MicroRNA 139-5p coordinates APLNR-CXCR4 crosstalk during vascular maturation

Irinna Papangeli1,*, Jongmin Kim1,2,*, Inna Maier3, Saejeong Park1, Aram Lee2, Yujung Kang1, Keiichiro Tanaka1, Omar F. Khan4, Hyekyung Ju1, Yoko Kojima1, Kristy Red-Horse5, Daniel G. Anderson4, Arndt F. Siekmann3 & Hyung J. Chun1

G protein-coupled receptor (GPCR) signalling, including that involving apelin (APLN) and its receptor APLNR, is known to be important in vascular development. How this ligand-receptor pair regulates the downstream signalling cascades in this context remains poorly understood. Here, we show that mice with Apln, Aplnr or endothelial-specific Aplnr deletion develop profound retinal vascular defects, which are at least in part due to dysregulated increase in endothelial CXCR4 expression. Endothelial CXCR4 is negatively regulated by miR-139-5p, whose transcription is in turn induced by laminar flow and APLN/APLNR signalling. Inhibition of miR-139-5p in vivo partially phenocopies the retinal vascular defects of APLN/APLNR deficiency. Pharmacological inhibition of CXCR4 signalling or augmentation of the miR-139-5p-CXCR4 axis can ameliorate the vascular phenotype of APLN/APLNR deficient state. Overall, we identify an important microRNA-mediated GPCR crosstalk, which plays a key role in vascular development.
A functional vascular system is fundamental for normal vertebrate development, as it is critical for delivery of oxygen and nutrients, while removing waste to ensure growth and differentiation of all tissues. The concept of tip and stalk cells has emerged over the past decade in the context of sprouting angiogenesis, and guides our understanding of the transition from vascular sprouting to maturation and ultimately quiescence. In this context, endothelial cell (EC) subsets can respond to molecular and biomechanical cues, and acquire non-proliferative tip cell identities which can extend numerous filopodia and guide vascular outgrowth, or become stalk cells that are considered proliferative and capable of forming lumens. Both the vascular endothelial growth factor (VEGF) and the Notch signalling pathways have been extensively described to regulate the decision between these cell populations during mouse development. Nevertheless, unresolved questions remain, and our understanding of the mechanisms by which these signalling processes are integrated remains incomplete.

Here we define a novel mechanism by which signalling pathways involving two of these GPCRs, namely APLNR and CXCR4, are intricately linked in the developing vasculature. We identify robust flow-induced endothelial expression of CXCR4 in both in vitro and in vivo models, which in turn leads to marked suppression of endothelial CXCR4 expression. This crosstalk was found to involve a key shear responsive microRNA (miRNA), miR-139-5p, which is positively regulated by APLN/APLNR signalling and directly targets CXCR4 in ECs. Moreover, we demonstrate novel pharmacological strategies to restore this signalling axis that rescue the vascular phenotypes associated with APLN/APLNR deficiency. Overall, these findings provide key mechanistic insights that involve a critical miRNA-based cross-talk between two GPCR signalling cascades, which regulate important early steps in vascular maturation.

Results
APLN/APLNR downregulates CXCR4 in developing retinal vessels. Our previous investigation of the APLN/APLNR signalling pathway included a gene expression profiling analysis of transcripts that are differentially regulated by APLN/APLNR knockdown in ECs (ref. 11). Of the transcripts that were increased, we found that CXCR4 was one of the most significantly upregulated transcripts in this context (data set GSE46825, Gene Expression Omnibus database (GEO NCBI)). We confirmed that both the messenger RNA (mRNA) and protein levels of CXCR4 are significantly increased in human umbilical vein ECs (HUVECs) subjected to AMD3100, a known selective CXCR4 inhibitor. First, we confirmed that AMD3100 can inhibit effects of SDF-1α in inducing endothelial sprouting in a fibrin gel bead assay (Supplementary Fig. 4). For our in vivo studies, as previous studies have demonstrated vascular abnormalities with high doses of AMD3100 (ref. 21), we chose a dose that was subphenotypic in control mice (Supplementary Fig. 5) and examined the retinal vasculature of the Apnhr−/− mice at P5. We found a significant increase in total vascularized area and vascular branchpoints of the Apnhr−/− mice treated with AMD3100 compared with the PBS injected controls (Fig. 1i); the radial growth of the retinas was not significantly changed, suggesting a partial rescue of the Apnhr−/− phenotype. Moreover, we also achieved partial rescue of the Apnhr−/−; Cdhs5-CreERT2 mice with AMD3100, with increase in total vascularized area and trends towards increase in number of vascular branchpoints and radial growth (Supplementary Fig. 6a,b).

