FGF-dependent metabolic control of vascular development

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Blood and lymphatic vasculatures are intimately involved in tissue oxygenation and fluid homeostasis maintenance. Assembly of these vascular networks involves sprouting, migration and proliferation of endothelial cells. Recent studies have suggested that changes in cellular metabolism are important to these processes1. Although much is known about vascular endothelial growth factor (VEGF)-dependent regulation of vascular development and metabolism2,3, little is understood about the role of fibroblast growth factors (FGFs) in this context1. Here we identify FGF receptor (FGFR) signalling as a critical regulator of vascular development. This is achieved by FGFR-dependent control of c-MYC (MYC) expression that, in turn, regulates expression of the glycolytic enzyme hexokinase 2 (HK2). A decrease in HK2 levels in the absence of FGFR signalling inputs resulted in decreased glycolysis, leading to impaired endothelial cell proliferation and migration. Pan-endothelial- and lymphatic-specific Hk2 knockouts phenocopy blood and/or lymphatic vascular defects seen in Fgfr1/Fgfr3 double mutant mice, while HK2 overexpression partly rescues the defects caused by suppression of FGFR signalling. Thus, FGFR-dependent regulation of endothelial glycolysis is a pivotal process in developmental and adult vascular growth and development.

FGFR1 is a widely expressed member of the FGFR family both in mouse and in human blood and lymphatic endothelial cells (LEC; Extended Data Fig. 1a, b). Knockdown of FGFR1 resulted in upregulation of FGF3 expression, while FGFR3 downregulation had no effect on other FGFR expression (Extended Data Fig. 1c, d). Cdh5-CreERT2 (ref. 5) and Proxl-CreERT2(BAC) (ref. 6) driver lines were crossed with Fgfr1i/ΔLEC(BAC) and Fgfr3−/− mice to generate pan-endothelial (Fgfr1iΔLEC(BAC)/Fgfr3−/−) and lymphatic endothelium (Fgfr1iΔLEC(BAC)/Fgfr3−/−) specific knockouts. The excision efficiency of both Cre drivers was assessed by crossing them with the mTmG reporter mice. Cre activation at embryonic day (E)12.5 and E13.5 resulted in a high degree of recombination in the skin lymphatic vessels at E15.5 with both Cre deleters (Extended Data Fig. 1e, f). When the deletion was activated a day later (E11.5), reduced migration and branching of lymphatics were still evident (Extended Data Fig. 2f–i). Analysis of LEC-specific Fgfr1/3 double knockout mice (tamoxifen treatment at E12.5 and E13.5) confirmed these findings, showing decreased LEC front migration, branching and fewer LECs in the skin (Fig. 1c–g). There was no appreciable difference in the size of the skin lymphatic vessels (Fig. 1f, h). To explore FGFR inhibition in suppression of pathological lymphangiogenesis, mice with orthotopic Panc02 tumours were orally treated with the SSRI128129E (SSRI) inhibitor4. There was a significant reduction of lymphangiogenesis in the peri-tumoural area in the inhibitor-treated mice compared with vehicle controls (Extended Data Fig. 2j, k), indicating a potential therapeutic value of FGFR inhibitors as anti-lymphangiogenic agents.

We next examined whether FGFR signalling plays a similar role in the blood vessel development. Analysis of Fgfr1ΔLEC;Fgfr3−/− mice after Cre activation at E10.5 showed a significant reduction in vessel branching and coverage in the skin at E15.5 (Extended Data Fig. 3a–d). Examination of the arterial vasculature, revealed by Connexin 40 (Cx40) staining, also showed a reduction in branching (Extended Data Fig. 3f, g) but no differences in capillary or arterial diameter (Extended Data Fig. 3e, h).

The involvement of FGFR signalling in blood vascular development was further confirmed by examining retinal vasculature in Fgfr1ΔLEC;Fgfr3−/− mice after Cre activation at postnatal day (P)0 (Extended Data Fig. 4a). There was a significant impairment of vascular growth and branching (Extended Data Fig. 4b, c), and marked reduction in the number of tip cells (Extended Data Fig. 4d, e) and the extent of proliferation (Extended Data Fig. 4f, g). There was no obvious difference between Fgfr1ΔLEC and Fgfr3−/− and between Fgfr1ΔLEC;Fgfr3−/− and Fgfr3−/− in vascular density and branching (Extended Data Fig. 4h–k). Knockdown of FGFR1 in human dermal lymphatic endothelial cells (HDLECs) significantly reduced cell proliferation and migration, while FGFR3 downregulation had no effect (Extended Data Fig. 5a–c). Effects of a double Fgfr1/Fgfr3

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knockdown were like those of FGFR1 knockdown (Extended Data Fig. 5a–c).

To establish the molecular basis of FGF-dependent regulation of vascular development, we performed RNA sequencing (RNA-seq) analysis of LECs after stimulation with FGF2 or FGFR1 knockdown. Gene Ontology analysis showed the expected statistical enrichment of molecular pathways related to cell proliferation and migration (Fig. 2a). Surprisingly, there was also enrichment among cellular metabolism processes and, especially, glucose metabolism pathways. To assess this aspect of FGF biology, we first examined contributions of major metabolic pathways to LEC energy generation. Flux analysis demonstrated that glycolysis was the most active process in LECs (Extended Data Fig. 6a), contributing to >70% of the total ATP generation. HDLEC treatment with FGF2 doubled their glycolytic flux (Fig. 2b) and significantly increased glucose uptake (Extended Data Fig. 6b). Conversely, knockdown of FGFR1 reduced the flux rate (Fig. 2b) and significantly increased glucose uptake (Extended Data Fig. 6b). Data represent mean ± s.e.m., *P < 0.05, **P < 0.01, NS is non-significant, calculated by unpaired t-test (d, e, h) and unpaired t-test with Welch's correction (g).

Figure 1 | Inhibition of FGF signalling impairs lymphatic development.

a. Embryonic anterior dorsal skin (green area) was used to analyse lymphatic vessel development. b. Progressive ingrowth of lymphatic vessels (midline, dotted lines). c. Representative images of anterior dorsal skin from E15.5 embryos. Double-headed arrows indicate the distance between the leading fronts of the lymphatic vessels. Bottom: high-magnification images (scale bars, 100 μm) of boxed regions in top panels (scale bars, 250 μm). Ctrl, control. d, e, Quantification of the distance between the leading fronts of lymphatic vessels (d; n = 3 litters) and the number of lymphatic branch points per square millimetre of skin area (e; n = 4 embryos for control (Fgfr1flox/flox,Fgfr3−/−); n = 10 embryos for Fgfr1flox/flox,Fgfr3−/−). f, Representative images for VEGFR3 and PROX1 staining in the skin of E15.5 embryos. Scale bar, 150 μm. g, h, Quantification of the number of LECs per square millimetre of skin area (g) and lymphatic vessel diameter (h). n = 4 embryos for control (Fgfr1flox/flox,Fgfr3−/−); n = 10 embryos for Fgfr1flox/flox,Fgfr3−/−. Data represent mean ± s.e.m., *P < 0.05, **P < 0.01, NS is non-significant, calculated by unpaired t-test (d, e, h) and unpaired t-test with Welch's correction (g).

