PLATELETS AND THROMBOPOIESIS

Podoplanin and CLEC-2 drive cerebrovascular patterning and integrity during development

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Key Points

- Podoplanin and CLEC-2 critically drive the formation and integrity of developing cerebral blood vessels.
- Loss of cerebrovascular integrity is influenced by the loss of αIIb-mediated platelet aggregation and platelet secretion.

Mice with a constitutive or platelet-specific deletion of the C-type lectin-like receptor (CLEC-2) exhibit hemorrhaging in the brain at mid-gestation. We sought to investigate the basis of this defect, hypothesizing that it is mediated by the loss of CLEC-2 activation by its endogenous ligand, podoplanin, which is expressed on the developing neural tube. To induce deletion of podoplanin at the 2-cell stage, we generated a podoplanin<sup>fl/fl</sup> mouse crossed to a PGK-Cre mouse. Using 3-dimensional light-sheet microscopy, we observed cerebral vessels were tortuous and aberrantly patterned at embryonic (E) day 10.5 in podoplanin- and CLEC-2-deficient mice, preceding the formation of large hemorrhages throughout the fore-, mid-, and hindbrain by E11.5. Immunofluorescence and electron microscopy revealed defective pericyte recruitment and misconnections between the endothelium of developing blood vessels and surrounding pericytes and neuro-epithelial cells. Nestin-Cre-driven deletion of podoplanin on neural progenitors also caused widespread cerebral hemorrhaging. Hemorrhaging was also seen in the ventricles of embryos deficient in the platelet integrin subunit glycoprotein Iib or in embryos in which platelet α-granule and dense granule secretion is abolished. We propose a novel role for podoplanin on the neuro-epithelium, which interacts with CLEC-2 on platelets, mediating platelet adhesion, aggregation, and secretion to guide the maturation and integrity of the developing vasculature and prevent hemorrhage. (Blood. 2015;125(24):3769-3777)

Introduction

Thrombocytopenia is the most common risk factor of intraventricular hemorrhage (IVH) in premature infants, which effects >12,000 infants every year.1 IVH in neonates causes substantial morbidity and mortality, the onset of which can be immediate or significantly delayed into adulthood. Strikingly, there has been no change in mortalities resulting from IVH over the last 3 decades, and although diagnostics are improving, no preventative therapeutic strategies currently exist.2,3 This relies on a better understanding of the molecular mechanisms that regulate cerebral vascular integrity during development. The cardiovascular system is the first functional organ system to develop in the mammalian embryo with angioblasts emerging around E7.0 to form the initial primitive vascular plexuses through vasculogenesis.4 The perineural vascular plexus (PNVP) develops around the neural tube between E8.5 and E9.5 and provides essential nutrients and oxygen to developing neural tissue.5 Uniquely, the entire vascularization of the neural tube subsequent to the formation of the PNVP is derived through angiogenesis. Blood vessels invade the neural tube at E10.0 in response to vascular endothelial growth factor (VEGF) released by cells of the neuroepithelium and migrate along a preformed lattice network of neuroepithelial cells.4,6 Capillary stabilization, branching, and remodeling are aided by the recruitment of a wide range of extracellular matrix (ECM) proteins and their intimate association with surrounding neurons, glial cells, and pericytes to form multicellular complexes termed neurovascular units (NVUs). The NVUs provide the anatomical basis for the formation of the blood-brain barrier, a tightly regulated interface between the central nervous system and the circulation.5 Within the NVUs, tight junctions between endothelial cells restrict movement of molecules. Many studies have shown that impaired development of NVUs through the loss of key molecules or signaling pathways leads to fatal hemorrhaging in mid-gestation as a result of abnormal vascular patterning and aberrant associations with pericytes and ECM components.7-11

CLEC-2 is a C-type lectin-like receptor, which is expressed at high levels on megakaryocytes and platelets, with no evidence for significant expression on any other circulating hematopoietic cell types outside of the vasculature. In addition, podoplanin is itself...
a receptor, which signals through the ezrin, radixin, and moesin (ERM) family of actin-binding proteins. Thus, binding of podoplanin to CLEC-2 generates reciprocal signals that regulate the function of both of the interacting cells.

