Hepatic leukemia factor-expressing paraxial mesoderm cells contribute to the developing brain vasculature

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ABSTRACT
Recent genetic lineage tracing studies reveal heterogeneous origins of vascular endothelial cells and pericytes in the developing brain vasculature, despite classical experimental evidence for a mesodermal origin. Here we provide evidence through a genetic lineage tracing experiment that cephalic paraxial mesodermal cells give rise to endothelial cells and pericytes in the developing mouse brain. We show that Hepatic leukemia factor (Hlf) is transiently expressed by cephalic paraxial mesenchyme at embryonic day (E) 8.0-9.0 and the genetically marked E8.0 Hlf-expressing cells mainly contribute to the developing brain vasculature. Interestingly, the genetically marked E10.5 Hlf-expressing cells, which have been previously reported to contain embryonic hematopoietic stem cells, fail to contribute to the vascular cells. Combined, our genetic lineage tracing data demonstrate that a transient expression of Hlf marks a cephalic paraxial mesenchyme contributing to the developing brain vasculature.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Hepatic leukemia factor, Cephalic mesenchyme, Lineage tracing, Endothelial cells, Pericytes

INTRODUCTION
During vertebrate development, the segmented paraxial mesoderm of the somite gives rise to different mesodermal derivatives including vascular cells. The quail-chicken chimera and labeling system demonstrated that vascular progenitors derive from the somite and contribute to the trunk (Pardanaud et al., 1996; Pouget et al., 2006; Sato et al., 2008) and limb vasculature (Huang et al., 2003; Kardon et al., 2002). Genetic lineage tracing experiments with the Cre-loxP system clearly demonstrated the contribution of the somatic vascular progenitors into the trunk and limb vasculature: paraxial mesoderm-derived angioblasts are assembled to form perineural vascular plexus (PNVP) around the neural tube (Hogan et al., 2004; Jukkola et al., 2005). Subsequently, sprouting vessels from the PNVP invade the CNS tissues and extend the branches from the plexus towards the ventricle (Gupta et al., 2021; Paredes et al., 2018; Tata et al., 2015). In addition to the mesodermal origin, recent genetic lineage tracing studies with the Cre-loxP system demonstrated that erythro-myeloid progenitors (EMPs) contribute to brain endothelial cells (Plein et al., 2018), although contradictory data were reported (Feng et al., 2020; Palis and Yoder, 2020). The cephalic neural crest cells penetrate and differentiate into pericytes in the forebrain vasculature (Korn et al., 2002; Reyahi et al., 2015; Yamanishi et al., 2012). Genetic lineage tracing studies have revealed heterogeneous origins of CNS vascular cells from distinct populations, but CNS vascular cells derived via a classical pathway of mesodermal differentiation into endothelial cells and pericytes were not examined.

Here, we studied the developmental timing of angiogenic cephalic paraxial mesenchyme in the developing vasculature of the CNS and various tissues using the Cre-loxP-based lineage tracing system. First, we found a unique expression of hepatic leukemia factor (Hlf), the proline and acid rich basic region leucine zipper (Par-bZip) transcription factor, in the cephalic paraxial mesenchyme at embryonic day (E) 8.5. Second, the lineage tracing experiments using Hlf-CreERT2; ROSA-LSL-tdTomato embryos, with tamoxifen administration at E8.0, revealed that a transient expression of Hlf marked angiogenic cephalic mesenchyme, which mainly contributes to vascular cells in the developing CNS tissues at E15.5. Interestingly, a transient expression of Hlf at E10.5 failed to mark vascular cells at E15.5, suggesting that Hlf marks an angiogenic paraxial mesenchyme subpopulation from an early stage in vascular development.

