Erythro-myeloid progenitors contribute endothelial cells to blood vessels

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The earliest blood vessels in mammalian embryos are formed when endothelial cells differentiate from angioblasts and coalesce into tubular networks. Thereafter, the endothelium is thought to expand solely by proliferation of pre-existing endothelial cells. Here we show that a complementary source of endothelial cells is recruited into pre-existing vasculature after differentiation from the earliest precursors of erythrocytes, megakaryocytes and macrophages, the erythro-myeloid progenitors (EMPs) that are born in the yolk sac. A first wave of EMPs contributes endothelial cells to the yolk sac endothelium, and a second wave of EMPs colonizes the embryo and contributes endothelial cells to intraembryonic endothelium in multiple organs, where they persist into adulthood. By demonstrating that EMPs constitute a hitherto unrecognized source of endothelial cells, we reveal that embryonic blood vascular endothelium expands in a dual mechanism that involves both the proliferation of pre-existing endothelial cells and the incorporation of endothelial cells derived from haematopoietic precursors.

Blood vessels distribute oxygen, nutrients, hormones and immune cells through the vertebrate body and help to remove waste molecules. Accordingly, the formation of functional blood vessels during embryogenesis is a prerequisite for vertebrate life. Endothelial cells (ECs) form the inner lining of blood vessels; they first arise from mesenchymal precursors termed angioblasts on embryonic day (E)7.0 in mice. After condensing into the yolk sac vasculature and paired dorsal aortae, ECs proliferate within existing endothelium to increase vascular diameter, sprout into avascular tissue areas or remodel into smaller vessels by intussusceptive growth. The current consensus is therefore that embryonic ECs are a self-contained cell lineage that expands without contribution from new angioblasts or circulating precursors. By contrast, circulating endothelial progenitors have been proposed to exist in adult vertebrates, although their relationship to myeloid cells remains controversial.

In addition to their primary roles in the innate immune system, myeloid cells such as monocytes and macrophages also modulate vascular growth. For example, the tissue-resident macrophages of the embryonic mouse brain, termed microglia, contact ECs at the tips of neighbouring vessel sprouts to promote their anastomosis into perfused vessel loops. By contrast, no direct contribution of myeloid cells to embryonic vascular endothelium has been reported; thus, genetic lineage tracing with myeloid Vav promoter activity and accordingly did not contain CSF1R protein.

Csflr lineage tracing identifies an EC subset
To target early EMPs7,10,12, microglia16,17 and other differentiated myeloid cells18, we and others have used a transgene that expresses CRE recombinase under the promoter for the myeloid lineage gene Csflr (also known as Pims), which encodes the colony-stimulating factor 1 receptor CSF1R. Microglia appear as single YFP+ cells in hindbrains from Csflr-iCre mouse embryos carrying the Rosa26SVP expansion (Fig. 1a–c; Extended Data Fig. 1a). Csflr-iCre targeting of vessel-bound cells was not an artefact caused by spontaneous Rosa26YFP recombination or unspecific immunostaining, because littermates that did not carry Csflr-iCre lacked YFP staining (Fig. 1a). Furthermore, hindbrain imaging from mice carrying Csf1r-iCre with CAG-Cat-Egfp or Rosa26Tom as alternative recombination reporters confirmed targeting of both microglia and vessel-bound elongated cells (Extended Data Fig. 1b, c). The tamoxifen-induced activation of CRE, expressed from an independently generated Csf1r-I-Cre-Mer transgene that targets myeloid cells19, also targeted vessel-bound cells in addition to microglia (Fig. 1d). Corroborating the endothelial identity of Csflr-iCre-targeted, elongated vessel-bound cells, these cells expressed the EC markers ERG and PECAM1, had a similar morphology to ECs targeted with the endothelium-specific Cdh5-CreER22 transgene, formed junctions with neighbouring ECs via the endothelial cadherin CDH5 and lacked both myeloid and pericyte markers (Fig. 1e, Extended Data Fig. 1d–f).

Csflr-iCre-mediated EC targeting was not explained by Csf1r expression in bone ECs, because hindbrain ECs, unlike microglia, lacked expression of a Csf1r-Egfp transgene that faithfully reports Csflr promoter activity20,21 and accordingly did not contain CSF1R protein.
between YFP Csf1r-Mer-iCre-Mer;RosatdTom embryos of the indicated genotypes, whole-mount labelled with the determination; goodness of fit, PHindbrain, vasculature. lineage tracing identifies ECs in developing brain Csf1r-iCre (Fig. 1 | 224 | NA t U re  | VO l  562 | 11 O ct OB  | 2018)

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Csf1r lineage-traced ECs derive from EMPs

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mation of Csf1r-iCre-targeted ECs was spatiotemporally linked to the emergence of Csf1r-expressing ECs from yolk sac haemogenic endothelium, which was visualized by staining for the EC marker VEGFR2. At E8.5, yolk sacs from Csf1r-Egfp embryos contained clusters of round EGFP+VEGFR2+ cells that protruded from the endothelium into the vascular lumen (Fig. 2a), consistent with previous work showing that FACS-isolated EMPs express both Csf1r [2] and VEGFR2 [2], and that EMPs bud from the yolk sac endothelium [11]. Csf1r-iCre lineage

tracing in yolk sacs at E8.5 similarly identified round YFP+ cells that protruded into the vascular lumen, expressed VEGFR2, persisted in PU.1-deficient yolk sacs and expressed the EMP marker KIT [7] (Fig. 2b, c; Extended Data Fig. 3a, b). Even though EGFP expression could not be detected in Csf1r-Egfp yolk sac endothelium (Fig. 2a), Csf1r-iCre;RosaYfp also targeted a subset of yolk sac ECs that lacked obvious