Figure 2 | FGF signalling controls glycolysis and HK2 expression.

a. Enriched nested Gene Ontology (nGO) categories (left) in the FGF signalling-regulated genes, identified by RNA-seq analysis of FGF2 and FGFR1 short interfering RNA (siRNA)-treated HDLECs, and violin plots (right) showing the log2-fold change distributions of differentially expressed genes for each enriched nGO term. The width of violin plot indicates relative gene frequency at specific log2-fold change. b, Left: measurement of glycolytic flux in control and FGF2-treated HDLECs (n = 4 wells of samples for each condition, representative of three independent experiments). Right: glycolytic flux measurement in HDLECs transfected with control or FGFR1 siRNA (n = 4 wells of samples for each condition, representative of two independent experiments). c, d, Mass spectrometry measurement of glycolytic intermediates and lactate in control and FGF2- and FGFR1 siRNA-treated HDLECs (n = 6 wells of samples for each condition, representative of two or three independent experiments). G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-BP, fructose-1,6-biphosphate; DHAP, dihydroxyacetone phosphate; 3-PG, 3-phosphoglycerate; 2-PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate. e, f, Mass spectrometry measurement of ATP generation (n = 6 wells of samples for each condition, representative of two independent experiments). g, h, Western blot analysis (g) and densitometric quantification (h) of glycolytic enzyme expression in control or FGF2-treated HDLECs (n = 6 experiments). L, J, Immunoblot analysis (i) and densitometric quantification (j) of glycolytic enzyme expression in HDLECs treated with control siRNA or FGFR1 siRNA (n = 3 independent experiments). k, qPCR analysis of Hk1 and Hk2 expression in dermal LECs isolated from E15.5 Fgfr1flox/flox,Fgfr3−/− and control embryos with tamoxifen injection at E12.5 and E13.5 (n = 2 litters including four control and two Fgfr1flox/flox,Fgfr3−/− embryos). Data represent mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001, calculated by unpaired t-test (b–f, h, j, k). For gel source data, see Supplementary Fig. 1.
enzymes, including hexokinase (HK1 and HK2), phosphofructokinase (PFKP) and pyruvate kinase (PKM2). We also assessed 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), which regulates blood vessel sprouting9. HDLEC stimulation with FGF2 induced a robust increase in HK2 expression, with minimal expression changes of other enzymes (Fig. 2g, h), while FGFR1 knockdown led to a significant reduction in HK2 (Fig. 2i, j). Examination of skin LECs isolated from E15.5 Fgfr1ΔLEC(BAC)/Fgfr3ΔLEC(BAC) embryos confirmed the reduction in HK2 expression (Fig. 2k). The importance of HK2 was supported by analysis of RNA-seq data: it was the only glucose metabolic gene among the top 20 transcripts (ranked by fold change) induced by FGF2 and downregulated by FGFR1 knockdown (Extended Data Fig. 6c).

HK2 knockdown significantly reduced, while adenosinergic-mediated HK2 overexpression increased, the glycolytic flux (Extended Data Fig. 6d–f). Since the knock out of Fgfr1/Fgfr3 in the endothelium also reduced angiogenesis, we examined whether FGFR signalling regulates glycolysis and enzyme expression in blood endothelial cells (BECs). As in LECs, treatment of human umbilical vein endothelial cells (HUVECs) with FGF2 enhanced glycolysis and selectively induced HK2 expression (Extended Data Fig. 6g–i), indicating that FGF regulation of angiogenesis and lymphangiogenesis share similar metabolic mechanisms.

Besides FGF2, several other growth factors including VEGFC and insulin-like growth factors 1 and 2 (IGF1 and IGF2) can regulate lymphangiogenesis10. We tested whether any of them also influences glycolysis in HDLECs. While all were effective in increasing cell proliferation, only VEGFC stimulation increased glycolytic flux, albeit to a lesser extent than FGF2 (Extended Data Fig. 6j). Consistent with these findings, only VEGFC increased HK2 expression without affecting other enzymes (Extended Data Fig. 6k).

FGF2 stimulation of HDLEC proliferation and migration was significantly reduced by HK2 knockdown (Extended Data Fig. 7a, b), while FGFR1 knockdown-induced decrease in HDLEC proliferation and migration was rescued by adenosinergic HK2 expression (Extended Data Fig. 7c, d). FGF2-induced LEC sprouting was almost completely blocked by HK2 knockdown (Extended Data Fig. 7e). FGFR1 knockdown in HDLECs also fully blocked FGF2-induced sprouting, while transduction of HK2 into HDLECs after FGFR1 knockdown partly restored sprouting (Extended Data Fig. 7f).

We next generated a mouse line with an endothelial-specific deletion of HK2 (HK2ΔEC) by crossing Hk2fl/fl mice11 with Cdh5-CreERT2. When examined at E15.5 after E12.5 Cre activation, HK2ΔEC embryos displayed lymphoedema (Extended Data Fig. 8a, b) and a reduction in the extent of lymphatic vessel branching and migration towards the midline (Extended Data Fig. 8c–e), while blood vascular development was largely normal (Extended Data Fig. 8f, g). Analysis of cell cycle progression in LYVE1+PECAM1+ LECs from the embryonic skin of HK2ΔEC and littermate control mice demonstrated a higher proportion of G1 and smaller proportion of S-phase cells in HK2-deficient LECs (Extended Data Fig. 8h).

To confirm that these defects in lymphatic development do not secondarily to any effects of HK2 deletion in the blood endothelium, we crossed Hk2fl/fl mice with Prox1-CreERT2(BAC). Immunostaining with anti-VEGFR3 antibody demonstrated a significant reduction in the skin lymphatic vessel development and branching at E15.5 after E12.5 Cre activation (Fig. 3a–c).

To examine the role played by HK2 in adult lymphangiogenesis, FGF2-containing pellets were implanted into corneas of Prox1-CreERT2(BAC) mice (referred to as Hk2ΔEC(KI)) and control mice (Fig. 3d). The Cre recombine, which was activated at the adult stage to bypass the early lymphatic defects caused by HK2 knockout, was highly efficient in driving recombination in cornea lymphatics (Fig. 3e). While adult mice corneas are devoid of both lymphatic and blood vasculature53, FGF2 pellet implantation led to robust stimulation of lymphangiogenesis in control mice that was significantly reduced in Hk2ΔEC(KI) mice (Fig. 3f, g). Similar to its role in the lymphatic development, pan-endothelial HK2 deletion at early developmental stage (E10.5) significantly reduced angiogenesis (Extended Data Fig. 8i–l) as well as arterial development and branching (Extended Data Fig. 8m, n) in the embryonic mouse skin. As in the case of Fgfr1/Fgfr3, activation of endothelial HK2 excision at P0 led to a significant reduction in the extent of development of the retinal vasculature (Extended Data Fig. 9a–c). The number of tip cells was reduced (Extended Data Fig. 9d, e) as was endothelial cell proliferation (Extended Data Fig. 9f, g). Vascular regression was not changed (Extended Data Fig. 9h, i).

Given RNA-seq demonstration of FGF-dependent regulation of HK2 messenger RNA (mRNA) levels and a previous observation of MYC binding to the regulatory region of the HK2 gene15, we examined whether MYC links FGF signalling to HK2 transcription in HDLECs. Chromatin immunoprecipitation (ChIP) confirmed MYC binding to conserved E-boxes in the first intron of the HK2 gene (Extended Data Fig. 10a, b). Moreover, knockdown of MYC decreased, while its overexpression increased, HK2 mRNA levels (Extended Data Fig. 10c, d). MYC knockdown also reduced glycolysis (Extended Data Fig. 10e, f).
mean
while MYC overexpression increased glycolytic activity (Extended Data Fig. 10g).

FGF2 treatment of HDLECs increased MYC protein expression (Fig. 4a), while FGFFR1 knockdown reduced it (Fig. 4b). Furthermore, ChIP–quantitative PCR (ChIP–qPCR) showed that the amount of MYC binding to the HK2 E-boxes was increased by FGF2 treatment and reduced by FGFFR1 knockdown (Fig. 4c). MYC knockdown selectively reduced HK2 expression and prevented FGF2-induced increase in HK2 levels (Fig. 4d). Finally, the decrease in HK2 expression after FGFFR1 knockdown was completely rescued by overexpression of MYC (Fig. 4e).