Endothelial APLNR expression is flow-dependent. We evaluated the retinal vasculature of P5 mice and confirmed that the expression of Aplnr was restricted to the stalk cells and maturing veins (Fig. 2a), whereas Apln was predominantly expressed by tip cell population (Fig. 2b), as previously reported. We found that the expression of Aplnhr was highest in the subset of ECs that surround the newly formed lumen, based on the perfusion pattern observed in fluorescein isothiocyanate (FITC) dextran injected P5 mice (Fig. 2c). Given the restricted Aplnhr expression in the retinal vasculature to the leading edge of the lumenez network, which would be the endothelial subset exposed to early onset of blood flow, we evaluated whether shear stress may be a positive regulator of APLNR expression. The type of flow in this context has been modelled in silico, but remains poorly characterized in vivo. We tested the effects of a number of different flow conditions on APLNR expression in HUVECs (Supplementary Fig. 7a). Of the different conditions of flow tested, we found the highest upregulation of APLNR with orbital shear stress (estimated to induce 10.7 dynes cm−2)24. We also conducted a serial time course to determine the effect of duration of flow on APLNR expression levels, and found that the highest induction was observed at 48 h, while the expression levels began to decrease beyond that time (Supplementary Fig. 7b). Moreover, we found that upregulation of APLNR was at least in part dependent on the shear responsive transcription factors KLF2...
and KLF4, as knockdown of KLF2, KLF4 or both, markedly inhibited the shear-mediated APLNR upregulation (Fig. 2d). In addition, ERK5 knockdown, a known shear responsive MAP kinase upstream of KLF2 and KLF4 (ref. 25), also abrogated induction of APLNR expression under shear conditions (Fig. 2e). In contrast to APLNR, APLN expression was significantly downregulated after exposure of HUVECs to shear stress (Supplementary Fig. 8). Lastly, we investigated the role of the second APLNR ligand, namely Toddler (ELABELA)26,27 and found no effect on CXCR4 expression levels by either its endogenous knockdown or stimulation with exogenous peptide in HUVECs (Supplementary Fig. 9a,b).
Figure 2 | APLNR is expressed in newly lumenized vessels and its expression is flow-dependent. (a) Aplnr expression as determined by in situ hybridization (blue) in P5 retinal vasculature with Isolectin B4 staining (white). The boxed area in the middle image is magnified on the right to show higher magnification of the adjacent panel. Scale bar, 50 μm. n = 3 retinas. (b) Aplnr expression as determined by in situ hybridization (blue) in P5 retinal vasculature with Isolectin B4 staining (white). The boxed area is magnified on the right to show higher magnification of the adjacent panel. Scale bar, 100 μm (low magnification) or 50 μm (high magnification). n = 4 retinas. Tip cells are marked by the asterisks. (c) Localization of FITC Dextran in the P5 retinal vasculature (green) with Isolectin B4 staining (red) and Erg123 (endothelial nuclei) staining (blue). The higher magnification images are shown in the right panels. Scale bar, 50 μm. n = 4 retinas. (d) APLNR transcript expression in response to shear stress alone or with concurrent KLF2, KLF4 or combined knockdown via siRNA. (e) APLNR transcript expression in response to shear stress alone or with concurrent ERK5 knockdown via siRNA. (f) Aplnr expression in the trunk and tail of zebrafish embryos at 50 hpf with or without nifedipine (2 h treatment). Arrows (DMSO) and arrowheads (nifedipine) demarcate aplnr expression. (g) Aplnr expression in the trunk and tail of zebrafish embryos at 48 hpf with sih or control morpholino (MO) injection. Asterisks mark absence of aplnr staining in intersegmental vessels (ISVs). Staining in the posterior cardinal vein (PCV) is also reduced. In situ hybridization with the pan-endothelial marker vascular endothelial cadherin (ve-cadherin) shows normal vascular morphology in all conditions. Arrows mark intersegmental blood vessels. Scale bar, 50 μm. **P ≤ 0.01, ***P ≤ 0.001, t-test. Error bars represent s.e.m.
To further validate the shear-mediated APLN regulation in vivo, we utilized the zebrafish embryonic system to evaluate the expression of the two paralogous apln receptors, namely aplnra and aplnrb, under disrupted flow conditions. At baseline, aplnrb expression was localized to the intersegmental vessels (ISVs) of 48–50 h post fertilization (hpf) embryos, as determined by in situ hybridization (Fig. 2f,g). Following treatment of the embryos with nifedipine, which is known to impede blood flow38, or by injection of the silent heart (sih) morpholino39, there was a significant decrease in aplnrb expression in the ISVs; demonstrating in vivo that blood flow is a critical regulator of aplnrb expression (Fig. 2f,g). The ISV vascular structure was unaffected, as confirmed by ve-cadherin staining. Aplnra expression in the ISVs, which was at baseline markedly lower than aplnrb, was either slightly increased or unchanged by nifedipine treatment or sih morpholino injection, respectively (Supplementary Fig. 10a,b).

APLN/APLNR regulation of CXCR4 is miR-139-5p dependent. Previous studies have demonstrated that shear stress can inhibit endothelial CXCR4 expression.40 We found that the shear-induced downregulation of CXCR4 was abrogated by concurrent knockdown of APLNR (Fig. 3a,b), suggesting that APLNR signalling is a critical mediator of flow-induced CXCR4 inhibition. Given the inverse relation between endothelial expression of APLNR and CXCR4, we sought to determine the mechanism by which APLNR regulates CXCR4 expression. Multiple mechanisms are known to affect CXCR4 expression, including emerging data suggesting an important role for miRNA-mediated regulation in different contexts such as breast31, prostate32, colon33 and thyroid34 cancer. The role of miRNAs as potential regulators of CXCR4 expression in ECs has not been thus far investigated. We first determined whether miRNAs in general may be playing a role in regulation of CXCR4 in ECs. We subjected HUVECs to Argonaute 2 (AGO2) knockdown, a key protein of the RNA-induced silencing complex, and found a significant increase in CXCR4 expression, suggesting that basal expression of CXCR4 may be miRNA regulated (Fig. 3c). We additionally found that shear-induced downregulation of CXCR4 was also abrogated by concurrent knockdown of AGO2 (Fig. 3d), demonstrating that a miRNA-based mechanism may be critical in the regulation of CXCR4 expression in the maturing vasculature.