Extended Data Fig. 2a, b). Moreover, our analysis of published transcriptomic datasets [22] showed that Csf1r is not expressed in ECs from embryonic brain, liver or lung, whilst quantitative PCR with reverse transcription (RT-qPCR) analysis of tdTomato+ ECs isolated by fluorescence-activated cell sorting (FACS) confirmed that they expressed Cdh5, but not Csf1r or the myeloid gene Spi1, which encodes the PU.1 transcription factor (Extended Data Fig. 2c–g). The lack of endothelial Csf1r expression suggests that Csf1r-iCre-targeted brain ECs arise from precursors in which Csf1r is activated before their incorporation into hindbrain vasculature. These precursors cannot be differentiated myeloid cells such as microglia, whose formation is PU.1-dependent, because PU.1 deficiency did not reduce the number of Csf1r-iCre-targeted ECs in the hindbrain at E11.5 (Fig. 1f–h) or the striatum at postnatal day (P)0 (Extended Data Fig. 2h). We therefore investigated whether Csf1r-iCre-targeted ECs are derived from PU.1-independent, Csf1r-expressing precursors.

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KIT expression (Extended Data Fig. 3b), showing that they were not haemogenic ECs\(^{a,4}\). Similar to Csf1r-iCre-targeted hindbrain ECs, the lineage-traced yolk sac ECs were PU.1-independent (Fig. 2b, c).

The finding that EMP formation correlates with the emergence of Csf1r-iCre-targeted yolk sac ECs was corroborated by temporally restricted Csf1r-Mer-iCre-Mer-mediated lineage tracing. As tamoxifen-induced, CRE-mediated reporter recombination peaks approximately 6 h and ends 24 h after tamoxifen injection\(^{26}\), we activated Csf1r-Mer-iCre-Mer:Rosa\(^{tdTom}\) in discrete temporal windows by single injections at E8.5, E9.5 or E10.5 before identifying lineage-traced cells in E12.5 yolk sacs (Extended Data Fig. 3c). Induction at all three stages labelled yolk sac macrophages (Extended Data Fig. 3d), consistent with their origin from Csf1r-expressing EMPs\(^{5}\) and their maintenance of Csf1r expression\(^{10,12}\). In addition, induction at E8.5 or E9.5 yielded tdTomato\(^{+}\) ECs, whereas induction at E10.5 did not (Extended Data Fig. 3d). As EMPs are present in the yolk sac at E8.5 and E9.5, but move to the liver thereafter\(^{13}\), their local availability makes them plausible precursors of Csf1r-iCre-labelled yolk sac ECs. Consistent with this, tamoxifen induction of a Kit\(^{CreERT2}\) knock-in allele at E8.5, when KIT+ early EMPs are still present in the yolk sac\(^{5}\), lineage-traced both yolk sac ECs and macrophages (Extended Data Fig. 3e, f).

In contrast to early wave EMPs that remain in the yolk sac, the late wave EMPs that populate the embryo are reported to lack Csf1r expression, at least when they form in the yolk sac\(^{7}\). We therefore investigated whether late wave EMPs begin to express Csf1r after homing to the liver and whether they are the precursors of the Csf1r-iCre-targeted ECs that appear in the hindbrain from E10.5 onwards. Thus, we combined the Csf1r-Egfp expression reporter with Csf1r-Mer-iCre-Mer:Rosa\(^{tdTom}\) and

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**Fig. 3** Csf1r-iCre-targeted hindbrain ECs emerge from intraembryonic EMPs. a, b, A pregnant Csf1r-Egfp:Csf1r-Mer-iCre-Mer:Rosa\(^{tdTom}\) dam was injected with a single dose of tamoxifen on E10.5 (a) before FACS of E11.5 liver and blood cells (b) to gate the CDM5\(^{hi}\)/KIT\(^{+}\) differentiated myeloid cell (MCs; blue) and CD45\(^{hi}\)/KIT\(^{+}\) EMP/myeloid progenitor (MPs; pink) populations for EGFP and tdTomato (Extended Data Fig. 3d). As EMPs are present in the yolk sac at E8.5 and E9.5, but move to the liver thereafter\(^{13}\), their local availability makes them plausible precursors of Csf1r-iCre-labelled yolk sac ECs. Consistent with this, tamoxifen induction of a Kit\(^{CreERT2}\) knock-in allele at E8.5, when KIT+ early EMPs are still present in the yolk sac\(^{5}\), lineage-traced both yolk sac ECs and macrophages (Extended Data Fig. 3e, f).

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**Fig. 4** EMPs in the liver and blood give rise to ECs in vitro. a, b, FACS strategy to separate the differentiated myeloid cell and EMP/MP populations from E12.5 Csf1r-iCre-Rosa\(^{tdTom}\) liver (a) and blood (b) using antibodies for CD45 and KIT after excluding PECAM1\(^{+}\) cells to prevent EC contamination. c, d, tdTomato\(^{+}\) proportions in the FACS-isolated myeloid cell versus EMP/MP populations from liver and blood shown in a, b and Giemsa–Wright staining of representative cells. Mo, monocyte; GC, granulocyte; Mn, macrophage. e–h, Bright-field images of myeloid (white) and erythroid (rust-coloured) colonies (e, f) and immunofluorescence of adherent cells (g, h) after three days in methocult (met.) on fibronectin. Adherent cells were immunolabelled for ERG and VEGFR2, counterstained with DAPI and shown together with tdTomato fluorescence (Tom). Arrows, tdTomato\(^{+}\) ECs; arrowheads, tdTomato\(^{+}\) myeloid cells; solid and clear symbols indicate high and low marker expression, respectively. Scale bars, 20 μm. n = 3 independent experiments.
induced CRE-mediated recombination at E10.5; 24 h later, we used FACS to separate the differentiated myeloid cells from EMPs and EMP-derived myeloid progenitors contained in the liver or blood (Fig. 3a, b). The differentiated myeloid cell populations from both sources contained tdTomatoEGFP cells, as expected, but tdTomatoEGFP cells were also present in the EMP/myeloid progenitor populations from both liver and blood (Fig. 3a, b; Extended Data Fig. 3g–i). These findings suggest that a subset of intraembryonic EMPs expresses Csf1r and can access organs such as the hindbrain via the circulation.