RESULTS
Hlf-expressing cells in the cephalic mesoderm but not yolk sac
Previous studies revealed that Hlf expression marks embryonic hematopoietic stem cell (HSC) precursors within the dorsal aorta of the aorta-gonad-mesonephros (AGM) region at E10.5 and maturing HSCs in the fetal liver between E11.5 and E14.5 (Yokomizo et al., 2019). Moreover, Hlf expression does not mark EMPs within the...
yolk sac or endothelial cells of the yolk sac, AGM, or fetal liver (Yokomizo et al., 2019). These observations were supported by the recent report by Tang et al. (2021). Compared with the unique Hlf expression in embryonic HSCs from E10.5 to E14.5, Hlf expression in non-hematopoietic cells remains elusive. To address this, we initially analyzed the published single cell RNA-sequence data set of E8.0 and E8.5 mouse embryo (Pijuan-Sala et al., 2019). At these stages, most hematopoietic progenitors emerge in the yolk sac and migrate to intra-embryonic organs via circulation (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015; Lux et al., 2008). Indeed, the clusters of blood progenitors and erythrocytes express RUNX family transcription factor 1 (Runx1), which is known to be expressed by hematopoietic progenitors and HSCs (Utz et al., 2020), while few blood progenitors and erythrocytes express Hlf (Fig. S1A-F). In contrast, Hlf expression is highly enriched in the paraxial mesoderm in both E8.0 and E8.5 embryos (Fig. S1A-F). To confirm the expression pattern of Hlf in E8.5 embryos, we first performed RNA whole-mount in situ hybridization chain reaction (HCR). Hlf expression was clearly detectable in the cephalic region, where the angiogenic cephalic mesenchyme was found in the quail-chicken chimera and labeling experiment (Couly et al., 1995) (Fig. 1A-J). Most of Hlf-expressing cells express the paraxial mesoderm labels Pax3 or Eomes or both (Fig. 1A-J), indicating that these Hlf-expressing cells are cephalic paraxial mesodermal cells. These findings were consistent with the published single cell RNA-sequence data set (Pijuan-Sala et al., 2019). We further performed the whole-mount immunohistochemical analysis of Hlf-tdTomato knock-in reporter embryos at E8.5 (Fig. 1K-U). Hlf-tdTomato-expressing cells were clearly detectable in the cephalic region (Fig. 1L-O; Fig. S2A-H), but these cells are negative for the endothelial cell marker PECAM-1 in the head or the yolk sac vasculature at E8.5 (Fig. 1P and Q, head; Fig. 1R-U, yolk sac). Combined, these data suggest that Hlf-expressing cells at E8.5 represent a mesenchyme subpopulation in the cephalic region.

Transient expression of Hlf marked cephalic mesenchyme

We next examined differentiation potentials of Hlf-expressing cephalic mesenchyme cells using a tamoxifen-induced Hlf-CreERT2 knock-in mice in combination with a Cre-mediated ROSA-LSL-tdTomato reporter mice (Fig. 2A). Like Hlf-tdTomato knock-in reporter mice (Yokomizo et al., 2019), Hlf-CreERT2 mice were generated by inserting a T2A-CreERT2 gene fusion before the endogenous stop codon within exon 4 (Fig. 2A). We first induced tdTomato expression in Hlf-CreERT2; ROSA-LSL-tdTomato embryos at E8.0 and used high-resolution whole-mount imaging to analyze the distribution of tdTomato-expressing Hlf lineages at E9.0 (Fig. 2A). Double RNA whole-mount in situ HCR using probes to Hlf and tdTomato revealed that Hlf and tdTomato signals have been largely overlapping in the cephalic region (Fig. S21-P). Consistent with the analysis of Hlf-tdTomato reporter embryos at E8.5, we found the majority of Hlf-lineage cells were detectable in the cephalic region (Fig. 2B-G; Fig. S21-P); a few Hlf-lineage cells were found in the rostral trunk region (Fig. 2B-G). Hlf-lineage cells were not detectable in the head, trunk, and yolk sac vasculature at E9.0 (Fig. 2C,D,F,G,H,I and J, head and trunk; K, L, and M, yolk sac). These data suggest that most Hlf-expressing cells at E8.0-9.0 represent cephalic mesenchymal cells.