We next set out to identify the miRNA that may be instrumental in APLN/APLNR-mediated CXCR4 regulation. We previously conducted a miRNA expression profiling array instrumental in APLN/APLNR-mediated CXCR4 regulation. expression in the maturing vasculature. based mechanism may be critical in the regulation of CXCR4 AGO2 downregulation of CXCR4 was also abrogated by concurrent knockdown, a key protein of the RNA-induced silencing complex, and found a significant increase in CXCR4 expression, respectively targeting oligonucleotide delivery to the endothelium 37. Intravenous injection of anti-miR-139-5p loaded 7C1 nanoparticles led to partial phenocopying of the Apln−/− phenotype, with significantly reduced vascularized area and radial expansion and trend towards reduced number of branchpoints, compared with mice injected with 7C1 loaded with non-targeting control (cel-miR-67-3p; Fig. 4a,b).

Restoring miR-139-5p-CXCR4 axis rescues Apln−/− phenotype. To further validate the role of miR-139-5p as a key downstream element of APLN/APLNR signalling in vascular maturation, we sought to identify a pharmacological strategy to restore its endothelial expression. We screened multiple Food and Drug Administration (FDA) approved compounds to determine whether any of them can induce miR-139-5p expression. Of the six compounds examined, we found that atorvastatin, belonging to the class of 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors38, induced the highest increase in miR-139-5p expression in HUVECs (Fig. 4c). We further investigated the association between APLN/APLNR signalling and miR-139-5p expression (Supplementary Fig. 12). In addition, we found that APLNR knockdown decreases the transcripts of both PDE2A, the host gene for miR-139-5p, as well as pri-miR-139-5p, the primary transcript of miR-139-5p (Supplementary Fig. 13), suggesting a direct transcriptional relationship between APLNR and miR-139-5p. We next tested the efficacy of miR-139-5p in regulating endothelial CXCR4 expression. Overexpression of miR-139-5p in HUVECs resulted in significant decrease in the protein levels of CXCR4 (Fig. 3f). Conversely, we found that inhibition of miR-139-5p via an anti-miR inhibitor resulted in increased CXCR4 protein levels (Fig. 3f). Moreover, a luciferase reporter activity containing the wild-type CXCR4 3' UTR was significantly decreased by miR-139-5p overexpression in HUVECs, whereas the same construct with mutagenized miR-139-5p binding sites was unaffected (Supplementary Fig. 14).

Endothelial CXCR4 is closely regulated by miR-139-5p. To further elucidate the relevance of our findings to the developing vascular context, we examined whether there is a relationship between shear stress and miR-139-5p. We found that miR-139-5p was significantly induced by exposure of ECs to shear stress (Fig. 3g). Moreover, upregulation of miR-139-5p in response to shear exposure was abrogated by concurrent knockdown of APLNR, demonstrating the key role of APLNR in mediating shear response in ECs (Fig. 3g). In addition, shear-stress-mediated decrease in CXCR4 expression was abrogated by concurrent inhibition of miR-139-5p (Fig. 3h).

We carried out additional functional studies to investigate the role of miR-139-5p in ECs. Sprouting of ECs in response to SDF-1α was significantly reduced in ECs overexpressing miR-139-5p, whereas sprouting in response to VEGF 165 was unaffected (Fig. 3i). Similarly, HUVECs transfected with an anti-miR against miR-139-5p sprouted more efficiently in response to SDF-1α compared with control transfected cells, while VEGF-induced sprouting was not affected by anti-miR transfection (Supplementary Fig. 15a). Moreover, cell migration in response to SDF-1α was also significantly impeded in HUVECs overexpressing miR-139-5p (Fig. 3j), whereas HUVECs transfected with anti-miR-139-5p had enhanced migration to SDF-1α stimulation (Supplementary Fig. 15b). The migratory response to VEGF 165 was not affected by anti-miR-139-5p (Supplementary Fig. 15b).