To determine whether the intraembryonic presence of Csf1r-expressing late wave EMPs correlated with the emergence of Csf1r-iCre-targeted hindbrain ECs, we visualized tdTomato expression in hindbrains from E12.5 Csf1r-iCre;Hoxa fl/fl mice after tamoxifen induction at E8.5, E9.5 or E10.5 (Fig. 3c). The hindbrain vasculature contained tdTomato ECs following induction at E10.5, but not at E8.5 or E9.5, even though the Csf1r-iCre-expressing microglia were targeted at all stages (Fig. 3d). KitCreERT2 induction at E8.5 also caused microglia targeting (Fig. 3e, f), consistent with microglia arising from yolk sac macrophages generated at around E8.5 from KIT+ early wave EMPs. Induction of KitCreERT2 at E8.5, when late wave EMPs begin to arise in the yolk sac, also yielded tdTomato ECs in the hindbrain at E12.5 (Fig. 3e, f), confirming that yolk sac-born EMPs can give rise to intraembryonic ECs. Lineage tracing from three independent Cre alleles therefore suggests that EMPs give rise to both yolk sac and hindbrain ECs.

Csf1r-expressing EMPs give rise to ECs in vitro

The myeloid and erythroid potential of EMPs has been demonstrated through in vitro differentiation.11,26 Using similar assays, we compared the endothelial potential of FACS-isolated differentiated myeloid cell and EMP/myeloid progenitor populations from E12.5 Csf1r-iCre;Rosa tdTom liver and blood, while ensuring that we were excluding contamination by PECAM1+ ECs (Fig. 4a, b). Both cell populations were mostly tdTomato+ (Fig. 4c, d). As expected, the EMP-containing population comprised round cells with a large nucleus and little cytoplasm, whereas the myeloid cell population contained granulocytes, in addition to monocytes in the liver and macrophages in the blood (Fig. 4c, d). For cell culture, we used methylcellulose to promote the formation of haematopoietic colonies, but included a fibronectin substrate to facilitate EC differentiation. Differentiated myeloid cells persisted in these cultures as single round or amoeboid cells (Fig. 4e, f) that were tdTomato+ ERGlowVEGFR2low (Fig. 4g, h; antibody controls in Extended Data Fig. 4a, b). By contrast, both liver and blood EMPs formed myeloid and erythroid cell colonies in suspension (Fig. 4e, f) and additionally gave rise to single adherent cells that appeared spindle-shaped, were tdTomato+ ERGhighVEGFR2high and lacked myeloid cell markers, consistent with an EC identity (Fig. 4g, h; Extended Data Fig. 4c). Together, these experiments demonstrate that EMPs have endothelial potential alongside their known haematopoietic capacity.

Csf1r lineage ECs support blood vessel growth

Hoxa cluster genes modulate haematopoiesis27 and are upregulated in perinatal ECs compared to adult ECs (Fig. 5a); HOXA9 also promotes EC differentiation from progenitors in ischaemic disease in adults.29 Our analysis of published transcriptomic data revealed that Hoxa transcripts are enriched in E10.25 EMPs compared to E9.0 EMPs and macrophages (Fig. 5a). To investigate whether Hoxa deficiency impairs the formation of EMP-derived hindbrain ECs, we combined Csf1r-iCre with a conditional null Hoxa cluster mutation (Hoxa) (Extended Data Fig. 5a). Gene copy analysis showed effective gene targeting in KItCreERT2;Csf1r-iCre:Hoxa+/- mutants at E12.5 compared to control livers, but the number of liver CD45+ cells, including differentiated myeloid cells, was not reduced (Extended Data Fig. 5b–f). Hoxa genes are therefore dispensable for myeloid cell specification from late wave EMPs. By contrast, fewer tdTomato+ ECs, also derived from late wave EMPs, had formed in Rosa tdTom-carrying Csf1r-iCre:Hoxa+/- mutant hindbrains compared to control hindbrains; moreover, SVP complexity was reduced in mutant hindbrains (Fig. 5b–d). Although we observed 20% fewer microglia in mutant hindbrains than in control hindbrains (Extended Data Fig. 5g–i), this is not likely to have contributed to the vascular defect, because even a 50% microglia reduction in Csf1r-/- mutants did not reduce SVP complexity (Extended Data Fig. 5j–l). Together, these findings suggest that Hoxa cluster genes promote the formation of EMP-derived brain ECs, which in turn support normal brain vascular development.