MYC is highly enriched in embryonic dermal LECs (Extended Data Fig. 10h). Examination of the lymphatic vasculature in the anterior dorsal skin at E15.5 in \( \text{Cdh5-CreERT}^{\text{fl}}/\text{Myc}^{\Delta \text{LEC}} \) mice after Cre activation at E11.5 showed, similarly to \( \text{Fgffr1}/\text{Fgffr3} \) and \( \text{Hk2} \) knockout mice, the presence of oedema (Fig. 4f) and a reduction in lymphatic vessel growth (Fig. 4g, h). LEC-specific MYC deletion using \( \text{Prox1-CreERT}^{\text{fl}}/\text{BAC} \) confirmed these findings (Extended Data Fig. 10i–k).

In agreement with the study demonstrating MYC involvement in blood vasculature development \(^{15} \), MYC knockdown in HUVECs reduced HK2 mRNA and protein expression (Extended Data Fig. 10l–n), while its overexpression increased HK2 levels (Extended Data Fig. 10o). Consistently, BECs isolated from \( \text{Myc}^{\text{ΔLEC}} \) embryos showed reduced expression of HK2 but not \( \text{Hk1} \) (Extended Data Fig. 10p). FGF2 treatment of HUVECs increased MYC expression (Extended Data Fig. 10q, r). Finally, FGF regulation of MYC was confirmed by the reduction of MYC expression in retinal vasculature of \( \text{P5 Fgf1}^{1}\text{ΔEC}, \text{Fgf3}^{1/2} \) mice (Extended Data Fig. 10s, t). Collectively, these results suggest that FGF-dependent regulation of MYC expression underlies control of HK2 levels in LECs and BECs.

These data indicate that FGF signalling plays a pivotal role in both blood and lymphatic vascular development and it is also required for lymphangiogenesis in tumours. At the molecular level, FGFs control glycolysis through an MYC-dependent regulation of HK2 expression. FGF stimulation increased HK2 levels leading to induction of glycolysis and increased production of glycolytic metabolites, while its suppression had the opposite effect. MYC mediates FGF2 effects on HK2 expression by directly binding to HK2 regulatory elements and controlling its transcription. Pan-endothelial and LEC-specific knockout of MYC induces a phenotype closely resembling those seen in \( \text{Fgf1}^{1}\text{ΔEC}, \text{Fgf3}^{1/2} \), \( \text{Fgf1}^{1}\text{ΔEC}(\text{BAC}), \text{Fgf3}^{1/2} \), \( \text{Hk2}^{\Delta \text{EC}} \) and \( \text{Hk2}^{\text{ΔLEC}(\text{BAC})} \) mice. Thus, the FGF–MYC–HK2 axis is the crucial driver of glycolytic metabolism in the endothelium. The dominant role of HK2 in cellular metabolism seems to be restricted to cells predominantly relying on glycolysis (rather than oxidative metabolism) for energy generation. This agrees with the reported HK2 role in cancer metabolism (another highly glycolytic environment) \(^{11} \).

The FGF/MYC/HK2-dependent regulation of vascular development is unexpected. Previously, FGF activity has been linked to prevention of endothelium-to-mesenchymal transition both in the lymphatic and in the systemic vasculature \(^{16} \), injury response \(^{18} \) and maintenance of vascular integrity \(^{19} \). While the FGF1 and FGF3 are the receptors involved, which of the 22 FGF family members is responsible for the required FGF signalling input is not known. In summary, FGF signalling regulates blood and lymphatic vascular development through control of endothelial metabolism driven by MYC-dependent regulation of HK2 expression. Therapeutic targeting of this FGF–MYC–HK2 pathway may open new possibilities for treatment of diseases associated with insufficient or excessive vascular growth.

Online Content
Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions P.Y. performed most of the experiments and prepared figures. K.W. analysed Myc mutant mice and performed other Myc-related experiments. A.D. performed retina analysis and sprouting assays. J.K.T. performed sprouting assay and western blotting analysis. T.C.A., J.S.F., and A.D. performed western blotting analysis. Z.C., L.S., H.S. and T.W.C. contributed to RNA-seq analysis. N.H. provided Hk2flox mice. S.-W.J., R.G.K.,...
culture plates were coated with 0.1% gelatin (Sigma) for 30 min at 37 °C and mycoplasma in Lonza. Culture medium was changed every other day. Tissue the 'loss-of-FGF signaling' dataset were first identified (1,999 transcripts). If a gene changes between groups. Genes observed to change significantly (false discovery number value was measured with an Agilent Bioanalyzer. One microgram were analysed. RNA isolation was performed using an RNeasy Mini Kit (Qiagen). For each treatment, nine replicates prepared from three independent experiments finally used to generate a list of differentially expressed genes between FGFR1 construction following the Illumina TruSeq RNA Sample Preparation protocol. One microgram total RNA was quantitated by NanoDrop and RNA integrity assay the effect of FGFR1 knockdown on glycolytic enzyme expression, HDLECs, transfected with control or FGFR1 siRNA three days in advance, were re-plated and collected for protein expression approximately 24 hours after the cell confluency reached ~80%. To examine the influence of growth factor treatment on glycolytic enzymes, freshly plated HDLECs were starved overnight with EBM2 plus 0.1% PBS and then stimulated with FGFR (100 ng ml−1 or 200 ng ml−1), VEGF (100 ng ml−1), VEGFD (100 ng ml−1), IGf (100 ng ml−1) and PDGFBB (100 ng ml−1) before lysis in RIPA buffer for protein extraction. For western blot analysis, the following antibodies were used: HK2 (Cell Signaling Technology, 2024), HK2 (Cell Signaling Technology, 2867), PFKFB3 (Proteintech, 13763-1-AP), PFKP (Cell Signaling Technology, 12746), PFKM2 (Cell Signaling Technology, 4053), MYC (Abcam, ab32072; Cell Signaling Technology, 9402), FGFR1 (Cell Signaling Technology, 9740), β-actin (Sigma, A5316) and tubulin (Cell Signaling Technology, 2148). ImageJ was used for densitometry quantification of western blot bands.

RNA-seq experimental design and sample preparation. HDLECs treated with or without FGFR2 for 14h were lysed for RNA extraction and were eventually used to generate a list of differentially expressed genes between FGFR2 and control, named the 'gain-of-FGF signaling' dataset. Similarly, HDLECs treated with FGFR1 siRNA or negative control siRNA for 3 days were lysed for RNA extraction and were finally used to generate a list of differentially expressed genes between FGFR1 siRNA and negative control siRNA, the so-called 'loss-of-FGF signaling' dataset. For each treatment, nine replicates prepared from three independent experiments were analysed. RNA isolation was performed using an RNeasy Mini Kit (Qiagen). RNA-seq. Extracted total RNA was quantitated by NanoDrop and RNA integrity number value was measured with an Agilent Bioanalyzer. One microgram of qualified RNA (RNA integrity number > 8.0) was used as input for library construction following the Illumina TruSeq RNA Sample Preparation protocol. RNA library was sequenced on an Illumina HiSeq® platform, paired-end 2 × 150 base pairs. The average data yield for each sample was 20 million paired-end reads with percentage of Q30 bases > 90.

