Platelet Activation Receptor CLEC-2 Regulates Blood/Lymphatic Vessel Separation by Inhibiting Proliferation, Migration, and Tube Formation of Lymphatic Endothelial Cells*

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*Running title: Mechanisms of CLEC-2-regulated lymphangiogenesis
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Keywords: CLEC-2; platelets; lymphangiogenesis; bone morphogenetic protein

Conclusion: Granule contents including BMP-9 released upon platelet activation by CLEC-2-podoplanin interaction may contribute to the separation in vivo.

Significance: We proposed a novel mechanism of platelet-mediated blood/lymphatic vessel separation.
SUMMARY

The platelet activation receptor CLEC-2 plays crucial roles in thrombosis/hemostasis, tumor metastasis, and lymphangiogenesis, although its role in thrombosis/hemostasis remains controversial. An endogenous ligand for CLEC-2, podoplanin, is expressed in lymphatic endothelial cells (LECs). We and others have reported that CLEC-2-deficiency is lethal at mouse embryonic/neonatal stages associated with blood-filled lymphatics, indicating that CLEC-2 is essential for blood/lymphatic vessel separation. However, its mechanism, and whether CLEC-2 in platelets is necessary for this separation, remains unknown. We found that specific deletion of CLEC-2 from platelets leads to the misconnection of blood/lymphatic vessels. CLEC-2<sup>-/-</sup> platelets, but not by CLEC-2<sup>+/+</sup> platelets, inhibited LEC migration, proliferation and tube formation but had no effect on human umbilical vein endothelial cells. Additionally, supernatants from activated platelets significantly inhibited these three functions in LECs, suggesting that released granule contents regulate blood/lymphatic vessel separation. Bone morphologic protein-9 (BMP-9), which we found to be present in platelets and released upon activation, appears to play a key role in regulating LEC functions. Only BMP-9 inhibited tube formation, although other releasates including transforming growth factor β and platelet factor 4 inhibited proliferation and/or migration. We propose that platelets regulate blood/lymphatic vessel separation by inhibiting proliferation, migration, and tube formation of LECs mainly owing to BMP-9 released upon activation by CLEC-2/podoplanin interaction.

We have previously identified C-type lectin-like receptor 2 (CLEC-2) as a receptor for the platelet activating snake venom, rhodocytin (1). CLEC-2 is a novel class of platelet activation receptor that belongs to the C-type lectin super family and elicits robust activation signals in conjunction with the Src family kinases, Syk, and phospholipase C (PLC) γ2. The adapter protein SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) and the linker for activation of T cells are also necessary for full activation of platelets induced by CLEC-2 (1). We also identified podoplanin as an endogenous ligand for CLEC-2, which was later confirmed by other groups (2,3). Podoplanin is expressed on the surface of tumor cells and induces platelet aggregation by binding to CLEC-2, which facilitates hematogenous tumor metastasis (2,4). Podoplanin is also expressed in kidney podocytes, the origin for its nomenclature, type I lung alveolar cells, and lymphatic endothelial cells (LECs), but not in vascular endothelial cells (reviewed in (5)). Since podoplanin in these cells cannot interact with CLEC-2, which facilitates hematogenous tumor metastasis (2,4). Podoplanin is also expressed in kidney podocytes, the origin for its nomenclature, type I lung alveolar cells, and lymphatic endothelial cells (LECs), but not in vascular endothelial cells (reviewed in (5)). Since podoplanin in these cells cannot interact with CLEC-2 in the blood stream, roles for CLEC-2/podoplanin interaction in the latter three cell types have remained unknown. Recently, we and others independently generated CLEC-2-deficient mice and reported that CLEC-2-deficient mice showed embryonic/neonatal lethality, severe edema, and
blood-filled lymphatic vessels due to impaired
blood/lymphatic separation (6,7).
CLEC-2-deficient mice showed
embryonic/neonatal lethality and almost all of
the CLEC-2-/- pups die shortly after birth,
probably due to respiratory failure. These
studies revealed that the CLEC-2/podoplanin
interaction facilitates blood/lymphatic vessel
separation in the developmental stages, when
primary lymph sacs are derived from the
cardinal vein. However, since other blood cells
including neutrophils, monocytes, and dendritic
cells express CLEC-2 in mice, it is not known
whether this separation requires CLEC-2
expression in platelets. A misconnection
phenotype was also observed in mice deficient
in podoplanin and the signaling molecules
necessary for platelet activation downstream of
CLEC-2, including Syk, SLP-76, and PLCγ2
(8,9). Moreover, platelet aggregate formation,
which is an indicator of platelet activation, was
observed in the connection between the cardinal
vein and primary lymph sacs in normal mice,
but not in SLP-76- or podoplanin-deficient mice
(6,10). These findings suggest that platelet
activation is necessary for blood/lymphatic
vessel separation. However, the mechanism by
which CLEC-2 in platelets regulates
blood/lymphatic vessel separation remains to be
elucidated.

In the present study, we demonstrated
that CLEC-2 in platelets is required for the
blood/lymphatic vessel separation using mice
with CLEC-2 specifically deleted from platelets.
We found that co-culture of platelets, or
supernatants from activated platelets,
significantly inhibited cell migration,
proliferation and tube formation of LECs in a
manner dependent on CLEC-2. These findings
suggest that granule contents released upon
platelet activation inhibit lymphangiogenesis.

We found that activated platelets released
the transforming growth factor (TGF)-β family
protein bone morphogenetic protein-9 (BMP-9)
and that this was involved in platelet-mediated
inhibition of lymphangiogenesis. We proved
that BMP-9 is an important mediator and that
the combination of the platelet releasates
(BMP-9, TGF-β, platelet factor 4 (PF4),
angiostatin and endostatin) appears to account
for platelet-mediated inhibition of
lymphangiogenesis in vivo. We have previously
reported some of these results in Abstract form
for the 2010 Annual Meeting of the American
Society of Hematology (11).

**EXPERIMENTAL PROCEDURES**

*Reagents* - PF4-Cre transgenic mice were kindly donated by Prof. Radek C. Skoda (University Hospital Basel, Switzerland) (12). The glycoprotein (GP) VI agonist poly(PHG) was a generous gift from JNC Corporation (Yokohama, Japan). Lotrafiban was donated by GlaxoSmithKline (UK). Recombinant VEGF165, TGF-β, and BMP9 were purchased from R&D systems (MN, USA). Recombinant PF4 was from Hematologic Technologies, Inc. (VT, USA). Recombinant angiostatin was purchased from Enzo Life Sciences, Inc. (NY, USA). Recombinant endostatin was from CEDARLANE (NC, USA). Recombinant extracellular domain of human CLEC-2
expressed as a dimeric rabbit immunoglobulin Fc domain fusion protein (hCLEC-2-rFc2) was generated as described previously (2). Anti-BMP-9 neutralizing antibody was from R&D systems (MN, USA). Other reagents were from sources described below or previously (13,14).