Transcriptional signature of Csf1r lineage ECs

Csf1r-iCre-targeted ECs not only appeared morphologically similar to neighbouring ECs (Fig. 1), but also had similarly slow proliferation and overall cell cycle kinetics (Extended Data Fig. 6). Moreover, RNA sequencing (RNA-seq) analysis of FACS-isolated tdTomato ECs from E12.5 Csf1r-iCre;Rosa tdTom embryos showed that they had largely similar transcriptomes, with only a few differentially expressed genes, including the expected difference in the tdTomato transcript (Fig. 6a–c; Extended Data Fig. 7a). Corroborating their endothelial identity, tdTomato+ ECs lacked markers for differentiated myeloid cells and other non-EC lineages, but expressed core EC transcripts at similar levels to tdTomato+ ECs (Fig. 6d, e). Amongst the differentially expressed genes, markers typical of EC specialization, such as ephrins and EPH receptors regulating arteriovenous differentiation, were under-represented in tdTomato+ ECs (Fig. 6e). This observation is consistent with Csf1r-iCre-targeted ECs being derived from progenitors that are recruited into preformed vascular endothelium. Whereas brain EC markers (for example, Slc2a1) were under-represented in the embryo-wide tdTomato+ EC population, liver EC markers (for example, Oit3, Mrc1) were over-represented, including early markers of liver sinusoidal differentiation (Stab2, Lyve1) (Fig. 6c, f; Extended Data Fig. 7b, c). Similar expression of Oit3 and Mrc1 in tdTomato+ and tdTomato- liver ECs (Extended Data Fig. 7d) suggests that the over-representation of liver EC transcripts in the total embryonic tdTomato+ EC population reflects their preferential contribution to
liver vasculature. Immunostaining and FACS of Csf1r-iCre;Rosa<sup>tdTom</sup> E12.5 and E18.5 embryos confirmed that tdTom<sup>+</sup> ECs were more prevalent than tdTom<sup>−</sup> ECs in liver endothelium (Fig. 6g, i; Extended Data Figs. 8, 9a, b). As liver EC specialization markers were present in both tdTom<sup>−</sup> and tdTom<sup>+</sup> liver ECs at E12.5 (Fig. 6g; Extended Data Fig. 8a), liver ECs from two distinct origins appear to undergo similar organ-specific EC differentiation.

**Csf1r** lineage ECs persist in multiple adult organs

Immunostaining and FACS analyses at E12.5 and E18.5 showed that Csf1r-iCre-targeted ECs were also present in the heart and lung vasculature at similar levels to the brain (Fig. 6i; Extended Data Figs. 8, 9a, b). Corresponding immunostaining and FACS analyses showed that tdTom<sup>+</sup> ECs persisted in the brain, heart, lung and liver of adults and continued to dominate the adult liver sinusoidal endothelium (Fig. 6h, j; Extended Data Figs. 9c, 10a). Accordingly, all adult organs examined contained EMP-derived ECs.

**Discussion**

The heterogeneous origin of blood vascular mural cells from distinct populations of mesodermal progenitors, haematopoietic and neural crest cells has been established. Here we have shown that embryonic
vascular endothelium has two major origins. Thus, ECs emerge via a classical pathway of angiblast differentiation into ECs and the pathway described in this report, which entails differentiation of ECs from the EMP lineage (Extended Data Fig. 10b). Multiple previous investigations have used Csf1r-Cre together with recombination reporters to follow the embryonic myeloid lineage10,12. These studies predominantly used FACS with haematopoietic markers, which precluded observation of Csf1r-Cre-targeted ECs. By contrast, we included EC markers in FACS protocols to additionally isolate Csf1r-Cre-targeted ECs. In addition, immunostaining was previously used to identify Csf1r-Cre-targeted cells in the retina17, liver and colon18, but without description of EC targeting, possibly because of the close spatial proximity of ECs and perivascular macrophages19,20. We overcame this limitation by performing high-resolution imaging of tissues immunostained with both EC and myeloid cell markers. The contribution of EMP-derived ECs to the yolk sac, brain, heart and lung vasculature is proportionally smaller than that of ECs of classical origin, whereas EMP-derived ECs predominate in the liver, particularly the sinusoidal endothelium. Liver endothelium was previously reported to be heterogeneous in origin, with an endoderm lineage contribution of approximately 15% and the remainder of the liver EC population attributed to a venous origin14. Our results suggest that liver endothelium contains approximately 60% EMP-derived ECs. Preferential homing of EMPs to the liver after their entry into the embryonic circulation15, and the dependence of liver growth on rapid vascular expansion25, may explain the relatively large contribution of EMP-derived ECs to this organ. Ultimately, the discovery that EMPs provide a source of ECs for organ vasculature may open up new therapeutic avenues for vessel-dependent organ repair and regeneration. For example, EMPs or EMP-like EC progenitors, derived from human stem cells by modulating the expression of factors such as Hoxa genes, might be delivered systemically to support vascular growth in ischaemic diseases or to provide angiocrine signals that stimulate tissue stem cells.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41586-018-0552-x.

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29. Author contributions A.P, A.F. and C.R. conceived and planned this study, analysed data and co-wrote the manuscript. L.D. performed genetic crosses and genotyping. A.P and A.F. either performed experiments together or replicated each other’s experiments, except for the cell cycle and Hoxa studies, which were carried out by A.P and A.F., respectively. J.W.P. provided mouse strains. C.R. supervised the project. All authors reviewed and edited the manuscript.
30. Competing interests The authors declare no competing interests.
31. Additional information Extended data is available for this paper at https://doi.org/10.1038/s41586-018-0552-x. Supplementary information is available for this paper at https://doi.org/10.1038/s41586-018-0552-x. Reprints and permissions information is available at http://www.nature.com/reprints. Correspondence and requests for materials should be addressed to C.R. Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
ReSeArcH

Article reSeArcH using the BD Influx cell sorter (BD Biosciences); FlowJo software (FlowJo LLC) was (Life Technologies) or LIVE/DEAD Fixable Violet (Life Technologies). Single-cell

terms, doublets were eliminated using pulse geometry gates (FSC-H versus FSC-A PE/Cy7-conjugated rat anti-PECAM1 and performing FACS analysis. In all experi-

10

tissue, Cy5.5-conjugated rat anti-CD11b (clone M1/70, cat 101227) (all BioLegend).