The binding of podoplanin to CLEC-2 on platelets activates a Src and Syk tyrosine kinase-dependent signaling cascade that regulates phospholipase C (PLC)γ2 and platelet activation.13 Mice deficient in podoplanin, CLEC-2, and other key signaling proteins, including Syk and PLCγ2, exhibit blood-lymphatic shunts at mid-gestation and are embryonic lethal.14-19 This is thought to be mediated by a combination of lymphatic-venous connections and retrograde flow through the thoracic duct.20-22 They additionally have a number of other developmental defects, including hemorrhaging in the brain at E12.5 and the absence of lymph nodes.18,23,24 These developmental defects are believed to be due to loss of platelet activation, as they are seen in mice with a megakaryocyte/platelet-specific deletion of CLEC-2 or its signaling kinase Syk.18,21,25 However, the neurovascular defects are believed to be unrelated to defects in lymphatic development because the lymphatic system is absent in the brain, and cerebral hemorrhages are observed before the presence of blood-lymphatic mixing.

In the characterization of CLEC-2- and Syk-deficient mice, we localized brain hemorrhages to the ventricles and parenchyma at E12.5 and reported that hemorrhages in Clec-2fl/flPF4-Cre mice were restricted to the parenchyma.18 Although cerebral hemorrhages have not been reported in podoplanin-deficient mice, the loss of T-synthese, a key glycosyltransferase enzyme responsible for glycosylation of the podoplanin extracellular domain, results in the formation of a disorganized microvasculature network, with the defective recruitment of ECM and pericytes, leading to hemorrhaging throughout the brain by E12.0 and lethality by E14.0.26 However, hemorrhages were not observed in endothelial specific T-synthese-deficient mice, which exhibited blood-lymphatic mixing, consistent with the neurovascular defect being independent of the defect in lymphatic development.27

In the present study, we investigated the mechanism of developmental defects within the central nervous system of CLEC-2-deficient mice. We generated a novel podoplanin-floxed mouse and crossed this to mice expressing PGK-Cre to delete the transmembrane protein at the 2-cell stage. We demonstrate that constitutive deletion of CLEC-2 or podoplanin induce a similar pattern of hemorrhaging from E11.5 in association with defective angiogenesis of cerebral blood vessels. A similar defect is also observed following nestin-Cre (Nes-Cre)-driven deletion of podoplanin specifically on neuro-epithelial cells. We also show the presence of hemorrhages in the ventricles of mice deficient in the subunit αIIb of the major platelet integrin αIIbβ3 and in mice where platelet-α-granule and dense-granule secretion is abolished. This leads us to propose a model in which expression of podoplanin on the neuro-epithelium interacts with CLEC-2 on platelets to regulate the maturation and integrity of cerebral blood vessels. During the development of these nascent, fragile vessels, we propose that CLEC-2-induced αIIbβ3-dependent aggregation drives the formation of small platelet aggregates to plug the vessel wall to prevent hemorrhage and maintain vascular integrity. Meanwhile, platelet-secreted molecules may function to recruit mural cells to the endothelium to aid blood vessel maturation and integrity, as well as to reinforce αIIbβ3-dependent platelet aggregation.