Hlf-expressing cephalic mesenchymal cells contribute to the brain vasculature

We next examined the contribution of Hlf-expressing cephalic mesenchymal cells to the developing brain vasculature. In addition to the Hlf-CreERT2; ROSA-LSL-tdTomato lineage tracing mouse model, we used a tamoxifen-inducible Runx1-CreERT2; ROSA-LSL-tdTomato lineage tracing mouse model as a reference for the lineage tracing experiments in both vascular and hematopoietic cells: Runx1 is known to be expressed by endothelial cells as well as HSCs and hematopoietic progenitors including the yolk sac EMPs at E8.0 (Hoeffel et al., 2015; Ng et al., 2010; Samokhvalov et al., 2007). Runx1-CreERT2 transgenic mice were generated by introducing multiple copies of the Runx1 enhancer element eR1-driven CreERT2 cassette (Matsuo et al., 2017). We induced tdTomato expression in Hlf-CreERT2; ROSA-LSL-tdTomato and Runx1-CreERT2; ROSA-LSL-tdTomato embryos at E8.0 and analyzed the distribution of tdTomato-expressing Hlf- and Runx1-lineage cells in the developing brain parenchyma such as hindbrain, midbrain, and diencephalon at E15.5 (Fig. 3A;B, and S3A). Interestingly, all tdTomato-expressing Hlf-lineage cells were found in the developing vasculature of the hindbrain (10.2±2.4% of blood vessels were positive for tdTomato), the midbrain (5.5±2.3%), and the diencephalon (8.1±2.8%) of Hlf-CreERT2; ROSA-LSL-tdTomato brain at E15.5 (Fig. 3C-K, arrowheads; Fig. 4R). Hlf-lineage cells were rarely found in the cerebral cortex. High-resolution imaging revealed the tdTomato expression in PECAM-1+ endothelial cells as well as PDGFRβ+ pericytes (Fig. 3L-P, arrows, tdTomato/PDGFβ) (PECAM-1+ endothelial cells; 3Q-U, open arrows, tdTomato/PDGFβ+ pericytes). Flow cytometric analysis of Hlf-CreERT2; ROSA-LSL-tdTomato brain at E15.5 also revealed that Hlf-lineage cells contribute to both endothelial cells and pericytes in the developing brain vasculature (Fig. 3V, 1.3±0.34% of endothelial cells from whole-brain were positive for tdTomato and 0.90±0.44% of pericytes from whole-brain were positive for tdTomato). These results clearly demonstrate that cephalic mesenchyme contributes to the developing brain vasculature. In contrast, tdTomato-expressing Runx1-lineage cells contribute to both vascular cells and non-vascular cells in the brain parenchyma (Fig. S3B-J, arrowhead, vascular cells; open arrowheads, non-vascular cells; Fig. S4M) such as the hindbrain (18.1±5.8% of blood vessels were positive for tdTomato), the midbrain (7.4±2.3%), and the diencephalon (8.9±1.4%) of E15.5 Runx1-CreERT2; ROSA-LSL-tdTomato brain (Fig. S4M). Like Hlf-CreERT2; ROSA-LSL-tdTomato brain, we found tdTomato expression in PECAM-1+ endothelial cells as well as PDGFRβ+ pericytes (Fig. S3K-O, arrows, tdTomato/PECAM-1+ endothelial cells; S3P-T, open arrows, tdTomato/PDGFβ+ pericytes). Both Hlf- and Runx1-lineage cells contribute to the developing vasculature in three major regions of the brain parenchyma, albeit with different levels of contribution to vascular cells (Fig. 4R versus Fig. S4M). All these results indicate that, compared with Runx1-lineage cells, the differentiation potential of Hlf-expressing cephalic mesenchymal cells at E8.0 appears to be restricted to the developing brain vasculature.

No contribution of Hlf-expressing cephalic mesenchymal cells to the muscular tissues

