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ANGPTL4– $\alpha\beta3$ interaction counteracts hypoxia-induced vascular permeability by modulating Src signalling downstream of vascular endothelial growth factor receptor 2

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Abstract

Dynamic control of endothelial cell junctions is essential for vascular homeostasis and angiogenesis. We recently provided genetic evidence that ANGPTL4 is a key regulator of vascular integrity both during developmental and in hypoxia-induced pathological conditions. The purpose of the present study was to decipher the molecular mechanisms through which ANGPTL4 regulates vascular integrity. Using surface plasmon resonance and proximity ligation assays, we show that ANGPTL4 binds integrin $\alpha\beta3$. *In vitro* and *in vivo* functional assays with *Angptl4*-deficient mice demonstrate that ANGPTL4– $\alpha\beta3$ interaction is necessary to mediate ANGPTL4 vasoprotective effects. Mechanistically, ANGPTL4– $\alpha\beta3$ interaction enhances Src recruitment to integrin $\alpha\beta3$ and inhibits Src signalling downstream of vascular endothelial growth factor receptor 2 (VEGFR2), thereby repressing hypoxia-induced breakdown of VEGFR2–VE-cadherin and VEGFR2– $\alpha\beta3$ complexes. We further demonstrate that intravitreal injection of recombinant human ANGPTL4 limits vascular permeability and leads to increased adherens junction and tight junction integrity. These findings identify a novel mechanism by which ANGPTL4 counteracts hypoxia-driven vascular permeability through integrin $\alpha\beta3$ binding, modulation of VEGFR2–Src kinase signalling, and endothelial junction stabilization. We further demonstrate that *Angptl4*-deficient mice show increased vascular leakage *in vivo* in a model of laser-induced choroidal neovascularization, indicating that this newly identified ANGPTL4– $\alpha\beta3$ axis might be a target for pharmaceutical intervention in pathological conditions. Copyright © 2016 Pathological Society of Great Britain and Ireland. Published by John Wiley & Sons, Ltd.

Keywords: hypoxia; vascular permeability; angiotensin-like 4; VEGFR2; integrin $\alpha\beta3$; VE-cadherin

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Conflicts of interest statement DMV, AJM, GDY and GT are employees of Regeneron Pharmaceuticals. The remaining authors declare no competing financial interests.

Introduction

Increased vascular permeability is a key feature of ischaemic pathologies, and triggers plasma leakage and protein extravasation, thereby leading to the formation of oedema and eventually to tissue damage. Pharmacological treatment aimed at targeting microvascular dysfunction in order to restore proper control of vascular

integrity in pathologies such as cancer, stroke, acute myocardial infarction and ischaemic retinopathies is still an unmet medical need [1–4]. Anti-vascular endothelial growth factor (VEGF) agents have been used clinically, in particular for exudative age-related macular degeneration (AMD), diabetic retinopathy, retinal vein occlusion, and retinopathy of prematurity, which are major causes of visual impairment, to inhibit retinal

angiogenesis. Despite encouraging results in halting the disease and improving vision, intravitreal injection of anti-VEGF agents may also be associated with systemic adverse events and devastating ocular complications. Safety concerns regarding intravitreal injection of common anti-VEGF agents have recently been raised [5]. Therefore, the identification of new molecular mechanisms that restrict vascular permeability is needed.

Full-length ANGPTL4, a secreted angiopoietin-like glycoprotein, and its fibrinogen-like domain (FLD or C-terminal ANGPTL4) have been characterized as context-dependent modulators of vascular permeability [6,7] and cancer metastasis [8–10], making the identification of the relevant endothelial cell receptor through which ANGPTL4 mediates its pathological effects necessary for the development of therapies. Using *Angptl4*-deficient mice [11], we have provided genetic evidence that ANGPTL4 protects vascular integrity in developmental angiogenesis [12] and in models of myocardial infarction [13] and stroke [14]. Indeed, loss of *Angptl4* resulted in increased lesion size, which was rescued by intravenous administration of human recombinant ANGPTL4.