390, cat 102418, lot B212262), FITC-conjugated rat anti-CD45 (clone 30-F11, (Sigma), incubated for 5 min with 0.5 mg/ml rat Fc block (Becton Dickinson)

µ FACS and cell culture.

confocal microscope (Zeiss) and processed using LSM image browser (Zeiss) and Photoshop IB4 (L2140, lot 085M4032V , Sigma) followed by Alexa-conjugated streptavidin or -rat IgG (Jackson ImmunoResearch). Note that CDH547, ERG48, EMCN49, chick, -rabbit or -rat IgG (Life Technologies), or, for primary antibodies raised in

Systems). Secondary antibodies used included Alexa Fluor-conjugated goat anti-

MCA497R, lot 1605, Serotec), chicken anti-GFP (1:1,000; GFP-1020, lot 0511FP12,

 skulls46. We used the following antibodies and dilutions: goat anti-CDH5 (1:200; (ref. 37) CDH5, (ref. 40) as well as endothelial-specific Cdh5-CreERT2 (ref. 41) and KitCreERT2 (ref. 42) used for subsequent analyses. In some experiments, a fraction of each population

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deviation of the mean (for details, see legends). Comparison of medians against

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Statistical analyses were performed with Excel 12.2.6 (Microsoft Office) or Prism 7 (GraphPad Software).

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

**Data availability**
All sequence data used in this study have been deposited in the NCBI Gene Expression Omnibus database (accession number GSE117978) and are listed in the Source Data for Fig. 6.

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Extended Data Fig. 1 | Endothelial Csf1r-iCre-targeting is observed with different recombination reporters, and targeted ECs are distinguishable from macrophages and pericytes. a–c, Csf1r-iCre;RosaYfp (a), Csf1r-iCre;CAG-Cat-Egfp (b) and Csf1r-iCre;RosatdTom (c) hindbrains (n = 3 each) at the indicated stages were whole-mount labelled with IB4 and for YFP (a) or GFP (b) or are shown with tdTomato fluorescence (c). In a, the white squares indicate areas that were imaged at higher magnification for Fig. 1a. The indicated single channels are also shown individually. d, Csf1r-iCre;RosatdTom E12.5 hindbrains (n = 3), whole-mount labelled for ERG and CDH5 and shown including tdTomato fluorescence to demonstrate that Csf1r-iCre targets ECs that form junctions with neighbouring non-targeted ECs. e, f, E12.5 Csf1r-iCre;RosaYfp hindbrains, labelled for YFP and the microglia marker F4/80 (e) or the pericyte marker NG2 (f) together with IB4, show that Csf1r-iCre-targeted vessel-bound cells are neither microglia nor pericytes; n = 3 each. In e, the boxed area is shown in higher magnification and as single channels adjacent to the panel. In f, a single optical y/z cross section at the position indicated with the yellow line is displayed at higher magnification with single channels. Arrowheads, microglia; arrows, ECs; double arrowheads, pericytes; curved arrow, junctional CDH5 staining; solid and clear symbols indicate the presence or absence of marker expression, respectively. Scale bars: 100 µm (a), 20 µm (b, c, e, f), 50 µm (d).

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Extended Data Fig. 2 | Endothelial Csf1r-iCre-targeting is not caused by endothelial Csf1r expression and occurs independently of myeloid differentiation. a, b, Csf1r-Egfp (a) and Csf1r-iCre;RosaYfp (b) E11.5 hindbrains (n = 3 each), whole-mount labelled for CSF1R and EGFP or YFP together with IB4, show lack of Csf1r promoter activity and CSF1R protein in ECs. c, Relative Cdh5 and Csf1r expression levels in our analysis of published E14.5 brain or pooled lung/liver EC microarrays; n = 5 each; ***, P < 0.0001 (two-tailed unpaired t-test). d–g, FACS separation of tdTomato+ cells from Csf1r-iCre;RosaYfp embryos (n = 3) for gene expression analysis, including representative gating strategy to exclude dead cells and doublets in this and subsequent experiments (d) and sorting into PECAM1+CD45− ECs versus CD45+PECAM1− MCs (e).

f, Representative RT-qPCR gene amplification graphs for Csf1r and Actb from tdTomato+ MCs and ECs; △Rn, normalized reporter value for SYBR Green minus baseline instrument signals. g, Graphic representation of the fold change in RT-qPCR amplification of the indicated genes relative to Actb for both cell populations; each data point represents one embryo; *, P = 0.0242, ***, P < 0.0001 (two-tailed unpaired t-test). h, Csf1r-iCre;RosaYfp P0 striatum on a Pu.1+/+ versus Pu.1−/− background (n = 3 brains each), cryosectioned and labelled for YFP and F4/80 together with IB4 to show that Csf1r-iCre-targeted ECs are Pu.1-independent and persist postnatally. Arrowheads, microglia; arrows, YFP+ ECs; clear arrows, YFP+ ECs that are CSF1R− and F4/80−. Scale bars, 20 µm.
Extended Data Fig. 3 | Lineage tracing of yolk sac and liver EMPs.