We next examined a contribution of Hlf-lineage cells to muscular tissues in the embryonic head such as tongue in the oral cavity (Fig. 4A). Given that tdTomato-expressing Hlf-lineage cells were found in PECAM-1+ vasculature in the tongue of Hlf-CreERT2; ROSA-LSL-tdTomato embryos at E15.5, Hlf-lineage cells were not detectable in muscle progenitors which express Desmin, a muscle-specific intermediate filament (Fig. 4B-D). Likewise, tdTomato-expressing Runx1-lineage cells were found in PECAM-1+ vasculature but not in Desmin+ muscle progenitors in the tongue of E15.5 Runx1-CreERT2; ROSA-LSL-tdTomato embryos (Fig. S4A-
Fig. 1. Hlf-expressing cells in the cephalic mesenchyme at E8.5. (A–J) Triple RNA whole-mount in situ hybridization chain reaction of E8.5 wild-type (WT) embryos. (A,F) The position of E8.5 embryos is indicated on the bright field image. The boxed region in A is magnified in F. Scale bars: 100 µm. (B–E,G–J) Maximum intensity projection images from triple RNA whole-mount in situ hybridization chain reaction using probes to Hlf (B and G, green; C and H, white) and the paraxial mesoderm marker Pax3 (B and G, blue; D and I, white) and Eomes (B and G, red; E and J, white). The boxed region in B is magnified in G. The boxed region in G is magnified in the bottom right. Scale bars: 100 µm. (K) Generation of Hlf-tdTomato reporter mice (Yokomizo et al., 2019). (L) The position of E8.5 embryo is indicated on the autofluorescence image of 488-channel. Scale bars: 100 µm. (M–O) Maximum intensity projection images from whole-mount immunofluorescent labeling of E8.5 Hlf-tdTomato reporter embryos using antibodies to tdTomato (red) and the pan-endothelial cell marker PECAM-1 (green). Scale bars: 100 µm. (P–U) Representative Z-slice images from whole-mount immunofluorescent labeling of E8.5 Hlf-tdTomato reporter embryos. The boxed regions in P, R and T are magnified in Q, S and U, respectively. Scale bars: 100 µm. n=2.
Fig. 2. Transient expression of Hlf marks cephalic mesenchymal cells. (A) Generation of Hlf-CreERT2 mice and Hlf-CreERT2; ROSA-LSL-tdTomato embryos. Tamoxifen was administered by oral gavage at E8.0 and embryos were harvested at E9.0 for analysis. (B-G) Representative Z-slice images from whole-mount immunofluorescent labeling of E9.0 Hlf-CreERT2; ROSA-LSL-tdTomato embryos with antibodies to tdTomato (red) and PECAM-1 (green). The boxed regions in C and F are magnified in D and G, respectively. Arrowheads indicate tdTomato-expressing Hlf-lineage cells. Scale bars: 100 µm, n=6. (H-M) Maximum intensity projection images from whole-mount immunofluorescent labeling of Hlf-CreERT2; ROSA-LSL-tdTomato embryos at E9.0 with antibodies to tdTomato (H and K, red; J and M, white) and PECAM-1 (H and K, green; I and L, white). Scale bars: 100 µm, n=6.
Fig. 3. See next page for legend.
expressing endothelial cells can be a source of the liver vasculature. Runx1 is expressed by embryonic endothelial cells at E8.0, Runx1-ttdTomato quantification of tdTomato+ blood vessels in the hindbrain, midbrain, and in (F-K). Arrowheads indicate tdTomato+ vascular cells. Note that pericyte marker PDGFRß (C-H, green) and the endothelial cell marker PECAM-1 (C-H, blue; I-K, white). The boxed regions in (C-E) are magnified in (F-K). Arrowheads indicate tdTomato+ vascular cells. Note that expression of tdTomato+ blood vessels in the hindbrain, midbrain, and diencephalon is shown in Fig. 4R. Scale bars: 100 µm (C-E) and 20 µm (F-K). n = 3. (L-U) High-resolution images of E15.5 Hlf-CreERT2; ROSA-LSL-ttdTomato brain sections stained with antibodies to tdTomato (red) together with PDGFRß (L, N, Q, and S, green; P and U, white) and PECAM-1 (L, M, Q, and R, blue; O and T, white). Arrows indicate tdTomato+/PECAM-1+ endothelial cells; open arrows indicate tdTomato+/PDGFRß+ pericytes. Scale bars: 10 µm. n=3. (V) Representative flow cytometry data analyzing E15.5 Hlf-CreERT2; ROSA-LSL-ttdTomato mouse model, the results presented here identify angiogenic cephalic and rostral trunk mesenchymal cells. Another subset of angiogenic cephalic mesenchyme cells. Another contribution of Hlf-expressing cells in the rostral trunk to the tissue vasculature Since a few Hlf-lineage cells were found in the rostral trunk region, we next examined whether Hlf-lineage cells also contribute to the vasculature of the lung, liver, and heart at E15.5 (Fig. 4E,F). Immunostaining analysis showed a significant contribution of tdTomato-expressing Hlf-lineage cells to the vasculature of lung (5.5±1.2% of blood vessels were positive for tdTomato), but not liver (0.95±0.44%) and heart (2.2±1.5%) in Hlf-CreERT2; ROSA-LSL-ttdTomato embryos at E15.5 (Fig. 4G-O,R). These data suggest that Hlf-expressing cells in the rostral trunk at E8.0 are angiogenic mesenchymal cells. Note that some Hlf-expressing cells in the trunk are also Hlf-lineage cells but not non-vascular cells such as cardiomyocytes in the heart (Fig. 4P-Q). Meanwhile, tdTomato-expressing Runx1-lineage cells largely contribute to the vasculature of liver (33.7±3.5% of blood vessels were positive for tdTomato) and heart (12.2±4.7%), but not lung (1.1±0.2%) in E15.5 Runx1-CreERT2; ROSA-LSL-ttdTomato embryos (Fig. 4D-L,M). Since Runx1 is expressed by embryonic endothelial cells at E8.0, Runx1-expressing endothelial cells can be a source of the liver vasculature.