RNA-seq data analysis. RNA-seq reads from each sample were aligned to human genome (build 38) using short reads aligner STAR (version 2.5.1b)23. Gene expression quantification was then performed using RSEM24 with GENCODE annotation (release 24: http://www.gencodegenes.org). Differential analysis was performed using edgeR25 to identify genes with significant expression changes between groups. Genes observed to change significantly (false discovery rate < 1 × 10−5) in their expression in both the 'gain-of-FGF signaling' dataset and the 'loss-of-FGF signaling' dataset were first identified (1,999 transcripts). Among those genes, those were truly regulated by FGF signalling, it should have shown opposite changing direction between the 'gain-of-FGF signaling' and 'loss-of-FGF signaling' datasets. Therefore, next-step analysis was focused on the 929 transcripts whose expression was upregulated by FGFR2 treatment but decreased after FGFR1 knockdown, and the 828 transcripts whose expression was reduced by FGFR2 stimulation but increased after FGFR1 knockdown. Those 1,757 transcripts were used for functional enrichment analysis by running GOseq, an algorithm controlling gene length bias in next-generation sequence data26. nGOseq, a modified version of the nEASE algorithm27,28 which also controls for gene length bias, was used to assess functional enrichment of nested GOseq terms. In brief, each enriched upper-level GOseq term was used for nested GOseq (nGOseq) analysis to identify statistically enriched nested GO terms driving upper-level functional enrichment of non-specific GOseq terms. For gene clustering, the Extended Data Fig. 6c, the top 50 transcripts that were increased by FGFR2 treatment (ranked by fold change) and the top 50 transcripts that were reduced after FGFR1 knockdown (ranked by fold change), among those 1,999 transcripts mentioned above, were first identified. Comparing the two lists of top hits resulted in an overlap containing 24 protein-coding genes. Those 24 genes were further ranked by the sum of their absolute log(fold changes) in FGFR2 treatment and FGFR1 knockdown conditions. After this ranking, the top 20 genes were shown in Extended Data Fig. 6c.

Measurement of glycolysis, glucose oxidation, glutamine oxidation, fatty-acid oxidation and glucose uptake. Glycolysis was measured as previously described29. In brief, subconfluent HDLECs cultured in 12-well plates were incubated with 1 ml per well EBM2 medium (containing appropriate amounts of serum and supplement) with 80 μCi mmol−1 [5-3H]-glucose (Perkin Elmer) for 2–3 h. Then 0.8 ml per medium was transferred into glass vials with hanging wells and filter papers soaked with H2O. After incubation in a cell culture incubator for at least two days to reach saturation, filter papers were taken out and the amount of evaporated 3H2O was measured in a scintillation counter. Glucose oxidation, glutamine oxidation and fatty-acid oxidation were measured essentially as reported30. For measurement of glucose uptake, HDLECs were incubated with [2-13C]-deoxy-glucose (2.5 μCi ml−1, Perkin Elmer) for 10 min before PBS washing (at least three times to remove all radioactive medium) and then lyzed with 500 μl 0.1 N NaOH. Four hundred microlitres of NaOH cell lysate for each sample was transferred to scintillation vials containing scintillation liquid and measured. Mass spectrometry analysis of metabolites. HDLECs were quenched by a rapid wash with ice-cold PBS and then collected in 150 μl of an ice-cold solution containing 20% methanol, 0.1% formic acid, 1 mM phenylalanine, 3 mM NaF and 100 μM EDTA. [3H]Taurine (10 μM, CDN Isotopes) was used as a loading control. All the samples were lyophilized and resuspended in 50 μl of water before the liquid chromatography–tandem mass spectrometry analysis. Samples were injected onto a Cognet Diamond Hydride column (2.2 μm particle size, 2.1 mm × 10 cm) at a flow rate of 0.5 ml min−1. Glycolytic intermediates were eluted isocratically with a 95% aqueous/5% organic solvent mixture. The aqueous solution contained 15 mM ammonium formate. The organic solution contained 60% acetonitrile, 35% isopropl alcohol and 15 mM ammonium formate. Samples were ionized by electrospray into an ABSCIEX 5500 QTRAP equipped with a SelectiON for differential mobility separation and acquired using multiple reaction monitoring in negative mode, as described previously29. Differential mobility separation-based separation of fructose-6-phosphate from glucose-6-phosphate, as well as the separation of ATP, ADP and AMP nucleotides, was achieved using no modifier. Isopropl alcohol was used as modifier for the differential mobility separation-based separation of the remaining glycolytic intermediates. Retention times were confirmed with known standards and peaks integrated using Multiquant (ABSCIEX) using the following multiple reaction monitoring transition pairs (Q1/Q2): 506/159 for ATP, 426/79 for ADP, 346/79 for AMP, 259/97 for glucose-6-phosphate, 259/97 for fructose-6-phosphate, 339/97 for fructose-1,6-bisphosphate, 169/97 for dihydroxyacetone phosphate (DHAP), 185/97 for 3-phosphoglycerate (3PG), 185/97 for 2-phosphoglycerate (2PG), 167/97 for phosphonoxyruvate (PYP), 89/89 for lactate and 124/80 for endogenous taurine. Endogenous taurine was used as internal control for cell density as measured by Cell Counting Kit-8.0 (Promega).

Seahorse assays. Metabolic analyses in HDLECs were performed with a Seahorse XF96 analyser (Agilent Seahorse) according to the manufacturer’s recommendations. In brief, siRNA-transfected or adenovirus-transduced HDLECs (40,000 cells per well of a 96-well plate) were seeded on fibronectin-coated XF96 microplates. After 2 h, cell culture medium was changed to a non-buffered assay medium and cells were maintained in a non-CO2 incubator for 1 h. A Glycolysis Stress Test kit (Agilent Seahorse) was used to monitor the extracellular acidification rate under various conditions. Three baseline recordings were made, followed by sequential injection of glucose (10 mM), the mitochondrial/ATP synthase inhibitor oligomycin (3 μM), and the glycolysis inhibitor 2-deoxy-D-glucose (2DG; 100 mM).

qPCR analysis. RNA was extracted from cells using the RNeasy Mini Kit or the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s instructions. cDNA synthesis was performed using the M-MLV reverse transcriptase (Invitrogen) or the
iScript cDNA synthesis kit (Bio-Rad). qPCR was performed either with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) and TaqMan probes (Thermo Fisher Scientific), or with iQ SYBR Green Supermix (Bio-Rad). For the TaqMan method, the following assays were used: human ACTB Hs99999903_m1; human MYC Hs00153408_m1; human HK2 Hs00606681_m1; mouse Actb Mm00468068_m1; mouse Hk2 Mm012619580_g1; mouse Hk1 Mm00439344_m1; mouse Hk2 Mm00433853_m1.

For SYBR method, qPCR primers for human FGFR1–FGFRA, human GAPDH, human β-actin, mouse Fgfr1–Fgfr4, mouse Hk1 and mouse β-actin were ordered from Qiagen. For qPCR primers both purchased from Invagen and designed in-house were used to generate data for Fig. 2k. The sequences of in-house designed qPCR primers are (5’ to 3’): Mouse Hk2 (CCGTCATCCTAATGACCAT CGGA; TCCACAGGATGCTGTTAC; human RPLP0 (TCTGCTATT CGTCTCTG; CAGGATCTGTTTGGACGCG).