**Generation of Mice** - To generate conditional CLEC-2-deficient mice, CLEC-2 flox/+ mice were generated using a targeting vector designed so that part of exon 1 flanked by two loxP sites could be deleted by expression of Cre protein (7). We crossed a CLEC-2 flox/+ mouse with a PF4-Cre transgenic mouse that specifically expresses Cre recombinase in platelets/megakaryocytes to generate PF4-Cre; CLEC-2 flox/+ mice. These mice were crossed to generate PF4-Cre; CLEC-2 flox/flox mice that have CLEC-2 specifically deleted from platelets. Genotypes of CLEC-2 floxed mice and PF4-Cre transgenic mice were analyzed as previously described (7,12). Since CLEC-2-null mice are embryonic/neonatal lethal, CLEC-2-deficient irradiated-chimeric mice were generated as described previously (7). Briefly, adult C57BL/6 male mice were given two irradiations of 500 rads from a 60Co source, 3 h apart. The mice were then rescued by intravenous injection of 1×10⁶ fetal liver cells from CLEC-2⁻/⁻ (CLEC-2 chimera) or CLEC-2⁺/+ embryos (WT chimera) at E13.5–E15.5. The reconstituted mice were used for experiments no sooner than 7 weeks following irradiation. This study was approved by the Animal Care and Use Committee in University of Yamanashi.

**Flow cytometry** - Whole blood drawn from mice as described (7) was diluted 15-fold using modified Tyrode’s buffer. Twenty-five micro liters of the diluted whole blood was incubated with Cy2-labeled anti-mouse CLEC-2 and Cy2-labeled control rabbit IgG, both of which were generated as described previously (7). Reactions were terminated by the addition of 400 μl PBS, and the samples analyzed using a FACScan (BD Biosciences, NJ, USA) and CellQuest software (BD Biosciences, NJ, USA).

**Microscopy** - Mice were anesthetized by diethyl ether and the small intestine and mesentery photographed using an Optio WG-1 digital camera (Pentax, HOYA Corporation, Tokyo, Japan). Mesentery was removed from euthanized mice and fixed in 3.7% formalin and embedded in paraffin. Sections were stained with rabbit anti-mouse LYVE-1 antibody (Abcam Inc., UK) using Simple Stain mouse MAX-PO (rabbit; Nichirei Corp.), according to the manufacturer’s instructions. Immunohistochemical analysis of embryonic back skin was performed as described previously (7).

**Cells** - Human umbilical vein endothelial cells (HUVECs) and human lymphatic endothelial cells (hLECs) were purchased from Lonza (Basel, Switzerland) and maintained on culture dishes in endothelial growth medium-2 (EGM-2) (Lonza, Basel, Switzerland) supplemented with 5% FBS and EGM-2 microvascular (MV) set (0.5 ml human EGF, 0.2 ml hydrocortone, 25 ml FBS, 0.5 ml VEGF, 2 ml human FGF-B, 0.5 ml R3-IGF-1, 0.5 ml ascorbic acid and 0.5 ml GA-1000).
Cultures were maintained at 37 °C, 5% CO₂ and 100% humidity. Conditionally immortalized, murine lymphatic endothelial cells (mLECs) were prepared as described previously (15). mLECs were maintained on gelatinized culture dishes in EGM-2 supplemented with 5% FBS and EGM-2MV set at 33 °C, 5% CO₂ and 100% humidity.

**Cell migration assay** - Transwell migration assays were performed in a modified Boyden-type Blind well chamber (Neuro Probe Inc, Gaithersburg, USA) with a 12-μm (for hLECs) or 8-μm (for HUVECs) nucleopore polycarbonate membrane separating the upper compartment from the bottom chamber containing serum-free medium (SFM) for endothelial cells, Human Endothelial SFM (Invitrogen, Life Technologies Corp., CA, USA). HUVECs or hLECs were kept in Human Endothelial SFM for 1 h, harvested, and resuspended in SFM at 3×10⁵ cells/ml. One hundred and eighty micro liters of cells were loaded into the upper compartment with 20 μl of washed platelets or indicated reagents. Where indicated, washed platelets were pretreated with 10 μM lotrafiban for 10 min. After incubation for 6 h at 37 °C with 5% CO₂, the membrane was fixed for 30 min in 70% ethanol and stained with Wright-Giemsa. Non-migrated cells from the upper surface of the filter were scraped off with a cotton bud. The membrane was mounted bottom-side-up on glass slides, and the number of migrated cells per well was determined by counting cells in five randomly selected microscopic view fields at 200× magnification.

For wound closure assay, a confluent monolayer of HUVECs or hLECs was scratched with a sterile pipette tip across the plate. The plates were washed twice with supplemented EBM-2 medium. After the wounds were marked and photographed, cells were incubated with washed platelets or indicated reagents for 20 h. Where indicated, washed platelets were pretreated with 10 μM lotrafiban for 10 min. The same place was photographed and the distance of migration was determined by measuring the width of the wound and subtracting this value from the initial width of the wound.

**Cell proliferation assay** - Cell proliferation assay was performed by Click-iT™ EdU Flow Cytometry Assay kits (Invitrogen, Life Technologies Corp., CA, USA). Sub-confluent HUVECs or hLECs were washed and kept in Human Endothelial SFM for 2 h or 6 h, respectively. After addition of a thymidine analogue, EdU together with washed platelets or indicated reagents, HUVECs and hLECs were incubated for 2 h or 4 h, respectively. Where indicated, washed platelets were pretreated with 10 μM lotrafiban for 10 min. Incorporation of EdU into the cells was measured according to the manufacturer’s instructions.

**Tube formation assay** - Sub-confluent HUVECs, hLECs, or mLECs were pre-stained with 10 μg/ml DiIC12(3) Fluorescent dye (BD Biosciences, NJ, USA) at 37 °C for 1 h, then harvested with trypsin/EDTA and detached cells collected by centrifugation at 1000 rpm for 5 min. Cell suspensions (HUVECs 5×10⁵/ml, hLECs 4×10⁵/ml, mLECs 1.25×10⁵/ml) were then prepared in supplemented EGM-2. Where
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indicated, cells were pretreated with human washed platelets or indicated reagents. Then, 50 μl of cell suspension was added to each well of a 96-well BD BioCoat Angiogenesis Plate (BD Biosciences, NJ, USA) that had been pre-coated with Matrigel Matrix. After incubation for 16 h at 37 °C (for mLECs at 33 °C), the tube-like network was visualized by a fluorescent microscopy IX71 (Olympus, Tokyo, Japan) and photographed by a digital camera DP-70 (Olympus, Tokyo, Japan). The tube-like network was traced using a BAMBOO tablet (WACOM, Saitama, Japan) and total length was quantified using Image J software.