No contribution of Hlf-expressing cephalic mesenchymal cells to tissue-localized macrophages Having established that cephalic mesenchyme contains hemogenic endothelial cells (Gama Sosa et al., 2021; Li et al., 2012), we next examined the differentiation potential of Hlf- and Runx1-lineage cells into tissue-localized macrophages. No significant contribution of Hlf-lineage cells was found in F4/80+ macrophages in brain, liver, lung, and heart in Hlf-CreERT2; ROSA-LSL-ttdTomato embryos at E15.5 (Fig. 5A). Consistent with previous studies demonstrating that EMPs contribute to tissue-localized macrophages in multiple organs (Gomez Perdiguero et al., 2015; Hoeffel et al., 2015), Runx1-lineage cells contributed to F4/80+ macrophages in brain (61.7±12.0% of F4/80+ macrophages were positive for tdTomato in the midbrain, 61.0±2.1% in the hindbrain, 64.7±5.4% in the diencephalon), liver (29.0±2.9%), lung (40.4±6.5%), and heart (13.6±6.9%) in E15.5 Runx1-CreERT2; ROSA-LSL-ttdTomato embryos (Fig. S5M-X,Y). These data suggest that the differentiation potential of Hlf-expressing mesenchymal cells at E8.0 appears to be restricted to vascular cells, while Runx1-expressing cells at E8.0 can differentiate into both vascular cells and hematopoietic cells including tissue-localized macrophages.

Hlf-expressing cells at E10.5 are devoid of the angiogenic potential Previous studies demonstrated that Hlf expression marks hematopoietic clusters in the AGM but not EMPs in the yolk sac, and endothelial cells of the yolk sac, AGM, and fetal liver at E10.5 (Yokomizo et al., 2019), but the studies did not examine what Hlf expression marks in the head. We first examined whether Hlf-expressing cells at E10.5 have an angiogenic potential. We induced tdTomato expression in Hlf-CreERT2; ROSA-LSL-ttdTomato embryos at E10.5 and analyzed the distribution of tdTomato-expressing Hlf-lineage cells at E15.5 (Fig. S6A). Given that brain microglia are mainly derived from yolk-sac EMPs but not AGM hematopoietic progenitors (Hoeffel et al., 2015), Hlf-lineage cells hardly contributed to microglia in the brain parenchymal region (Fig. S6B-J,L); a few Hlf-lineage cells contributed to F4/80+ meningeal macrophages (Fig. S6C,F, open arrowheads). Likewise, Hlf-lineage cells were not detectable in the developing brain vasculature (Fig. S6B-J,K). The observation that Hlf-expressing cells at E8.0 are angiogenic but the cells at E10.5 are devoid of the angiogenic potential suggest that a transient expression of Hlf marks angiogenic mesenchymal cells.

DISCUSSION

With the genetic lineage tracing experiments using Hlf-CreERT2; ROSA-LSL-ttdTomato mouse model, the results presented here identify angiogenic cephalic and rostral trunk mesenchymal cells. Previous genetic lineage tracing experiments using paraxial mesoderm-specific Cre lines demonstrated that paraxial mesoderm contributes to trunk and limb vasculature, but these experiments did not show the developmental timing of the paraxial mesoderm commitment into vascular lineage. Given that Hlf expression marks vascular cells but not muscle cells in the head and neck regions, Hlf-expressing cephalic mesenchymal cells at E8.0 are mainly committed to vascular lineage (Fig. 5).