ChIP-qPCR. ChIP assays were performed using SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling) according to the manufacturer’s protocol with some minor modifications. Cells cultured on 20-cm dishes were fixed for 10 min by adding 37% formaldehyde solution to the culture medium to a final concentration of 1%. Fixation was quenched with glycine for 5 min at room temperature. Cells were washed twice with ice-cold PBS, scraped into 2 ml PBS and centrifuged at 5,000 rpm for 10 min. The cell pellets from two 20-cm dishes were combined and lysed in 1 ml lysis buffer. The lysate was then centrifuged at 5,000 rpm for 5 min at 4°C and the pellet was resuspended in 100 μl nuclease digestion buffer. The DNA was digested with 0.5 μl of micrococal nuclease for 20 min at 37°C to a length of approximately 150–900 base pairs (checked by agarose gel electrophoresis). Lysates were centrifuged and the pellet was resuspended in 500 μl ChIP buffer and sonicated for 5 × 30 s at power level 2 and 40% constancy. The solution was centrifuged at 10,000 rpm for 10 min, and the supernatant was collected, which was the cross-linked chromatin. For ChIP, 150 μl of cross-linked chromatin was used for each immunoprecipitation and mixed with rabbit anti-MYC antibody (Abcam, 1:50) or same amount of rabbit IgG control at 4°C overnight. Two per cent of cross-linked chromatin was saved as input control for later qPCR reaction. Thirty microlitres of Protein G magnetic bead slurry was added to each immunoprecipitation reaction and incubated for 2 h at 4°C with rotation. The magnetic beads were washed three times with ChIP low salt buffer and once with ChIP high salt buffer. The bound chromatin on the beads was released in ChIP elution buffer by heating at 65°C for 30 min with vortex at 1,200 r.p.m. The chromatin was then digested with Protease K and purified using a spin column. The DNA was eventually eluted in 50 μl DNA elution buffer. The amount of precipitated DNA from each sample was quantified by qPCR using primers flanking the MYC binding element in the HK2 gene. The reading was normalized to that of DNA purified from the previously saved cross-linked chromatin (2% input). The reading by DNA from immunoprecipitation using MYC antibody against that from IgG purified from the previously saved cross-linked chromatin (2% input). The reading was normalized to that of DNA binding element in the HK2 gene. The reading was normalized to that of DNA purified from the previously saved cross-linked chromatin (2% input). The reading by DNA from immunoprecipitation using MYC antibody against that from IgG purified from the previously saved cross-linked chromatin (2% input).

Three-dimensional bead sprouting assay. HDLECs were trypsinized and mixed with collagen-coated Cytox microcarrier beads (Sigma) in a ratio of 2:500 beads to 1 × 106 cells in warm EGM-2 medium in a 15-ml Falcon tube. The mixture was incubated at 37°C for 4 h, with shaking every 20 min to ensure even coating of the beads. After 4 h, the coated beads were transferred to a six-well plate in 2 ml of EGM-2 medium per well and incubated at 37°C overnight. The next day, coated beads were embedded into a fibrinogen gel. For each well of a 24-well plate, 300 μl of 3 mg ml −1 fibrinogen in PBS was used, along with 100 μg ml −1 of aprotinin (Sigma) and 1.5 units per millilitre of thrombin (Sigma). Approximately 250 coated beads were embedded in each well. The plate was then incubated at 37°C for 1 h to generate a clot. After the gel solidified, human lung fibroblasts in EGM-2 medium were seeded on top at a concentration of 20,000 cells per well. The medium was changed every other day (full EGM-2 medium with 200 ng ml −1 of FGFR2), and the plates were imaged on day 6 using a spinning disc confocal microscope (Perkin Elmer). ImageJ was used to measure the sprout length for the data analysis.

Adenovirus. Adenovirus encoding green fluorescent protein (GFP), empty CMV vector, HK2 (ref. 31) or MYC (from Vector Bioslabs) for in vitro experiments was amplified in 293A cells according to the user manual of the ViraPower Adenoviral Expression System (Life Technologies). Virus was titrated using an Adeno-X Rapid Titer Kit (Clontech Laboratories). Analysis of lymphatic development. To induce Cre activity during embryonic stages, each pregnant mouse was injected intraperitoneally with 2 mg tamoxifen (Sigma, T5648) for 2 consecutive days (E10.5–E11.5, E11.5–E12.5 or E12.5–E13.5). Skin tissues were collected at E15.5. Standard whole-mount immunohistochemistry procedure was performed to stain the skin with antibodies against PECAM1 (BD Pharmingen, 553370), VEGFR3 (R&D Systems, AF743), PROX1 (AngioBio, 11-002), Cx40 (ALPHA DIAGNOSTIC, CX40-A), EGFSP (Life Technologies, A-11122; Abcam, ab13970) and Alexa fluorescent secondary antibodies (Life Technologies). A spinning disk confocal microscope (Perkin Elmer) and a Leica SP5 confocal microscope were used to generate high-resolution images of immunostained samples. Stitch imaging mode was chosen to image samples of large size. ImageJ was used to crop a representative area from large, stitched images for exhibition. For quantification of lymphatic development in the anterior dorsal skin, comparable regions (on the basis of the blood vessel pattern) between different samples were selected and cropped out of those stitched images as regions of interest for further analysis. Lymphatic development parameters, for example, branching points and LEC nucleus numbers were measured using ImageJ with the ‘Lymphatic Vessel Analysis’ plugin. If a considerably large area within a region of interest was destroyed during skin dissection, that sample was excluded for analysis.

Retinal vasculature development. Dorsal skin explants were collected from E15.5 mouse embryos, and minced into ice-cold Dulbecco’s Modified Eagle’s Medium supplemented with 20% fetal bovine serum and 1.25 mg ml −1 collagenase. Samples were incubated for 30 min at 37°C, and mechanically dissociated by repeated pipetting until a single cell suspension was achieved. Samples were then centrifuged for 1 min at 2,000 rpm at 4°C, and cell pellet was resuspended in PBS supplemented with 10% FBS, 5.5 mM glucose and 20 mM HEPES. Cells were incubated in the presence of Hoechst 33342 (25 μg ml −1) for 30 min at 37°C, and then additionally for 15 min at 37°C in the presence of Procion Y (0.5 mg ml −1) as well as the fluorescently conjugated antibodies PECAM1–FITC (BD Pharmingen, 553372) and LYVE1–Alexa Fluor 488 (eBioscience, 50-0443-82). Samples were washed and resuspended in 0.5 ml PBS on ice for subsequent analysis. BECs and LECs were identified by flow cytometry as PECAM1-LYVE1− and PECAM1-LYVE1+ events respectively, and, for each population, cell cycle distribution was determined by relative DNA (Hoechst) and RNA (Procion Y) content.
Fluorescence-activated cell sorting (FACS) to sort LECs for qPCR analysis. Dorsal skin explants were collected from E15.5 mouse embryos in ice-cold Dulbecco's Modified Eagle's Medium supplemented with 20% fetal bovine serum and 1 mg ml⁻¹ collagenase. Samples were incubated for 1 h at 37°C, and mechanically dissociated by repeated pipetting until a single-cell suspension was achieved. Samples were then centrifuged for 1 min at 2,000g at 4°C, and the cell pellet was resuspended in Hank's Buffered Saline Solution supplemented with 10% FBS, 5.5 mM glucose and 20 mM HEPES. Cells were incubated in the presence of the fluorescently conjugated antibodies PECAM1- FITC (BD Pharmingen, 553372), CD45-PECy7 (eBioscience, 25-0451-82) and LYVE1-Alexa647 (eBioscience, 50-0443-82) for 30 min at 37°C. Samples were pelleted for 1 min at 2,000g at 4°C, resuspended in 0.5 ml PBS on ice, and filtered through a 35 μm nylon mesh before flow cytometry analysis and cell sorting. BECs were identified by FACS as PECAM1⁺CD45⁻LYVE1⁻, LECs were identified as PECAM1⁺CD45⁺LYVE1⁺ events and cells from each population were sorted into RLT lysis buffer (Qiagen) for mRNA preparation (RNeasy Micro kit), cDNA library construction and subsequent qPCR analysis. When analysing qPCR results, wells with a sample melting curve peak at the incorrect temperature were excluded.

**Corneal lymphangiogenesis model.** Slow-releasing pellets containing FGF2 were made as previously described. Surgery to implant the pellets into the mouse cornea was performed as reported. Adult Prox1-CreERT2/KI, Hk2floxed and control female mice were injected intraperitoneally with tamoxifen (150 μg per g body weight) every other day (seven injections in total) before the corneal surgery. One week after the pellet implantation, eyeballs were collected for cornea dissection and immunostaining with LYVE1 (Angiobio, 11-034) and PECAM1 (BD Pharmingen, 553370) antibodies and then used for imaging (spinning disk confocal microscopy) and quantification analysis (ImageJ).