Mouse strains

All animal experimentation was performed under an approved license from the UK Home Office. Clec-2fl/fl mice have been previously described.28 Podoplaninfl/fl (Pdpnfl/fl) mice were generated at Taconic Artemis by insertion ofloxP sites flanking exon 3 of the podoplanin allele using standard methods and back-crossed to C57BL/6 mice. Pdpnfl/fl mice were crossed to PGK-Cre, Nes-Cre, or Tie2-Cre mice for constitutive, neural-, or endothelial-specific deletion of the podoplanin allele, respectively.29-31 PGK-Cre mice were purchased from Harlan Laboratories. Nes-Cre mice were obtained from Cancer Research UK (London, UK). Tie2-Cre mice were purchased from Jackson Laboratories. Nheal-2+/−, Unc13d−/−, and Nbeal-2−/−, Unc13d−/− embryos were provided by Bernhard Nieswandt.32,33 (Deppermann et al, unpublished data). Spkh1fl/flSpkh2−/− and Spkh1fl/flSpkh2−/−PF4-Cre embryos were provided by Eric Camerer (Mariko et al, unpublished data). aIib−/− mice were provided by John Frampton and have been previously described.34

Immunolabeling and microscopy

Immunohistochemistry and immunofluorescence are described in detail in the supplemental Materials.

Brieﬂy, sections of paraffin-embedded embryos were either processed for hematoxylin and eosin (H&E) staining or for podoplanin staining, whereby sections were boiled in citric acid buffer (pH 6) before permeabilizing, blocking, and incubating with hamster anti-podoplanin (clone eBio8.1.1; eBiosciences), followed by goat anti-hamster Cy3 (ab6969; Abcam) secondary antibody. H&E sections were analyzed using a Zeiss Axiostar Zoom brightﬁeld microscope. Confocal imaging was performed using a Leica SP2 confocal microscope.

For immunofluorescence staining of frozen embryo tissue, sections were quenched with ammonium chloride and permeabilized before blocking and incubating with the following primary antibodies: rat anti-mouse platelet endothelial cell adhesion molecule-1 (PECAM-1; clones 5D2.6 and 1G5.1, provided by Dr S. Butz25), rabbit anti-NG2 (clone 132.39; Millipore), anti-nestin (Rat 401; Developmental Studies Hybridoma Bank), rat anti-TER119 (Cambridge Bioscience), rat anti-mouse CD41 (clone MWReg30; BD Pharmigen), or hamster anti-podoplanin (clone eBio8.11.1; eBiosciences). Sections were then incubated in the appropriate secondary antibodies: goat anti-hamster Cy3 (ab6969; Abcam), goat anti-rat Alexa Fluor 488 (A-11006; Invitrogen), or goat anti-rat Alexa Fluor 647 (A-21247; Invitrogen), and where applicable, further incubated with TO-PRO-3-Iodide (Invitrogen). Confocal imaging was performed using a Leica SP2 confocal microscope.

Whole mount immunofluorescence and ultramicroscopy preparation and imaging have been previously described35 and are detailed in the supplemental Materials. Brieﬂy, embryos were permeabilized, blocked, and immunostained with rat anti-mouse PECAM-1 (clones 5D2.6 and 1G5.1) primary antibodies, followed by goat anti-rat Alexa Fluor 488 secondary antibody. Embryos were embedded in agarose, successively dehydrated, and chemically cleared for imaging using a LaVision Ultramicroscope (La Vision BioTec; Bielefeld). Images were analyzed using IMARIS Vantage software (Version 7.6.0; Bitplane).

Electron microscopy

Electron microscopy is described in detail in the supplemental Materials (reagents supplied by Agar Scientific). Brieﬂy, embryos were ﬁxed in 2.5% glutaraldehyde and washed in 0.1 M sodium cacodylate buffer (pH 7.4; Na Cacodylate) before treatment with 1% osmium tetroxide. Samples were washed in Na Cacodylate before successive dehydration and emersion in propylene oxide:resin (1:1), followed by resin only overnight. Samples were polymerized at 80°C before sectioning and staining with lead citrate/uranyl acetate for imaging using a Jeol 2100 200-kV LaB6 TEM.

Statistical analysis

The number of NG2-positive pericytes was quantified using a 1-way analysis of variance with a Tukey posttest, where *P < .05 and **P < .01. Data are means ± standard error of the mean.