Platelet preparation - Venous blood from healthy drug-free volunteers was collected into 10% sodium citrate. This study was approved by the Ethical Committee in University of Yamanashi, and written informed consent was provided according to the Declaration of Helsinki. Wild-type chimeras or CLEC-2 chimeras were killed with diethyl ether, and blood was drawn by post caval puncture and collected into 100 μl of acid-citrate-dextrose. Washed human or murine platelets were obtained by centrifugation as described previously using prostacyclin to prevent activation during the isolation procedure (14). Both platelets were resuspended in modified (calcium-free) Tyrode’s buffer (CFT) (14) at the indicated cell densities.

Generation of platelet activated supernatants - Washed human platelets (2 × 10^9/ml) were stimulated with CFT or 1 μg/ml of poly(PHG). Poly(PHG) is synthetic collagen fiber made by polycondensation of Pro-Hyp-Gly, which spontaneously assume polymeric structure with molecular weights greater than 10^5. Poly(PHG) has been proved to potently stimulate platelets through GPVI (16). After stimulation with poly(PHG) for 10 min without stirring, platelets were removed by centrifuge and the resultant supernatant was filtered using a 0.22-μm filter to completely remove platelets from the supernatant. The effect of the supernatant was examined at a final concentration of 10%. For BMP-9 western blotting, centrifugation was performed in the presence of 0.1 μg/ml prostaglandin I2 and 15% acid-citrate-dextrose.

Western blotting - Western blotting was performed as described previously (14). Briefly, platelet supernatants, washed human platelets (1×10^9/ml), and recombinant human BMP-9 were dissolved in SDS sample buffer, separated by 4–12% SDS-PAGE, electrotransferred, and western-blotted with rabbit anti-human BMP-9 antibody (GeneTex, CA, USA).

Statistics – Statistical significance was evaluated by Student’s t test. In each case, p values < 0.05 were taken as the minimum to indicate statistical significance.

RESULTS

Mice with CLEC-2 specifically deleted from platelets have impaired blood/lymphatic vessel separation. - In mice, CLEC-2 expression has been reported in neutrophils, monocytes and dendritic cells in addition to platelets (17,18). To examine the role of platelets in
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blood-lymphatic vascular separation, we generated mice that have tissue-specific, conditional knockout of CLEC-2 from platelets and megakaryocytes with the use of a PF4-Cre transgene (12). We previously generated CLEC-2 flox/flox mice, in which part of CLEC-2 exon 1 was flanked by two loxP sites (7). We crossed a CLEC-2 flox/flox mouse with a mouse that expresses Cre recombinase in megakaryocytes/platelets to generate PF4-Cre; CLEC-2 flox/flox mice. In contrast to CLEC-2-null mice, these mice were not embryonic/neonatal lethal. Flow cytometric analysis showed that CLEC-2 was deleted from platelets (Supplemental Fig. 1), but not from neutrophils in PF4-Cre; CLEC-2 flox/flox mice (data not shown). Mice with CLEC-2 specifically deleted from platelets have the macroscopic and histological signs of blood/lymphatic misconnection. We observed blood-filled red lymphatic vessels in the mesentery and small intestine in the conditional knockout mice, but not in the control mice (Fig. 1A). Moreover, the small intestine of the knockout mice, but not that of the control mice, was severely edematous (Fig. 1A). Histological analysis of the mesentery confirmed blood-filled lymphatic vessels in the absence of CLEC-2 (Fig. 1B).

We next performed whole-mount triple fluorescence confocal microscopic examination of embryonic back skin (E17.5) using antibodies to PECAM-1, LYVE-1, and TER-119 (a molecular marker of erythrocytes). Triple staining revealed that the dilated lymphatic vessels in embryos with CLEC-2-deficient platelets contained erythrocytes, whereas those in control embryos did not. In platelet-specific CLEC-2-deficient embryos, lymphatic vessels stained for LYVE-1 and PECAM-1 exhibited dilated, tortuous, and rugged appearances, whereas lymphatic vessels in control embryos had a narrow, straight and smooth appearance (Fig. 1C). Taken together, CLEC-2 is required for normal blood/lymphatic separation in platelets, but not in other cells.

Platelets inhibit cell migration and proliferation of hLECs, but not those of HUVECs in a CLEC-2-dependent manner. - We next investigated the mechanism of how CLEC-2 regulates blood/lymphatic vessel separation. It has been reported that platelet aggregates are observed in the connection between the lymph sac and the vein in wild-type mice but not in SLP-76 or podoplanin-deficient mice (6,10). It is possible that platelet aggregates physically occlude the connection between the lymph sac and the vein. However, it is also possible that platelets have some effect on LEC function close to platelet aggregates. Here we investigated whether platelets affect LEC migration, proliferation or tube formation.

In the Boyden chamber, hLECs or HUVECs in the upper chamber randomly migrate and pass through the micropores into the lower chamber without chemoattractant. With co-culture of human or wild-type mouse platelets, hLEC migration significantly decreased by 30% compared with the platelet-free control (Fig. 2Ai). Interestingly, CLEC-2-deficient platelets did not inhibit hLEC migration (Fig. 2Ai).
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migration, co-culture of platelets did not inhibit but rather increased HUVEC migration (Fig. 2Aii). Migration of both hLECs and HUVECs increased in the presence of VEGF-A (Fig. 2A), indicating that cells were still capable of migration during the experiment.

We also investigated migration using the wound closure assay, in which migration was measured by a shortening of the scratch width across a confluent monolayer of endothelial cells. Twenty hours after making scratches, the scratch width decreased as a result of cell migration. Co-culture of human or wild-type mouse platelets significantly inhibited hLEC migration by 50% and 30%, respectively, whereas co-culture of CLEC-2-deficient platelets did not inhibit hLEC migration (Figs. 2Bi and iii). In contrast, co-culture of platelets increased HUVEC migration (Fig. 2Bii). These findings are consistent with the results from the Boyden chamber, suggesting that platelet inhibit of hLECs, but not HUVEC migration, is dependent on CLEC-2.

Proliferation of hLECs or HUVECs was investigated by a thymidine analog incorporation assay. Co-culture of human or wild-type mouse platelets significantly inhibited hLEC proliferation by 20% (Figs. 3A, 3B). However, co-culture of CLEC-2-deficient platelets did not inhibit hLEC proliferation. Platelets did not affect proliferation of HUVECs (Figs. 3A, 3C).