**Mouse orthotopic pancreatic tumour model.** The mouse pancreatic tumour cell line Panc02 was obtained from B. Wiedenmann. One million tumour cells were pelleted for 1 min at 2,000g at 4°C, resuspended in 0.5 ml PBS on ice, and filtered through a 35 μm nylon mesh before flow cytometry analysis and cell sorting. BECs were identified by FACS as PECAM1⁺CD45⁻LYVE1⁻, LECs were identified as PECAM1⁺CD45⁺LYVE1⁺ events and cells from each population were sorted into RLT lysis buffer (Qiagen) for mRNA preparation (RNeasy Micro kit), cDNA library construction and subsequent qPCR analysis. When analysing qPCR results, wells with a sample melting curve peak at the incorrect temperature were excluded.

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Extended Data Figure 1 | Expression of FGFRs in mouse and human LECs and the effect of single knockout of Fgfr1 or Fgfr3 on lymphatic development in the embryonic skin. a, b, qPCR analysis of FGFR expression in mouse dermal LECs (isolated from E15.5 embryos by FACS) (a; n = 3 embryos) and HDLECs (b; n = 3 technical replicates, representative of two experiments). c, d, qPCR analysis of FGFR expression in HDLECs with FGFR1 (c) or FGFR3 (d) knockdown. FGFR mRNA levels in FGFR1- or FGFR3-deficient cells were presented as values relative to those of control siRNA-treated cells. n = 6 replicates (two independent experiments, technical triplicates per experiment). e, f, Activation of mTmG reporter by Cdh5-CreERT2 (e; scale bar, 100 μm) or Prox1-CreERT2(BAC) (f; scale bar, 250 μm) in dermal LECs of E15.5 mouse embryos. g, Anterior dorsal skin with VEGFR3 and PECAM1 staining from E15.5 Fgfr1ΔLEC(BAC) and Fgfr1 flox/flox embryos, which were treated with tamoxifen at E12.5 and E13.5. e, Arrowheads indicate lymphatic vessels marked by EGFP. Scale bar, 250 μm. h, Quantification of the distance between the two leading fronts of the lymphatic vessels (n = 6 embryos for Fgfr1 flox/flox, n = 5 embryos for Fgfr1 ΔLEC(BAC)). i, Anterior dorsal skin stained for VEGFR3 and PECAM1 from E15.5 Fgfr1 flox/flox, Fgfr3 Δ/Δ, Fgfr3 Δ/+ and Fgfr1 flox/flox, Fgfr3 Δ−/− embryos. Scale bar, 250 μm. j, Quantification of the distance between the two leading fronts of the lymphatic vessels (n = 4 embryos for Fgfr1 flox/flox, Fgfr3 Δ/Δ; n = 6 embryos for Fgfr1 flox/flox, Fgfr3 Δ−/−; n = 2 embryos for Fgfr1 flox/flox, Fgfr3 Δ−/−). Note that these embryos were not treated with tamoxifen. Dotted lines indicate the midline in g and i. Data represent mean ± s.e.m., ***P < 0.001, NS is non-significant, calculated by unpaired t-test (c, d, h) and one-way ANOVA plus Tukey’s multiple comparisons test (j).
Extended Data Figure 2 | Endothelial Fgfr1/Fgfr3 deletion impairs dermal lymphatic development and inhibition of FGF signalling suppresses pathological lymphangiogenesis. a, Experimental strategy. b, Bright-field images of E15.5 Fgfr1\textsuperscript{ΔEC};Fgfr3\textsuperscript{−/−} and control (Fgfr1\textsuperscript{flox/flox};Fgfr3\textsuperscript{+/-}) embryos with tamoxifen treatment at E10.5 and E11.5. Arrowhead denotes area with lymphoedema. c, Representative images of anterior dorsal skin with VEGFR3 and PECAM1 staining from E15.5 Fgfr1\textsuperscript{ΔEC};Fgfr3\textsuperscript{−/−} and control embryos treated with tamoxifen at E10.5 and E11.5. d, e, Quantification of the distance between the two leading fronts of the lymphatic vessels (d; n = 3 litters) and the number of lymphatic branch points per square millimetre of skin area (e; n = 9 embryos for control (Fgfr1\textsuperscript{flox/flox};Fgfr3\textsuperscript{+/-}); n = 7 embryos for Fgfr1\textsuperscript{ΔEC};Fgfr3\textsuperscript{−/−}). f, Experimental strategy. g, Representative images of anterior dorsal skin with VEGFR3 and PECAM1 staining from E15.5 Fgfr1\textsuperscript{ΔEC};Fgfr3\textsuperscript{−/−} and control embryos with tamoxifen treatment at E11.5 and E12.5. h, i, Quantification of the distance between the two leading fronts of the lymphatic vessels (h; n = 4 litters) and the number of lymphatic branch points per square millimetre of skin area (i; n = 9 embryos for control (Fgfr1\textsuperscript{flox/flox};Fgfr3\textsuperscript{+/-}); n = 8 embryos for Fgfr1\textsuperscript{ΔEC};Fgfr3\textsuperscript{−/−}). In c, g, bottom panels (scale bar, 100 μm) for each genotype are high-magnification images of boxed regions in the top panels (scale bar, 250 μm). Double-headed arrows indicate the distance between the two leading fronts of the lymphatic vessels, which is larger in Fgfr1\textsuperscript{ΔEC};Fgfr3\textsuperscript{−/−} skin than in control. j, Representative images of the peri-tumoural area of orthotopic Panc02 tumours stained for LYVE1 after vehicle (control) or FGFR-inhibitor treatment (SSR). k, Quantification of the area of LYVE1\textsuperscript{+} lymphatics per peri-tumoural area (n = 3 mice for each condition). Data represent mean ± s.e.m., *P < 0.05, **P < 0.001, calculated by unpaired t-test (d, e, h, i, k).
Extended Data Figure 3 | Endothelial Fgfr1/Fgfr3 are essential for blood vessel development in the embryonic skin. 

**a.** Schematic of the experimental strategy. 

**b.** Representative images of anterior dorsal skin with PECAM1 staining from E15.5 Fgfr1ΔEC;Fgfr3−/− and control embryos. Scale bar, 250 μm.

**c–e.** Quantification of the number of blood vessel branch points per square millimetre of skin area (**c**; n = 9 embryos for control (Fgfr1fl/fl;Fgfr3+/-); n = 7 embryos for Fgfr1ΔEC;Fgfr3−/−), blood vessel covered area relative to skin area (**d**; n = 9 embryos for control (Fgfr1fl/fl;Fgfr3+/-); n = 7 embryos for Fgfr1ΔEC;Fgfr3−/−), and capillary diameter (**e**; n = 6 embryos for control (Fgfr1fl/fl;Fgfr3+/-); n = 3 embryos for Fgfr1ΔEC;Fgfr3−/−). Data represent mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001, calculated by unpaired t-test (**c–e**).