Next, we further validated our findings in vivo, using the recently described 7C1 nanoparticles that are capable of selectively targeting oligonucleotide delivery to the endothelium37. Intravenous injection of anti-miR-139-5p loaded 7C1 nanoparticles led to partial phenocopying of the Apln−/− and Aplnr−/− phenotype, with significantly reduced vascularized area and radial expansion and trend towards reduced number of branchpoints, compared with mice injected with 7C1 loaded with non-targeting control (cel-miR-67-3p; Fig. 4a,b).
Figure 3 | APLNR regulates CXCR4 expression through miR-139-5p. (a,b) CXCR4 expression in response to shear stress exposure in HUVECs, with or without concurrent APLNR knockdown. mRNA (a) and protein levels (b) are shown. (c) CXCR4 expression in response to AGO2 knockdown. (d) CXCR4 expression in response to shear stress exposure in HUVECs, with or without concurrent AGO2 knockdown. (e) Determination of miR-139-5p levels in FACS sorted ECs from P5 retinas of Aplnr+/C0/C0, Apln+/C0/C0 mice and respective littermates. n = 3 retinas per genotype. (f) CXCR4 expression in HUVECs overexpressing miR-139-5p mimic or non-targeting control anti-miR. (g) Expression levels of miR-139-5p in response to shear stress, with or without concurrent APLNR knockdown. (h) CXCR4 expression in HUVECs in response to shear stress exposure, with or without concurrent miR-139-5p inhibition via anti-miR. (i) Sprouting assay using HUVEC covered beads transduced with miR-139-5p or control lentivirus in response to SDF-1α or VEGF 165. Scale bar, 175 μm. (j) Migration assay of HUVECs transduced with miR-139-5p or control lentivirus in response to SDF-1α. Scale bar, 200 μm. *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001, ###P ≤ 0.001 against NC v/PBS, †††P ≤ 0.001 against NC v/SDF-1α, t-test. Error bars represent s.e.m. n = 3 experiments per condition.
whether atorvastatin can regulate CXCR4 expression; we found that treatment of HUVECs with atorvastatin led to marked inhibition of CXCR4 expression (Fig. 4d).

Our previous studies demonstrated that statins can augment APLN/APLNR signalling, in an APLNR-dependent manner. Given the present data, we sought to investigate whether atorvastatin can potentially rescue the retinal vascular phenotype of mice with Apln or Aplnr deficiency. We found that atorvastatin treatment of Apln−/− mice led to robust improvement in retinal vascularized area, radial expansion and increased the number of branchpoints (Fig. 4e,f). The phenotypic rescue of either the Apln−/− or the Aplnrfl/fl;Cdh5-CreERT2 mice were more modest (Supplementary Fig. 16a,b), likely in part due to the more severe phenotype of these mice, and supporting our previous data that endothelial signalling cascades induced by statin are at least in part APLNR dependent.

**Discussion**

Vascular development and maturation is a complex process involving crosstalk among multiple independent, yet closely integrated signalling cascades. VEGF and Notch signalling pathways have been extensively characterized in this context as key factors that provide an intricate orchestration of endothelial identities and promote vascular network formation. A distinct set of signalling pathways whose characterization remains at a relative infancy in this context is that mediated by GPCRs. The

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**Figure 4** Atorvastatin augments apelin/APLNR signalling and improves the Apln−/− retinal phenotype. (a,b) In vivo inhibition of miR-139-5p postnatally with 7C1 nanoparticles loaded with anti-miR to miR-139-5p results in reduced vascularized area and radial expansion of retinal vasculature at P5. n = 6 retinas per genotype. (c) Drug screen using clinically approved compounds that affect miR-139-5p expression levels in HUVECs. n = 3 experiments. (d) CXCR4 mRNA expression in HUVECs in response to atorvastatin stimulation. n = 3 experiments. (e,f) P5 retinal phenotype of Apln−/− pups in response to treatment with atorvastatin. Vascularized area, vascular branchpoints and radial expansion are shown. Scale bar, 200 μm. n ≥ 4 retinas per genotype. (g) Schematic representation of the proposed signalling cascade between APLNR, CXCR4 and the respective ligands in the developing vasculature. *P<0.05, **P≤0.01, ***P≤0.001, t-test. Error bars represent s.e.m.
importance of GPCR signalling in vascular development is highlighted by the embryonic lethal phenotypes of several GPCRs and their respective ligand knockout mice, described to display a spectrum of cardiovascular defects.11,13,14,40–43. However, their downstream targets and interacting partners remain incompletely defined. Adding to the complexity are emerging studies that demonstrate GPCR activation by mechanical forces such as cellular stretch and shear stress in a ligand-independent manner, which may in part explain the phenotypic differences between ligand and receptor knockout mice.15,44–46.

CXCR4 is a GPCR with widespread functional roles in a number of contexts, including angiogenesis, hematopoiesis, neurogenesis and immune function.17. In the context of vascular development, it has been established as a tip cell-specific gene in the developing mouse and zebrafish vasculature.10,48. Interestingly, it is one of a few tip cells enriched endothelial genes that is also known to be markedly downregulated by shear stress in vitro30, and by blood flow in the zebrafish in vivo68,69. These findings suggest that CXCR4 needs to be strictly regulated in the developing vasculature, to restrict the cellular response to its ligands such as SDF-1α, and avoid the potential detriment of inappropriate EC behaviour resulting from aberrantly expressed CXCR4. Although this possibility has not been specifically examined in the vascular context, whole embryo overexpression of Cxcr4a in zebrafish results in significant craniofacial and cartilage defects, highlighting the requirement for stringent CXCR4 regulation.50 In addition, although multiple studies have suggested pro-angiogenic effects of augmenting CXCR4 signalling,39,51,52, its restricted expression pattern in the developmental context10,48 suggests the importance of limiting expression to endothelial subsets for normal angiogenesis. Mechanisms that regulate its expression, however, remain inadequately defined. The current body of work provides key insights into a novel mechanism that integrates flow, a secondary GPCR signalling mediated by APLN/APLNR, and miR-139-5p, to restrict endothelial CXCR4 expression in the context of vascular development and maturation (Fig. 4g for schematic).