Taken together, platelets inhibit hLEC migration and proliferation in a CLEC-2-dependent manner.

Platelets inhibit tube formation of LECs, but not those of HUVECs in a CLEC-2-dependent manner. Since tube formation is an integrated process of cell migration and proliferation, we next investigated the effects of platelets in tube formation of LECs. We observed that normal mouse platelets inhibit HUVEC tube formation as well as hLEC tube formation (data not shown), although HUVEC migration and proliferation were not affected (Figs. 2, 3). We have no explanation for this phenomenon, and this needs to be elucidated elsewhere. We utilized mLECs to investigate the effects of CLEC-2-deficient platelets on LEC tube formation.

Co-culture of human platelets significantly inhibited hLEC tube formation by 80% (Figs 4A, C). On the other hand, it had no inhibitory effects on HUVEC tube formation (Figs 4B, C), although a slight thickening of tube walls was observed in the presence of platelets (Fig. 4B). Figs. 4D and E showed that normal mouse platelets, but not CLEC-2-/- platelets, significantly inhibited tube formation of mLECs, as was the case in hLEC migration and proliferation. Taken together, it is suggested that platelets inhibit migration, proliferation and tube formation of LECs, but not those of HUVECs, in a CLEC-2-dependent manner.

There still remains the possibility that podoplanin generates inhibitory signals for LEC migration by binding to CLEC-2, since podoplanin directly interacts with ezrin and increased cell migration and invasiveness in MDCK cells (19). To address this possibility, we investigated the effects of recombinant extracellular domain of human CLEC-2
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expressed as a dimeric rabbit immunoglobulin Fc domain fusion protein (hCLEC-2-rFc2). hCLEC-2-rFc2 significantly inhibited migration of hLECs, but not that of HUVECs (supplemental Fig.2A). On the other hand, the recombinant CLEC-2 did not affect proliferation of hLECs/HUVECs (supplemental Fig.2B) or hLEC tube formation (supplemental Fig.2C). These findings suggest that podoplanin cross-linking also regulates LEC function at least in part.

Integrin αIIbβ3 blocker did not attenuate platelet-induced inhibition of hLEC migration, proliferation and tube formation, whereas supernatants from activated platelets inhibited hLEC migration, proliferation and tube formation. - Previous studies observed the misconnection phenotype in mice deficient in Syk, SLP-76 or PLCγ2, all of which are necessary signaling molecules in CLEC-2-mediated signal transduction (8,9). Podoplanin-deficient mice also show the same phenotype (10). Moreover, platelet aggregates are observed in the orifice of the lymph sacs in wild-type mice, but not in SLP-76- or podoplanin-deficient mice (6,10). Together, these findings suggest that platelet activation induced by CLEC-2-podoplanin interaction is necessary for blood/lymphatic vessel separation. Platelet activation leads to two events, granular release and platelet aggregate formation. We next sought to clarify which of these events is important for blood/lymphatic vessel separation. To address this question, we utilized an integrin αIIbβ3 blocker, lotrafiban, to inhibit platelet aggregation. The αIIbβ3 blocker itself did not have any effect on migration, proliferation and tube formation of hLECs (Figs. 5A, B, C; middle bars). Pretreatment of platelets with lotrafiban did not attenuate platelet-induced inhibition of hLEC migration, proliferation and tube formation (Figs. 5A, B, C; compare right and left bars), suggesting that platelet aggregation is not required for lymph/blood vessel formation.

Since αIIbβ3 blocker did not attenuate platelet-induced inhibition of hLEC migration, proliferation and tube formation, we next investigated the effects of released granule contents from platelets upon platelet activation. Platelets are activated by the GPVI agonist, poly(PHG). After separation of the activated platelets, the resultant supernatants were filtered and added to hLECs. We chose a GPVI agonist to stimulate platelets, since LECs do not express GPVI. The supernatant significantly inhibited migration, proliferation and tube formation of hLECs, while the agonist alone did not affect migration and proliferation (Figs. 6A, B, C). These findings suggest that the contents of granules released from activated platelets, but not platelet aggregates, inhibit LEC migration, proliferation and tube formation.

Platelet granule contents inhibited hLEC migration, proliferation and tube formation. - To characterize the molecule(s) that is responsible for the inhibition of LEC migration, proliferation and tube formation, we heated the supernatants from activated platelets to 100 °C and examined the effects of the heated supernatants on hLEC function. Heated supernatants lost their inhibitory effect on hLEC
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migration (Supplemental Fig. 3A) and proliferation (Supplemental Fig. 3B). Heated control supernatant (poly(PHG)-containing buffer) also had no effect on migration (Supplemental Fig. 3A) and proliferation (data not shown). These findings suggest that proteins present in the activated platelet supernatants, which are capable of being inactivated by heating, are responsible for the inhibition of hLEC function.

We next investigated a molecule responsible for platelet-mediated inhibition of lymphangiogenesis. We identified TGF-β, PF4, angiostatin and endostatin as probable candidates, since it has been reported to inhibit lymphangiogenesis (20-22), and platelets contain these molecules in α granules. BMP-9 is a member of the TGF-β family that binds to type I and type II serine-threonine kinase receptors (23). BMP-9 inhibits basic fibroblast growth factor-stimulated migration and proliferation of bovine aortic endothelial cells (24). BMP-9 also inhibits the migration and growth of human dermal microvascular endothelial cells (25), suggesting that BMP-9 has an inhibitory effect on endothelial cells. BMP-9 is highly expressed in the liver (26,27), but its expression in platelets has not been elucidated to date. Western blotting with an antibody against BMP-9 showed that platelets express both BMP prepropeptides of 400–525 amino acids (28,29) (Fig. 7A, lane c, an arrow head) and mature BMP of 100–140 amino acids (30) (Fig. 7A, lane c, an asterisk). The recombinant BMP-9 made in Chinese hamster ovary cells is approximately 13 kDa (Fig. 7A, lane d), which is consistent with the molecular weight of mature BMP. In addition to 50 and 13 kDa bands, platelets have a 25 kDa BMP-9 band (Fig. 7A, lane c, an arrow), which appears to be due to human-specific post-translational modifications. The 25 kDa BMP-9 was also detected in supernatants from activated platelets, whereas only a negligible band was detected in supernatants from resting platelets (Fig. 7A, a, b), suggesting that BMP-9 is released from activated platelets.

