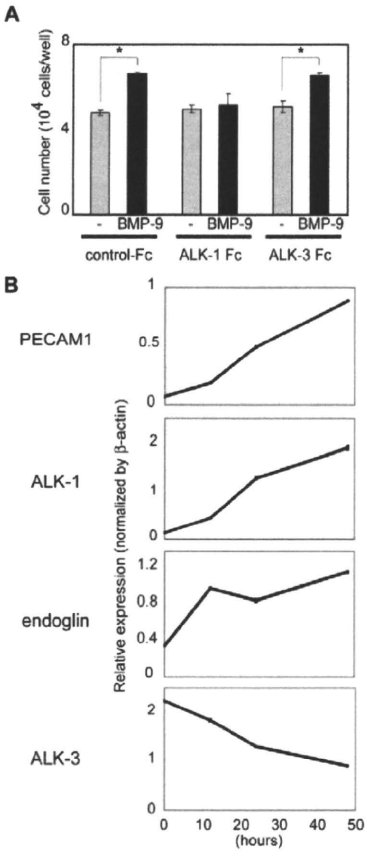

Fig. 4. Involvement of ALK-1 in the BMP-9-mediated increase in number of MESECs. (**A**) VEGFR2+ cells (5×10^4/well) derived from CCE cells were cultured in serum-free SFO medium containing 30 ng/ml of VEGF in the absence (–) or presence of BMP-9 (1 ng/ml) in combination with control-Fc (30 ng/ml), ALK-1-Fc (30 ng/ml) or ALK-3-Fc (30 ng/ml) for 2 days, followed by cell-proliferation analysis. Each value represents the mean of three determinations; error bars, s.d. *$P<0.004$. (**B**) Expression of vascular markers and ALKs during in vitro differentiation of ESC-derived VEGFR2+ cells. Quantitative RT-PCR analysis of PECAM1, ALK-1, endoglin and ALK-3 of VEGFR2+ cells stimulated with 30 ng/ml of VEGF in the absence of serum. Error bars, s.d.

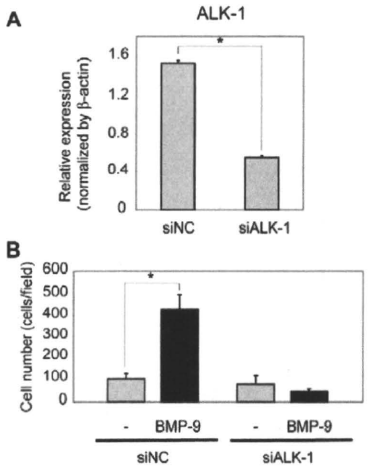

Fig. 5. Effects of decreased ALK-1 expression on MESEC proliferation. MGZ5 ESC-derived VEGFR2+ cells were transfected with siRNA for *ALK-1* (siALK-1) or negative control siRNA (siNC), and cultured in the absence (–) or presence of BMP-9 (1 ng/ml) in combination with VEGF (30 ng/ml) and serum for 4 days. (**A**) Expression of endogenous level of *ALK-1* was determined by quantitative RT-PCR analyses. Each value represents the mean of three determinations; error bars, s.d. *$P<0.002$. (**B**) Roles of ALK-1 on the proliferation of MESECs. In order to determine the number of endothelial cells, ESC-derived vascular cells containing both endothelial and mural cells were stained for PECAM1 and SMA, followed by counting the numbers of endothelial cells in each field. Each value represents the mean of five determinations; error bars, s.d. *$P<0.00002$.

of MESECs is mediated by ALK-1. We therefore knocked down ALK-1 expression in ESC-derived VEGFR2+ cells using siRNA during endothelial cell differentiation in the presence of serum. As shown in Fig. 5A, the level of transcripts encoding ALK-1 was significantly decreased by its specific siRNA.

Although the number of MESECs was not significantly altered by knocking down ALK-1 expression, BMP-9 failed to increase the number of MESECs only when ALK-1 expression was knocked down (Fig. 5B). These findings suggest that ALK-1 is required for BMP-9 to increase the number of MESECs.

Expression of constitutively active ALK-1 increases the number of MESECs

We next examined whether the expression of constitutively active ALK-1 (caALK-1) regulates the proliferation of MESECs in a fashion similar to the addition of BMP-9. Because we wished to induce the expression of caALK-1 in differentiating endothelial progenitor cells instead of undifferentiated ESCs, we established ESC lines carrying a tetracycline (Tc)-regulatable caALK-1 transgene (Tc–caALK-1) or no transgene (Tc-empty) (Masui et al., 2005; Mishima et al., 2007).

We differentiated the Tc-empty and Tc–caALK-1 ESCs into VEGFR2+ vascular progenitor cells in the presence of Tc, so that no transgene expression would be induced. Sorted VEGFR2+ cells were re-differentiated in the presence or absence of Tc. As shown in Fig. 6A, in endothelial cells derived from Tc–caALK-1 ESCs, expression of the caALK-1 transgene was induced only in the absence of Tc. When caALK-1 was expressed, the number of MESECs was significantly increased (Fig. 6B), consistent with the effects of addition of BMP-9. When caALK-1 transgene was expressed in MESECs, Id1 expression was significantly increased (Fig. 6C), a finding also observed when BMP-9 was added to culture of MESECs (Fig. 7). These findings suggest that activation of Smad1/5/8 signaling by expression of caALK-1 mimics the signaling induced by BMP-9.

Induction of endothelial cell proliferation by BMP-9–ALK-1 signaling via stimulation of VEGF-VEGFR2 and Ang-1–Tie2 signaling

We next attempted to identify the targets of BMP-9–ALK-1 signaling that induce endothelial cell proliferation. VEGF-VEGFR2 signaling pathways are known to stimulate endothelial proliferation, survival and migration. Ang-1 is an agonist for the Tie2 tyrosine kinase receptor, which inhibits apoptosis of endothelial cells (Maisonpierre et al., 1997; Holash et al., 1999). We previously showed that BMP-4 induces the expression of transcripts for

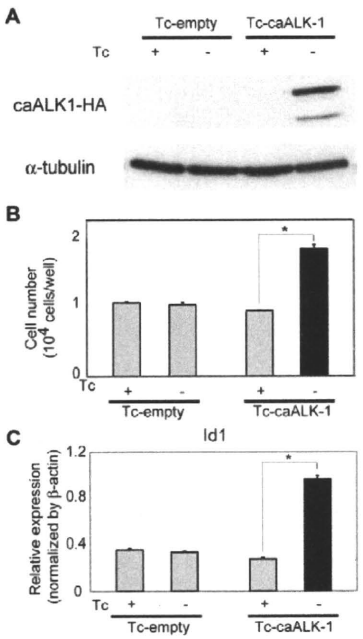

Fig. 6. Effects of constitutively active ALK-1 expression on MESEC proliferation. VEGFR2+ vascular progenitor cells derived from ESCs carrying a tetracycline (Tc)-regulated transgene encoding hemagglutinin (HA)-tagged constitutively active ALK-1 (Tc–caALK-1) or control transgene (Tc-empty) were cultured in the presence (+) or absence (–) of Tc. After 2 days of culture in the absence of serum, the MESECs obtained were subjected to immunoblotting for HA-epitope to determine the level of expression of *ALK-1* transgene (**A**), to cell-proliferation assay (**B**) or to quantitative RT-PCR analysis for Id1 expression (**C**). Error bars, s.d. $*P<0.003$.

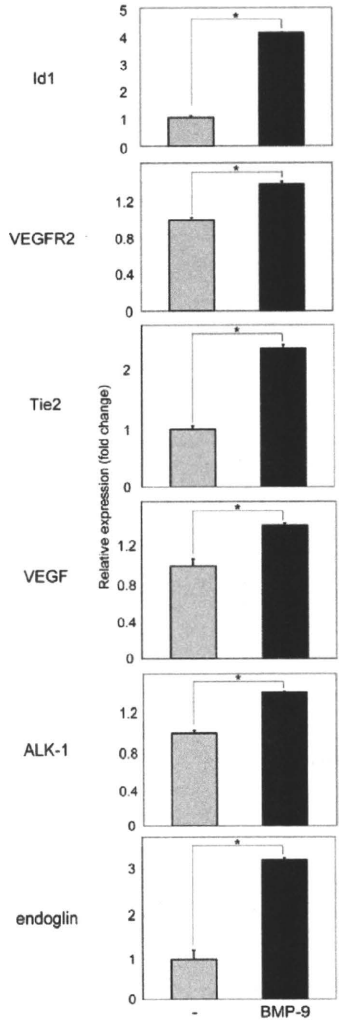

Fig. 7. Effects of BMP-9 on the expression of various angiogenesis-related factors. VEGFR2+ cells derived from CCE cells were cultured in serum-free SFO medium containing 30 ng/ml of VEGF in the absence (–) or presence of 1 ng/ml of BMP-9 for 2 days, and subjected to quantitative RT-PCR analyses for the expression of Id1, VEGFR2, Tie2, VEGF, ALK-1 and endoglin. Quantified mRNA values were normalized to the amount of β-actin mRNA, and results are given as fold change. Error bars, s.d. $*P<0.006$.

VEGFR2 and Tie2, suggesting that activation of VEGF-VEGFR2 and Ang-1–Tie2 signaling by BMP-4 leads to the activation of MESECs (Suzuki et al., 2008).

In addition to increasing Id1 levels, BMP-9 increased the levels of transcripts for VEGFR2 and Tie2 (Fig. 7). Furthermore, we identified VEGF as a putative target of BMP-9–ALK-1 signaling in MESECs, suggesting that the BMP-9-mediated increase in the number of MESECs is promoted by the activation of VEGF-VEGFR2 and Ang-1–Tie2 signaling. In order to examine the roles of VEGF-VEGFR2 signals in the BMP-9-mediated promotion of angiogenesis, we treated allantoic explants with SU1498, a specific inhibitor of VEGFR2 kinase. As shown in supplementary material Fig. S2, the addition of SU1498 reduced the formation of blood vessels from allantoic explants, showing that blockade of VEGF signaling inhibits angiogenesis. Although BMP-9 enhanced blood-vessel formation in control explants, this augmentation was completely abrogated by SU1498. These results further suggested that VEGF-VEGFR2 signals play important roles in the promotion of angiogenesis by BMP-9.

Interestingly, expression of *ALK-1* transcripts increased upon the addition of BMP-9 to culture of MESECs, suggesting that BMP-9–ALK-1 signaling is self-activating as a result of a positive-feedback mechanisms. In addition, we found that the expression of endoglin is upregulated by BMP-9; this might modulate BMP-9–ALK-1 signaling.

BMP-9 enhances in vivo angiogenesis in Matrigel plug assays

We next attempted to examine whether present ex vivo and in vitro findings that BMP-9 activates the proliferation of endothelial cells can be applied to angiogenesis in adults. Adult angiogenesis includes not only neo-angiogenesis, which involves the proliferation of existing endothelial cells, but also vasculogenesis, which involves the differentiation of endothelial progenitor cells circulating in peripheral blood (Asahara and Kawamoto, 2004). As a model of angiogenesis in adults, we chose a Matrigel plug assay.