Methods

An expanded methods section is available in the supplemental Materials available on the Blood Web site.
Results

As part of the characterization of CLEC-2-deficient mice, we reported extensive hemorrhaging in the fore-, mid-, and hindbrain regions at E12.5, which we hypothesized were mediated by the activation of platelets with the podoplanin-expressing choroid plexus.18 In the present study, we extensively mapped the time of onset of hemorrhaging through investigation of earlier time points in development and found that vascular defects and hemorrhage occur before the development of the choroid plexus. Cerebral hemorrhages were found to develop between E10.5 and E11.5 (supplemental Figure 1A). At this stage of development, the endogenous ligand for CLEC-2, podoplanin, is widely expressed along with the intermediate of development, the endogenous ligand for CLEC-2, podoplanin, is between E10.5 and E11.5 (supplemental Figure 1A). At this stage investigation of earlier time points in development and found that vascular defects and hemorrhages form among aberrantly patterned vascular networks (supplemental Videos 1-3). Complete spatial reconstruction of the entire embryonic brain that is provided by our ultramicroscopy approach (supplemental Videos 1-3). Higher-magnification images of the vasculature at E12.5 revealed hemorrhages forming among aberrantly patterned vascular networks in Clec-2-/- and Pdpn-/-PGK-Cre embryos compared with the organized, characteristic branching patterns of littermates (Figure 2A). By E12.5, hemorrhages were clearly visible throughout the developing fore-, mid-, and hindbrain in CLEC-2- and podoplanin-deficient mice, as indicated by dense areas of PECAM-1 staining (Figure 2B, red arrows). The size and localization of hemorrhages can only fully be appreciated by a complete spatial reconstruction of the entire embryonic brain that is provided by our ultramicroscopy approach (supplemental Videos 1-3).

To investigate a role for podoplanin in the development of cerebral hemorrhages, we generated a floxed podoplanin mouse (Figure 1B) and crossed this to a mouse expressing Cre recombinase driven by the PGK promoter (Pdpn-/-PGK-Cre) for deletion of podoplanin at the 2-cell stage.29 Pdpn-/-PGK-Cre embryos developed cerebral hemorrhages between E10.5 and E11.5, which were prominent by E12.5 (Figure 1C). Examination of H&E-stained histologic sections of Clec-2-/- and Pdpn-/-PGK-Cre embryos at E12.5 revealed hemorrhages that extended across vast regions of the neuro-epithelium, displacing surrounding neuro-epithelial cells and protruding into the ECM (Figure 1D; supplemental Figure 1B). Immunofluorescence of wild-type and Clec-2-/- embryos at E14.5 demonstrated that hemorrhages in the parenchyma and in the ventricles were rich in CD41+ platelets (supplemental Figure 3). The overall pattern of hemorrhaging was similar in Clec-2-/- and Pdpn-/-PGK-Cre embryos.