Here, BMP-9, TGF-β and PF4 significantly inhibited hLEC migration, whereas angiostatin and endostatin did not (Fig. 7B). BMP-9 and TGF-β also significantly inhibited hLEC proliferation, whereas PF4, angiostatin and endostatin did not (Fig. 7C). As expected, BMP-9, but not angiostatin and endostatin, significantly inhibited tube formation of hLECs (Fig. 7D). However, although TGF-β inhibited migration and proliferation, it did not inhibit tube formation (Fig. 7D). PF4 also inhibited hLEC migration, but did not inhibit tube formation (Fig. 7D). Under in vivo activation conditions, it is most likely that platelets release almost all the granule contents at the same time. The mixture of TGF-β, BMP-9, PF4, angiostatin and endostatin significantly inhibited tube formation (Fig. 7D); suggesting that BMP-9 was still able to inhibit tube formation in the presence of PF4 and endostatin.

Taken together, we propose that podoplanin in LECs activates platelets by binding to CLEC-2 in the connection between lymph sac and vein during developmental stages, and that released BMP-9 in conjunction with
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other releasates inhibit migration, proliferation and tube formation, which facilitates blood/lymphatic vessel separation.

Anti-BMP-9 neutralizing antibody cancelled the inhibitory effects of activated platelet supernatants on LEC migration, proliferation, and tube formation.

We next investigated whether BMP-9 is the major secreted mediator for inhibition of hLEC migration, proliferation and tube formation using an anti-BMP-9 neutralizing antibody. The antibody alone had no significant effects on hLEC migration, proliferation, and tube formation, although it showed tendency to stimulate these responses (Fig. 8). Inhibition of hLEC migration, proliferation and tube formation by platelet supernatants was cancelled in the presence of the neutralizing anti-BMP-9 antibody (Fig. 8), suggesting that the inhibitory effects of platelet supernatants are mainly owing to BMP-9.

DISCUSSION

In the present study, we have demonstrated that CLEC-2 in platelets, but not that in other cells, is required for blood/lymphatic vessel separation. We also propose podoplanin in LECs activates platelets by binding to CLEC-2 in the connection between lymph sacs and veins during the developmental stages, and that released BMP-9 from activated platelets in conjunction with other releasates inhibits migration, proliferation, and tube formation of LECs, which facilitates blood/lymphatic vessel separation.

In initial studies, RT-PCR analysis has shown CLEC-2 transcripts in peripheral blood mononuclear cells, bone marrow cells, monocytes, dendritic cells, and granulocytes (31). However, subsequent studies revealed that the surface expression of CLEC-2 is limited to in platelets/megakaryocytes (1,32) in blood cells in humans, although it is also expressed in the liver sinusoid (32,33). In mice, although analysis of Serial Analysis of Gene Expression library derived from mouse megakaryocytes revealed that CLEC-2 is one of the most megakaryocyte-specific and abundant molecules in megakaryocytes (34), CLEC-2 is expressed in other blood cells such as neutrophils (17), macrophages (18), and dendritic cells (35) in addition to platelets. Therefore, it is conceivable that CLEC-2 in blood cells other than platelets may play a role in blood/lymphatic vessel separation in mice. To address this issue, we generated mice with CLEC-2 specifically deleted from platelets. Specific deletion of CLEC-2 from platelets resulted in blood-filled, dilated, and tortuous lymphatic vessels (Fig. 1), proving that platelets regulate blood/lymphatic vessel separation through CLEC-2. CLEC-2-deficient mice die at the embryonic/neonatal stage (6,7), whereas mice that are specifically deleted of CLEC-2 from platelets survive, suggesting that CLEC-2 expressed in cells other than platelets play a crucial role in life maintenance at the embryonic/neonatal stage.

We next investigated the mechanism by which CLEC-2 facilitates lymph/blood vessel separation. We observed that normal platelets,
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but not CLEC-2-deficient platelets, significantly inhibited migration, proliferation, and tube formation of LECs, but not those of HUVECs (Figs. 2-4), suggesting that platelets inhibited lymphangiogenesis by way of CLEC-2. It is obvious that activation of platelets is required for blood/lymphatic vessel separation, since the lack of Syk and SLP-76, which are necessary in CLEC-2-mediated signal transduction, results in misconnection between blood and lymphatic vessels (8,9). Platelet activation is likely to be induced by the association between CLEC-2 in platelets and podoplanin in LECs, since mice deficient in either molecule has the misconnection phenotype (6,7,10). Platelet activation leads to two events: release of granule contents and platelet aggregate formation. A blocker of integrin \( \alpha \text{IIb}\beta3 \), lotrafiban, inhibits platelet aggregation. We found that pretreatment of platelets with lotrafiban did not affect platelet-induced inhibition of lymphangiogenesis (Fig.5). We observed CD62P expression and fibrinogen binding in platelets after seeding on the surface of coated with LECs, but not HUVECs, suggesting that platelets activation and fibrinogen binding were encountered in our in vivo system used in Fig.5, although fibrinogen binding was very weak probably due to absence of stirring (data not shown). Integrin \( \alpha \text{IIb}\beta3 \)-deficient mice, whose platelets can release granule contents but cannot form aggregates, reportedly lack the misconnection phenotype, which is consistent with our results. Hence, it is unlikely that platelet aggregates physically occlude the connection between the lymph sac and the vein.

Since it is impossible to specifically inhibit granule release without inhibiting aggregation, we utilized activated-platelet supernatants, which supposedly contain all the granule contents released upon platelet activation, in order to investigate the role of granule release. We did not use rhodocytin to obtain activated-platelet supernatants since it also binds to integrin \( \alpha2\beta1 \) (36,37), which is expressed in LECs and HUVECs (38,39). We did not use thrombin, either, since endothelial cells express thrombin receptors, proteinase-activated receptor 1 and 2 (40). Hence, we used supernatants from platelets stimulated by a GPVI agonist, poly(PHG), since GPVI is not expressed in LECs or HUVECs (41). The supernatants of platelets activated through GPVI significantly inhibited migration, proliferation, and tube formation of LEC. These findings suggest that contents released from granules facilitate blood/lymphatic vessel separation and that the inhibitory effect is not restricted to supernatants from platelets activated through CLEC-2. It has been reported that kindlin-3-deficient mice, whose platelets can release granule contents but not can form aggregates, have the misconnection phenotype (10,42). This report seems to be at odds with our hypothesis. However, it has recently been demonstrated that kindlin-3 is present in human endothelial cells derived from various anatomical origins and that kindlin-3 knockdown results in impaired formation of tube-like structures in Matrigel (43), leaving open the possibility that endothelial cell defects can contribute to the misconnection phenotype.
Mechanisms of CLEC-2-regulated lymphangiogenesis

We found that recombinant CLEC-2 inhibited hLEC migration, but not proliferation and tube formation (supplemental Fig.2), suggesting that cross-linking of podoplanin by recombinant CLEC-2 generates activation signals for cell migration. It has been recently reported that small interfering RNA depletion of podoplanin expression in human lung microvascular lymphatic endothelial cells (HMVEC-LLy) causes a dramatic reduction in directional migration compared with control siRNA-transfected cells (44). Podoplanin depletion causes Cdc42 activation and RhoA inhibition, leading to decrease in HMVEC-LLy migration (44). It may be possible that podoplanin clustering by recombinant CLEC-2 regulates activity of small GTPases and inhibits LEC migration. However, recombinant CLEC-2 did not inhibit proliferation and tube formation of LEC, suggesting that podoplanin signaling only partly contribute to inhibition of LEC functions.