Matrigels mixed with FGF-2 in combination with BMP-9 were subcutaneously injected to Balb/c mice. One week later, Matrigel plugs were harvested, and resulting vasculature was examined by staining for PECAM1. As shown in Fig. 8A and B, a significantly

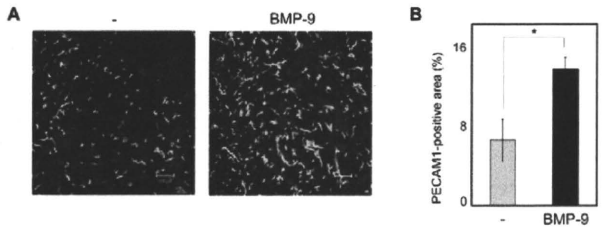

Fig. 8. Effects of BMP-9 on angiogenesis in a Matrigel plug assay in vivo. Matrigel plugs mixed with 1 μg/ml FGF-2 in the absence (–) or presence of 10 ng/ml of BMP-9 were subcutaneously injected in BALB/c mice. After 1 week, Matrigel plugs were harvested and examined for vascular density. (**A**) Immunostaining for PECAM1 (green) of sections obtained from both types of Matrigel plugs. Scale bars: 100 μm. (**B**) Percent PECAM1-positive area. Each value represents the mean of ten sections; error bars, s.d. *$P<0.005$.

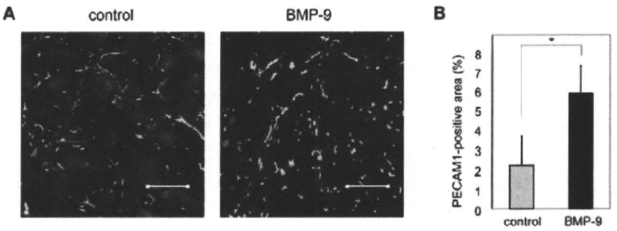

Fig. 9. Effects of BMP-9 on tumor angiogenesis in a mouse xenograft model of human pancreatic cancer. BxPC3 human pancreatic adenocarcinoma cells that were infected with lentiviruses encoding GFP (control) or BMP-9 were subcutaneously inoculated in BALB/c nude mice. After 5 weeks, tumors were excised and examined for vascular density. (**A**) Immunostaining for PECAM1 (green) of sections obtained from both types of tumors. Scale bars: 100 μm. (**B**) Percent PECAM1-positive area. Each value represents the mean of 15 sections; error bars, s.d. *$P<0.0000003$.

higher density of microvessels was observed in the Matrigels that contained BMP-9 than in the controls. These findings suggest that BMP-9 promotes in vivo adult angiogenesis.

Promotion of tumor angiogenesis by BMP-9 in a human pancreatic-cancer xenograft model

We next tried to extend our finding that BMP-9 enhances angiogenesis in Matrigel plugs to another model of angiogenesis. Because previous studies showed that BMP-2 induced tumor angiogenesis in human lung- and breast-cancer xenograft models (Langenfeld and Langenfeld, 2004; Raida et al., 2005), we examined whether BMP-9 also plays any roles in tumor angiogenesis.

In order to avoid effects on tumor cells themselves of BMP-9 signaling, we established BxPC3 pancreatic adenocarcinoma cells that express green fluorescence protein (GFP; control) or *BMP-9* transgenes. Because BxPC3 cells exhibit a homozygous deletion of the *SMAD4* gene (Kleeff et al., 1999), BMP-9 secreted from BxPC3-BMP-9 tumor cells cannot activate the Smad pathways by itself. We confirmed that BMP-9 expression does not affect the rate of growth of tumor cells in vitro (data not shown). When conditioned medium prepared from BxPC3-BMP-9 cells was added to MDA-MB-231 cells, the expression of Id1 was significantly induced compared with when medium from BxPC3-GFP cells was added (data not shown).

Both types of BxPC3 cells were subcutaneously grafted to immunocompromised Balb/c nude mice to obtain tumors. Tumor vasculature was examined by staining for PECAM1. As shown in Fig. 9A and B, a significantly higher density of microvessels was observed in the tumors derived from BxPC3-BMP-9 cells than in the controls. These findings suggest that BMP-9 also promotes in vivo tumor angiogenesis.

BMP-9 promotes the proliferation of primary cultured normal and tumor-associated endothelial cells derived from adult mice

Present findings that BMP-9 promotes angiogenesis in Matrigels and tumors transplanted to mice prompted us to investigate whether BMP-9 induces the proliferation of endothelial cells directly or indirectly via activation of surrounding cells, including inflammatory cells. Recent findings have revealed that tumor-associated endothelial cells (TECs) are different from normal endothelial cells (NECs) in many respects (Hida et al., 2004; Hida et al., 2008; Akino et al., 2009). In order to examine the effects of BMP-9 on the proliferation of both types of primary cultured endothelial cells, we prepared TECs from renal carcinoma xenografts (OSRC-2) in nude mice and NECs from dermal tissues as a normal counterpart of TECs (see Materials and Methods). When NECs and TECs were cultured in the absence or presence of BMP-9, proliferation of both types of cell was significantly promoted by BMP-9 (Fig. 10). These results suggest that BMP-9 induces angiogenesis via direct activation of endothelial cells in normal and cancerous conditions.

Discussion

In the present study, we demonstrated for the first time that BMP-9 induces ex vivo allantois vascular formation, proliferation of in-vitro-cultured MESECs, and in vivo angiogenesis. Using in vitro systems, we showed that ALK-1 is necessary and sufficient for the BMP-9-induced proliferation of MESECs, and that BMP-9 induces the expression of various targets, including VEGFR2 and Tie2, involved in the proliferation of endothelial cells.

We previously reported that BMP-4 induces the proliferation of MESECs via activation of VEGFR2 and Tie2 signaling (Suzuki et al., 2008). Although in the present study we showed that members of all three BMP subfamilies (BMP-2/4, OP-1 and BMP-9/10) induce the proliferation of MESECs (Fig. 3B), their effects on MESECs seem to differ, given the differential onset of receptor expression and induction of other target genes. Because the

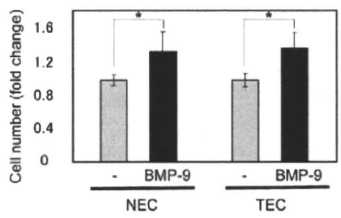

Fig. 10. Effects of BMP-9 on the number of normal and tumor-associated endothelial cells isolated from adult mice. Normal endothelial cells (NECs) prepared from dermal tissues and tumor-associated endothelial cells (TECs) prepared from renal carcinoma xenografts in nude mice were cultured in the absence (–) or presence of BMP-9 (5 ng/ml) for 3 days, followed by determination of cell numbers by MST assay. Each value represents the mean of five determinations; error bars, s.d. *$P<0.003$.

expression of ALK-3, a receptor for BMP-4, begins before mesoderm induction, BMP-4–ALK-3 signaling is involved in mesoderm induction (Lawson et al., 1999; Winnier et al., 1995; Dunn et al., 1997; Mishina et al., 1995; Ahn et al., 2001) and formation of VEGFR2+ angioblasts (Park et al., 2004). By contrast, ALK-1 expression parallels that of PECAM1, further confirming that ALK-1 is an endothelial marker. These findings highlight the difference between the effects of BMP-4 and BMP-9: BMP-4 signaling occurs in the early stages during differentiation of progenitors cells, whereas BMP-9 signaling occurs only in fully differentiated endothelial cells.

In addition, we identified ALK-1 as a target of BMP-9 signaling. The BMP-9-mediated positive-feedback mechanisms involved in the regulation of ALK-1 expression seem to play important roles during embryonic vascular formation, because BMP-9–ALK-1 signaling enhances the proliferation of endothelial cells. We also found that endoglin expression is induced by BMP-9 in MESECs, consistent with our previous finding that adenovirally transduced caALK-1 induced the expression of endoglin in human umbilical-vein endothelial cells (Ota et al., 2002). Endoglin serves as a co-receptor for TGFβ-family signals (Yamashita et al., 1994). In vitro studies have shown that endoglin protein is able to form complexes with ALK-1 and to enhance the effects of ALK-1 in endothelial cells (Blanco et al., 2005; Goumans et al., 2002; Lebrin et al., 2004). The phenotype of endoglin-deficient mice strongly resembles that of ALK-1-deficient mice, suggesting that endoglin also plays a role in ALK-1 signaling in angiogenesis (Li et al., 1999). Although both ALK-1 and endoglin are involved in the pathogenesis of HHT, the roles of endoglin in MESECs remain to be elucidated.

BMP-9 has been reported to inhibit the proliferation and migration of endothelial cells (David at al., 2007; Scharpfenecker et al., 2007), a finding that is inconsistent with our results that BMP-9 inhibits the proliferation of three kinds of endothelial cells: MESECs, NECs and TECs. However, as previously reported (David et al., 2007), we also found that BMP-9 inhibits proliferation of HMVEC-ds (data not shown), suggesting that the effects of BMP-9 on endothelial cell proliferation are cell-type dependent. Identification of the components that determine whether BMP-9 induces or inhibits endothelial proliferation is of great interest.

Consistent with our in vitro data, we found that BMP-9 enhanced the angiogenesis in ex vivo culture of allantois and in vivo Matrigel plug assay. We also found that BMP-9 secreted by BxPC3 carcinoma cells affected the tumor microenvironment and promoted angiogenesis by directly activating endothelial cells, which is crucial for tumor growth. However, the tumors derived from BMP-9-expressing BxPC3 cells were smaller than control tumors (data not shown). Because Smad4 is defective in BxPC3 cells, the BMP-9 secreted from BxPC3-BMP-9 cells is unable to activate Smad signaling in the tumor cells, although we cannot exclude the possibility that BMP-9 activated Smad-independent signaling in tumor cells and thereby attenuated tumor growth, which we were not able to observe in vitro (data not shown). It is also noteworthy that the newly formed vasculature in BxPC3-BMP-9 tumor (Fig. 9) seemed to be immature and non-functional compared with that in the controls. We observed that pericyte coverage of the newly formed vessels was decreased in the tumors derived from BxPC3-BMP-9 cells (data not shown). These observations suggest that the in vivo growth of BxPC3 cells does not principally depend on newly formed vasculature, which typically is the case for pancreatic tumors. It will be of great interest to examine the effects of BMP-9 on the growth of other types of tumor cells.

Angiogenesis plays crucial roles in numerous pathological conditions such as inflammation and tumorigenesis. In addition, regenerative medicine for vascular systems has attracted much attention as a potential means of eliminating the vascular-system defects accompanying diabetes and other diseases. The present findings are thus useful in suggesting BMP-9 as a putative target or other type of resource in the treatment of inflammation, cancer and vascular dysfunction.

Materials and Methods

Cells and cell culture

The CCE ESC line was obtained from Michael J. Evans (University of Cambridge, UK). MGZ5 and MGZRTcH ESC lines were obtained from Hitoshi Niwa (RIKEN CDB, Kobe, Japan). Maintenance, differentiation, culture and cell sorting of ESCs were performed as previously described (Yamashita et al., 2000; Suzuki et al., 2008; Kokudo et al., 2008). Establishment of Tc-inducible ESC lines from parental MGZ5TcH2 cells was as described (Masui et al., 2005; Mishima et al., 2007). VEGF (30 ng/ml), BMP-4 (60 ng/ml), BMP-6 (360 ng/ml), BMP-9 (1 ng/ml), TGFβ1 (1 ng/ml), control-Fc (30 ng/ml), ALK-1-Fc (30 ng/ml) and ALK-3-Fc (30 ng/ml) were purchased from R&D Systems. The BxPC3 human pancreatic adenocarcinoma cell line was obtained from American Type Culture Collection (Manassas, VA). BxPC3 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS).