Because of the limitations of 2-dimensional analysis, we used the planar illumination-based microscope modality, ultramicroscopy, to visualize the developing cerebral vasculature in 3 dimensions (3D). Ultramicroscopy creates stacks of optical sections that are digitally analyzed and reconstructed into a single 3D image.35,36 The cerebral vasculature was visualized at E10.5 and E12.5 in Clec-2-/- and Pdpn-/-PGK-Cre embryos stained with PECAM-1 (Figure 2). Before the appearance of hemorrhages, the vasculature at E10.5 appeared tortuous and abnormally patterned in Clec-2-/- and Pdpn-/-PGK-Cre embryos compared with the organized, characteristic branching patterns of littermates (Figure 2A). By E12.5, hemorrhages were clearly visible throughout the developing fore-, mid-, and hindbrain in CLEC-2- and podoplanin-deficient mice, as indicated by dense areas of PECAM-1 staining (Figure 2B, red arrows). The size and localization of hemorrhages can only fully be appreciated by a complete spatial reconstruction of the entire embryonic brain that is provided by our ultramicroscopy approach (supplemental Videos 1-3). Higher-magnification images of the vasculature at E12.5 revealed hemorrhages forming among aberrantly patterned vascular networks in Clec-2-/- and Pdpn-/-PGK-Cre embryos (Figure 2B, right, red dashed lines outline hemorrhages). Developing vessels were discontinuous and appeared prone to hemorrhage compared with the intricately branched sheets of vessels forming in littermate controls (Figure 2B, right). The length of vessels and the number of branch points were quantified using the hindbrain model developed by Fantin et al (supplemental Figure 4).37 Although a trend for increased vessel length and branch points was observed in CLEC-2- and podoplanin-deficient mice, the defect mainly lies in the tortuosity of vessels.
revealed areas of defective vascular integrity, resulting in hemorrhaging among visibly tortuous blood vessels, resulting in the vast accumulation of blood in the ventricles (supplemental Figure 5; supplemental Video 4). Although hemorrhages were consistent and equally widespread in Pdpnfl/flNes-Cre embryos, the size of parenchymal hemorrhages was often smaller, indicating a slightly milder phenotype than in Clec-2−/− and Pdpnfl/flPGK-Cre embryos. Although the lymphatic system is absent from the brain, we excluded a role for endothelial-derived podoplanin in maintaining cerebral vascular integrity, showing no evidence of hemorrhaging in Pdpnfl/flTie2-Cre embryos at E12.5 (supplemental Figure 6). These results provide strong evidence for a role of podoplanin specifically for maintaining cerebral vascular integrity.
often distended from the surrounding neuro-epithelium (Figure 4A, embryos compared with littermates (Figure 4). Blood vessels were arterioles, venules, and capillaries. Immunostaining of tissue sections cytes, which overlay endothelial cell junctions to support smaller ar-

tually supported vascular network through the recruitment of ECM which leaky, nascent angiogenic vessels are transformed into a struc-

tural support for developing vessels. In Clec-2−/−, Pdpnfl/flPGK-Cre, and Pdpnfl/flNes-Cre vessels, endothelial cell junctions are well established, with endothelial cell flaps clearly visible (Figure 5). Despite this, vascular lumens appear expanded, and the endothelium was enriched in vacuoles, appearing fragile, tortuous, and prone to hemorrhage. Large gaps were visible between the endothelial cell layer and the surrounding pericytes and neuro-epithelial cells, precluding their supportive role to the enclosed vessel (Figure 5).

We previously showed that constitutive deletion of the tyrosine kinase, Syk, also leads to development of hemorrhages in the brain by E12.5. A similar phenotype, albeit less severe, is seen when deleting Syk or CLEC-2 in the megakaryocyte/platelet lineage, supporting a role for CLEC-2-induced platelet activation in this phenotype. To dissect the mechanism by which CLEC-2- induced platelet activation safeguards the developing cerebral vasculature, we studied embryos from mice deficient in αIb, which together with β3 forms the major platelet integrin, as well as mice with secretion defects, including mice deficient in neurobeachin-like 2 protein (Nbeal-2−/−); which lack α-granules, mice deficient in Munc13-4 (Unc13d−/−), in which platelet dense-granule secretion is abolished and platelet α-granule secretion is reduced; and double-deficient Nbeal-2−/−Unc13d−/− mice.

Although there was no evidence of hemorrhaging in Unc13d−/− embryos (data not shown), hemorrhages were observed in 2 of 12 Nbeal-2−/− embryos (supplemental Figure 7) and in 6 of 8 Nbeal-2−/− Unc13d−/− embryos (Figure 6), whereas no hemorrhages were seen in littermate controls (n = 10). A comparable phenotype was observed in αIb−/− embryos, where blood was visible in the ventricles from E11.5 (data not shown), becoming more prominent by E12.5 in 6 of 6 embryos compared with the absence of significant hemorrhaging in 7 littermate controls (Figure 6). In all cases, hemorrhages were not identifiable by histology in the parenchymal tissue, suggesting a more discrete defect in vascular integrity that leads to the accumulation of blood in the ventriciles. Furthermore, hemorrhages were not observed on H&E sections at E14.5 in αIb−/− (n = 5) and Nbeal-2−/− (n = 10) embryos (data not shown), suggesting they had largely resolved, whereas hemorrhages were seen to persist past E14.5 in Clec-2−/− (n > 10), Pdpnfl/flPGK-Cre (n = 5), and Pdpnfl/flNes-Cre (n = 6) embryos (data not shown).