The abnormal vascular patterning of Aphp and Aphp null mice likely involves a multitude of downstream signalling targets, some of which have been described previously in other endothelial contexts.11. Our findings that CXCR4 expression is decreased by APLNR-mediated signalling, and that inhibition of CXCR4 activity with AMD3100 can achieve a partial rescue of the Aphp−/− phenotype highlights the importance of this mechanism, but also implicate other downstream targets that are also likely involved in regulating processes such as cellular proliferation and migration, lumen formation and maintenance, and regulation of vascular permeability.53 Ongoing studies will further delineate the role of this and other related GPCR signalling cascades in vascular maturation.

Onset of blood flow, and subjecting ECs to shear stress leads to a remarkable alteration in the endothelial behaviour and function. Multiple in vivo models have demonstrated the role of flow in vascular development in murine54,55, avian56 and fish models. A number of shear responsive endothelial elements have also been identified, including kinases (ERK5 (ref. 58), ERK1/2 (ref. 59) and AKT (ref. 60)), transcription factors (KLF2 (ref. 61), and KLF4 (ref. 62)) and other epigenetic modifiers (miR-126 (ref. 57)). Our current findings highlight the induction of APLNR expression in ECs subjected to shear stress, an effect that was dependent at least in part on ERK5, KLF2 and KLF4, which are genes with key flow responsive roles in the endothelium.

The specific characterization of the flow pattern in the developing vascular bed remains incompletely defined, although recent studies have used computational analyses to estimate the shear profiles.14 We found induction of APLNR in ECs in response to multiple types of shear stress, and furthermore showed that apminb, one of the two paralogous aphp receptors in the zebrafish, was markedly decreased with cessation of blood flow.

miRNAs are emerging as vital rheostats in multiple biological processes, and their key roles in the context of vascular development are only beginning to be appreciated. The intricacies of miRNA-mediated signalling integration in vascular development remain at infancy, although recent studies have demonstrated their key roles in angiogenesis.77 Although miR-139–5p has been described in a limited number of studies as a potential tumour suppressor miRNA63,64, little is known about the function or regulation of miR-139–5p in the endothelium. Our current findings demonstrate two key aspects of this highly regulated endothelial miRNA that is likely a critical mediator of vascular maturation. First, we identified miR-139–5p as a novel shear responsive endothelial miRNA, that is APLN/APLNR dependent. Second, we found that miR-139–5p is a key intermediary that links APLN/APLNR signalling with repression of endothelial CXCR4 expression, which appears to be essential for proper vascular maturation. Lastly, we show that in vivo inhibition of miR-139–5p postnatally results in partial phenocopying of the Aphp−/− and Aphp−/− phenotypes. We expect that our ongoing studies into the role of miR-139–5p in the endothelium, both in the developmental and the vascular disease context, will shed greater insights into its functions.

A key aspect of our current findings is the effect of statins in induction of miR-139–5p expression, inhibition of CXCR4 expression and pharmacological rescue of the retinal phenotype of the Aphp−/− mice. These findings provide support for our previous findings describing the augmentation of APLNR-mediated signalling by statins,39 the mechanism of which will require further investigation. From the perspective of statin pleiotropy, these findings also invoke new potential endothelial targets of statins, which may be playing a key role in mediating the vascular protective effects of these agents in atherosclerosis.

In summary, the crosstalk between APLNR and CXCR4-mediated pathways provide a novel mechanism that may be a crucial determinant of vascular maturation. Further studies to expand on these findings, as well as identification of potential relation between these elements and the role of VEGF–Notch signalling cascade, will advance our understanding of the signalling crosstalk involved in vascular maturation.