a, b, E8.5 wild-type (a) and Pu.1−/− (b) yolk sacs on a Csf1r-iCre;RosaYfp background (n = 3 yolk sacs each), whole-mount labelled for YFP and KIT, show Csf1r-iCre-targeted KIT+ round cells corresponding to EMPs and MPs as well as Csf1r-iCre-targeted KIT− flat cells corresponding to ECs. Scale bars, 20 µm. c–f, Pregnant Csf1r-Mer-iCre-Mer;RosaTdn (*c, d) and KitCreERT2;RosaTdn (e, f) dams were injected with a single tamoxifen dose on the indicated days; E12.5 yolk sacs were whole-mount labelled for the indicated markers to identify Csf1r-iCre-targeted ECs and macrophages (n = 3 yolk sacs for each genotype). Wavy arrows, EMPs; straight arrows, Csf1r-iCre-lineage-traced ECs; arrowheads, macrophages; solid and clear symbols indicate the presence or absence, respectively, of the indicated markers. Scale bars, 20 µm. g–i, Pregnant dams were injected with a single tamoxifen dose on E10.5 (g) before using the indicated markers for FACS analysis of E11.5 Csf1r-Egfp;Csf1r-Mer-iCre-Mer;RosaTdn (h) or Csf1r-Mer-iCre-Mer;RosaTdn control (i) livers (n = 4 each); the CD45highKIT− differentiated MC (blue), CD45loKIT+ EMP/MP (pink) and CD45− KIT+ (grey) populations were gated further for Csf1r-Egfp and tdTomato. CD45− KIT+ cells were neither MCs nor EMPs, because they lacked CD45, tdTomato and EGFP.
Extended Data Fig. 4 | Immunostaining controls for cultured Csf1r-iCre-targeted cells. The indicated cell populations were FACS-isolated from E12.5 Csf1r-iCre;Rosa<sup>tdTom</sup> liver or blood with the indicated markers and cultured for three days in methocult (met.) on fibronectin (FN); n = 1 experiment. a, b, Adherent cells from tdTomato<sup>+</sup> liver MC (a) and EMP/MP (b) cultures were stained for ERG and VEGFR2 (top) or with secondary antibodies only (bottom). c, Adherent cells from tdTomato<sup>+</sup> blood EMP and MP cultures were immunostained for CSF1R together with the myeloid markers CD45 (top) or F4/80 (bottom). In the first panel in each row, the phase contrast and DAPI images were merged. In panels 2–4 in each row, immunolabelled cells were visualized together with tdTomato fluorescence, with single channels for the indicated markers shown separately in greyscale. Arrows, tdTomato<sup>+</sup> ECs; arrowheads, tdTomato<sup>+</sup> MCs; solid and clear symbols indicate the presence or absence, respectively, of the indicated markers. Scale bars, 20 µm.
Extended Data Fig. 5 | Hoxa gene targeting with Csf1r-iCre. a, Schematic representation of the Hoxa gene cluster and adjacent Evx1 gene using the UCSC Genome Browser with the mouse December 2011 (GRCm38/mm10) Assembly, including position of the LoxP sites used for gene targeting. b, Validation of Hoxa targeting. b, FACS strategy to isolate KIT+ cells from E12.5 control (pooled Csf1r-iCre− or Csf1r-iCre+;Hoxa+/+; n = 14), Hoxa+/−;Csf1r-iCre (n = 6) and Hoxa−/−;Csf1r-iCre (n = 8) livers. c, qPCR analysis of Hoxa11 gene copy number relative to Evx1; mean ± s.d.; each symbol represents the value for one liver; *P = 0.0156, ***P < 0.001 (one-way ANOVA, Tukey’s multiple comparisons test). d–f, Representative FACS analysis (d) and quantification (e, f) of liver cell populations at E12.5 shows a similar number of total CD45+ and CD45+CD11b+ differentiated myeloid cells in Hoxa−/−;Csf1r-iCre mutants (n = 7 for CD45+; n = 6 for CD45+CD11b+) versus pooled Csf1r-iCre− and Csf1r-iCre+;Hoxa+/+ controls (n = 25 for CD45+, n = 17 for CD45+CD11b+); mean ± s.d. fold change in mutants compared to controls; each data point represents one liver; NS, not significant, P = 0.6519 (e) and P = 0.496 (f) (two-tailed unpaired t-test). g–i, E12.5 hindbrains of the indicated genotypes were immunolabelled to determine vascular complexity and quantify microglia. g, Schematic representation of a whole-mount embryonic hindbrain (left) and location of the hindbrain areas i–iv used for quantification (right); values for the four areas in each hindbrain were averaged to obtain the value for that hindbrain; EC quantifications are shown in Fig. 5c. h, Hindbrains were whole-mount labelled with IB4 and for RFP to visualize tdTomato and for F4/80 to visualize microglia; white boxes indicate areas shown in higher magnification in Fig. 5. i, Quantification of microglia in Hoxa−/−;Csf1r-iCre mutants (n = 9) versus controls (n = 10, pooled Csf1r-iCre−;Hoxa+/+ and Csf1r-iCre− of any Hoxa genotype); mean ± s.d. fold change in mutant compared to control hindbrain; each data point represents one hindbrain; **P = 0.0055 (two-tailed unpaired t-test). j–l, E11.5 Csf1+/+ and Csf1−/− littermate hindbrains, whole-mount labelled for F4/80 together with IB4 (j) before quantification of microglia number (k) and vascular branchpoints as a measure of vascular complexity (l). Mean ± s.d.; each data point represents one hindbrain, n = 3 each; NS, not significant, P = 0.808, **P = 0.0012 (two-tailed unpaired t-test). Scale bars: 200 µm (h), 100 µm (j).