Nevertheless, the relevant endothelial cell receptors through which ANGPTL4 mediates its pathological effects, the identification of which is a requirement for developing therapies, are still unknown. Interestingly, several studies have characterized interactions between ANGPTL4 FLD and integrins. Huang *et al* previously reported that ANGPTL4 FLD binds to integrin $\alpha 5\beta 1$ *in vitro* and induces endothelial cell barrier disruption [10]. In normal and tumour epithelial cells, ANGPTL4 FLD interacts with integrins $\alpha 5\beta 1$ and $\alpha v\beta 5$, leading to anoikis resistance [15]. ANGPTL4 FLD was also reported to modulate keratinocyte migration through interaction with integrins $\beta 1$ and $\beta 5$, but not integrin $\beta 3$ [16]. Given the major role played by $\beta 3$ integrins in regulating vascular permeability and VEGF signalling [17–21], we hypothesized that full-length ANGPTL4 might bind to integrin $\alpha v\beta 3$ at the endothelial cell surface. Using surface plasmon resonance (SPR) binding and proximity ligation assays (PLAs), we demonstrate here that ANGPTL4 binds directly to integrin $\alpha v\beta 3$. Mechanistically, binding of ANGPTL4 to integrin $\alpha v\beta 3$ leads to Src recruitment and its sequestration away from VEGF receptor 2 (VEGFR2), resulting in diminished Src signalling downstream of VEGFR2, and thereby inducing stabilization of both VEGFR2–VE-cadherin and VEGFR2– $\alpha v\beta 3$ complexes.

Materials and methods

Experimental procedures

The experiments were performed in accordance with the official regulation edict by the French Ministry of Agriculture. This study conforms to the standards of INSERM (the French National Institute of Health) regarding the care and use of laboratory animals

(Animal house agreement number: C-75-05-12), and was performed in accordance with European Union Council Directives (2010/63/UE).

Angptl4^{LacZ/LacZ} mice

Angptl4-deficient (*Angptl4*^{LacZ/LacZ}) mice were generated and characterized as described previously [12].

Adult retinal vascular network analysis

Adult mice were killed, and eyes were enucleated and fixed in 4% paraformaldehyde. Retinas were dissected and immunostaining was performed as described previously [22]. For VE-cadherin immunostaining, mice were perfused with 1% paraformaldehyde, and retinas were processed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ phosphate-buffered saline (1 mM CaCl_2 , 1 mM MgCl_2) as described previously [23]. Retinas were immunostained with: biotinylated isolectin B4 (Cat. no. B-1205; 1:50; Vector Laboratories, Burlingame, CA, USA), anti-NG2 (Cat. no. MAB5384; 1:40; Chemicon Merck Millipore, Darmstadt, Germany), anti-zonula occludens-1 (ZO-1) (Cat. no. 61–7300; 1:100; Thermo Fisher Scientific, Waltham, MA, USA), anti-VE-cadherin (BV13; 1:50; gift from E. Dejana, IFOM Milano), and anti-CD102 (Clone 3C4; Cat. no. 553326; 1:100; BD Biosciences, Franklin Lakes, NJ, USA). The corresponding streptavidin conjugate and secondary antibodies were purchased from Molecular Probes (Invitrogen, Eugene, OR, USA). Retinal flat mounts were then prepared in fluorescent mounting medium (Dako, Agilent Technologies, Glostrup, Denmark) and visualized under a Leica, Wetzlar, Germany TCS SP5 microscope with $\times 10/0.3$ numerical aperture (NA), $\times 20/0.7$ NA, $\times 40/1.25$ NA or $\times 63/1.4$ NA objectives. By the use of ImageJ, images were filtered and converted to binary images. Total vessel area was assessed by counting the number of white versus black pixels in the binary images. Quantifications were performed on six to eight images per retina ($n = 8$ per group).