During this manuscript being reviewed, Finney et al. reported that platelets inhibited LEC transmigration depending on CLEC-2 (45), which is consistent with our results (Fig. 2). However, they reported that application of platelet releasate did not affect LEC transmigration and that treatment with an anti-podoplanin plus a secondary cross-linking antibody decreased VEGF-C-induced LEC migration (45). These findings suggest that platelets inhibit LEC migration in vitro by contact-dependent mechanisms. We agree that LEC migration is inhibited partly due to contact-dependent mechanism since we also observed that recombinant CLEC-2 inhibited LEC migration (supplemental Fig. 2). In our hand, however, platelet releasate clearly inhibited not only LEC migration but also LEC proliferation and tube formation (Fig. 6). They added platelet releasate from platelets stimulated with rhodocytin to the transmigration well. However, we used supernatants from platelets stimulated with poly(PHG), but not those from platelets stimulated with rhodocytin, since rhodocytin also binds to integrin α2β1 (36, 37), which is expressed in LECs and HUVECs (38, 39). The different results may be due to some effects of rhodocytin in the supernatants on LEC migration in their experiment. Alternatively, it may be possible that different agonists cause different pattern of granule release from platelets, which leads to the different results. In the present study, we reported that BMP-9 is released from activated platelets for the first time, and kinetics of BMP-9 release is an issue to be addressed in the future.

The next question is which molecule(s) are required for blood/lymphatic vessel separation. We investigated the effects of TGFβ, PF4, angiostatin, and endostatin in lymphangiogenesis, since they are cytokines released from platelets and have been reported to inhibit lymphangiogenesis (20-22). In addition to these molecules, we identified BMP-9 as a component of platelet releasates, which affect LEC function. This finding has not been reported elsewhere, to the best of our knowledge. Among all the molecules, BMP-9 had the most potent inhibitory effects on migration, proliferation, and tube formation of
Mechanisms of CLEC-2-regulated lymphangiogenesis

hLECs (Fig. 7). Angiostatin and endostatin had no effect on these functions in hLECs (Fig. 7). On the other hand, TGF-β and PF4 showed inconsistent effects on the hLEC function; TGF-β inhibited migration and proliferation, but not tube formation. PF4 inhibited migration, but not proliferation and tube formation of hLECs. The differential effects of these cytokines on LEC function may have important physiologic implications, which would be future topics for research. The combination of all these molecules (BMP-9, TGF-β, PF4, angiostatin and endostatin) strongly inhibited tube formation of hLECs. The combined administration inhibited hLEC tube formation by 45%, which was almost equivalent to the inhibitory effect of platelet supernatants on tube formation (Figs 6C, 7D). Although other releasates may also be involved in the regulation of lymphangiogenesis, we suggest that this combination (BMP-9, TGF-β, PF4, angiostatin and endostatin) plays a key role in platelet-induced inhibition of lymphangiogenesis in vivo. Although our data shown in Fig. 7 suggest that BMP-9 plays a key role in platelet-mediated inhibition of lymphangiogenesis, this is not directly demonstrated. To directly prove a major role of BMP-9 in platelet-mediated inhibition of lymphangiogenesis, we utilized an anti-BMP-9 neutralizing antibody. The anti-BMP-9 blocking antibody cancelled the inhibitory effects of platelet supernatants on hLEC migration, proliferation, and tube formation (Fig. 8). Among them, hLEC tube formation was increased in the presence of supernatants plus antibody (Fig. 8C), proving the major role of BMP-9 in platelet-mediated inhibition of lymphangiogenesis. Generation of BMP-9-deficient mice is awaited to prove the major role of BMP-9 in vivo.

BMP-9 also reportedly inhibits migration or proliferation of vascular endothelial cells (24). However, we observed that platelet supernatant including BMP-9 and other releasates inhibited LEC function (Fig. 6), but not HUVEC function (data not shown). These findings suggest that the inhibitory effects of BMP-9 on HUVECs are cancelled by other releasates from activated platelets, which is supposed to occur in vivo.

Mice deficient in NF-E2 transcription factor show severe thrombocytopenia, but fail to develop the misconnection phenotype (46). This finding apparently contradicts the hypothesis that platelets regulate the separation. However, these mice produce a few platelet-like particles, which can express P-selectin, an α granule membrane protein, in response to thrombin (47). It is conceivable that a small number of platelets may be enough for blood/lymphatic separation, since the key factor for the separation is granule release, but not platelet aggregates, which may require a large number of platelets to physically seal the orifice between lymph sacs and veins. Myeloid ecotropic viral integration site 1 (Meis1)-deficient mice or PF4-Cre; Rosa26R-Laczbpα-DTA transgenic mice, both of which completely lack megakaryocyte/platelets show the misconnection phenotype (48), suggesting that the notion that a complete lack of platelets is responsible for misconnection.
Normal platelets, but not CLEC-2-deficient platelets, significantly inhibited migration, proliferation, and tube formation of LECs, whereas normal platelets as well as CLEC-2-deficient platelets did not inhibit those of HUVECs (Figs. 2-4). Platelets and platelet releasates have been shown to have proangiogenic effects in various models of angiogenesis (reviewed in (49)). For example, platelets or platelet releasates stimulate HUVEC proliferation, migration, and tube formation in vitro (50,51). We also observed that platelets significantly increased HUVEC migration (Fig. 2). Moreover, slightly thickened walls of HUVEC tubes were observed in the presence of platelets (Fig. 4B), which may be a part of proangiogenic effect of platelets. HUVEC tube formation was not inhibited either by intact platelets (Fig. 4B) or by supernatants from activated platelets (data not shown), suggesting that LECs and HUVECs respond differently to platelet releasates.

REFERENCES

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Acknowledgments - We are grateful to Ms. Chiaki Komatsu, Mr. Hisaichiro Nakazawa, Dr. Kumiko Nakazawa, and Mr. Tsutomu Yuminamochi, for their excellent technical assistance. Gratitude is expressed to Prof. Radek C. Skoda for his kind donation of PF4-Cre mice. We also thank Drs. Naohiro Kodama and Testuro Takehara for their help with the study.