Isolation and culture of TECs and NECs

Endothelial cells were isolated as previously described (Ohga et al., 2009). In brief, TECs were isolated from renal carcinoma xenografts (OSRC-2) in nude mice aged 8-12 weeks (Sankyo Laboratory, Tokyo, Japan). NECs were isolated from the dermal tissue as a control. Both types of endothelial cells were isolated using a magnetic cell-sorting system (Miltenyi Biotec) according to the manufacturer's instructions using fluorescein isothiocyanate (FITC)-anti-PECAM1 antibody. PECAM1-positive cells were sorted and plated onto 1.5% gelatin-coated culture plates and grown in EGM-2 MV (Clonetics) and 15% FBS. Diphtheria toxin (500 ng/ml; Calbiochem) was added to TEC subcultures to kill any remaining human tumor cells and to NECs to ensure technical consistency. The isolated endothelial cells were purified by a second round of purification, using FITC–Bandeirea Simplicifolia lectin 1-B4, and purity was determined by flow cytometry. Growth of TECs and NECs was quantified after 3 days of the culture in the absence or presence of BMP-9 by MTS assay using CellTiter 96 Aqueus One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol.

Cell-proliferation assays

The number of MESECs was counted in different ways depending on the conditions of differentiation from ESC-derived VEGFR2+ cells. When ESC-derived VEGFR2+ cells were re-differentiated in the absence of serum, we obtained only endothelial cells (Yamashita et al., 2000). Therefore, cell numbers were counted using a Coulter Counter (Yamato Kagaku). When ESC-derived VEGFR2+ cells were re-differentiated in the presence of serum, we obtained both endothelial cells and mural cells. Such mixed cell populations were immunostained for PECAM1, an endothelial cell marker, and smooth muscle α-actin (SMA), a mural cell marker, and subjected to manual counting of endothelial cells in the fields of photographs.

Luciferase assay

The transcriptional activation induced by BMPs and TGFβ1 was measured using BRE-Luc and 12×CAGA-Luc, which are luciferase reporter constructs containing BMP-responsive and TGFβ-responsive elements, respectively (Korchynskyi and ten Dijke, 2002; Dennler et al., 1998). ESC-derived VEGFR2+ vascular progenitor cells were seeded in 24-well plates and then transiently transfected with promoter-reporter constructs, followed by stimulation with ligands. Cell lysates were then prepared, and luciferase activities in the lysates were measured with the Dual-Luciferase reporter system (Promega) using a luminometer (MicroLumat Plus, Berthold). Values were normalized to *Renilla*-luciferase activity under control of cytomegalovirus promoter.

RNA interference

siRNAs for mouse *ALK-1* (Stealth RNAi Oligo ID MSS235821) and negative control (Stealth RNAi Negative Control Med GC) were purchased from Invitrogen, and were introduced into cells using HiPerFect reagent (QIAGEN) according to the manufacturer's instructions.

Ex vivo allantois assay

Explant culture of mouse allantois was carried out as previously described (Downs et al., 2001). Briefly, allantoises were excised from concepti of ICR mice at E8.25 in phosphate-buffered saline. Individual allantoises were cultured on eight-well culture slides in 250 μl of minimum essential medium (Invitrogen) supplemented with 10% FBS and β-mercaptoethanol. After 2 days of culture at 37°C in a 5% CO_2

incubator, allantois explants were subject to immunohistochemical examination. When SU1498 (Cosmobio) was added to the culture, dimethyl sulfoxide (DMSO) was used as vehicle control.

Matrigel plug assay

Balb/c male mice aged 5-6 weeks were obtained from CLEA Japan (Tokyo, Japan) and Sankyo Laboratory (Tokyo, Japan). All animal experimental protocols were performed in accordance with the policies of the Animal Ethics Committee of the University of Tokyo. Matrigel plug assays were carried out as previously described (Kano et al., 2005). Briefly, regular Matrigel (BD Biosciences) was mixed with FGF-2 (R&D Systems) and heparin (Aventis Pharma) in combination with 10 ng/ml BMP-9, and injected subcutaneously into the abdominal region of mice (n>5 mice per group). Matrigels were harvested 1 week after injection and were subject to immunohistochemistry for PECAM1.

Lentivirus production and infection

A lentiviral expression system was used to establish BMP-9-expressing BxPC3 cells (Shibuya et al., 2003). cDNA encoding human BMP-9 was cloned from liver cDNA of Multiple Tissue cDNA Panels (Clontech) using the following primers: forward, 5′-TTCCTTCAGAGCAAACAGCA-3′ and reverse, 5′-GTTGTGCTCAAATC-CCCATT-3′. *BMP-9* cDNA was subcloned into the pENTR vector and subsequently transferred into the pCS-EF-RfA lentiviral expression vector by the LR recombination reaction (Invitrogen). For production of lentiviral vectors, 293FT cells (Invitrogen) were transfected using Lipofectamine 2000 (Invitrogen) with three plasmids: vector construct, VSV-G- and Rev-expressing construct (pCMV–VSV-G–RSV–Rev) and packaging construct (pCAG-HIVgp). The culture supernatants were collected and viral particles were concentrated by centrifugation. For lentiviral infection, 1×10^5 BxPC3 cells were infected with lentivirus vectors in suspension and plated in six-well culture plates.

Balb/c nude mouse model of human pancreatic cancer

Balb/c nude male mice aged 5-6 weeks were obtained from CLEA Japan (Tokyo, Japan) and Sankyo Laboratory (Tokyo, Japan). A total of 5×10^6 BxPC3 tumor cells in 100 μl phosphate-buffered saline (n>5 mice per group) were injected subcutaneously into the left flank of each mouse and allowed to grow for 5 weeks, at which point the major axis of tumors was approximately 10-mm long.

Immunohistochemistry and immunoblot analysis

Staining of cultured cells was performed as previously described (Watabe et al., 2003; Kokudo et al., 2008). Excised mouse tissue samples from Balb/c mice grafted with BxPC3 cells were snap-frozen in a dry-ice acetone bath for immunohistochemistry. Frozen samples were further sectioned at 10-μm thickness in a cryostat and subsequently incubated with primary and secondary antibodies. Monoclonal antibodies to PECAM1 (Mec13.3) and SMA (1A4) for immunohistochemistry were purchased from BD Pharmingen and Sigma, respectively. Stained specimens were examined using a phase-contrast microscope (Model IX70; Olympus) or an LSM 510 META confocal microscope (Carl Zeiss). All images were imported into Adobe Photoshop as JPEGs or TIFFs for figure assembly. Images were processed using ImageJ (NIH) to quantify PECAM1-positive areas. Immunoblot analysis was performed as described (Watabe et al., 2003). Antibodies to HA and α-tubulin for immunoblot analysis and immunohistochemistry were obtained from Sigma. The bound antibody was detected using a chemiluminescent substrate (ECL; Amersham) and a LAS-4000 Luminescent image analyzer (Fuji Photo Film).

RNA isolation and RT-PCR analysis

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen). RNAs were reverse-transcribed by random hexamer priming using Superscript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR analysis was performed using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) and Power SYBR Green (Applied Biosystems). All expression data were normalized to those for β-actin. The primer sequences are shown in supplementary material Table S1.

Statistical analysis

Results were statistically examined using the two-sided Student's t-test. Differences were considered significant at P<0.05.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/10/1684/DC1

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CASE REPORT

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Multiple lymphadenopathy as an initial sign of extramammary Paget disease

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None declared.

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Summary

Extramammary Paget disease (EMPD) often develops in external genitalia. Paget cells can, however, adopt an invasive phenotype and metastasize to regional lymph nodes and beyond, leading to poor patient outcomes. Based on this clinical observation, multiple lymphadenopathy may represent an initial sign of EMPD. To address the potential significance of multiple lymph node swelling in EMPD, we report two patients with cutaneous primary EMPD who showed multiple lymphadenopathy as an initial sign during the clinical course of the disease as well as tumour metastasis. Significantly, marked lymphatic vessel growth was observed in regional lymph nodes that underwent massive tumour cell invasion. Therefore, nodal lymphangiogenesis may promote tumour cell invasion and metastasis to distant organs, including the lymph nodes, emphasizing the clinical relevance of multiple lymphadenopathy.

Extramammary Paget disease (EMPD) is a rare skin tumour that possibly arises from apocrine ductal cells.[1] Histological features of EMPD represent adenocarcinoma in the skin, and most cases are found as carcinoma in situ, indicating a good prognosis. However, invasive Paget disease often develops metastases to regional lymph nodes and/or distant organs, resulting in reduced patient survival. Recently, tumour-associated lymphangiogenesis was found within primary sites of EMPD and other malignant neoplasms such as cutaneous melanoma.[2–4] More importantly, lymphangiogenesis is strongly induced in sentinel and/or regional lymph nodes targeted by metastatic EMPD, suggesting that nodal lymphangiogenesis may facilitate tumour spread via lymphatic vessels. However, the significance of nodal lymphangiogenesis to clinical outcomes is not yet established. We thus report two cases of EMPD in which multiple lymphadenopathy was an initial sign of the disease, one that preceded detection of primary sites in the skin.

Case reports

Patient 1

A 53-year-old man undergoing a routine health check was found to show elevated serum levels of carcinoembryonic antigen (9·6 ng mL^{-1}; normal ≤ 3·4). Positron emission tomography combined with computed tomography (CT) revealed 2-(^{18}F)fluoro-2-deoxy-D-glucose accumulation in abdominal para-aortic and left inguinal lymph nodes (Fig. 1a, arrowheads). A CT image at the femoral joint level revealed swollen lymph nodes in the left inguinal region (Fig. 1b, circled), suggesting the presence of multiple lymph node metastases by a malignant neoplasm. An initial biopsy of a massive inguinal lymph node was obtained. Haematoxylin and eosin (H&E) staining of biopsy material indicated metastatic adenocarcinoma in the lymph node (Fig. 1c). However, general examination failed to identify the exact origin of metastatic tumours. Therefore, an additional examination was performed at a dermatology unit. The patient exhibited a demarcated depigmented macule in the perineum with focal erosions (Fig. 2a), a feature typical of EMPD. The patient was diagnosed with metastatic EMPD, and surgical resection was performed to remove the cutaneous primary lesion and regional lymph nodes. In addition to surgical resection, systemic chemotherapy was initiated with docetaxel combined with trastuzumab, a neutralizing antibody targeting the HER2 receptor, which is expressed by Paget cells (Fig. 2i). However, during treatment the patient developed distant organ metastases, including to liver, and died after surviving 24 months since the initial signs of lymphadenopathy emerged.

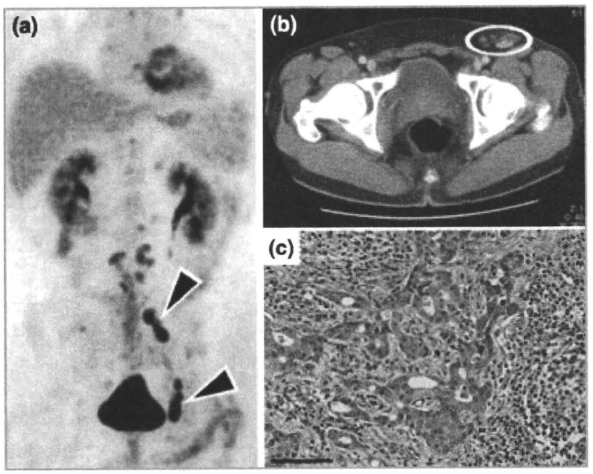

Fig 1. Multiple lymphadenopathies are seen in patient 1 with extramammary Paget disease. (a) A positron emission tomography–computed tomography (CT) image of the abdomen shows 2-(^{18}F)fluoro-2-deoxy-D-glucose accumulation among para-aortic and left inguinal lesions (arrowheads), indicating multiple metastases from the lymph nodes in patient 1. (b) A CT image at the femoral joint level shows multiple swollen lymph nodes in the left inguinal region (circle). (c) Haematoxylin and eosin staining of the inguinal lymph node from biopsy material shows metastatic foci representing adenocarcinoma of unknown origin. Scale bar = 100 μm.