For extravasation assays, fluorescein isothiocyanate (FITC)–dextran (25 mg/ml, M_r 70 000; Sigma-Aldrich, Saint-Louis, MI, USA) was injected into the tail vein prior to hypoxia. FITC–dextran leakage was examined under a Leica TCS SP5 microscope with a $\times 20/0.7$ NA oil immersion objective.

Laser-induced choroidal neovascularization

The irises of mice aged 2–3 months were dilated with 1% tropicamide (Laboratoires Alcon, Rueil Malmaison, France). An argon laser photocoagulator (532 nm) was used to create four burns situated from the optic nerve at 3, 6, 9 and 12 o'clock with the following parameters: power, 150 mW; spot size, 75 μm ; and duration, 0.1 s. Three weeks after laser treatment, mice were anaesthetized and injected intravenously with 70S FITC–dextran (Sigma-Aldrich, Saint-Louis, MI, USA), and then perfused through the aorta with physiological serum. Blood, dye and buffer exited

through an opening in the right atrium, and choroid flat mounts were generated. Choroidal neovascularization was stained for ICAM2, which is the most effective means of identifying choroidal neovascularization, as described previously [24], and FITC-dextran leakage was examined under a Leica TCS SP5 microscope with a $\times 20/0.7$ NA oil immersion objective.

Modified Miles assay

To assess hypoxia-induced permeability, mice were injected intravitreally with 1 μ l of 14 ng/ μ l recombinant human ANGPTL4 (350 pM, produced as described previously [25]) or vehicle or anti- α v β 3 (LM609, 1 mg/ml). Evans Blue was injected into the tail vein, and mice were placed under normobaric hypoxia (8% O₂, 92% N₂) for 4 h in a hypoxia chamber whose oxygen concentration was kept stable at 8% with a Pro-OX oxygen controller (BioSpherix, Parish, NY, USA). At euthanasia, mice were anaesthetized and then perfused through the aorta with citrate buffer. (pH 4). Blood, dye and buffer exited through an opening in the right atrium. Evans Blue was extracted from retinal lysates for 18 h at 70 °C in 1 ml of formamide. After centrifugation at 14000 g for 12 min at 25 °C, the absorbance at 620 nm was measured using a spectrophotometer (Biomate 3; Thermo Fisher Scientific, Waltham, MA, USA). Extravasated Evans Blue was determined from a standard curve and normalized to tissue weight.

Immunoprecipitation (IP) and immunoblotting analyses

For *in vivo* samples, mice subjected to 4 h of hypoxia were anaesthetized, and injected with 1 mM Na₃VO₄ and 2 mM H₂O₂ into the tail vein. Retinas were then harvested, dissected in 50 mM vanadate/100 mM H₂O₂ and 2 mM CaCl₂, and extracted on ice in 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.5% Triton X-100, 0.5% NP-40, vanadate/H₂O₂ (300 μ M/600 μ M), and a cocktail of protease inhibitors (PIC; Sigma). For each IP, four retinas were pooled in 500 μ l of extraction buffer; 100 μ l was used for protein content quantification (Bradford assay; BioRad, Hercules, CA, USA), and 400 μ l was used for IP. We obtained a mean (\pm standard deviation) protein concentration of 1.16 μ g/ μ l (\pm 0.2 μ g/ μ l) for *Angptl4*^{LacZ/+} samples and 1.19 μ g/ μ l (\pm 0.4 μ g/ μ l) for *Angptl4*^{LacZ/LacZ} samples. Extracts were precleared for 50 min with G-sepharose beads (Invitrogen, Carlsbad, CA, USA), and incubated overnight with G-sepharose beads coupled to 5 μ g of anti-VE-cadherin. After three washes in washing buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM CaCl₂), proteins were eluted by boiling for 10 min in reducing Laemmli sample buffer.