FOOTNOTES
*This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Sports, Science and Technology and the Japan Society for the Promotion of Science (JSPS) through the Funding Program for Next-Generation World-Leading Researchers (NEXT Program), initiated by the Council for Science and Technology Policy (CSTP).
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2The abbreviations used are: CLEC-2, C-type lectin-like receptor 2; PLCγ2, phospholipase Cγ2; SLP-76, The adapter protein SH2 domain-containing leukocyte protein of 76 kDa; HUVECs, human umbilical vein endothelial cells; LECs, lymphatic endothelial cells; BMP-9, bone morphogenetic protein-9; TGF-β, transforming growth factor-β; PF4, platelet factor 4
FIGURE LEGENDS

FIGURE 1. Blood/lymphatic vessel misconnection in mice with CLEC-2 specifically deleted from platelets. A. Mesentery (upper panels) and small intestine (lower panels) from wild type; CLEC-2 flox/+ (control) and PF2-Cre; CLEC-2 flox/flox (CLEC-2 conditional knockout (KO) from platelets (plts)/megakaryocytes (megs)). “A”, “V”, and “L” denotes artery, vein, and lymphatic vessel, respectively. An arrow in the right upper panel indicates lymphatic vessel. B. A mesenteric sections of 10-week-old WT;CLEC-2 flox/flox (left panel, control) and PF2-Cre;CLEC-2 flox/flox mice (right panel, CLEC-2 conditional KO in plts/megs) stained with LYVE-1. C. Whole-mount triple fluorescence confocal microscopy of embryonic back skin was performed with antibodies to PECAM-1 (red), LYVE-1 (green), and TER-119 (blue) at E15.5. Arrows indicated the distended lymphatic vessels containing erythrocytes.

FIGURE 2. Inhibitory effects of platelets on endothelial cell migration through CLEC-2. A. Cell migration of hLEC (i) and HUVEC (ii) in the presence of buffer, human washed platelets (hPlt, 1×10^8 /ml), wild-type (WT) murine washed platelets (mPlt WT, 1×10^8 /ml), CLEC-2-deficient murine washed platelets (mPlt KO, 1×10^8 /ml), or VEGF (20 ng/ml) was investigated by Boyden-type transwell migration assay. Quantification of the migration was performed as described in EXPERIMENTAL PROCEDURES. The graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). B. Cell migration of hLEC (i, iii) and HUVEC (ii) in the presence of buffer, hPlt (1×10^8 /ml), mPlt WT (1×10^8 /ml), mPlt KO (1×10^8 /ml) was investigated by wound closure assay. Quantification of the migration was performed as described in EXPERIMENTAL PROCEDURES (i, ii). The graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). One, two, and three asterisks denote P<0.05, P<0.01, and P<0.005, respectively. Wound in hLEC monolayer was photographed (iii). Black lines indicate a front of a hLEC monolayer.

FIGURE 3. Inhibitory effects of platelets on endothelial cell proliferation. Platelets inhibited hLEC proliferation, but not HUVEC proliferation, depending on CLEC-2. A. Cell proliferation of hLECs (upper panels) and HUVECs (lower panels) in the presence of buffer (left panels) and human washed platelets (right panels, hPlt, 1×10^8/ml) was investigated by thymidine analogue incorporation assay. A group of EdU incorporated cells is indicated by arrows. B,C. Cell proliferation of hLECs (B) and HUVECs (C) in the presence of buffer, human washed platelets (hPlt, 1×10^8/ml), wild-type murine washed platelets (mPlt WT, 1×10^8/ml), and CLEC-2-deficient murine washed platelets (mPlt KO, 1×10^8/ml) was investigated by thymidine analogue incorporation assay. Quantification of the
Mechanisms of CLEC-2-regulated lymphangiogenesis

proliferation was performed as described in EXPERIMENTAL PROCEDURES. The graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments).

**FIGURE 4.** Inhibitory effects of platelets on tube formation of endothelial cells through CLEC-2. Tube formation of hLECs (4×10^5/ml) (A) or HUVECs (5×10^5/ml) (B) in the presence (hPlt) or absence (buffer) of human washed platelets (1×10^8/ml). Images are representative of five different experiments. Quantification of tube formation is shown in (C). Tube formation of mLECs (1.25×10^7/ml) in the presence of buffer (buffer), wild-type murine washed platelets (mPlt WT, 1×10^7/ml), and CLEC-2-deficient murine washed platelets (mPlt KO, 1×10^7/ml) (D). Images are representative of three different experiments. Quantification of mLEC tube formation is shown in (E). The graphs (C, E) show quantification of tube formation as percent change ± SE from baseline (buffer) (n = 10 – 12 from three independent experiments). Three asterisks denote P < 0.005.

**FIGURE 5.** The lack of effect of integrin αIIbβ3 blocker on platelet-induced inhibition of hLEC migration, proliferation, or tube formation. A. Cell migration of hLEC in the presence of buffer, human washed platelets (hPlt 1×10^8 /ml), lotrafiban (αIIbβ3 blocker, final concentration; 1 μM), or human washed platelets pretreated with 10 μM lotrafiban (final concentration; 1×10^8 /ml platelets, 1 μM lotrafiban) was investigated by Boyden-type transwell migration assay. Quantification of the migration was performed as described in EXPERIMENTAL PROCEDURES. The graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). B. Cell proliferation of hLECs in the presence of buffer, platelets or lotrafiban as described in A. was investigated by thymidine analogue incorporation assay. Quantification of the migration was performed as described in EXPERIMENTAL PROCEDURES. The graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). C. Tube formation of hLECs in the presence of buffer, platelets or lotrafiban as described in A. was investigated. The images of tube formation were photographed (left images). Quantification of the migration was performed as described in EXPERIMENTAL PROCEDURES. The graph on the right illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). One, two, and three asterisks indicate P<0.05, P<0.01, and P<0.005, respectively.

**FIGURE 6.** Inhibitory effects of supernatants from activated platelets on hLEC migration, proliferation, and tube formation. A. Cell migration of hLECs in the presence of buffer, control supernatants (agonist only), or activated platelet supernatants (supernatants) was investigated by Boyden-type transwell migration assay. Quantification of the migration was performed as described in EXPERIMENTAL PROCEDURES. The graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). B. Cell proliferation of hLECs in the presence of buffer,
Mechanisms of CLEC-2-regulated lymphangiogenesis

control supernatant (agonist only), or activated platelet supernatant (supernatants) was investigated by thymidine analogue incorporation assay. Quantification of the migration was performed as described in EXPERIMENTAL PROCEDURES. The graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). C. Tube formation of hLECs in the presence of buffer (PBS), control supernatant (agonist only), or activated platelet supernatant (supernatants) was investigated and photographed (upper images). Quantification of tube formation was performed as described in EXPERIMENTAL PROCEDURES. The lower graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). One and three asterisks indicate P<0.05 and P<0.005, respectively.