In light of the clear redundancy between CLEC-2-induced platelet activation pathways that are involved in maintaining cerebral vascular integrity, we considered a role for the bioactive lipid sphingosine-1 phosphate (S1-P). Platelets store a rapidly deployable pool of S1-P. Platelets release S1-P in response to activation by agonists that trigger phosphatidylinositol-3,4,5-trisphosphate (PIP3) hydrolysis, which is a common mechanism for activating intracellular signaling pathways that contribute to platelet activation and aggregation. S1-P is a potent agonist of G-protein-coupled receptors (GPCRs) that are expressed on the surface of platelets, including CLEC-2.

S1-P is also stored in intracellular storage granules and released upon platelet activation. S1-P has been shown to promote platelet aggregation and adhesion to the endothelium, as well as mediate platelet activation and adhesion to endothelial cells through engagement of CLEC-2. Furthermore, S1-P has been shown to play a critical role in maintaining cerebral vascular integrity, as evidenced by the occurrence of hemorrhages in S1-P-deficient (Sphk1−/−) and Sphk2−/− mice. These findings suggest that S1-P plays a crucial role in maintaining cerebral vascular integrity through its interaction with CLEC-2.

In summary, our study provides insights into the mechanisms that underlie the development of hemorrhages in the brain and highlights the importance of CLEC-2 and S1-P in maintaining cerebral vascular integrity. Further studies are needed to elucidate the precise mechanisms by which S1-P regulates cerebral hemorrhage and to identify potential therapeutic targets for the prevention of cerebral hemorrhage.
Discussion

In our initial characterization of CLEC-2-deficient mice, we reported that mice deficient in the platelet C-type lectin-like receptor, CLEC-2, or the downstream-signaling tyrosine kinase, Syk, develop cerebral hemorrhages at E12.5 and speculated that this was mediated by the interaction of platelets with podoplanin on the choroid plexus.18 In the present study, we show that hemorrhages develop between E10.5 and E11.5, a critical time point for vascularization of the neural tube and before the choroid plexus has fully formed. We further show through the generation of a mouse model that allows inducible deletion of podoplanin at the 2-cell stage that mice deficient in podoplanin develop cerebral hemorrhages at the same stage of development. Furthermore, using 3D ultramicroscopy, we were able to show a similar location and scale of hemorrhages in Pdpnfl/flPGK-Cre and Clec-2-/- embryos in association with early developmental defects in vessel formation. The pattern of hemorrhaging and vascular defects was indistinguishable in the 2 sets of mutant embryos.

Podoplanin is widely expressed on neuro-epithelial cells in the developing neural tube and was selectively deleted on this cell population using the Nes-Cre transgene. Pdpnfl/flNes-Cre embryos developed hemorrhages and defects in vascular development at the same timescale as Pdpnfl/flPGK-Cre and Clec-2-/- embryos. For each n = 1, a minimum of 4 different sections with 8 images per section were analyzed. Statistical significance was measured by a 1-way analysis of variance with a Tukey posttest, where *P < .05 and **P < .01. Error bars show means ± standard error of the mean.
work by our group showed hemorrhages in the brains of mice with a megakaryocyte/platelet specific deletion of CLEC-2 or the downstream signaling molecule Syk. Taken together with the lack of significant CLEC-2 expression on other circulating hematopoietic cells during development, this provides strong evidence of a causal relationship for neuro-epithelial-expressed podoplanin in regulating platelet activation through CLEC-2 to safeguard the integrity of the developing cerebral vasculature.