Methods

Animals. All animal experiments were conducted in compliance with the relevant laws and institutional guidelines and were approved by Yale University Institutional Animal Care and Use Committee and local animal ethics committees of the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen. Aphp−/−, Aphpfl/fl, Aphp−/−, Cdh5-CreERT2 and Apln-CreER; Rosa26mTmG mice have been previously described.19,36,65 All animals were maintained on a C57Bl/6 background, except for Apln-CreER; Rosa26mTmG which were on a mixed background. Given the young age of the mice at the time of our studies, their gender was not determined. The Aphp−/− was not determined. The Aphp−/− and Aphp−/− were bred with a C57Bl/6 (Sigma-Aldrich) dissolved in PBS were injected intraperitoneally in mice at P0 and P1. Injection with PBS/DMSO was used as negative control. Atovalstatin (Cayman Chemical) was dissolved in dimethyl sulfoxide (DMSO) at 15 mg ml−1 and further diluted in PBS. Mice were injected intraperitoneally at P1, P2 and P3 with 40 mg kg−1. Injection with PBS/DMSO was used as negative control. To induce recombinatation of the Aphp allele with the Cdh5-CreERT2 driver or recombinatation of Rosa26mTmG with the Apln-CreER driver, mice received intragastric injection of 100 μg of Tamoxifen (Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich) at P1 and P2. FITC Dextran (MW = 2 × 106 Da; Sigma-Aldrich) was dissolved in water and injected in the left ventricle of anaesthetized P5 pups and allowed to perfuse for 1 min. G0/1 cells were stained with 0.1% PI (Sigma-Aldrich) in PBS at 1:400 diluted in PBS. Mice were injected intraperitoneally at P0, P2 and P3 with 5 mg kg−1. Injection with PBS/DMSO was used as negative control. To induce recombinatation of the Aphp allele with the Cdh5-CreERT2 driver or recombinatation of Rosa26mTmG with the Apln-CreER driver, mice received intragastric injection of 100 μg of Tamoxifen (Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich) at P1 and P2. FITC Dextran (MW = 2 × 106 Da; Sigma-Aldrich) was dissolved in water and injected in the left ventricle of anaesthetized P5 pups and allowed to perfuse for 1 min. 7C11 nanoparticles containing custom made miR-miR hairpin inhibitors for miR-139–5p or cel-miR-7–3p (Dharmacon) were injected daily intravenously at 1 mg kg−1 at P0–P2, via the superficial temporal facial vein.66 All mice for retinal vasculature studies were killed at P5.

For zebrafish blood flow blocking experiments, a stock solution of 10 mM nifedipine (Sigma-Aldrich) in DMSO was diluted 1:4000 (working concentration 2.5 μM) in E3 medium (diluted from a 60 × stock containing 34.8 g NaCl, 1.6 g
KGL, 5.8 g CatCl₂ × H₂O, 9.78 g MgCl₂ × 6H₂O dissolved in 2 l H₂O and pH adjusted to 7.2 with NaOH) containing 2 × tricine and 0.003% phenylthiourea to prevent pigmentation. Tricine (2 ×) was prepared by adding 84 ml of a stock solution (400 mg tricine powder, 97.9 ml H₂O, 2.1 ml Tris (pH9), pH adjusted to 7.0) to 100 ml of E3 medium. Embryos were incubated for 1 h and then fixed in 4% paraformaldehyde (PFA) for in situ hybridization experiments. For washout experiments, embryos were washed once with E3 medium, and then transferred to E3 medium without n福利depine.

Morpholinos targeting cardiac troponin T2 (silent heart (sh), 2 ng per embryo) were designed to bind the translation start codon and flanking 5' sequence (5'-CATGTTGTCGTCATGACGCA-3') and injected into embryos at the 1–4 cell stage.

**Immunostaining and in situ hybridization.** Isolcetin B4 staining (Life Technologies, working concentration 20 mg ml⁻¹) was performed at room temperature for 1 h at 4°C overnight. Alexa Fluor (Life Technologies, working concentration 10 μg ml⁻¹) secondary antibody was used. Retinal analysis of the different mice was performed on at least four mice per genotype. Retinas were analysed by DNAsp Eclipse 800 microscope, T7, T3 or SP6 polymerase. The Apln plasmid was a kind gift from A. Eichmann12, Apnr and Ccr2 plasmids were purchased from Dharmaco (MIM1013-20276618, clone ID: 4557726, MIM1013-202762695, clone ID: 3592479). For extensive washing the hybridized probes were detected with alkaline phosphatase–coupled anti-digoxigenin– alkaline phosphatase (Roche), working concentration 0.375 U ml⁻¹ at 4°C overnight and signal was visualized with BM purple alkaline phosphatase substrate (1144207001, Roche).

Retinas were dehydrated in me thanol series and stored at −20°C until required. Before hybridization, retinas were rehydrated to PBS/0.1% Tween and then digested (1 ml 0.375 U ml⁻¹ Proteinase K, Sigma-Aldrich) for 15 min at 37°C with agitation and fixation in 4% PFA/0.2% glutaraldehyde in PBS/0.1% Tween. Retinas were washed in PBS/0.1% Tween, pre-incubated in hybridization buffer at 70°C for 3 h and then incubated with RNA probes in hybridization buffer at 70°C overnight. Probes were labelled with digoxigenin-uridine triphosphate (Roche) using full-length complementary DNA (cDNA) clones and T7, T3 or SP6 polymerase. The labelled probe was a kind gift from A. Eichmann,12, Apnr and Ccr2 plasmids were purchased from Dharmaco (MIM1013-20276618, clone ID: 4557726, MIM1013-202762695, clone ID: 3592479). For extensive washing the hybridized probes were detected with alkaline phosphatase–coupled anti-digoxigenin– alkaline phosphatase (Roche), working concentration 0.375 U ml⁻¹ at 4°C overnight and signal was visualized with BM purple alkaline phosphatase substrate (1144207001, Roche). Retinas were mounted using Vectashield (Vector Labs) and imaged.