Patient 2

A 49-year-old man was referred for consultation because of general fatigue and right inguinal lymph node swelling, 2 cm in diameter. Initially, malignant lymphoma was suspected due to multiple lymphadenopathy detected by whole-body CT (Fig. 3a–c). Physical examination of the groin revealed well-demarcated, pinkish erythematous plaques with dotty scale and erosion on the scrotum (Fig. 3d). Histological examination of the cutaneous primary lesion revealed invasive EMPD. Pathological analysis identified nodal lymphangiogenesis and tumour cell invasion of lymphatic sinusoids within inguinal lymph nodes. Such multiple lymphadenopathy was determined to be due to lymph node metastases. Although systemic chemotherapy with 5-fluorouracil and cisplatin was administered in addition to trastuzumab treatment, the patient died of tumour progression 39 months after the primary consultation.

Materials and methods

Primary tumours or regional lymph nodes from patients were fixed in buffered formalin or embedded in OCT compound (Sakura Finetek, Torrance, CA, U.S.A.) and snap-frozen. Sections (5 μm) were immunostained as described,[2] using the primary antibodies: NZ-1 for podoplanin, OV-TL 12/30 for

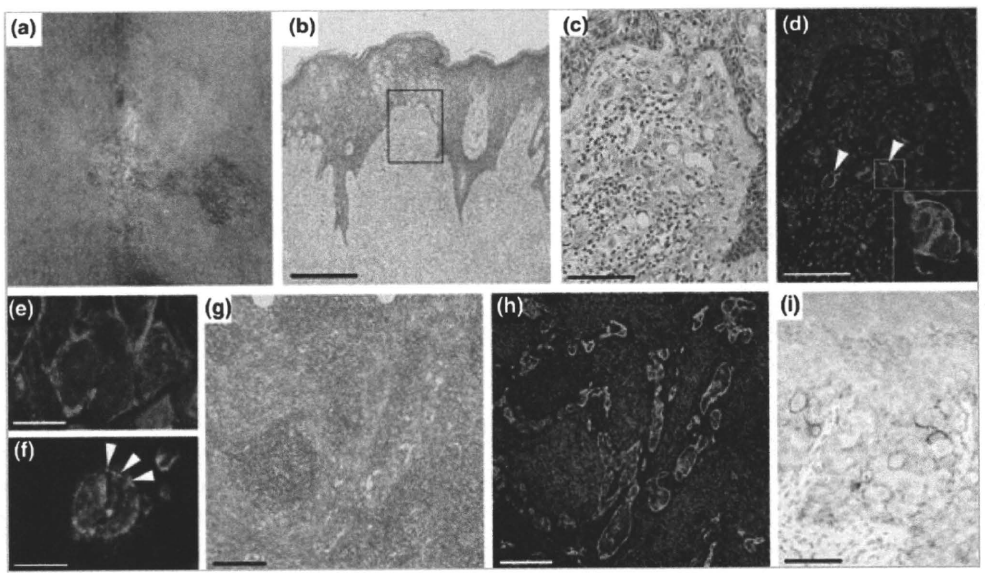

Fig 2. Noninvasive appearance and aggressive features of highly metastatic extramammary Paget disease. (a) Macroscopic appearance of the primary lesion in patient 1. Moderate and demarcated erythema was found with focal erosion in the perineum. (b) Haematoxylin and eosin (H&E) staining of tissue from the primary site shows epidermal hyperplasia with tumour cells spreading within the epidermis in a pagetoid pattern. (c) High-power analysis of boxed region in (b) shows that Paget cells invade the papillary dermis. (d) Double immunofluorescence staining of a serial section shows lymphatic invasion by cytokeratin 7-positive Paget cells (red) within podoplanin-positive tumour-associated lymphatic vessels (green, arrowheads). Inset shows a higher-power magnification of lymphatic invasion. (e, f) Double immunofluorescence for cytokeratin 7 (red) and N-cadherin (green) demonstrates that Paget cells in carcinoma *in situ* do not express N-cadherin (e), whereas invasive Paget cells show N-cadherin positivity on the cell surface (f, arrowheads). (g) H&E staining shows metastatic foci in regional/inguinal lymph nodes obtained from surgical resection. (h) Double staining for cytokeratin 7 (red) and podoplanin (green) indicates marked induction of sinusoidal lymphatic vessel growth, suggesting that Paget cells invade and metastasize via lymphatic vessels. Nuclei are stained blue with 4′,6-diamidino-2-phenylindole. (i) Immunohistochemical staining for HER2 shows that Paget cells express the receptor tyrosine kinase on their cell surface. Scale bars = 500 μm (b); 100 μm (c, d); 10 μm (e, f); 200 μm (g, h), 50 μm (i).

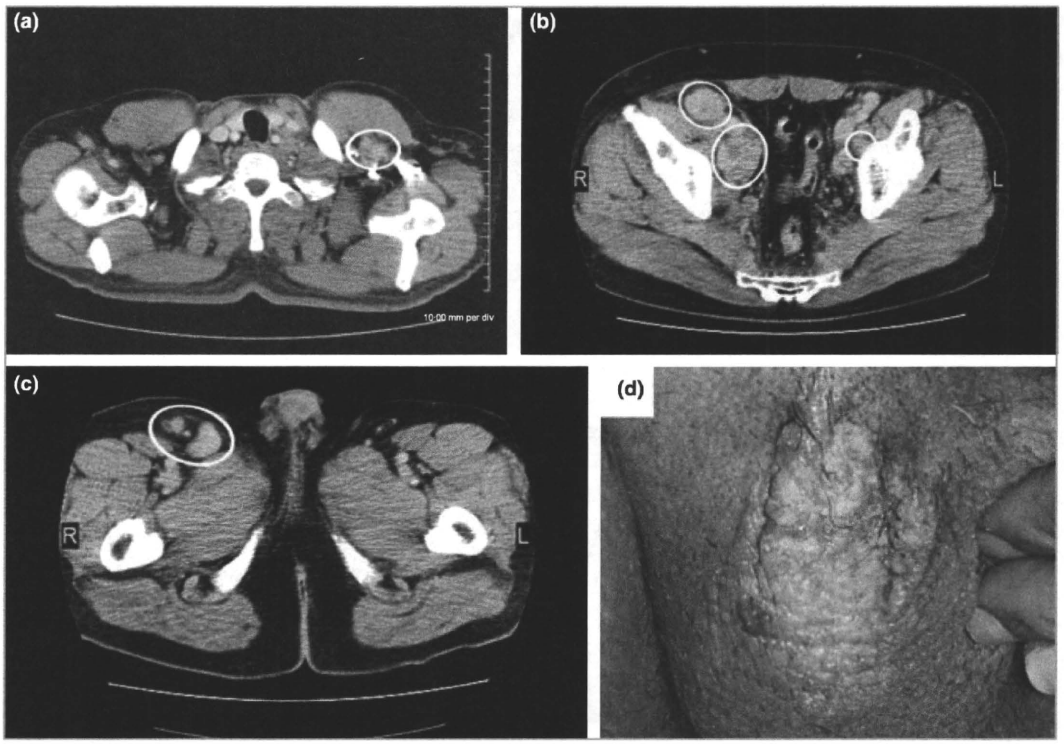

Fig 3. Multiple lymphadenopathy and clinical features in patient 2 with extramammary Paget disease. A whole-body computed tomographic image shows multiple swollen lymph nodes in the left supraclavicular (a), the bilateral external iliac (b) and the right inguinal regions (c), all indicated by circles. (d) Macroscopic appearance of cutaneous primary lesion shows demarcated, erythematous plaques with small erosions on the scrotum.

cytokeratin 7 (Dako, Carpinteria, CA, U.S.A.), 13A9 for N-cadherin (Millipore, Billerica, MA, U.S.A.) and EP1045Y for HER2 (Epitomics, Burlingame, CA, U.S.A.). The respective secondary antibodies were labelled with Alexa Fluor 488 or 594 (Molecular Probes, Eugene, OR, U.S.A.). Antigens were retrieved in paraffin sections by incubation with citrate buffer (pH 6·0 for 30 min at 95 °C) prior to immunostaining. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes). Sections were also immunohistochemically stained using a 3-amino-9-ethylcarbazole peroxidase substrate kit (Vector Laboratories, Burlingame, CA, U.S.A.). Respective control IgG was stained as a specificity control. Sections were examined and digital images were captured using a confocal laser scanning microscope (LSM510; Carl Zeiss, Jena, Germany). This study was approved by the institutional review boards of the Graduate Schools of Medicine at Ehime and Osaka Universities.

Results

Low-power microscopic evaluation of H&E-stained specimens from the primary lesion in patient 1 revealed adenocarcinoma within the epidermis (Fig. 2b). Higher-power analysis identified dermal invasion by Paget cells (Fig. 2c). Double immunofluorescence for cytokeratin 7 and podoplanin revealed invasion by Paget cells in the direction of tumour-associated lymphatic vessels within the stroma (Fig. 2d, arrowheads). Furthermore, double staining for cytokeratin 7 and the mesenchymal marker N-cadherin showed that invasive Paget cells expressed cell surface N-cadherin (Fig. 2f, arrowheads), whereas Paget cells seen in carcinoma in situ did not express N-cadherin (Fig. 2e). This finding suggests that Paget cells adopt an invasive phenotype during epithelial–mesenchymal transition (EMT) seen in tumour progression. Regional lymph nodes obtained by surgical resection indicated the presence of tumour metastasis (Fig. 2g). Differential staining for cytokeratin 7 and podoplanin revealed marked lymphangiogenesis and massive tumour cell invasion towards sinusoidal lymphatic vessels within lymph nodes (Fig. 2h).

Discussion

EMPD should be considered in differential diagnosis of lymphadenopathy of unknown origin. Multiple lymphadenopathy is a major symptom of patients with invasive EMPD. Cutaneous manifestations of EMPD include erythema and/or demarcated plaques resembling dermatitis or psoriasis.[5] Meanwhile, as a common site affected by EMPD is the external genitalia, clinical diagnosis may be delayed due to the unique location of the disease. Indeed, Paget cells can metastasize to regional and/or distant lymph nodes via lymphatic vessels. Nodal lymphangiogenesis has recently been shown to promote enhanced tumour metastasis, leading to progression of nodal metastasis in EMPD.[2] In this report, we observed marked lymphangio-

genesis in the sinusoidal lymphatic network, which was strikingly enhanced in metastatic lymph nodes. Therefore, multiple lymphadenopathy may be indicative of nodal lymphangiogenesis in patients with EMPD.

Tumour cell invasion of lymphatic vessels is one of the crucial events that predict decreased patient survival in cutaneous malignant neoplasms such as melanoma.[6–8] Clinically, lymphatic invasion of regional lymph nodes is positively correlated with reduced patient survival in EMPD.[2] In the present cases, metastatic Paget cells showed marked lymphatic invasion within regional lymph nodes. These observations corroborate our recent findings indicating the significance of this activity in relation to development of distant lymph node metastases and reduced patient survival.[2] We found that invasive Paget cells in patient 1 express N-cadherin, a mesenchymal marker indicative of EMT, suggesting a molecular mechanism underlying tumour invasion. EMT is reportedly required for invasive phenotypes of tumour cells of epithelial origin, including EMPD.[2,9] Therefore, EMT is likely to promote aggressive phenotypes characteristic of highly metastatic EMPD.