**f.** Anterior dorsal skin stained for Cx40 in E15.5 Fgfr1ΔEC;Fgfr3−/− and control embryos. Scale bar, 250 μm.

**g, h.** Quantification of the number of artery branch points (**g**; n = 6 embryos for control (Fgfr1fl/fl;Fgfr3+/-); n = 3 embryos for Fgfr1ΔEC;Fgfr3−/−) and artery diameter (**h**; n = 6 embryos for control (Fgfr1fl/fl;Fgfr3+/-); n = 3 embryos for Fgfr1ΔEC;Fgfr3−/−).
Extended Data Figure 4 | Requirement of endothelial Fgfr1/Fgfr3 for retinal angiogenesis. a, Schematic of the experimental strategy to assess early formation of the retinal vasculature. The red triangles indicate the intragastric injections of tamoxifen at P0, P1 and P2. b, Representative images of isolectin B4 (IB4)-stained retinal vessels in P5 Fgfr1<sup>ΔEC</sup>;Fgfr3<sup>−/−</sup> and control mice. Scale bar, 500 μm. c, Quantification of vascular progression (d is the distance between the vascular front and the optic nerve; D is the retina radius), vascular density (AU, arbitrary units) and the number of branch points per square millimetre of retina area. n = 16 retinas for control (Fgfr1<sup>flox/flox</sup>;Fgfr3<sup>−/−</sup>); n = 19 retinas for Fgfr1<sup>ΔEC</sup>;Fgfr3<sup>−/−</sup>. d, Angiogenic fronts of IB4- and Erg1/2/3-stained retinal vessels in P5 Fgfr1<sup>ΔEC</sup>;Fgfr3<sup>−/−</sup> and control mice. Scale bar, 50 μm. e, Quantification of the number of tip cells per 200-μm length (n = 6 retinas for control (Fgfr1<sup>flox/flox</sup>;Fgfr3<sup>−/−</sup>); n = 4 retinas for Fgfr1<sup>ΔEC</sup>;Fgfr3<sup>−/−</sup>) and the number of vascular front endothelial cells (ECs) per 0.04-mm<sup>2</sup> retina area (n = 4 retinas for control (Fgfr1<sup>flox/flox</sup>;Fgfr3<sup>−/−</sup>); n = 4 retinas for Fgfr1<sup>ΔEC</sup>;Fgfr3<sup>−/−</sup>). f, Retinal vessels stained for phospho-Histone H3 (PH3) and IB4 in P5 Fgfr1<sup>ΔEC</sup>;Fgfr3<sup>−/−</sup> and control mice. Scale bar, 50 μm. g, Quantification of the number of PH3<sup>+</sup>IB4<sup>+</sup> endothelial cells per vascular area (normalized to control mice; n = 4 for control (Fgfr1<sup>flox/flox</sup>;Fgfr3<sup>−/−</sup>); n = 6 for Fgfr1<sup>ΔEC</sup>;Fgfr3<sup>−/−</sup>). h, Representative images of IB4-stained retinal vessels in P5 Fgfr1<sup>ΔEC</sup> and Fgfr1<sup>flox/flox</sup> mice. Scale bar, 500 μm. i, Quantification of vascular density and the number of branch points per square millimetre of retina area (n = 18 retinas for Fgfr1<sup>flox/flox</sup>; n = 8 retinas for Fgfr1<sup>ΔEC</sup>). j, Representative images of IB4-stained retinal vessels in P5 Fgfr1<sup>flox/flox</sup>;Fgfr3<sup>+/−</sup> and Fgfr1<sup>flox/flox</sup>;Fgfr3<sup>−/−</sup> mice. Scale bar, 500 μm. k, Quantification of vascular density and the number of branch points per square millimetre of retina area (n = 16 retinas for Fgfr1<sup>flox/flox</sup>;Fgfr3<sup>+/−</sup>; n = 16 retinas for Fgfr1<sup>ΔEC</sup>;Fgfr3<sup>−/−</sup>). Data represent mean ± s.e.m., **P < 0.01, ***P < 0.001, calculated by unpaired t-test (c, e, g, i, k).
Extended Data Figure 5 | Proliferation and migration of HDLECs with FGFR1 and/or FGFR3 knockdown. a, Proliferation of HDLECs treated with indicated siRNAs was measured by using xCELLigence (see Methods) \((n = 4\) wells of samples for each condition; representative of two independent experiments). b, Wound-healing assay to assess the migration of HDLECs transfected with siRNAs as indicated. Red dotted lines outline wound area in the last time-point images of HDLECs with different treatments. c, Wound closure area between the first and last time points was measured and normalized to that of control siRNA-treated HDLECs \((n = 8\) imaging fields for control siRNA, FGFR1 siRNA and FGFR3 siRNA; \(n = 7\) imaging fields for FGFR1/FGFR3 siRNAs; representative of two independent experiments). Data represent mean ± s.e.m., ***\(P < 0.001\), calculated by one-way ANOVA plus Tukey’s multiple comparisons test (a, c).
Extended Data Figure 6 | Metabolic measurement and glycolytic enzyme expression in HDLECs and HUVECs. a, Measurement of flux rate of different metabolic processes in HDLECs (glucose oxidation, \( n = 3 \) samples; glycolysis, \( n = 11 \) samples, combined from three experiments; glutamine oxidation, \( n = 5 \) samples, combined from two experiments; and fatty-acid oxidation, \( n = 4 \) samples). b, Measurement of glucose uptake in HDLECs treated with or without FGF2 (\( n = 3 \) wells of samples for each condition). c, Top 20 protein-coding transcripts (ranked by fold change) that were increased by FGF2 and reduced by FGFR1 siRNA (see Methods for details). HK2, highlighted in red, is the only glucose metabolic gene in this list. d, Western blots showing the knockdown efficiency of HK2 siRNA. e, f, Measurement of glycolytic flux rate of HDLECs with indicated treatments. For e, \( n = 4 \) wells of samples for control siRNA, \( n = 4 \) wells of samples for HK2 siRNA, \( n = 3 \) wells of samples for control siRNA + FGF2, and \( n = 4 \) wells of samples for HK2 siRNA + FGF2. For f, \( n = 4 \) wells of samples for each condition. g, Measurement of glycolytic flux rate of HUVECs in the absence or presence of FGF2 (\( n = 4 \) wells of samples for each condition, representative of two independent experiments). h, i, Representative western blot analysis (h) and densitometric quantification (i; \( n = 5 \) replicates from three experiments) of glycolytic enzyme expression in control or FGF2-treated HUVECs. j, Measurement of glycolytic flux rate of HDLECs treated with or without different growth factors (\( n = 4 \) wells of samples for each condition). k, Densitometric quantification (\( n = 3 \) independent experiments) of glycolytic enzyme expression in HDLECs in the presence or absence of different growth factors. Data represent mean ± s.e.m., *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \), calculated by unpaired \( t \)-test (b, f, g, i) and one-way ANOVA plus Sidak’s (e, j) or Dunnett’s (k) multiple comparisons test. For gel source data, see Supplementary Fig. 1.
Extended Data Figure 7 | Role of HK2 in FGF-dependent cellular behaviours. a, b, Proliferation (a; n = 4 wells of samples for each condition) and migration (b; n = 4 imaging fields for each condition) of HDLECs with indicated siRNAs, which were serum-starved and treated with or without FGF2. c, d, Proliferation (c; n = 4 wells of samples for control siRNA + Ad-control, control siRNA + Ad-HK2 and FGFR1 siRNA + Ad-control; n = 3 wells of samples for FGFR1 siRNA + Ad-HK2) and migration (d; n = 8 imaging fields for each condition) of HDLECs with indicated siRNAs, which were cultured in fully supplemented medium and treated with control or HK2 adenovirus. Proliferation was measured using xCELLigence and migration was analysed through a wound-healing assay (see Methods). Wound closure area between the first time point and the last time point was measured and normalized to that of control siRNA-treated HDLECs (b) or HDLECs treated with control siRNA and control adenovirus (d). Red dotted lines outline wound area in the last time-point images of HDLECs with different treatments. e, f, Representative images and quantification of microcarrier beads coated with HDLECs under treatments as indicated. Total length of LEC sprouts per bead was quantified. e, f, Arrowheads indicate sprouts extending from the beads. For e, n = 14 beads for control siRNA, n = 14 beads for HK2 siRNA, n = 19 beads for control siRNA + FGF2 and n = 25 beads for HK2 siRNA + FGF2. For f, n = 25 beads for each condition. Data represent mean ± s.e.m., **P < 0.01, ***P < 0.001, calculated by one-way ANOVA plus Sidak’s multiple comparisons test (a–f).
Extended Data Figure 8 | Effect of endothelium-specific deletion of HK2 on the lymphatic and blood vessel development in the skin.