Extended Data Fig. 6 | Csf1r-iCre-targeted ECs proliferate in vivo. a, b, E12.5 Csf1r-iCre;Rosa<sub>tdTom</sub><sup>dTom</sup> yolk sac (a) or hindbrain (b), whole-mount stained for the proliferation marker pH3 and VEGFR2 or for pH3 together with IB4, respectively, and shown together with tdTomato fluorescence (n = 3 each). Areas indicated with white squares were imaged at higher magnification and are shown below the corresponding panels, with tdTomato and pH3 channels also shown separately in greyscale. Arrows, proliferating tdTomato<sup>+</sup> pH3<sup>+</sup> ECs; solid and clear symbols indicate the presence or absence, respectively, of tdTomato fluorescence; wavy arrow, a tdTomato<sup>−</sup> pH3<sup>+</sup> neural progenitor. Scale bars: 100 µm (top), 20 µm (bottom). c–e, Cell cycle distribution of tdTomato<sup>+</sup> and tdTomato<sup>−</sup> ECs. c, FACS strategy to isolate tdTomato<sup>+</sup> and tdTomato<sup>−</sup> PECAM1<sup>+</sup> ECs from E12.5 Csf1r-iCre;Rosa<sub>tdTom</sub><sup>dTom</sup> embryos (n = 3 embryos). d, Cell cycle distribution based on Hoechst 33342 fluorescence as a measure of DNA content; low and high staining intensity is observed in cells with a DNA ploidy of 2n (G0/G1 phase) or 4n (G2/M phase), respectively; intermediate staining intensity corresponds to S phase. e, Mean ± s.d. proportion of tdTomato<sup>+</sup> and tdTomato<sup>−</sup> ECs in G1, S and G2/M based on the area of the corresponding peaks in d; NS, not significant, P > 0.9999 (two-way ANOVA, Bonferroni’s multiple comparisons test).
Extended Data Fig. 7 | Validation of gene expression data from RNA-seq and microarray studies. ECs were FACS-isolated from E12.5 Csf1r-iCre;Rosa26Sor embryos (n = 3) as in Fig. 6a to validate the RNA-seq and microarray data shown in Fig. 6d–f. Slc2a1 was analysed as a representative brain EC-enriched transcript/differentiation marker, and Mrc1 and Oit3 as representative liver EC-enriched transcripts. a, Relative transcript levels of the Gt(ROSA)26Sor (tdTomato) transcript by RNA-seq of the E12.5 tdTomato+ and tdTomato− EC populations (analysis presented in Fig. 6a–f); mean ± s.d. of normalized counts, n = 3 each; **P = 0.0085 (two-sided unpaired t-test). b, RT-qPCR analysis for the indicated genes in tdTomato+ versus tdTomato− ECs isolated from whole E12.5 embryos (n = 5) to validate genes identified by RNA-seq in Fig. 6e,f as differentially expressed. Mean ± s.d. of fold change; ***P < 0.0001 (Slc2a1), ***P = 0.0008 (Mrc1) **P = 0.0056 (Oit3) (two-sided unpaired t-test). c, RT-qPCR analysis for the indicated genes in tdTomato− ECs isolated from the E12.5 brain versus liver (n = 3 for each organ) to validate organ-specific transcript enrichment identified via microarray analysis shown in Fig. 6f. Mean ± s.d. of fold change; *P = 0.019, **P = 0.0082, ***P < 0.0001 (two-sided unpaired t-test); ND, not detectable. d, RT-qPCR analysis for the indicated genes to directly compare the expression levels of brain and liver EC differentiation markers in tdTomato+ versus tdTomato− ECs isolated from brain (n = 3) or liver (n = 5). Mean ± s.d. of fold change; NS, not significant, P = 0.9398 (liver Slc2a1), P = 0.8045 (liver Mrc1), P = 0.6327 (liver Oit3), **P = 0.0073 (brain Slc2a1) (two-sided unpaired t-test); ND, not detectable.
Extended Data Fig. 8 | Csf1r-iCre-targeted ECs contribute to embryonic vasculature in multiple organs. a, 20-µm cryosections of the indicated E12.5 Csf1r-iCre;Rosa<sup>tdTom</sup> organs (n = 3 each) were immunolabelled for the indicated EC markers together with antibodies for RFP to identify tdTomato protein (top and bottom) or are shown with tdTomato fluorescence (middle); single channels are shown in greyscale. The white boxes indicate the positions of areas shown in higher magnification in Fig. 6g; some areas selected for higher magnification are not contained entirely within the field of view, and accordingly the boxes are shown incomplete. Scale bars, 200 µm. b, Gating strategy for FACS analysis of tdTomato<sup>+</sup> and tdTomato<sup>−</sup> ECs from E12.5 Csf1r-iCre;Rosa<sup>tdTom</sup> brain, lung, heart and liver versus control organs lacking iCre, using antibodies for CD11b, CD41, CD45, KIT and PECAM1; associated EC quantifications are shown in Fig. 6i. An analogous strategy was used for the quantifications shown in Fig. 6j and in Extended Data Fig. 9b.
Extended Data Fig. 9 | Csf1r-iCre-targeted ECs contribute to organ vasculature in late-stage embryos and adults. a, 20-µm cryosections of the indicated organs from E18.5 Csf1r-iCre;Rosa26× mice (n = 2 each) were immunolabelled for YFP, PECAM1 and IBA1; single channels are shown in greyscale. Arrowheads, YFP+IBA1+ macrophages; solid and empty arrows, ECs that are YFP+ and lack IBA1 expression, respectively. Scale bars, 20 µm. b, FACS analysis of dissociated cells from the indicated organs of E18.5 Csf1r-iCre;Rosa26× embryos after staining with antibodies for CD11b, CD41, KIT and PECAM1, using the gating strategy shown in Extended Data Fig. 8b; mean ± s.d., n = 5 each; ***P < 0.0001 (one-way ANOVA, Tukey’s multiple comparisons test). c, 20-µm cryosections of the indicated organs from 6-month-old adult Csf1r-iCre;Rosa26× mice (n = 3 organs each) were immunolabelled for YFP, PECAM1 and F4/80; single channels are shown in greyscale. Arrowheads and arrows as in a. Scale bars, 20 µm.
Extended Data Fig. 10 | Csf1r-iCre-targeted ECs contribute to adult organ vasculature. a, 20-µm cryosections of 3-month-old adult Csf1r-iCre;Rosa26tm1(RorCre) livers (n = 3) were immunolabelled for RFP, VEGFR2 and F4/80 or MRC1 and then counterstained with DAPI; single channels are shown in greyscale. The white box indicates an area shown in higher magnification in Fig. 6h. Scale bars, 100 µm. b, Working model for the role of EMPs in generating extra-embryonic yolk sac and intra-embryonic organ ECs alongside their known role in generating myeloid and erythrocyte/megakaryocyte lineage cells. It is not yet known whether EMP-derived and non-EMP-derived ECs have different functions to regulate normal organ physiology or pathological vascular responses in the adult.
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**Sample size**