EMPD is classified as either a primary or secondary disorder. Secondary Paget disease represents a cutaneous extension of an underlying adnexal adenocarcinoma.[1,5] In the present cases, no suspicious internal malignancies were detected by general examination. Therefore, both cases are likely to represent primary EMPD. In fact, EMPD is predominantly of cutaneous primary origin, as compared with a secondary disorder, in Asian countries.

What's already known about this topic?

- Tumour metastasis may occur via lymphatic vessels in patients with extramammary Paget disease (EMPD). Nodal lymphangiogenesis has recently been shown to play a key role in mediating distant lymph node and organ metastases in this disease.

What does this study add?

- EMPD should be considered as a differential diagnosis in patients with multiple lymphadenopathies that precede cutaneous manifestations. Lymphadenopathies may represent nodal lymphangiogenesis and lymph node metastases associated with EMPD.

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Invited review article

Regulation of pathological lymphangiogenesis requires factors distinct from those governing physiological lymphangiogenesis

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Vascular development

ABSTRACT

Physiological lymphangiogenesis requires key factors such as vascular endothelial growth factor (VEGF)-C and the homeodomain transcription factor Prox1 to induce the formation of primitive lymph sacs from veins during mammalian development. However, pathological lymphangiogenesis, defined as new lymphatic vessel growth resulting from pathogenic stimuli, may utilize additional signaling pathways and/or cell types in conditions such as tumor progression or inflammatory responses. In fact, although both physiological and pathological lymphatic vascular development share fundamental mechanisms, pleiotropic growth factors and/or pro-inflammatory cytokines mediate lymphangiogenesis in experimental models of pathologic lymphangiogenesis. This review summarizes molecular mechanisms underlying lymphangiogenesis in pathological conditions and focuses in particular on current findings relevant to tumor-associated lymphangiogenesis.

Contents

1. Introduction

Lymphatic vessels give rise to the lymphatic system in conjunction with lymph nodes to facilitate effective immune surveillance in the mammalian body. This structure allows lymphatic vessels to drain lymph, the interstitial fluid in peripheral organs, to the blood circulation [1]. However, pathological conditions such as tumor progression induce new lymphatic vessel growth within primary tumor sites and promote enhanced metastasis to draining lymph nodes [2–4]. Such tumor-associated lymphangiogenesis was first described in 2001 [2–5]. Since then, numerous experimental models and relevant human disease conditions have been analyzed in investigations of pathological lymphangiogenesis. On the other hand, key mechanisms underlying physiological lymphangiogenesis have been identified [1,6–8]. Functional studies of the homeodomain transcription factor Prox1 showed that lymphatic vessels originate from veins during mammalian embryonic development [9,10]. Furthermore, vascular

Abbreviations: COUP-TFII, chicken ovalbumin upstream promoter transcription factor; EMT, epithelial–mesenchymal transition; ESAM, endothelial specific adhesion molecule; LEC, lymphatic endothelial cell; LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1; CLEC-2, C-type lectin-like receptor 2; EMPD, extramammary Paget's disease; M-CSF, macrophage colony-stimulating factor; SCC, squamous cell carcinoma; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

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endothelial growth factor (VEGF)-C, a key growth factor in physiological lymphangiogenesis, was found to promote activation of VEGFR-3, a receptor tyrosine kinase expressed in lymphatic endothelium [11,12]. Inactivation of genes encoding either *Prox1* or *vegf-c* in mouse promotes loss of lymph sacs, the primitive organs for lymphatic vessels [9,13].

Physiological and pathological lymphangiogenesis likely share certain biological mechanisms. However, substantial evidence indicates that pathological lymphatic vessel growth requires additional factors. Cell types such as inflammatory cells, bone-marrow-derived progenitors, and/or tumor cells, which produce pleiotropic growth factors, pro-inflammatory cytokines, and/or chemokines, are recruited in pathological conditions. These molecules likely alter the structure and function of lymphatic vessels and contribute the pathogenesis of cutaneous diseases. The author has a long-term interest in tumor-associated lymphangiogenesis and the translation of basic research to clinical settings [14–17]. Therefore, this article will review events in tumor lymphangiogenesis and focus on pathological lymphangiogensis in comparison with physiological lymphangiogenesis.

2. Lymphatic vessels as a vital network in the mammalian vascular system

Lymphatic vessels are distributed to most organs including the skin, although some organs such as brain or bone are devoid of lymphatic vessels. Cutaneous lymphatic vessels consist of a dense network that begins by blunt end (Fig. 1, arrowheads). These thin-walled capillary-sized vessels are called 'initial lymphatics' and drain to collecting lymphatic vessels [18]. Furthermore, lymphatic vessels are composed of both peripheral lymphatics and the lymphatic trunk. These vessels communicate with each other to drain lymph into the systemic circulation. Lymphatic vessels have two major functions. One is to absorb and transport interstitial fluids and macromolecules that arise in the extracellular space of peripheral tissues. Lymphatic capillaries absorb interstitial fluid, whereas collecting lymphatics transport the protein-rich lymph to the proximal site. Subsequently, the thoracic duct, a major lymphatic trunk, drains to the venous circulation around the left subclavian or left branchiocephalic vein. The other function of lymphatic vessels is to promote chemo-attraction of immune cells for immune surveillance.

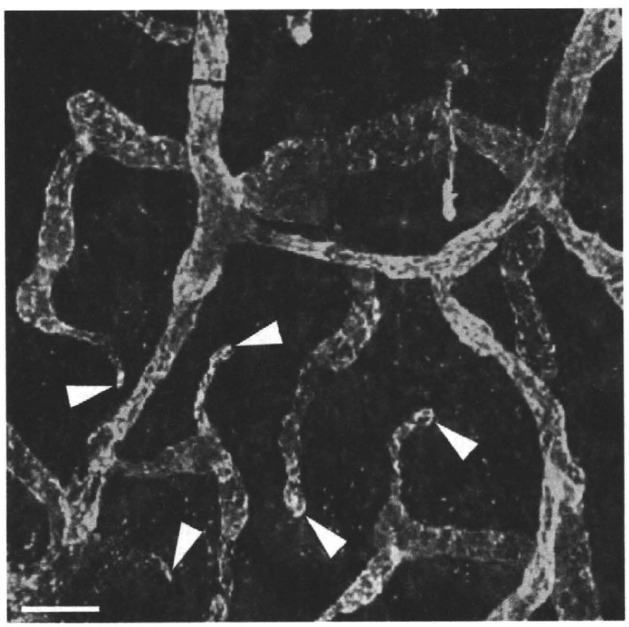

Fig. 1. LYVE-1 is a specific marker of lymphatic capillaries in mouse skin. Double immunofluorescence for LYVE-1 (red) and podoplanin (green). The podoplanin-positive vascular network indicates lymphatic vessels in the skin. Among them, initial lymphatic vessels beginning with a blunt ending (arrowheads) are positive for both LYVE-1 and podoplanin (merge), indicating that LYVE-1 is a marker of lymphatic capillaries. Scale bar: 100 μm.

Recent experimental studies demonstrate that lymphatic vessels play a crucial role in the pathogenesis of hypertension or obesity and undergo major alterations associated with circulatory factors or fat metabolism [19,20]. A high salt diet induces not only hypertension but also sodium accumulation in the skin. A recent study in rats showed that hypertonic stimuli induce cutaneous lymphatic vessel growth though activation of macrophages [20]. The newly formed lymphatic vessels control dermal extracellular volume by efficiently draining interstitial fluid and electrolytes into the circulation. Thus, lymphatic vessels regulate interstitial volume balance and blood pressure, providing a buffering system against experimentally-induced hypertension. Insufficient development of lymphatic vessels also causes adult-onset obesity in *Prox1*-heterozygous mice, which exhibit reduced Prox1 levels [19]. This condition likely results from abnormal leakage from poorly patterned or ruptured lymphatic vessels, promoting lymph accumulation in adipose tissue. A recent study further showed that a high fat diet induces macroscopic swelling and lipid accumulation in the skin of mice with hypercholesterolemia due to apolipoprotein-E deficiency [21]. Furthermore, these mice show markedly enlarged initial lymphatic vessels, and impaired pericyte coverage and valve formation, leading to decreased drainage and abnormal leakage by lymphatic vessels. These findings indicate a close association between fat metabolism and lymphatic vessel function.

3. Establishment of lymphatic-vessel specific markers

Recent progress in understanding lymphatic vascular development and pathology was facilitated by discovery of molecules specifically expressed in lymphatic vessels. In particular, antibodies against lymphatic vessel-specific glycoproteins such as podoplanin or the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) have been widely used to identify lymphatic vessels in physiological and pathological conditions. LYVE-1, a member of the link protein superfamily, was originally identified as a CD44 homologue [22]. LYVE-1 is expressed by lymphatic endothelial cells and is highly specific for lymphatic capillaries (Fig. 1, merge). In the skin, the first lymphatic vessels appear LYVE-1-positive capillaries (Fig. 1). LYVE-1 is one of the most useful markers to visualize lymphatic vessels in mouse experimental models such as that of chemically-induced skin carcinogenesis (Fig. 2C). Loss-of-function studies, however, reveal no specific phenotypes in LYVE-1 null mice [23,24]. In contrast, another study shows that functional inactivation of LYVE-1 in mice resulted in enlargement of capillary-sized lymphatic vessels and in constitutively increased interstitial-lymphatic flow. Therefore, conclusive proof of detailed function in LYVE-1 remains controversial [25]. Another marker of lymphatic vessels, podoplanin, is a sialomucin-type glycoprotein originally identified on the surface of podocytes in rat glomeruli [26]. Podoplanin shows differential expression in cutaneous lymphatic capillaries and collectors from blood vessels (Fig. 1, green). D2-40, a monoclonal antibody recognizing podoplanin, is widely used to diagnose human skin diseases (Fig. 3C). Of note, double staining for podoplanin and Prox1 defines lymphatic vessels, even in pathological conditions such as tumor progression (Fig. 3D). NZ-1, another monoclonal rat IgG against podoplanin, has been used to identify lymphatic vessels in several types of human skin tumors, such as cutaneous malignant melanoma [27]. Recent loss-of-function studies demonstrate that

