that blockade of relevant signaling inhibitors might enhance the efficacy of BMP-4 treatment. Previous *in vivo* studies have described anti-tumorigenic effects of BMP-4 for several tumor types (41, 46-48) – as well as pro-tumorigenic effects for some - but thus far only one other study, using a model of glioblastoma multiforme, has demonstrated an anti-tumor effect of therapeutically administered recombinant BMP-4 (49). While the authors identified a pro-differentiation effect on tumor stem cells, we noted that VEGF-D is highly expressed in glioblastoma multiforme (50). Our study adds weight to the potential of BMP-4 as an anti-tumor agent by showing that it can inhibit tumor growth driven by two different lymphangiogenic/angiogenic factors through action on both tumor cells and stroma.
The context-specific nature of BMP-4 signaling does compel careful tuning of BMP-4 targeting and dosage to ensure a robust anti-tumor effect. A more constant dosage of BMP-4, or a delivery system more targeted to the LN, may help clarify whether therapeutically-administered BMP-4 can reverse HEV remodeling or inhibit metastasis. Nevertheless, reduction of BMP-4 expression in HEVs is an important early molecular indicator of remodeling, as it precedes loss of MECA-79 upon incorporation into the vasculature of the tumor deposit (7). Clinical studies will establish whether BMP-4 may represent a convenient surrogate marker of HEV remodeling in cancer. Further, BMP-4 or HEV remodeling may serve as indicators of systemic or distant effects of pro-metastatic tumor-derived factors such as VEGF-D, and provide prognostic information relevant to metastasis, treatment response or patient outcome. Our data further highlight the need to better understand the functional and prognostic significance of the LN, and in particular its vasculature, to cancer metastasis, as well as the potential of BMP-4 as a multipotent anti-tumor agent.

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References


### Tables

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<td>0.034</td>
</tr>
<tr>
<td><em>Hs3st1</em></td>
<td>heparan sulfate (glucosamine) 3-O-sulfotransferase 1</td>
<td>3.19</td>
<td>0.034</td>
</tr>
<tr>
<td><em>Nova1</em></td>
<td>neuro-oncological ventral antigen 1</td>
<td>-3.80</td>
<td>0.035</td>
</tr>
</tbody>
</table>

Table 1: Genes differentially expressed in ECs of LNs draining non-metastatic versus metastatic tumors – microarray analysis

<sup>a</sup> Fold change in abundance: non-metastatic over metastatic

<sup>b</sup> Benjamini-Hochberg adjusted p value

### Figure legends

**Figure 1. Isolation of ECs from tumor-draining LNs.**

*A*, schematic of approach to investigate differentially-expressed genes in enriched ECs from LNs draining metastatic or non-metastatic tumors.  
*B*, immunomagnetic selection for podoplanin enriched populations of LN ECs, as confirmed by flow cytometry. Gray line, isotype control; percentages represent proportions within podoplanin<sup>+/−</sup> gate (isotype control
proportion subtracted). C, enriched EC populations from LNs draining metastatic tumors were analyzed for ICAM-1 and endoglin expression by immunofluorescence or flow cytometry.

Figure 2. Identification of differentially-expressed genes in LN ECs.
A, ECs from LNs draining metastatic or non-metastatic tumors (labeled non-metastatic or metastatic LN EC) were compared by microarray. B, a volcano plot of log odds of differential expression against fold change illustrates significantly differentially-expressed genes. C, for selected genes, differential expression was validated by qRT-PCR. Shown are two representative examples (1 and 2) of pairwise comparisons. Data are mean ± standard deviation of triplicate reactions. *p<0.05, **p<0.01, ***p<0.001

Figure 3. Localization and differential expression of BMP-4 protein in LNs.
Draining LNs of metastatic and non-metastatic tumors (labeled metastatic and non-metastatic LNs) or axillary LNs of immunocompetent mice were sectioned and stained with BMP-4 and MECA-79 antibodies by immunofluorescence (A) or by standard immunohistochemistry in serial sections (B). Scale bar is 50 µm (A) or 100 µm (B). In (B), arrows denote BMP-4\textsuperscript{high} and arrowheads BMP-4\textsuperscript{low} HEVs. Proportional BMP-4 expression in HEVs was quantitated as BMP-4-stained/total MECA-79-stained endothelial area, and the proportion of BMP-4\textsuperscript{high} HEVs. n=16-17 sections; ***p<0.001 (C).

Figure 4. HEV remodeling in VEGF-D-driven tumor metastasis.
Luminal and basolateral edges of immunostained HEVs were traced manually to create binary masks (A; scale bar 20 µm), allowing morphometric quantitation of lumen area and vessel wall width in draining LNs of metastatic and non-metastatic tumors (metastatic and non-metastatic LNs; B). n=18-20 for lumen area and n=6 for vessel wall width; *p<0.05, **p<0.01, ***p<0.001. To measure proliferating HEV ECs, LNs (n=5) were co-stained for
PCNA (C). Serial sections of LNs were stained for VEGFR-2 and VEGFR-3 (D). Arrows: HEVs; arrowheads: lymphatics, scale bar: 50 µm. Tumor-associated LNs from breast cancer patients or control LNs were immunostained with MECA-79 antibody. Arrows denote examples of remodeled HEVs. Scale bar: 20 µm.

Figure 5. Involvement of VEGF-D and VEGFR-2 in HEV remodeling

Mouse ears were injected with recombinant VEGF-D protein or PBS. Draining LNs were analyzed using MECA-79/BMP-4 (n=10) and MECA-79/BrdU (n=5) immunofluorescence for effects on HEV lumen area, vessel wall width and proliferation (A); and BMP-4 expression (B). Mice bearing metastatic tumors were treated with antibodies to VEGFR-2 or VEGF-D. HEV morphology and proliferation (C) and BMP-4 expression (D) were assessed as above in tumor-draining LNs (n=5-6). *p<0.05, **p<0.01, ***p<0.001. E, schematic of the proposed mechanisms of HEV remodeling.

Figure 6. Therapeutic administration of BMP-4.

A, BMP-4 or vehicle control was administered to mice from day 1 until day 12 or experiment termination, and tumor volume measured (n=9-11). B, detection of BMP-4 in serum by ELISA (n=3). C, LNs were scored histologically positive or negative for metastatic cells. D, immunohistochemistry detecting BMPR-II expression on multiple tumor types including blood vessels, inset. E, Western blot detecting BMPR-II in cultured tumor cells and metastatic (VEGF-D) tumor lysates, and densitometric quantitation of expression (n=3; full length blot, Fig. S5).
Figure 1

A

Non-metastatic

Primary tumour

No metastasis

Metastatic

secreted VEGF-D

Draining lymph node

Enriched endothelial cell population

Microarray comparison

Candidate genes

Validation & functional investigation

B

Digested metastatic LN

150

100

60

Podoplanin

0

10^2

10^3

10^4

Enriched metastatic LN EC

100

60

12%

10%

10^2

10^3

10^4

C

ICAM-1

150

endoglin

50

0

10^2

10^3

10^4
Figure 4

A. MECA-79 + BMP-4

B. HEV lumen area (log_{10}-transformed)

C. % PCNA vs. HEV EC

D. MECA-79, VEGFR-2, VEGFR-3

E. Non-tumor-associated LN, Metastatic tumor-draining LN
A role for bone morphogenetic protein-4 in lymph node vascular remodeling and primary tumor growth

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