For experiments with human umbilical vein endothelial cells (HUVECs), 40 000 cells/cm² were seeded in complete culture medium (Promocell, Heidelberg, Germany) for 72 h. Cells were then starved for 16 h in 1% bovine serum albumin in basal culture medium. They were treated for 5 min with human recombinant VEGF₁₆₅ (Sigma) or with a mix containing VEGF

and human recombinant ANGPTL4, as described previously [13]. Proteins were extracted on ice in 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% deoxycholate, 0.5% NP-40, 10% glycerol, 1 mM β -glycerophosphate, 1 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, and a cocktail of protease inhibitors (Calbiochem Merck Millipore, Darmstadt, Germany). Lysates were split for IP and for total extract immunoblotting. For IP, extracts were precleared for 60 min with protein G-agarose beads, incubated overnight with anti-VEGFR2 [goat anti-mouse VEGFR2, 2 μ g/IP, clone AF644 (R&D Systems, Minneapolis, MN, USA) and rabbit anti-human VEGFR2, 2 μ g/IP, clone 55B11 Cat. no. 2479 (Cell Signaling Technology, Danvers, MA, USA)], anti-VE-cadherin [rat anti-mouse VE-cadherin, 2 μ g/IP (400 μ l), clone BV-13 (a gift from E. Dejana) and mouse anti-human VE-cadherin, clone BV-9, Cat. no. sc-52751 (Santa Cruz Biotechnology, Dallas, TX, USA)] or anti- α v β 3 (mouse anti- α v β 3, 2 μ g/IP, clone LM609, Cat. no. MAB1976; Millipore, Guyancourt, France) antibodies, and the immunocomplexes were collected on protein G-agarose beads for 3 h. Proteins were eluted by boiling for 10 min in reducing Laemmli buffer. Samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis followed by western blotting with rabbit anti-VEGFR2 (clone 55B11; Cat. no. 2479; 1:1000; Cell Signaling), rabbit anti-phospho-VEGFR2 (Y1175) (clone 19A10; Cat. no. 2478; 1:1000; Cell Signaling), goat anti-VE-cadherin (clone C-19; Cat. no. sc-6458; 1:300; Santa Cruz), rabbit anti-Src kinase family (Cat. no. 2108; 1:1000; Cell Signaling), rabbit anti-phospho-Src family Tyr416 (Cat. no. 2101; 1:1000; Cell Signaling) and rabbit anti- α v (Cat. no. AB1930; 1:1000; Chemicon Millipore) antibodies. Signals were revealed by Attophos chemiluminescence (Promega, Fitchburg, WI, USA), and band intensity was quantified with Quantity One 1-D Analysis software (BioRad).

SPR binding assays

SPR binding assays were performed to study the interactions of full-length immobilized integrin α v β 3 (C1021; Millipore) with ANGPTL4. They were carried out in a Biacore T100 system (GE Healthcare, Lyon, France). Full-length integrin α v β 3 was immobilized onto a CM5 sensor chip via amine coupling, as described previously [26]. An immobilization level ranging between 2600 and 5600 resonance units (RU) was obtained. The running buffer was 10 mM Hepes (pH 7.4) containing 150 mM NaCl, 50 mM octyl- β -D-glucopyranoside, 1 mM MnCl₂, and 1 mM MgCl₂. A control flow cell was prepared by omitting the integrin. Several concentrations of ANGPTL4 (6.25–100 nM) were injected over immobilized integrins at 30 μ l/min and 25 °C. The association and dissociation rate constants (k_a and k_d , respectively) and the equilibrium dissociation constant (K_D) were calculated with the Biacore T100 evaluation software.