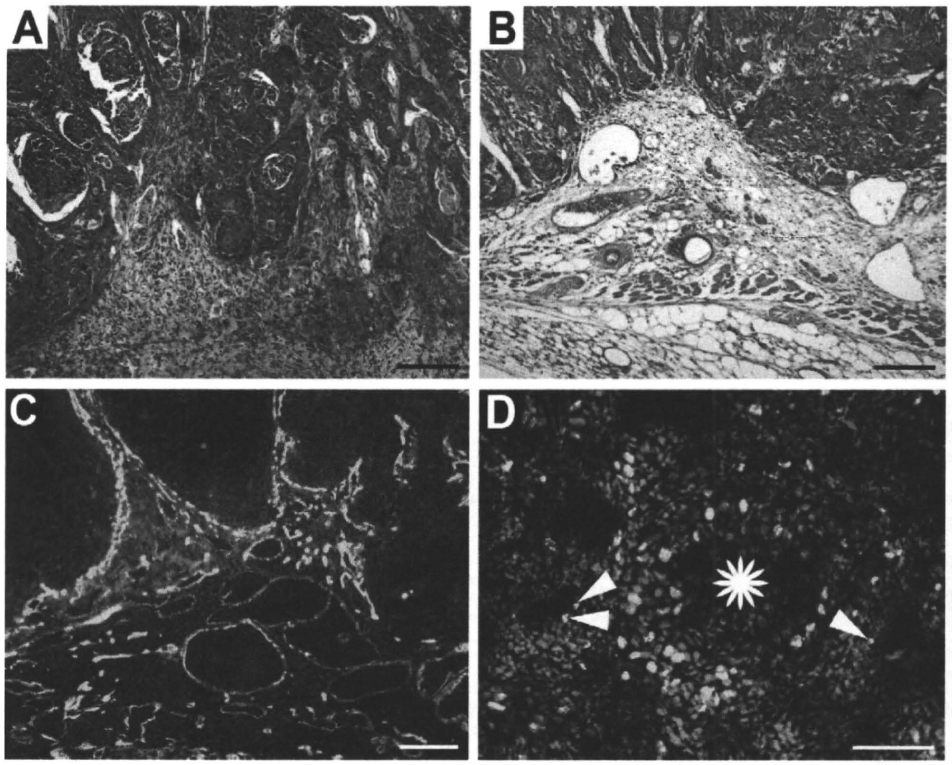

Fig. 2. Targeted VEGF-A overexpression enhances edema formation and tumor lymphangiogenesis in chemically-induced mouse skin carcinogenesis. (A) and (B) Invasive front of squamous cell carcinomas in wild-type mice (A) or Keratin 14-promoter-driven VEGF-A transgenic mice (B). Note that stromal edema is prominent in VEGF-A-overexpressing SCCs compared with controls. (C) Double immunofluorescence for CD31 (green) and LYVE-1 (red) shows marked enlargement of tumor-associated blood and lymphatic vessels in VEGF-A-overexpressing SCCs. (D) Double immunofluorescence staining for BrdU (green) and LYVE-1 (red) shows active proliferation of tumor-associated lymphatic endothelial cells (arrowheads) surrounding tumor nests overexpressing VEGF-A (asterisk), indicating that VEGF-A is a potent lymphangiogenesis factor in tumor progression. Nuclei are stained in blue (DAPI). Scale bars: 200 μm (A–C); 100 μm (D).

podoplanin plays an essential role in separating lymphatic vessels from blood vessels during mouse embryogenesis [28–30]. C-type lectin-like receptor 2 (CLEC-2), a functional podoplanin receptor, is abundantly expressed by platelets [31]. During embryogenesis, communication between blood circulation and primitive lymph sacs allows platelets to contact lymphatic endothelial cells expressing podoplanin. Subsequently, podoplanin/CLEC-2 interaction activates downstream signaling leading to blood coagulation. These clots are essential for separation of lymph sacs from blood vessels during mouse embryogenesis. Accordingly, podoplanin- or CLEC-2-null embryonic mice show defects in blood aggregation adjacent to primitive lymph sacs and exhibit altered lymphatic vessels filled with blood. Indeed, SLP-76 and Syk76, downstream effectors of the podoplanin/CLEC-2 axis, play a key role to separate lymphatic from blood vessels [32] (Fig. 4).

4. Lymphatic vessels connect the skin and lymph nodes in the immune system

The mammalian epidermis maintains a barrier to protect an organism from pathogens and immunogenic antigens. For this purpose, Langerhans cells are responsible for capturing hapten and then migrate to lymph nodes for antigen presentation. In fact, dendritic cells including Langerhans cells can enter the lymphatic vessels in the skin. Recent studies show that lymphatic endothelial cells in initial lymphatic vessels form a 'button-like junction', which represents a flap enabling dendritic cells to enter the lymphatic vessels without enzymatic digestion of the lymphatic endothelium [18,33].

Lymphatic vessels and lymph nodes cooperatively function as an immune system. Lymphatic vessels show a sinusoidal network pattern in lymph nodes. A recent study indicates that lymphatic endothelial cells and/or primitive lymph sacs are required for formation of the lymph node capsule in *Prox1* conditional null embryos [34]. Furthermore, these mice show altered positioning of hematopoietic cells within developing lymph nodes. In fact, lymph sacs are not required to initiate lymph node formation during mouse embryonic development; however, sinusoidal lymphatic endothelial cells are required for mesenchymal cell differentiation and attraction of lymphoid tissue inducer cells in developing mouse lymph nodes. Of interest, a recent study showed that CCL21, the chemokine responsible for attracting lymphoid tissue inducer cells, promotes formation of a new lymphoid-like reticular stromal network in B16-F10 melanoma-derived peri-tumoral stroma and recruitment of regulatory T-cells [35]. Such tumor cell-based manipulation of the host immune system leads to formation of a tolerogenic tumor microenvironment, facilitating tumor progression. In contrast, another recent study showed that a subset of lymphoid tissue-inducer cells that express the natural cytotoxicity receptor NKp46 contribute to antitumor immunity in B16 melanoma-associated tumor vasculature [36]. Importantly, NKp46-positive lymphoid tissue-inducer cells are activated by interleukin-12, and upregulate adhesion molecules in the tumor vasculature, resulting in recruitment of leukocytes and tumor suppression.

5. Pathological lymphangiogenesis requires diverse cell types

Florence Sabin, a twentieth century pioneer in anatomy, proposed in 1902 that lymphatic vessels arise from veins during mammalian embryogenesis [37]. This elegant concept was confirmed in 1999 by knockout of mouse *Prox1*. Null mice exhibited arrest of budding and sprouting of lymphatic endothelial cells from veins at E11.5–12.0, leading to absence of lymphatic

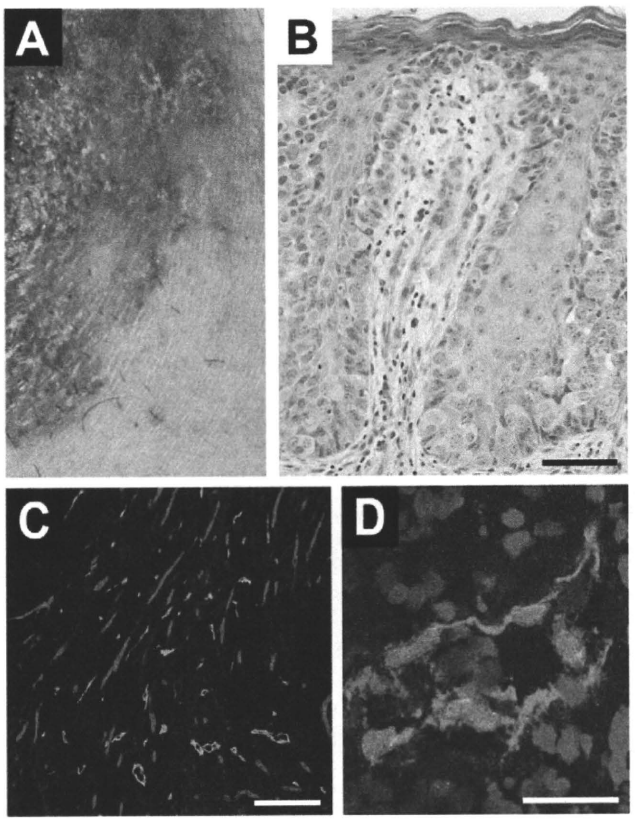

Fig. 3. Tumor lymphangiogenesis in extramammary Paget's disease (EMPD). (A) Macroscopic appearance of genitalia of a 68-year-old man with EMPD. A demarcated erythema was found with scale-crusts and small-sized erosions. (B) H&E stains show that Paget cells appear rounded in the hyperplastic epidermis and that dermal papillae are markedly elongated. Marked edema and penetration of capillary-sized blood vessels are observed within the papillary dermis, indicating that tumor-associated angiogenesis is induced in EMPD. (C) Double immunofluorescence for podoplanin (green) and von Willebrand factor, a blood vessel marker (red) show a marked increase in lymphatic and blood vessels within primary EMPD sites. (D) Podoplanin staining of tumor-associated lymphatic vessels (green) was also Prox1-positive (red), confirming their lymphatic identity. Nuclei are stained in blue (DAPI). Scale bars: 50 μm (B); 100 μm (C); 20 μm (D).

vasculature and embryonic lethality [9]. Thus, in embryonic mouse, physiological venous development is sufficient for lymphatic vascular development and other lymphatic precursors of hematopoietic or mesenchymal origin make marginal contribution to the process [38].

To gain insight into the significance of veins to lymphatic vascular development, a recent study showed that COUP-TFII, a key transcription factor functioning in specification of veins from arteries, plays an essential role to activate Prox1 expression during murine embryogenesis. COUP-TFII physically interacts with Prox1 to regulate lineage-specific genes in cultured lymphatic endothelial cells [39,40], Furthermore, the SRY-related HMG domain transcription factor Sox18 was shown to be an upstream regulator of Prox1 in venous lymphatic endothelial cell progenitors [41]. Thus, emerging evidence has shed light on molecular mechanisms underlying transcriptional regulation of lymphatic vessel development.

Although it resembles physiological lymphangiogenesis, pathological lymphangiogenesis following human renal transplantation employs additional lymphatic endothelial cells from bone marrow-derived precursors [42]. Trans-differentiating CD11b-positive macrophages also function in pathological lymphangiogenesis following penetrating keratoplasty in mouse cornea [43] or during cutaneous wound healing in a diabetic mouse model [44]. In addition, little or no incorporation of bone-marrow-derived endothelial progenitor cells was observed in experimental tumor models, indicating that pre-existing lymphatic vessels are largely required for tumor lymphangiogesis [45]. Activated macrophages are commonly acknowledged to be a major source of VEGF-C and/or VEGF-D, both of which potently induce new lymphatic vessel development during tumor progression. Tumor-associated macrophages were originally identified to promote tumor lymphangiogenesis in human uterine cervical cancers [46]. Later, several experimental or pathological studies indicate that tumor-associated macrophages contribute to new lymphatic vessel growth [47–49]. Of note, a mouse orthotopic xenograft model revealed that F4/80-positive macrophages are a major source of VEGF-C and VEGF-D, and that the cyclooxygenase-2 inhibitor etodolac reduces draining lymph node metastases in addition to tumor lymphangiogenesis [49]. Furthermore, mice harboring a mutation in the *csf-1* gene, which encodes macrophage colony-stimulating factor (M-CSF), demonstrated that M-CSF deficiency suppressed lymphangiogenesis and lymph node metastasis in experimental tumor models [50], supporting the concept that cells of the monocyte lineage play a fundamental role in promoting tumor-associated lymphangiogenesis.

6. Tumor lymphangiogenesis: inflammatory conditions enhance lymphatic vessel growth in a tumor microenvironment

Sentinel and/or regional lymph node metastasis are often the first indicators of spread of malignant tumors from the primary lesion. Furthermore, lymph node metastases in distant sites indicate disease progression in certain types of cancer. Therefore, the significance of lymph node metastasis is well documented in malignant neoplasms such as cutaneous melanoma [51]. Recent pathological studies show that tumor lymphangiogenesis promotes enhanced sentinel or regional lymph node metastasis in several types of malignant neoplasm, including cutaneous melanoma [52,53]. Importantly, tumor-associated lymphangiogenesis in melanoma patients is not only correlated with enhanced sentinel lymph node metastasis but also predicts reduced patient survival [47].