For zebrafish in situ hybridization, aplnra and aplb plasmids were amplified from CDNA of 19 somite stage-old embryos using the following primers: aplnra forward 5'-GAAAGGCGGTAAGCACCATAGG-3' and aplnra reverse 5'-GAGTGTATCTCTGATGCTCAAACCA-3'. Apln plasmid was a kind gift from A. Eichmann,12, Apnr and Ccr2 plasmids were purchased from Dharmaco (MIM1013-20276618, clone ID: 4557726, MIM1013-202762695, clone ID: 3592479). For extensive washing the hybridized probes were detected with alkaline phosphatase–coupled anti-digoxigenin– alkaline phosphatase (Roche), working concentration 0.375 U ml⁻¹ at 4°C overnight and signal was visualized with BM purple alkaline phosphatase substrate (1144207001, Roche). Retinas were mounted using Vectashield (Vector Labs) and imaged.

Cells and reagents. HUVECs (Yale VBT Core) were cultured at 37°C in a 5% CO₂ incubator in EGM-2 medium (Lonza). For experimental treatments, HUVECs were passed and incubated for 15 min at 37°C in EBM-2 containing Collagenase A (1.5 mg ml⁻¹, 103578, Roche) and DNAsa 1 (25 ng ml⁻¹, 104159, Roche) before cells were dissociated by gentle trituration. Cells were stained with FITC-conjugated anti-CD31 antibody (558738, BD Biosciences, working concentration 5 μg ml⁻¹) or APC-conjugated anti-CXCR4 antibody (558644, BD Biosciences, working concentration 2 μg ml⁻¹). Stained cells were sorted using a FACSArta flow cytometer (BD Biosciences) or an LSRII flow cytometer (BD Biosciences). When cells were collected, both the negative and positive selections were collected in ice cold PBS and processed for RNA extraction and real-time (RT) PCR as described above. Data quantification was performed with FlowJo 7.6 software.

**HUVECs** were transfected with GAPDH or miR-139-5p mimics using small interfering RNAs (siRNAs) against miR-139-5p (Life Technologies). RT-PCR was performed with TaqMan probes for both genes and miR-139-5p (Ambion). 

HUVECs were transfected with GFP or APLNR-GFP (Origene) plasmids for 48 h. HUVECs were treated with miR-139-5p mimic or negative control miRNA. At 24 h after transfection, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay kit (Promega) according to the manufacturer’s instructions.
mRNA samples, ribosomal 18S was used as internal control. Individual RT-PCRs were performed on a CFX96 (Bio-Rad) according to the manufacturer’s instructions. TaqMan probes were used for all genes.

Protein extraction and western blotting. HUVECs were lysed with RIPA lysis buffer (Thermo Scientific) containing Halt Protease and Phosphatase Inhibitor cocktail (Thermo Scientific). Total protein contents of soluble cell lysate were measured using a Micro BCA Protein assay kit (Thermo Scientific). Western blotting was performed using standard methods. Briefly, total protein from each sample was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Bio-Rad) and probed with antibodies specific to CXCR4 (ab2074, Abcam, 1:2,000) and GAPDH (2118, Cell Signaling, 1:3,000). Subsequently, the membranes were incubated with HRP-conjugated secondary antibodies (7074, Cell Signaling, 1:3,000) and developed using an enhanced chemiluminescence detection method (Thermo Scientific). Uncropped scans of key western blots are provided as a Supplementary Fig. 17.

Statistical analyses. Unless otherwise noted, all cell treatments, cell migration and sprouting assays, western blots, quantitative PCR assays and in situ hybridizations are representative of at least three independent experiments. Results are reported as mean ± s.e.m. Unpaired Student’s t-test was used to determine statistical significance. Post hoc analysis was performed using the Holm–Sidak method. P<0.05 was considered significant.