Proximity ligation assays

PLAs were performed with the Duolink In Situ red Starter Kit Mouse/Rabbit (Sigma-Aldrich, Uppsala, Sweden), according to the manufacturer's instructions. Briefly, HUVECs were grown on six-channel Ibidi microslides for 5 days under normoxia (37 °C, 5% CO₂, 21% O₂) or hypoxia (37 °C, 5% CO₂, 1%

O₂). Cells were fixed with 4% paraformaldehyde, and permeabilized with Triton X-100 (0.1%). Integrin $\alpha\beta3$ was detected with LM609 (Cat. no. MAB1976; 1:100; Millipore), and ANGPTL4 was detected with rabbit anti-human antibody (Cat. no. 18374-1-AP; 1:100; ProteinTech, Manchester, UK). The kit included the corresponding probes of

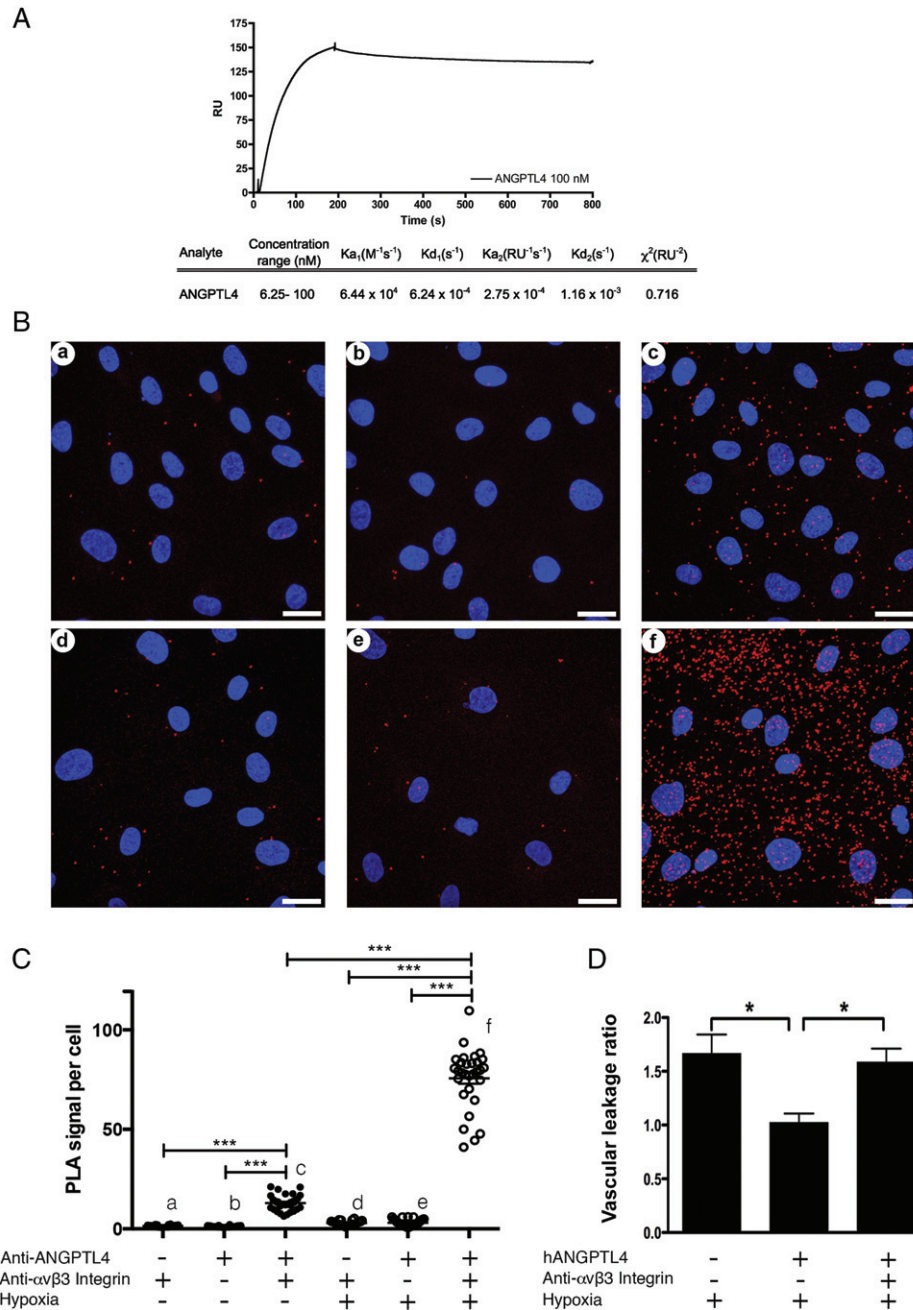


Figure 1. ANGPTL4 inhibits hypoxia-induced vascular leakage by integrin $\alpha\beta3$ binding. (A) Representative sensorgram showing the binding of ANGPTL4 to immobilized full-length integrin $\alpha\beta3$. The experimental data were best fitted to a bivalent analyte model, which allowed the calculation of rate constants (k_{d1} , k_{d2} , k_{a1} , and k_{a2}). (B) PLA performed *in situ* for ANGPTL4 and integrin $\alpha\beta3$ in HUVECs cultured under hypoxia or normoxia for 5 days. Scale bar: 20 μ m. Conditions a, b, c, d, e and f are shown in (C). (C) Quantification of PLA experiments; $n = 3$ independent experiments. (D) Hypoxia-induced vascular leakage after intravitreal injection of vehicle or 3.5×10^{-7} M human ANGPTL4 (hANGPTL4) in *Angptl4^{lacZ/lacZ}* and *Angptl4^{lacZ/+}* control mice ($n = 5$ per group). * $p < 0.05$ for *Angptl4^{lacZ/lacZ}* versus *Angptl4^{lacZ/+}* control mice; § $p < 0.05$ for hANGPTL4-injected versus saline-injected mice. Intravitreal injection of hANGPTL4 inhibits hypoxia-induced Evans Blue leakage in control *Angptl4^{+/+}* mice, whereas co-delivery of hANGPTL4 with anti- $\alpha\beta3$ blocking antibody (LM609) restores acute hypoxia-induced vascular permeability. Injection of anti- $\alpha\beta3$ blocking antibody (LM609) alone did not have a significant effect on hypoxia-induced vascular leakage. * $p < 0.05$; $n = 4$ per group.