Vascular endothelial growth factor (VEGF)-C and its receptor VEGFR-3/Flt4 are essential for lymphatic vascular development (Fig. 5) [13,54]. Emerging evidence shows that VEGF-C/VEGFR-3 signaling also play a key role in tumor lymphangiogenesis [2,4,5]. VEGFR-3 blockade by neutralizing antibodies or a soluble form of VEGFR-3 markedly reduces VEGF-C-dependent tumor lymphangiogenesis and draining lymph node metastasis in experimental tumor models [55–57]. Based on this finding, a monoclonal single chain fragment targeting human VEGF-C was recently generated using phase display techniques [58]. This small fragment inhibits binding of VEGF-C not only to VEGFR-3/Flt4 but also to VEGFR-2/KDR. Intriguingly, recent studies indicate that VEGFR-2 is an endogenous inhibitor of lymphangiogenesis [59,60]. *VEGFR-2/KDR* yields two splicing variant forms with or without the transmembrane and intracellular tyrosine kinase domains. In fact, avascular areas such as mouse epidermis or cornea abundantly express the soluble form of VEGFR (sVEGFR)-2/KDR, which binds to VEGF-C, demonstrating that sVEGFR-2 is an endogenous VEGF-C antagonist *in vivo* [59]. Meanwhile, membrane-bound VEGFR-2 has been shown to internalize VEGF-C by inducing formation of a VEGFR-2/VEGF-C complex in blood vascular endothelial cells, leading to delayed lymphangiogenesis in a mouse corneal micropocket assay [60]. However, it should be noted that lymphatic endothelial cells express VEGFR-2, which responds to VEGF-A *in vitro* and *in vivo* (Fig. 5) [14,61–63].

In addition to VEGF-C, pathological lymphangiogenesis is induced by pro-inflammatory cytokines. As noted, physiological

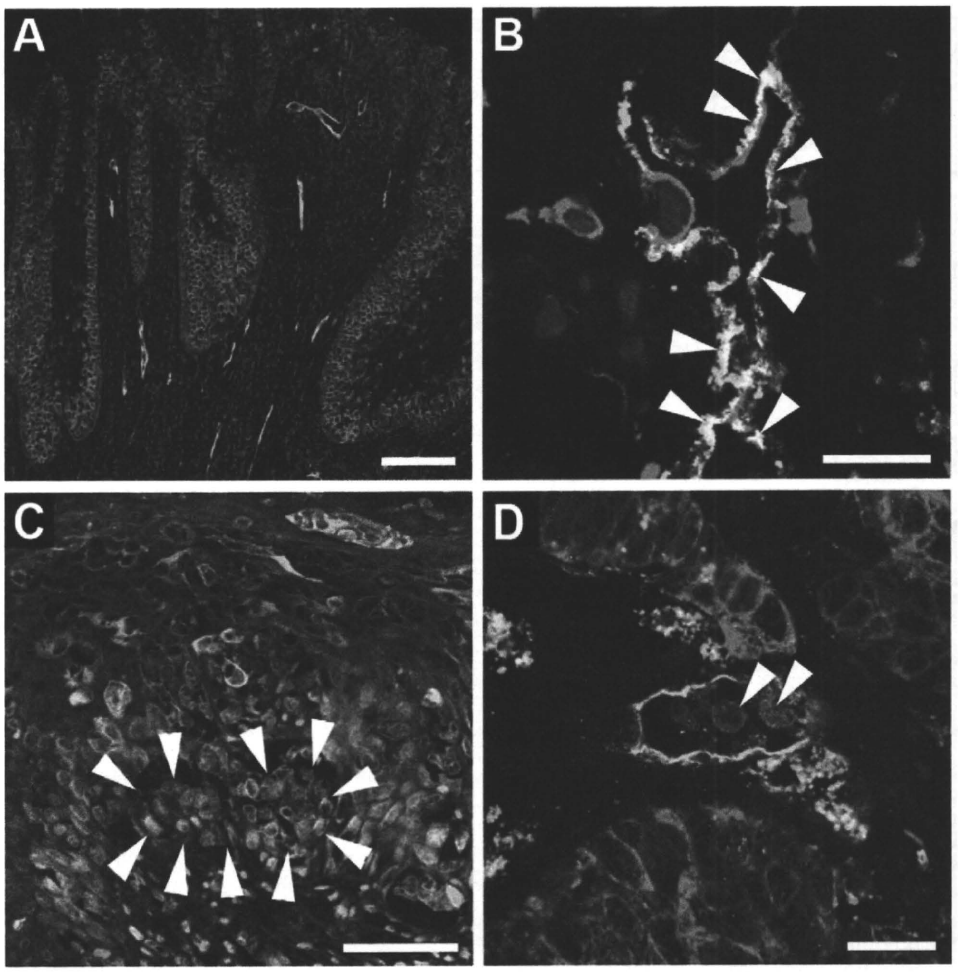

Fig. 4. Phenotypic alterations in invasive Paget cells and tumor-associated lymphatic vessels in EMPD. (A) Double immunofluorescence for cytokeratin 7 (red) and podoplanin (green) in carcinoma *in situ*. Respective stains are mutually exclusive, indicating no association between Paget cells and tumor-associated lymphatic vessels. (B) Double immunofluorescence for CXCL12 (red) and LYVE-1 (green) in carcinoma *in situ*. Tumor associated-lymphatic endothelial cells express CXCL12 (arrowheads, yellow), a chemoattractant for invasive tumor cells. (C) Double immunofluorescence for E-cadherin (green) and N-cadherin (red) in invasive EMPD. Little or no E-cadherin was observed in invasive Paget cells, whereas these tumor cells express N-cadherin, a mesenchymal marker in dermo-epidermal junction (arrowheads), indicating that invasive Paget cells undergo epithelial-to-mesenchymal transitions. Nuclei are stained in blue (DAPI). (D) Double immunofluorescence for cytokeratin 7 (red) and podoplanin (green) in invasive EMPD. Among massive invasion by Paget cells within the stroma, podoplanin-positive lymphatic vessels are enlarged and invaded by Paget cells (arrowheads). Nuclei are stained in blue (DAPI). Scale bars: 100 μm (A); 50 μm (B and C); 30 μm (D).

lymphatic vessel development is regulated by transcription factors such as Prox1 and by the growth factor VEGF-C. Furthermore, several growth factors and cytokines have been shown to induce experimental and/or pathological lymphangiogenesis in animal models. Recent studies show that tumor necrosis factor-α and interleukin-1β play a critical role in bacteria-induced airway inflammation [64,65]. Furthermore, transgenic overexpression of hepatocyte growth factor induces marked density and enlargement of lymphatic vessels in the skin [66]. Meanwhile, functional blokade of transforming growth factor (TGF)-β leads to marked increases in new lymphatic vessel growth in the inflammatory tissue response or in experimental mouse tumor models, indicating that TGF-β negatively regulates pathological lymphangiogenesis [67]. In mouse, targeted VEGF-A overexpression in skin promotes lymphangiogenesis within primary tumor sites in a chemically-induced mouse skin carcinogenesis regimen (Fig. 3), leading to enhanced draining lymph node metastasis [14]. Indeed, epidermal overexpression of VEGF-A promotes significant enlargement of lymphatic vessels in cutaneous delayed-type hypersensitivity reactions [68], UVB irradiation [69] or cutaneous wound healing [63]. Accordingly, adenoviral expression of VEGF-A in mouse skin also promotes lymphangiogenesis as well as angiogenesis [62], supporting the idea that pathological lymphangiogenesis is mediated by pro-inflammatory cytokines and/or growth factors such as VEGF-A. Importantly, the inflammatory tissue response likely promotes enhanced tumor-associated lymphangiogenesis. Indeed, marked lymphangiogenesis has been observed in cutaneous malignant melanoma [52], extramammary Paget's disease [16], inflammatory breast cancer [70], and in an experimental ovarian cancer model [48] in which an inflammatory condition is likely induced.

7. Molecular profiles of lymphatic endothelium in pathological conditions

Tumor-associated lymphatic vessels are different from normal lymphatic vessels in the skin. Recent studies show that tumor-associated lymphatic endothelial cells show molecular profiles distinct from normal lymphatic endothelium [71]. Tumor-associated lymphatic endothelial cells were originally isolated from an experimental tumor model bearing T241 fibrosarcoma cells overexpressing VEGF-C. Comparative gene profiles showed that the endothelial specific adhesion molecule (ESAM), the transforming growth factor-β co-receptor endoglin, and CD200 are up-regulated, whereas the extracellular matrix molecule biglycan is down-regulated in cultured tumor-associated lymphatic endothe-

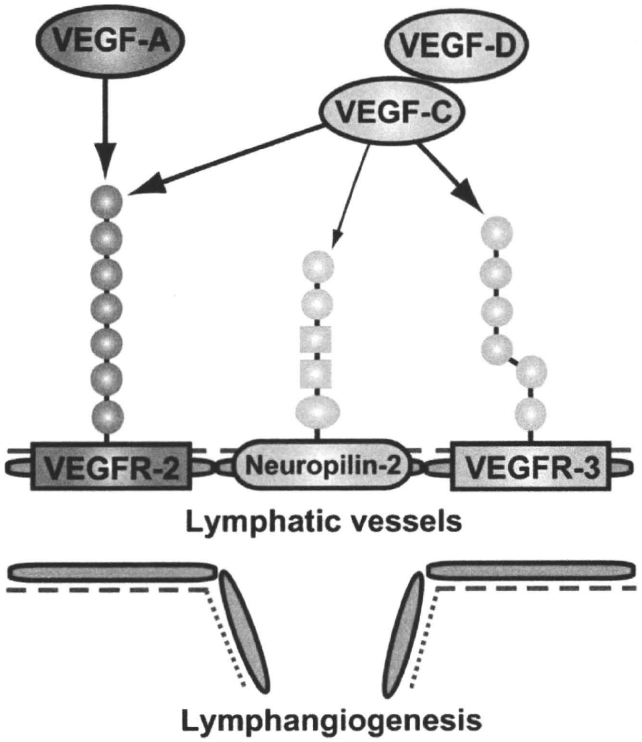

Fig. 5. VEGF family members and their cognate receptor tyrosine kinases in the tumor micoenvironment. VEGF-C and VEGF-D, potent VEGFR-3 ligands, promote lymphangiogenesis. Neuropilin-2, the VEGF-C co-receptor, is induced in lymphatic endothelial cells during tumor progression. Pro-inflammatory cytokines such as VEGF-A also promote pathological lymphangiogenesis.

lial cells relative to dermal lymphatic endothelial cells. Accordingly, ESAM was found specifically expressed in tumor-associated lymphatic vessels in VEGF-C-expressing T241 tumors and in human cancers such as head and neck squamous carcinomas (HNSCC). Importantly, a striking correlation was observed between the incidence of ESAM-positive, tumor-associated lymphatic vessels and the presence of lymph node metastases in patients with HNSCC, indicating that ESAM may have a prognostic value in relation to tumor lymphangiogesis [71].

Neuropilin-2, the VEGF-C co-receptor, is expressed in lymphatic vessels during mouse embryonic development [72]. Recent studies in either experimental tumor models [73] or human skin cancers [16] show that neuropilin-2 is expressed in tumor-associated lymphatic vessels. Importantly, functional blockade of neuropilin-2 using a neutralizing antibody decreased tumor lymphangiogenesis and induced significant delay of sentinel lymph node metastasis in experimental tumor models, indicating that neuropilin-2 may be an alternative target to prevent lymph node metastasis through lymphatic vessels [73].

Cutaneous lymphatic vessels attract immune cells such as leukocytes or dendritic cells to promote immune surveillance and the inflammatory tissue response. LECs, but not normal blood vascular endothelial cells, are a major source of CCL21, a functional ligand and chemokine for CCR7, while dendritic cells and T leukocytes express the CCR7 receptor [74,75]. Importantly, VEGF-C or the pro-inflammatory cytokine TNF-α increases CCL21 secretion in cultured lymphatic endothelial cells, leading to enhanced transmigration of either mature dendritic cells or CCR7-positive tumor cells towards lymphatic endothelial cells *in vitro* [76,77]. In fact, the CCR7/CCL21 axis reportedly promotes lymph node metastasis in malignant neoplasms such as breast cancer or malignant melanoma [78–80]. Furthermore, another chemokine network, the CXCR4/CXCL12 axis, has been demonstrated to promote lymphatic trafficking of dendritic cells in inflamed mouse skin, indicating that lymphatic vessels likely engage CXCR4-positive dendritic cells by producing CXCL12 [81]. In several types of cancer, metastatic tumor cells reportedly express CXCR4, leading to increased incidence of sentinel and/or regional lymph node metastasis [78,82]. In fact, tumor-associated rather than normal lymphatic vessels express CXCL12 during tumor progression (Fig. 4B) [16]. Thus, lymphatic vessels in pathologic conditions employ physiological mechanisms to attract immune cells.