With this in mind, we sought to identify the mechanism by which activated platelets guide and maintain vascular integrity during development. Strikingly, we saw a hemorrhagic phenotype in mice deficient in one of the subunits of the major platelet integrin, αIIb, from E11.5. Thus, hemorrhaging is presumably influenced by a loss of integrin αIIbβ3-mediated platelet adhesion and/or aggregation. Although this role of the integrin is a component of classical hemostatic function of platelets, this is the first description to our knowledge of hemorrhaging associated specifically with loss of this hemostatic pathway during development. Remarkably, however, hemorrhages in αIIb-deficient mice appeared to resolve by E14.5, whereas in CLEC-2- and podoplanin-deficient mice, hemorrhages were seen to persist. This suggests that an additional mechanism is influenced by the loss of CLEC-2 activation.

Platelets are powerful secretory cells and release a range of bioactive molecules on activation. In this study, a specific role for α-granule secretion was considered, given their enrichment in growth factors, such as VEGF and various hemostatic proteins including von Willebrand factor. However, the mild hemorrhagic phenotype observed in NBEAL-2-deficient embryos, with <20% exhibiting hemorrhaging, argues against a major role of platelet α-granule secretion in development and is consistent with the mild bleeding diathesis observed in adult NBEAL-2 mice. We further considered a role for platelet-dense granules, which release a range of nonprotein molecules that can promote platelet activation, but saw no defect in vascular development.

To further support a role for neuro-epithelial podoplanin, we showed both a reduction in pericyte recruitment and a marked disruption in the association of mural cells to the visibly tortuous endothelium of cerebral vessels in Pdpn$^{fl}Nes$-Cre, Pdpn$^{fl}PGK$-Cre and Clec-2$^{-/-}$ embryos. This phenotype is characteristic of a defect in vessel maturation and has been observed in other studies where key signaling pathways that guide mural cell and ECM recruitment are disrupted.5,9,11

Having established that podoplanin on neuro-epithelial cells is critical for the development and integrity of the cerebral vasculature, we sought to investigate the mechanism that governs this process. Previous
in Unc13d−/− embryos.33 However, we did observe hemorrhaging in 75% of embryos where both α-granule and dense-granule secretion was abolished. These data strongly suggest a significant level of redundancy exists between different platelet activation pathways to compensate for the targeted loss of a single pathway.

Recent studies by our group have demonstrated the ability of CLEC-2 to cluster podoplanin on the surface of cells, which is proposed to induce podoplanin signaling through ERK proteins, as well as supporting platelet adhesion.45 A number of in vitro studies have associated podoplanin-ERM signaling with changes in cell motility, adhesion, and shape change, raising the possibility for platelets to induce neuro-epithelial cell behavior and influence their interactions with surrounding mural cells and the endothelium.46–48 This supports the concept of reciprocal signaling between CLEC-2 and podoplanin on platelets and neuro-epithelial cells, respectively, which may account for the enhanced phenotype in CLEC-2- and Syk-deficient platelets relative to platelets deficient in the major platelet integrin or in granule secretion.

In conclusion, we propose that during the initial vascularization of the neural tube, the activation of CLEC-2 by podoplanin is critical in maintaining cerebral vascular integrity. Although platelet aggregates plug the vessel wall to prevent hemorrhage, we propose that secreted molecules function to recruit cells and matrix components to developing vessels. In the absence of this interaction, vessels become tortuous and prone to hemorrhage, as shown in the accompanying model (supplemental Figure 9). It was recently reported that platelet inflations in high-risk preterm infants enhance hemostasis and reduce the risk of IVH.39 In this context, the present observations are of great interest as they provide a potential explanation for IVH and identify new pathways that can be targeted for noninvasive therapeutic intervention of IVH.

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Authorship

Contribution: K.L.L. and B.A.F. designed the research, performed the research, and analyzed data; K.L.L. wrote the manuscript; R.H. and F.K. assisted with microscopy and provided essential reagents; C.D. and S.L.G. performed experimental work; B.N., E.C., J.F., and C.B. contributed essential reagents; and S.P.W. contributed to experimental design, discussion, and manuscript preparation.

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