anti-mouse MINUS and anti-rabbit PLUS. After PLA, 4',6-diamidino-2-phenylindole-containing mounting medium was added to each six-channel Ibidi microslide, and images were taken with a Leica confocal SP-5 inverted microscope with a $\times 63/1.4$ NA objective.

Statistical analysis

Mann–Whitney tests were used to assess the statistical differences between measurements in all experiments except PLAs, where one-way ANOVA with Tukey's multiple comparison tests was performed with using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA).

Results

ANGPTL4 inhibits vascular leakage by binding to integrin α v β 3

Previous studies have revealed binding of ANGPTL4 FLD to integrin α 5 β 1 or integrin α v β 5 in keratinocytes, epithelial cells, and endothelial cells [10,15,16]. As integrin α v β 3 present on the surfaces of endothelial cells is a critical modulator of angiogenesis and vascular permeability [17,20,27], we sought to determine whether ANGPTL4 might interact with integrin α v β 3 by using PLAs in endothelial cells and SPR [26], as well as functional and biochemical analyses.

We demonstrated first that human ANGPTL4 bound to immobilized human integrin α v β 3, and formed a very stable complex with a low dissociation rate (Figure 1A). Kinetic analysis was carried out following injection of different concentrations of ANGPTL4 over immobilized integrin α v β 3. The experimental data were best fitted to a bivalent analyte model, corresponding to the presence of two binding sites for the integrin on ANGPTL4. This model is consistent with data reported by us and others indicating that soluble ANGPTL4 forms oligomers through intermolecular disulphide bonds [28,29], and suggests that there is one integrin-binding site in each ANGPTL4 monomer. The affinity constant of the first binding site ($K_{D1} = k_{d1}/k_{a1}$) of integrin was lower (9.68 nM) than the K_{D1} of heparan sulphate that we previously reported (124 nM) [29], showing that ANGPTL4 bound to this site with a higher affinity than to heparan sulphate.