Invasion is a biological process that promotes tumor growth and survival within primary sites. Recent studies show that tumor cells of epithelial origin may switch to a mesenchymal phenotype promoting stromal invasion (Fig. 4C). A pathological epithelial-to-mesenchymal transition (EMT) is induced by transcription factors such as Snail. Our recent study showed that A431 cells increase their CXCR4 expression following ectopic expression of Snail. Importantly, CXCR4 levels are further enhanced by TGF-β1, another key regulator of EMT, indicating that EMT may significantly increase CXCR4 expression during tumor cell invasion of stroma or tumor-associated vasculature (Fig. 4D). Thus, multiple chemokine signals likely mediate tumor cell invasion of lymphatic vessels in the tumor microenvironment and further dissemination in the lymph nodes

8. Lymph node lymphangiogenesis that facilitates metastases during tumor progression

Experimental tumor models analyzed by us and others indicate that draining lymph nodes induce a new sinusoidal lymphatic network prior to tumor metastasis [14,15,83,84]. This nodal lymphangiogenesis likely contributes to enhanced distant lymph node and/or distant organ metastases. Importantly, lymph node lymphangiogenesis is induced in certain human cancers, such as extramammary Paget's disease (EMPD) (Fig. 6A and B) [16]. Our recent study of 116 patients, the largest EMPD cohort analyzed, revealed markedly increased lymph node lymphangiogenesis during lymph node metastasis, and that event was significantly correlated with progression of nodal metastasis. Furthermore, tumor cell invasion of the sinusoidal lymphatic network indicates the presence of distant lymph node and/or distant organ metastasis, leading to reduced patient survival. In fact, marked progression of nodal lymphangiogenesis may be seen as multiple lymphadenopathy in EMPD, indicating clinical relevance [17]. Most importantly, new lymphatic vessel growth is likely induced in draining lymph nodes, even before tumors metastasize in EMPD (Fig. 6A). Thus, the functional role of the 'lymphvascular niche' needs to be clarified in order to understand how the newly formed lymphatic network contributes to tumor spread via lymphatic vessels [27]. Notably, recent studies using experimental mouse models also show that nodal lymphangiogenesis is induced in cutaneous inflammatory tissue response [85–87]. Lymph node lymphangiogenesis likely contributes to increased migration of cutaneous dendritic cells to draining lymph nodes, maintenance of lymphatic flow, and resolution of inflammation.

9. Perspectives

Recent advances in molecular technologies have enabled researchers and clinicians to visualize lymphatic vessels using specific antibodies. However, a fundamental question remains as to whether lymphatic vessels play a functional role in the pathogenesis of cutaneous diseases. In 1627 the anatomist and surgeon Gasparo Aselli discovered physiological flow in lymphatic vessels using vivisection of the mesentery in dogs [88]. His observations revealed that one function of lymphatic vessels was to transport nutrients from the intestine to proximal organs. His

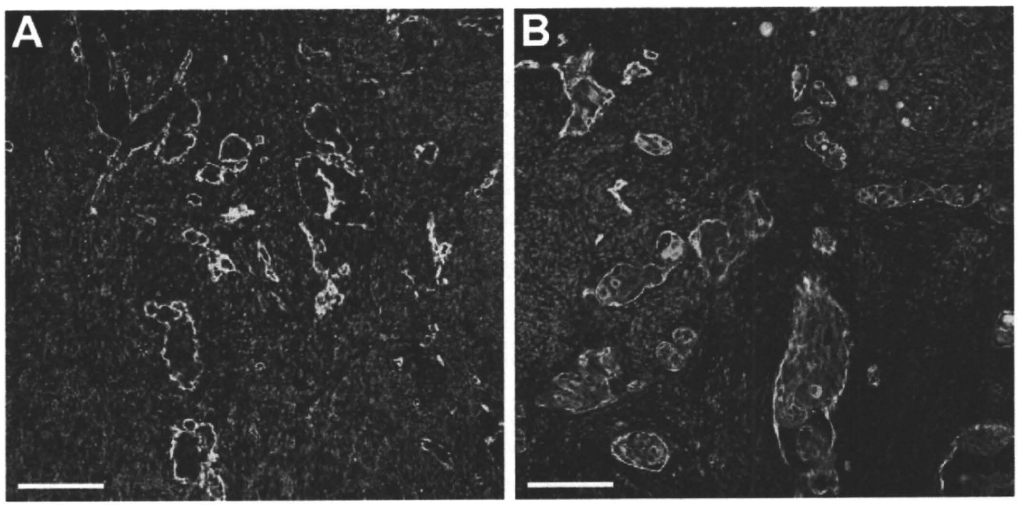

Fig. 6. Lymph node lymphangiogenesis in EMPD. Double immunofluorescence stains for von Willebrand factor (red) and podoplanin (green) in regional lymph nodes prior to tumor metastasis. New lymphatic vessel growth was observed prior to tumor cell metastasis, indicating induction of a "lymphvascular niche" for metastatic Paget cells. H&E staining shows no metastatic foci in serial sections. (B) Double immunofluorescence for cytokeratin 7 (red) and podoplanin (green) in metastatic lymph nodes. Note that metastatic Paget cells invade the tumor-associated sinusoidal lymphatic network. Nuclei are stained in blue (DAPI). Scale bars: 200 μm (A and B).

visualization of invisible vessels using white-colored lymph was a historic scientific achievement. Today, it is necessary to re-visualize the lymphatic flow to identify a functional role of lymphatic vessels in pathologic conditions such as the immune response [89] or cancer progression [90]. This experimental approach will provide a fundamental understanding of mechanisms and pathogenesis of diseases associated with lymphatic vessels. Another important question is whether lymphatic vessels could be a therapeutic target to slow disease progression. Recently, lymphatic vessel alteration was shown to be a major cause of UVB or bacterial pathogen-induced acute skin inflammation [87,91] and chronic inflammation in K14-VEGF-A transgenic mice [92]. Importantly, VEGF-C-induced VEGFR-3 activation induced marked reduction of the inflammatory tissue response to baseline levels in those experimental models [87,91,92], indicating that lymphatic vessels can be targeted to control inflammatory human skin diseases, including psoriasis vulgaris.

Pathological lymphangiogenesis is a stepwise process requiring proliferation and migration of lymphatic endothelial cells. However, it remains unclear whether lymphatic vessels initially undergo vascular hyperpermeability, potentially induced by altered cell–cell contact. Furthermore, it remains unclear whether enzymatic digestion of basement membranes occurs in lymphatic vessels during neovascularization. Therefore, mechanisms underlying pathological lymphangiogenesis must be clarified to better understand lymphatic vessel alterations that may underlie the pathogenesis of cutaneous diseases.

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Genome-Wide Approaches Reveal Functional Interleukin-4-Inducible STAT6 Binding to the Vascular Cell Adhesion Molecule 1 Promoter▽†

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Endothelial cell activation and dysfunction underlie many vascular disorders, including atherosclerosis and inflammation. Here, we show that interleukin-4 (IL-4) markedly induced vascular cell adhesion molecule 1 (VCAM-1), both in cultured endothelial cells and in the intact endothelium in mice. Combined treatment with IL-4 and tumor necrosis factor alpha (TNF-α) resulted in further, sustained induction of VCAM-1 expression. IL-4-mediated induction of VCAM-1 and secondary monocyte adhesion was predominantly regulated by the transcription factor STAT6. Genome-wide survey of IL-4-mediated STAT6 binding from sequential chromatin-immunoprecipitation with deep sequencing (chromatin immunoprecipitation sequencing [ChIP-seq]) in endothelial cells revealed regions of transient and sustained transcription factor binding. Through the combination of DNA microarrays and ChIP-seq at the same time points, the majority of IL-4-responsive genes were shown to be STAT6 dependent and associated with direct STAT6 binding to their promoter. IL-4-mediated stable binding of STAT6 led to sustained target gene expression. Moreover, our strategy led to the identification of a novel functionally important STAT6 binding site within 16 kb upstream of the VCAM-1 gene. Taken together, these findings support a critical role for STAT6 in mediating IL-4 signal transduction in endothelial cells. Identification of a novel IL-4-mediated VCAM-1 enhancer may provide a foundation for targeted therapy in vascular disease.

Endothelial cells are highly responsive to their extracellular milieu. Endothelial cell activation is a term used to describe the phenotypic response of endothelial cells to inflammatory mediators, including lipopolysaccharide, tumor necrosis factor alpha (TNF-α), and interleukin-1 (IL-1). The activation phenotype typically includes some combination of increased leukocyte adhesiveness, reduced barrier function, a shift in hemostatic balance toward the procoagulant side, and altered vasomotor tone. Many of these properties are mediated by changes in gene expression.

Vascular cell adhesion molecule 1 (VCAM-1) is a 110-kDa cell surface glycoprotein that is expressed in cytokine-activated endothelial cells. VCAM-1 is also expressed in other cell types, including smooth muscle cells and fibroblasts (8). The VCAM-1 promoter represents a potentially valuable tool for dissecting the molecular mechanisms of endothelial cell activation. Previous studies have implicated a role for NF-κB (17, 28, 29), GATA (28, 29, 44), Sp1 (34), activating protein 1 (2), interferon regulatory factor 1 (35), and SOX18 (13) in mediating inducible expression of VCAM-1. Several transcription factors have been shown to interfere with NF-κB-dependent expression of VCAM-1, including KLF4 and Oct1 (7, 12).

IL-4 is a 20-kDa pleiotropic cytokine expressed by T helper 2 (Th2) lymphocytes, eosinophils, basophils, and mast cells (reviewed in references 38 and 42). IL-4 has been shown to be necessary for stabilization of the Th2 phenotype and promotes the synthesis of IgE (reviewed in references 6 and 22). IL-4 has been implicated in the pathogenesis of atherosclerosis (reviewed in reference 21) and allergic asthma (reviewed in reference 6). Signaling of IL-4 in endothelial cells occurs via a heterodimeric IL-4 receptor (IL-4R), consisting of IL-4Rα and IL-13Rα subunits (36). Activation of the receptor results in Janus kinase 1/2 (JAK-1/2)-dependent tyrosine phosphorylation and subsequent dimerization of signal transducer and activation of transcription 6 (STAT6), which then translocates to the nucleus and binds to consensus sequences ($TTCN_{3-4}GAA$) found within promoters of IL-4-regulated target genes (14, 27). Previous studies with endothelial cells have demonstrated that IL-4 induces the expression of CXCL-8, inducible nitric oxide synthase (iNOS) (15), urokinase-type plasminogen activator (u-PA) (46), vascular endothelial growth factor (VEGF) (15), P-selectin (20, 32, 47), monocyte chemoattractant protein 1 (MCP-1) (39), CCL26 (18), IL-6 (25), 15-lipoxygenase (24), and osteoprotegerin (41). In addition, previous studies have shown that IL-4 upregulates the expression of VCAM-1 in endothelial cells (4, 10, 14, 23, 26, 37, 40). In contrast, IL-4 does not lead to increased expression of intercellular adhesion molecule 1 (ICAM-1) (10, 43) and has a variable effect on E-selectin expression (3, 10, 15).

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