Given that the recombination efficiency of Hlf-CreERT2 is high, based on the observation that the expression of tdTomato reporter is present in the vast majority of Hlf-expressing cells in Hlf-CreERT2; ROSA-LSL-ttdTomato embryos at E9.0 (24 h after tamoxifen administration) (Fig. S2I-P), the Hlf-lineage contribution in the brain vascular cells at E15.5 is relatively low (Fig. 3V, the contribution of Hlf-lineage cells in the whole-brain vasculature; Fig. 4R, the contribution of Hlf-lineage cells in the vasculature of hindbrain, midbrain, and diencephalon). One potential explanation is that non-Hlf-lineage cells are the major contributors to the brain vascular cells and Hlf-lineage cells merge at some point to contribute to the brain vascular cell populations. Indeed, PECAM-1+ endothelial cells in the nascent vascular plexus around the open neural tube (the future PNVP) are negative for tdTomato in Hlf-CreERT2; ROSA-LSL-ttdTomato embryos at E9.0 (Fig. 2D), suggesting that Hlf-lineage cells marked at E8.0 do not contribute to these endothelial cells. In this scenario, Hlf marks a subset of angiogenic cephalic mesenchyme cells. Another
Fig. 4. See next page for legend.
Fig. 4. Contribution of Hlf-expressing cells to the vasculature of head, liver, lung, and heart. (A) A representative immunofluorescent image of sagittal sections of E15.5 head stained with anti-PECAM-1 antibody (white). The yellow box indicates oral cavity. (B-D) Representative immunofluorescent images of oral cavity sections from E15.5 Hlf-CreERT2; ROSA-LSL-tdTomato stained with antibodies to tdTomato (red) together with the muscle progenitor marker Desmin (green) and the endothelial marker PECAM-1 (blue). The boxed regions in B are magnified in C and D. Arrowheads indicate tdTomato+ vascular cells. Scale bars: 100 µm, n=3. (E-F) A representative immunofluorescent image of transverse sections of the trunk region of E15.5 mouse embryos including liver (E), lung (F), and heart (F) stained with anti-PECAM-1 antibody (white). The yellow boxes show the region of liver, lung, and heart in the trunk region. (G-O) Representative immunofluorescent images of liver, lung and heart sections of E15.5 Hlf-CreERT2; ROSA-LSL-tdTomato embryos stained with antibodies to tdTomato (red), PDGFRα (green) and PECAM-1 (G-L, blue; M-O, white). The boxed regions in G-I are magnified in J-O. Arrowheads indicate tdTomato+ vascular cells; open arrowheads indicate tdTomato+ non-vascular cells. Scale bars: 100 µm (G-I) and 20 µm (J-O). n=3. (P-O) Representative immunofluorescent images of heart sections of E15.5 Hlf-CreERT2; ROSA-LSL-tdTomato embryos stained with antibodies to tdTomato (red), cardiac troponin T (CTNT, green) and PECAM-1 (blue). The boxed region in P is magnified in Q. Open arrowheads indicate tdTomato+ cardiomyocytes. (R) Quantification of tdTomato+ blood vessels in each tissue. The results are shown as the mean±s.e.m. n=3 in each group.

An explanation is that Hlf-expressing cells earlier than E8.0 may initiate the first wave of brain vascularization including PNVP; these Hlf-expressing cells turn off the Hlf expression by E8.0, so these cells are not genetically marked in Hlf-CreERT2; ROSA-LSL-tdTomato embryos with tamoxifen administration at E8.0. In this scenario, angiogenic cephalic mesenchyme cells are heterogeneous and transiently express Hlf at different developmental time points.

Although the transient expression of Hlf marks an angiogenic mesenchyme, it is not clear whether Hlf is required for the differentiation of mesenchymal cells into vascular cells. Previous studies demonstrated that Hlf homozygous mutants are morphologically normal and fertile (Gachon et al., 2004). Given that Hlf is specifically expressed in HSCs in the AGM and fetal liver (Yokomizo et al., 2019) and the adult bone marrow (Komorowska et al., 2017; Wahlestedt et al., 2017), Hlf is dispensable for HSC generation. Likewise, given that Hlf marks a classical mesodermal origin of CNS vascular cells, Hlf appears not to be required for CNS vascular development. It is possible that other Par-bZip transcription factors (Dbp and Tef), which share a similar DNA binding motif with Hlf, may compensate for loss of Hlf in the vascular development. What controls Hlf expression may provide an insight in understanding the transcriptional machinery of mesodermal differentiation into vascular cells.