Sample sizes were not pre-calculated. For histological analyses in Figs. 1, 2, 3 and 4, at least three, but typically more, independent samples (i.e. hindbrains or yolk sacs; for n, see figure legends), were qualitatively analysed by immunostaining, with each experimental repeat yielding highly similar results. Tissue culture experiments were repeated three times with independent samples, with qualitative results obtained by histological examination without associated statistical tests, but results from each experiment being highly similar (Fig. 5). In some experiments (Fig. 6), sample sizes were based on prior published protocols (e.g. Fantin et al., Nature Protocols 2013) and analogous experiments carried out with other strains in our lab (e.g. Fantin et al., Blood 2013). In some experiments (Fig. 2), sample sizes were based on analogous experiments carried out in other genetic background strains in our lab (Fantin et al., Cell Reports 2015). In other experiments, we found that using three independent samples per group gave us sufficient power to detect significant differences between groups, which were then validated by comparing results to published microarray data (i.e. Fig. 7A-E).

**Data exclusions**

No data exclusions.

**Replication**

We have reproduced all our results in a minimum of three independent experimental repeats and using independent samples (i.e. by using embryonic materials from several litters). Many experiments were repeated independently by two investigators in the lab, in particular experiments shown in Fig. 1, 2, 3, 4 and 5.

**Randomization**

Randomisation was not required for our study.

**Blinding**

Genotype determined group allocation. Data analysis were performed by different staff members responsible for genotyping and analysis, respectively, to avoid conscious and unconscious bias.

Reporting for specific materials, systems and methods

| Materials & experimental systems |
|----------------------------------|
| n/a | Involved in the study |
| [ ] | Unique biological materials |
| [ ] | Antibodies |
| [ ] | Eukaryotic cell lines |
| [ ] | Palaeontology |
| [ ] | Animals and other organisms |
| [ ] | Human research participants |

| Methods |
|---------|
| n/a | Involved in the study |
| [ ] | ChiP-seq |
| [ ] | Flow cytometry |
| [ ] | MRI-based neuroimaging |

**Antibodies**

For all antibodies used, we have provided the catalogue number and supplier. For polyclonal antibodies, which may show batch variation, we have listed the lot number. For monoclonal antibodies, we have listed the clone name.

**Validation**

All antibodies were sold by the manufacturer with validation data and citations, and they detected the specified targets in our study as expected. Specificity was confirmed by obtaining the expected pattern of tissue staining with the respective antibodies. For example, an antibody to the vascular endothelial protein CDH5 stained only blood vessels, and the F4/80 antibody for macrophages/microglia stained only those cells (e.g. Fig. 1x). YFP and EGFP staining was validated by comparing tissues expressing these proteins or lacking these proteins based on their genetic status (e.g. see Fig. 1A).

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We have used laboratory mice for our studies and have reported the strain, genetic background and age for each experiment.
Laboratory animals

The strains have all been published previously and are described in the methods section with appropriate references. Ages are specified in the figures and figure legends. The vast majority of work was carried out with embryonic material of unknown sex, but, based on Mendelian genetics, all material would be comprised of equal proportions of male and female embryonic tissues, as none of the strains we have used have reported sex-related lethality that would eliminate one or the other sex from litters. Experiments with adult material for Fig. 7 and Extended Data Fig. 5 were also carried out with a mixture of male and female tissues; the sex of the tissues has been recorded and can be provided if required, but is considered not relevant in the context of our study.

Wild animals

The study did not involve wild animals.

Field-collected samples

The study did not involve samples collected from the field.

Flow Cytometry

Plots

Confirm that:

- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

listed in the methods

Instrument

listed in the methods

Software

listed in the methods

Cell population abundance

We have specified the relative abundance of all sorted cell populations in each figure panel containing FACS data. Purity was confirmed by qPCR analysis for representative genes in Fig. 2.

Gating strategy

The gating strategy is described in each figure or associated extended data figure. The FSC/SSC gates were determined as described in the methods section. The boundaries between positive and negative populations were determined with FMOs, as described in the methods section.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.