FIGURE 7. Effects of platelet granule contents on hLEC migration, proliferation and tube formation.
A. Western blotting with anti-BMP-9 antibody. An asterisk indicates mature BMP-9. An arrow indicates putative glycosylated mature BMP-9. An arrowhead indicates BMP-9 prepropeptides. B. Cell migration of hLECs in the presence of buffer, 10 ng/ml BMP-9 (BMP), 10 ng/ml TGF-β (TGF), 1.5 μg/ml PF4 (PF4), 1 ng/ml angiostatin (angio), or 150 ng/ml endostatin (end) was investigated by wound closure assay. Quantification of the migration was performed as described in EXPERIMENTAL PROCEDURES. The graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). C. Cell proliferation of hLECs in the presence of buffer, 10 ng/ml BMP-9 (BMP), 10 ng/ml TGF-β (TGF), 1.5 μg/ml PF4 (PF4), 1 ng/ml angiostatin (angio), or 150 ng/ml endostatin (end) was investigated by thymidine analogue incorporation assay. Quantification of the proliferation was performed as described in EXPERIMENTAL PROCEDURES. The graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). D. Tube formation of hLECs in the presence of 10 ng/ml BMP-9 (BMP), 10 ng/ml TGF-β (TGF), 1.5 μg/ml PF4 (PF4), 1 ng/ml angiostatin (angio), 150 ng/ml endostatin (end), or the mixture of these molecules (mix). Quantification of the tube formation was performed as described in EXPERIMENTAL PROCEDURES. The graph illustrates percent change ± SE from baseline (buffer) (n=10 from four independent experiments). One and three asterisks indicate P<0.05 and P<0.005, respectively.

FIGURE 8. Effects of the anti-BMP-9 neutralizing antibody on inhibition of hLEC migration, proliferation and tube formation by platelet granule contents. A. Cell migration of hLECs in the presence of buffer, 10 μg/ml of anti-BMP-9 neutralizing antibody (BMP-9 Ab), supernatants from activated platelets (supernatants), supernatants plus 10 μg/ml of anti-BMP-9 neutralizing antibody was investigated by wound closure assay. B. Cell proliferation of hLECs in the presence of buffer, 1 μg/ml of the anti-BMP-9 neutralizing antibody (BMP-9 Ab), supernatants from activated platelets (supernatants), supernatants plus 1 μg/ml of the anti-BMP-9 neutralizing antibody was investigated by
Mechanisms of CLEC-2-regulated lymphangiogenesis

thymidine analogue incorporation assay. C. Tube formation of hLECs in the presence of buffer, 1 μg/ml of anti-BMP-9 neutralizing antibody (BMP-9 Ab), supernatants form activated platelets (supernatants), supernatants plus 1 μg/ml of anti-BMP-9 neutralizing antibody was investigated. Quantification of migration, proliferation, and tube formation was performed as described in EXPERIMENTAL PROCEDURES (migration assay: n=6 from three independent experiments, proliferation assay: n=8 from four independent experiments, tube formation assay: n=16 from three independent experiments). The graph illustrates percent change ± SE from baseline (buffer). One and three asterisks indicate P<0.05 and P<0.005, respectively.
A) control vs CLEC-2 conditional KO in plts/megs

B) Lyve-1

C) PECAM-1/Ter-119 vs Lyve-1 vs merge

Fig. 1
A) Boyden chamber

i) hLEC

- Increased migration
- Decreased migration

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- Increased migration
- Decreased migration

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B) wound closure

i) hLEC

- Increased migration
- Decreased migration

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- Increased migration
- Decreased migration

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iii) hLEC

- Control
- hPlt
- mPlt WT
- mPlt KO

0 hrs

20 hrs

Fig. 2
A) hLEC  

B) hLEC

C) HUVEC

Fig. 3
Fig. 4

A) hLEC

B) HUVEC

C) increased (%)

D) mLEC

E) mLEC

buffer hPlt

buffer mPlt

WT

KO

increased production

decreased production

hLEC  HUVEC

mPlt

WT

KO

***

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A) migration

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hPlt: + - +
αIIBβ3 blocker: - + +

B) proliferation

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hPlt: + - +
αIIBβ3 blocker: - + +

C) tube formation

buffer

hPlt

increased (％)

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<th>decreased</th>
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hPlt: + - +
αIIBβ3 blocker: - + +

Fig. 5
A) migration

B) proliferation

C) tube formation

Fig. 6
Fig. 7

A) WB: BMP-9

B) migration (Wound closure)

C) proliferation (Edu incorporation assay)

D) tube formation

**A)**

- a. resting-platelet supernatants
- b. activated-platelet supernatants
- c. platelet whole cell lysate
- d. recombinant BMP9

**B)**

- **BMP**
- **TGF**
- **PF4**
- **endo**

**C)**

- **BMP**
- **TGF**
- **PF4**
- **endo**

**D)**

- **BMP**
- **TGF**
- **PF4**
- **endo**
- **mix**
A) migration (wound closure)

- Increased (%): 20
- Increased: 10
- Increased: 0
- Decreased: -10
- Decreased: -20
- Decreased: -30
- Decreased: -40
- Decreased: -50
- Decreased: -60

BMP-9 Ab: + - +
Supernatants: - + +

B) proliferation (Edu incorporation assay)

- Increased (%): 20
- Increased: 15
- Increased: 10
- Increased: 5
- Increased: 0
- Decreased: -5
- Decreased: -10
- Decreased: -15
- Decreased: -20
- Decreased: -25

BMP-9 Ab: + - +
Supernatants: - + +

C) tube formation

- Increased (%): 60
- Increased: 50
- Increased: 40
- Increased: 30
- Increased: 20
- Increased: 10
- Decreased: 0
- Decreased: -10
- Decreased: -20
- Decreased: -30
- Decreased: -40

BMP-9 Ab: + - +
Supernatants: - + +

Fig. 8
Platelet activation receptor CLEC-2 regulates blood/lymphatic vessel separation by inhibiting proliferation, migration, and tube formation of lymphatic endothelial cells
Makoto Osada, Osamu Inoue, Guo Ding, Toshiaki Shirai, Hirotake Ichise, Kazuyoshi Hirayama, Katsuhiro Takano, Yutaka Yatomi, Masanori Hirashima, Hideki Fujii, Katsue Suzuki-Inoue and Yukio Ozaki
J. Biol. Chem. published online May 3, 2012

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