MATERIALS AND METHODS
Mice
All animal procedures were approved by the Animal Care and Use Committee of Kumamoto University; the National Heart, Lung, and Blood Institute (NHLBI) Animal Care and Use Committee in accordance with NIH research guidelines for the care and use of laboratory animals. The following mice were used in this study: Hlf-tdTomato knock-in mice (Yokomizo et al., 2019), Runx1-CreERT2 transgenic mice (Matsuo et al., 2017), and ROSA-LSL-tdTomato mice (Madisen et al., 2010). The targeting strategy of Hlf-CreERT2 is the same as the one of Hlf-tdTomato reporter mice as described previously (Yokomizo et al., 2019). Hlf-CreERT2 knock-in mice were generated in the Kumamoto University by inserting a T2A-CreERT2 gene fusion before the endogenous stop codon within exon 4. The targeting construct was knocked into the locus using the CRISPR/Cas9 method. The detailed procedure will be reported elsewhere (Yokomizo et al., Nature in press). The Cre-mediated excision was induced by administrating 2.5 mg tamoxifen (T-5648, Sigma-Aldrich) by oral gavage at embryonic days (E) 8.0 or 10.5 and embryos were harvested at E9.0 or E15.5. Hlf-CreERT2, ROSA-LSL-tdTomato or Runx1-CreERT2, ROSA-LSL-tdTomato double heterozygous embryos were used for all experiments.

Whole-mount immunostaining of embryos
Staining was performed essentially as described previously (Yokomizo et al., 2019, 2012). Staining was performed using anti-PECAM-1/CD31 antibody (Clone MEC 13.3, BD Pharmingen, 1:500) to detect endothelial cells and anti-RFP antibody (Rockland, 1:1000) to detect tdTomato. For immunofluorescent detection, either Cy3 or Alexa-647 conjugated secondary antibodies were used. All confocal microscopy was carried out on an Olympus FV1200 confocal equipped with GaAsP PMT detectors (Olympus).

Section immunostaining of embryos
Staining was performed essentially as described previously (Li et al., 2013). Embryos were fixed with 4% paraformaldehyde/ PBS at 4°C overnight, sunk in 30% sucrose/ PBS at 4°C and then embedded in OCT compound. Embryos were cryosectioned at 25 µm thickness and collected on pre-cleaned slides (Matsunami, Japan). Staining was performed using Armenian hamster anti-PECAM-1/CD31 antibody (Clone 2H8, Chemicon, 1:200) to detect endothelial cells, Rat anti-PDGFRα antibody (Clone AB5, eBioscience, 1:100) to detect pericytes, Rabbit anti-DsRed antibody (Takara, 1:1000) to detect tdTomato, Rat anti-F4/80 antibody (Clone BM8, Invitrogen, 1:500) to detect macrophages, and Mouse anti-Desmin antibody (Clone D33, Dako, 1:500). For immunofluorescent detection,

Fig. 5. Contribution of Hlf-expressing cephalic mesenchyme to the brain vasculature. Graphical summary was created with BioRender.com.
either Alexa-488, Alexa-568, or Alexa-647 conjugated secondary antibodies (Jackson ImmunoResearch or Thermo Fisher Scientific, 1:250) were used. All confocal microscopy was carried out on a Leica TCS SP5 confocal (Leica). Area of tdTomato positive blood vessels and macrophages were quantified using ImageJ (NIH). The percentage of tdTomato-positive blood vessels was based on the area of tdTomato-positive blood vessels within the area of PECAM-1-positive blood vessels (Fig. 4R; Figs S4M, S6K). Likewise, the percentage of tdTomato-positive macrophages was based on the area of tdTomato-positive macrophages within the area of F4/80-positive macrophages (Figs S5Y, S6L).

Cell preparation and flow cytometry
Single-cell suspensions of embryonic brains were prepared by treating tissues with 0.1% collagenase/0.3% Dispase/0.05% DNasel solution for 45 min at 37°C. Cells were stained with fluorescence-conjugated antibodies: CD45-BB708 (Clone 30-F11, BD Biosciences), Ter119-FITC (Clone TER-119, BioLegend), PECAM-1/CD31-BV421 (Clone MEC 13.3, BD Biosciences), and PDGFRa-APC (Clone AP5B1, eBioscience). Non-staining controls were used to define positive gates. Cells were analyzed by FACS AriaIII (BD Biosciences).

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