a, Schematic of the experimental strategy.
b, Bright-field images of E15.5 Hk2ΔEC and control embryos treated with tamoxifen at E12.5 and E13.5. Arrowhead denotes area with lymphoedema.
c, Representative images of anterior dorsal skin with VEGFR3 and PECAM1 staining from E15.5 Hk2ΔEC and control (Hk2fl/fl) embryos with tamoxifen treatment at E12.5 and E13.5. Double-headed arrows indicate the distance between the two leading fronts of the lymphatic vessels, which is larger in Hk2ΔEC skin than in control. Lower panels (scale bar, 100 μm) for each genotype are high-magnification images of boxed regions in top panels (scale bar, 250 μm).
d, e, Quantification of the distance between the two leading fronts of the lymphatic vessels (d; n = 4 litters) and the number of lymphatic branch points per square millimetre of skin area (e; n = 5 embryos for control (Hk2fl/fl); n = 6 embryos for Hk2ΔEC).
f, g, Quantification of the number of blood vessel branch points per square millimetre of skin area (f) and area covered by blood vessels (g).

h, LECs were isolated from E15.5 control (Hk2fl/fl) and Hk2ΔEC embryos and analysed for cell cycle distribution. Percentage of cells in different cell cycle phases was quantified (n = 12 embryos for control (Hk2fl/fl) and n = 9 embryos for Hk2ΔEC).
i, The experimental strategy.
j, Representative images of anterior dorsal skin with PECAM1 staining from E15.5 Hk2ΔEC and control embryos with tamoxifen treatment at E10.5 and E11.5. Scale bar, 250 μm.
k, l, Quantification of the number of blood vessel branch points per square millimetre of skin area (k) and blood vessel covered area relative to skin area (l). n = 4 embryos for control (Hk2fl/fl); n = 7 embryos for Hk2ΔEC.
m, Anterior dorsal skin stained for Cx40 in E15.5 Hk2ΔEC and control embryos treated with tamoxifen at E10.5 and E11.5. Scale bar, 250 μm.
n, Quantification of the number of artery branch points (n = 4 embryos for control (Hk2fl/fl); n = 7 embryos for Hk2ΔEC). Data represent mean ± s.e.m., *P < 0.05, **P < 0.01, calculated by unpaired t-test (d, f–h, k, l, n).
Extended Data Figure 9  |  Endothelial Hk2 is required for retinal angiogenesis. 

**a**, Schematic of the experimental strategy to assess early formation of the retinal vasculature (P0–P5). The red triangles indicate the intragastric injections of tamoxifen at P0, P1 and P2. 

**b**, Representative images of IB4-stained retinal vessels in P5 Hk2ΔEC and control mice. Scale bar, 500 μm. 

**c**, Quantification of vascular progression (d is the distance between the vascular front and the optic nerve; D is the retina radius), vascular density and the number of branch points per square millimetre of retina area. n = 18 retinas for control (Hk2floxed/floxed) and n = 24 retinas for Hk2ΔEC. 

**d**, Angiogenic fronts of IB4- and Erg1/2/3-stained retinal vessels in P5 Hk2ΔEC and control mice. Scale bar, 50 μm. 

**e**, Quantification of the number of tip cells per 200-μm length (n = 4 retinas for control (Hk2floxed/floxed); n = 4 retinas for Hk2ΔEC) and the number of vascular front ECs per 0.04-mm² retina area (n = 4 for control (Hk2floxed/floxed); n = 4 for Hk2ΔEC). 

**f**, Retinal vessels stained for PH3 and IB4 in P5 Hk2ΔEC and control mice. Scale bar, 50 μm. 

**g**, Quantification of the number of PH3⁺IB4⁺ ECs per vascular area (normalized to control mice; n = 4 for control (Hk2floxed/floxed); n = 4 for Hk2ΔEC). 

**h**, Staining for collagen IV (Col4) and IB4 in the retinas of P5 Hk2ΔEC and control mice. Scale bar, 50 μm. 

**i**, Quantification of Col4⁺ area per IB4⁺ area (n = 6 retinas for control (Hk2floxed/floxed); n = 8 retinas for Hk2ΔEC). Data represent mean ± s.e.m., **P < 0.01, ***P < 0.001, calculated by unpaired t-test (c, e, g, i).
Extended Data Figure 10 | Characterization of FGF–MYC–HK2 signalling in endothelial cells. a, Schematic showing that E-boxes, which are MYC binding elements, localize in the first intron of human and mouse HK2 genes. Primers were designed to amplify the E-box-containing region (green bar) in ChIP–qPCR assay. b, ChIP–qPCR analysis of DNA immunoprecipitated with MYC antibody or IgG (n = 3 independent experiments) in HDLECs. c, qPCR analysis of HK2 expression in HDLECs transfected with control siRNA or MYC siRNA (n = 4 experiments). d, qPCR analysis of HK2 mRNA in HDLECs infected with control or MYC adenovirus (n = 6 experiments). e, Glycolytic flux measurement of HDLECs transfected with control siRNA or MYC siRNA (n = 4 wells for samples from each treatment, representative of two independent experiments). f, Extracellular acidification rate (ECAR) in HDLECs transfected with control siRNA or MYC siRNA (n = 4 replicates for each treatment, duplicates per experiment). g, Extracellular acidification rate in HDLECs infected with control or MYC adenovirus. n = 4 replicates (two independent experiments, duplicates per experiment). h, Anterior dorsal skin was dissected from E15.5 mouse embryos and immunostained with anti-VEGFR3 and anti-MYC antibodies. Arrowheads indicate LECs with Myc expression. i, Confocal images of anterior dorsal skin with VEGFR3, PROX1 and PECAM1 staining from E15.5 Myc^ΔLEC(BAC) and control embryos. Scale bar, 250 μm. j, k, Quantification of the distance between the leading fronts of ingrowing lymphatics (j; n = 7 embryos for control (Myc^fl/fl)); n = 9 embryos for Myc^ΔLEC(BAC)) and the number of LECs (PROX1 staining) per 100-μm length of lymphatic vessels (k; n = 6 embryos for control (Myc^fl/fl)); n = 8 embryos for Myc^ΔLEC(BAC)). l, qPCR analysis of HK2 expression in HUVECs transfected with control siRNA or MYC siRNA (n = 4 experiments). m, n, Representative immunoblot analysis (m) and densitometric quantification (n) of HK2 expression in HUVECs transfected with control siRNA or MYC siRNA (n = 4 independent experiments). o, qPCR analysis of HK2 mRNA in HUVECs infected with control or MYC adenovirus (n = 2–4 experiments). p, qPCR analysis of Hk1 and HK2 expression in HUVECs isolated from E15.5 Myc^ΔEC and control embryos with tamoxifen treatment at E11.5 and E12.5 (n = 2–4 embryos). q, r, Representative western blot (q) and densitometric quantification (r) of MYC expression in HUVECs treated with or without FGF2 (n = 5 replicates from three experiments). s, Representative images showing Myc expression was reduced in retinal vasculature of P5 Fgfr1^ΔEC;Fgfr3^−/− compared with control (tamoxifen treatment from P0 to P2). Arrowheads indicate retinal BECs with Myc expression. Scale bar, 50 μm. t, Quantification of the percentage of MYC+ retinal endothelial cells in the vascular fronts (n = 4 retinas for each genotype). Data represent mean ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001, calculated by unpaired t-test (b–g, j–l, n–p, r, t). For gel source data, see Supplementary Fig. 1.