References

1. Siekmann, A. F., Affolter, M. & Belting, H. G. The tip cell concept 10 years after: new players tune in for a common theme. Exp. Cell Res. 319, 1255–1263 (2013).
2. Blanco, R. & Gerhardt, H. VEGF and Notch in tip and stalk cell selection. Cold Spring Harb. Perspect. Med. 3, a006569 (2013).
3. Gerhardt, H. et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. J. Cell Biol. 161, 1163–1177 (2003).
4. Hoey, T. et al. Dll4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. Cell Stem Cell 5, 168–177 (2009).
5. Tachibana, K. et al. An endothelial apelin-APJ-Gi3 link mediated by mI-424 and mI-503 is disrupted in pulmonary arterial hypertension. Nat. Med. 19, 74–82 (2013).
6. Chen HI, S. B. et al. The sinus venosus contributes to the coronary vasculature through VEGF-C stimulated angiogenesis. Development 141, 4500–4512 (2014).
7. McLean, D. L. et al. Apelin/APJ signaling is a critical regulator of statin effects in vascular endothelial cells—brief report. Arterioscler. Thromb. Vasc. Biol. 32, 2640–2643 (2012).
8. Shufstik, S. et al. The Notch ligand Delta-like 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc. Natl Acad. Sci. USA 104, 3225–3230 (2007).
9. Tachibana, K. et al. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature 393, 591–594 (1998).
10. Strasser, G. A., Kaminker, J. S. & Tessier-Lavigne, M. Microarray analysis of retinal endothelial tip cells identifies CXCR4 as a mediator of tip cell morphology and branching. Blood 115, 5102–5110 (2010).
11. Kang, Y. et al. Apelin-APJ signaling is a critical regulator of endothelial MEF2 activation in cardiovascular development. Circ. Res. 113, 22–31 (2013).
12. del Toro, R. et al. Identification and functional analysis of endothelial tip cell-enriched genes. Blood 116, 4025–4033 (2010).
13. Xu, C. et al. Arteries are formed by vein-derived endothelial tip cells. Nat. Commun. 5, 5758 (2014).
14. Liu, Y. et al. Edg-1, the G protein-coupled receptor for sphingosine-1-phosphate, is essential for vascular maturation. J. Clin. Invest. 106, 951–961 (2000).
15. Jung, B. et al. Flow-regulated endothelial tip S1P receptor-1 signaling sustains vascular development. Dev. Cell 23, 600–610 (2012).
16. Gaengel, K. et al. The sphingosine-1-phosphate receptor S1PR1 restricts sprouting angiogenesis by regulating the interplay between VE-cadherin and VEGFR2. Dev. Cell 23, 587–592 (2012).
17. Kasai, A. et al. Retardation of retinal vascular development in apelin-deficient mice. Arterioscler. Thromb. Vasc. Biol. 28, 1717–1722 (2008).
18. Sakimoto, S. et al. A role for endothelial cells in promoting the maturation of astrocytes through the apelin/APJ system in mice. Development 139, 1327–1335 (2012).
19. Wang, Y. et al. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. Nature 465, 483–486 (2010).
20. Hatse, S., Princen, K., Bridger, G., De Clercq, E. & Schols, D. Chemokine receptor inhibition by AMB3100 is strictly confined to CXCR4. FEBS Lett. 527, 255–262 (2002).
21. Unoki, N. et al. SDF-1/CXCR4 contributes to the activation of tip cells and microglia in retinal angiogenesis. Invest. Ophthalmol. Vis. Sci. 51, 3362–3371 (2010).
53. Kidoya, H., Naito, H. & Takakura, N. Apelin induces enlarged and nonleaky blood vessels for functional recovery from ischemia. Blood 115, 3166–3174 (2010).
54. Yashiro, K., Shiratori, H. & Hamada, H. Haemodynamics determined by a genetic programme govern asymmetric development of the aortic arch. Nature 450, 285–288 (2007).
55. Lucitti, J. L. et al. Vascular remodeling of the mouse yolk sac requires hemodynamic force. Development 134, 3317–3326 (2007).
56. Buschmann, I. et al. Pulsatile shear and Gja5 modulate arterial identity and remodeling events during flow-driven arteriogenesis. Development 137, 2187–2196 (2010).
57. Nicolì, S. et al. MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. Nature 464, 1196–1200 (2010).
58. Yan, C., Takahashi, M., Okuda, M., Lee, J. D. & Berk, B. C. Fluid shear stress stimulates big mitogen-activated protein kinase 1 (B MK1) activity in endothelial cells. Dependence on tyrosine kinases and intracellular calcium. J. Biol. Chem. 274, 143–150 (1999).
59. Takahashi, M. & Berk, B. C. Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for a herbimycin-sensitive kinase. J. Clin. Invest. 98, 2623–2631 (1996).
60. Dimmeler, S., Assmus, B., Hermann, C., Haendeler, J. & Zeiher, A. M. Fluid shear stress stimulates phosphorylation of Akt in human endothelial cells: involvement in suppression of apoptosis. Circ. Res. 83, 334–341 (1998).
61. Huddleston, J. P., Ahmad, N., Srinivasan, S. & Lingrel, J. B. Induction of KLF2 by fluid shear stress requires a novel promoter element activated by a phosphatidylinositol 3-kinase-dependent chromatin-remodeling pathway. J. Biol. Chem. 280, 23371–23379 (2005).
62. Clark, P. R. et al. MEK5 is activated by shear stress, activates ERK5 and induces KLF4 to modulate TNF responses in human dermal microvascular endothelial cells. Microcirculation 18, 102–117 (2011).
63. Shen, K. et al. MiR-139 inhibits invasion and metastasis of colorectal cancer by targeting the type 1 insulin-like growth factor receptor. Biochem. Pharmacol. 84, 320–330 (2012).
64. Wong, C. C. et al. The microRNA miR-139 suppresses metastasis and progression of hepatocellular carcinoma by down-regulating Rho-kinase 2. Gastroenterology 140, 322–331 (2011).
65. Charo, D. N. et al. Endogenous regulation of cardiovascular function by apelin-APJ. Am. J. Physiol. Heart Circ. Physiol. 297, H1904–H1913 (2009).
66. Gombash Lamp, S. E., Kaspar, B. K. & Foust, K. D. Intravenous injections in neonatal mice. J. Vis. Exp. e52037 (2014).
67. Helker, C. S. The hormonal peptide Elabela guides angioblasts to the midline during vasculogenesis. eLife 4, e06729 (2015).

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Author contributions
I.P., J.K. and H.J.C. conceived the study. I.P., J.K., I.M., S.P., A.L., Y.K., K.T., H.J., Y.K., A.F.S. and H.J.C. performed the experiments. O.F.K. and D.G.A. developed the 7Cl particles. K.R.H generated mouse lines. I.P., J.K., A.F.S., and H.J.C. analysed the data. I.P. and H.J.C. wrote the manuscript.

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