We next investigated whether ANGPTL4 and integrin α v β 3 might also interact at the endothelial cell surface by using an *in situ* PLA [30] in HUVECs cultured under hypoxia to induce ANGPTL4 expression, and by using specific antibodies against ANGPTL4 and integrin α v β 3 as proximity probes and secondary proximity PLA probes. Interactions were then imaged with fluorescence microscopy. This allowed the detection of ANGPTL4- α v β 3 complexes on the surfaces of HUVECs (Figure 1B). As a control, individual proximity probes failed to give rise to detection signals (Figure 1C). We next sought to assess the functional consequences

of ANGPTL4- α v β 3 interaction and its requirement for ANGPTL4 inhibition of hypoxia-induced vascular permeability *in vivo*. Control mice were injected with ANGPTL4 alone or in combination with anti- α v β 3 (LM609) blocking antibody (Figure 1D), and this was followed by hypoxic challenge. Quantification of Evans Blue extravasation showed that the protective effect of ANGPTL4 on vascular permeability was abolished upon co-injection with a blocking anti- α v β 3 antibody. These results therefore show that integrin α v β 3 is required for ANGPTL4-induced inhibition of hypoxia-induced vascular permeability.

ANGPTL4 modulates Src recruitment and phosphorylation at integrin α v β 3 and VEGFR2 complexes in hypoxic retinas *in vivo*

We next sought to investigate the signalling pathway downstream of ANGPTL4- α v β 3 interaction, and therefore performed IP analyses of integrin α v β 3 complexes in mouse retinas. Because of the well-documented role of Src in regulating vascular permeability [31,32], we analysed Src signalling downstream of integrin α v β 3 complexes in mouse retinas, as in [33]. Immunoprecipitates obtained with anti- α v β 3 antibody were analysed for Src recruitment by immunoblotting for total Src and for Src activation with a Tyr416 phospho-Src antibody in both *Angptl4^{LacZ/LacZ}* mice and *Angptl4^{LacZ/+}* control mice. Src and phospho-Src were abundant downstream of integrin α v β 3 in the retinas of control mice upon hypoxia, whereas the levels of both were diminished (and thus Src activation was decreased) in *Angptl4^{LacZ/LacZ}* mice as compared with controls (Figure 2A, B, left panel). Importantly, intraocular administration of ANGPTL4 prior to the hypoxia challenge rescued Src activation downstream of integrin α v β 3 complexes in *Angptl4^{LacZ/LacZ}* retinas (Fig. 2C, D, left panel).

We also investigated whether ANGPTL4 might modulate the VEGFR2–Src signalling pathway, which constitutes a major cascade implicated in induction of vascular permeability upon hypoxia [32]. IP of VEGFR2 showed an increase in Src signalling in *Angptl4^{LacZ/LacZ}* mice (Figure 2A, B, central panel), which was suppressed upon human ANGPTL4 injection (Figure 2C, D, central panel). Conversely, a decrease in Src activation was seen downstream of VE-cadherin in *Angptl4^{LacZ/LacZ}* mice (Figure 2A, B, right panel, bottom); this was also suppressed by injecting ANGPTL4 (Fig. 2C, D, right panel, bottom). In *Angptl4^{LacZ/LacZ}* mice, increased Src signalling downstream of VEGFR2 at the expense of Src signalling downstream of VE-cadherin complexes could result from either modulation of Src recruitment or modulation of Src phosphorylation. To distinguish between these possibilities, we analysed Src recruitment downstream of VEGFR2 and VE-cadherin (Figure 2A). Our results showed that Src recruitment was increased downstream of both complexes in *Angptl4^{LacZ/LacZ}* mice as compared with control mice. Mechanistically, we therefore showed

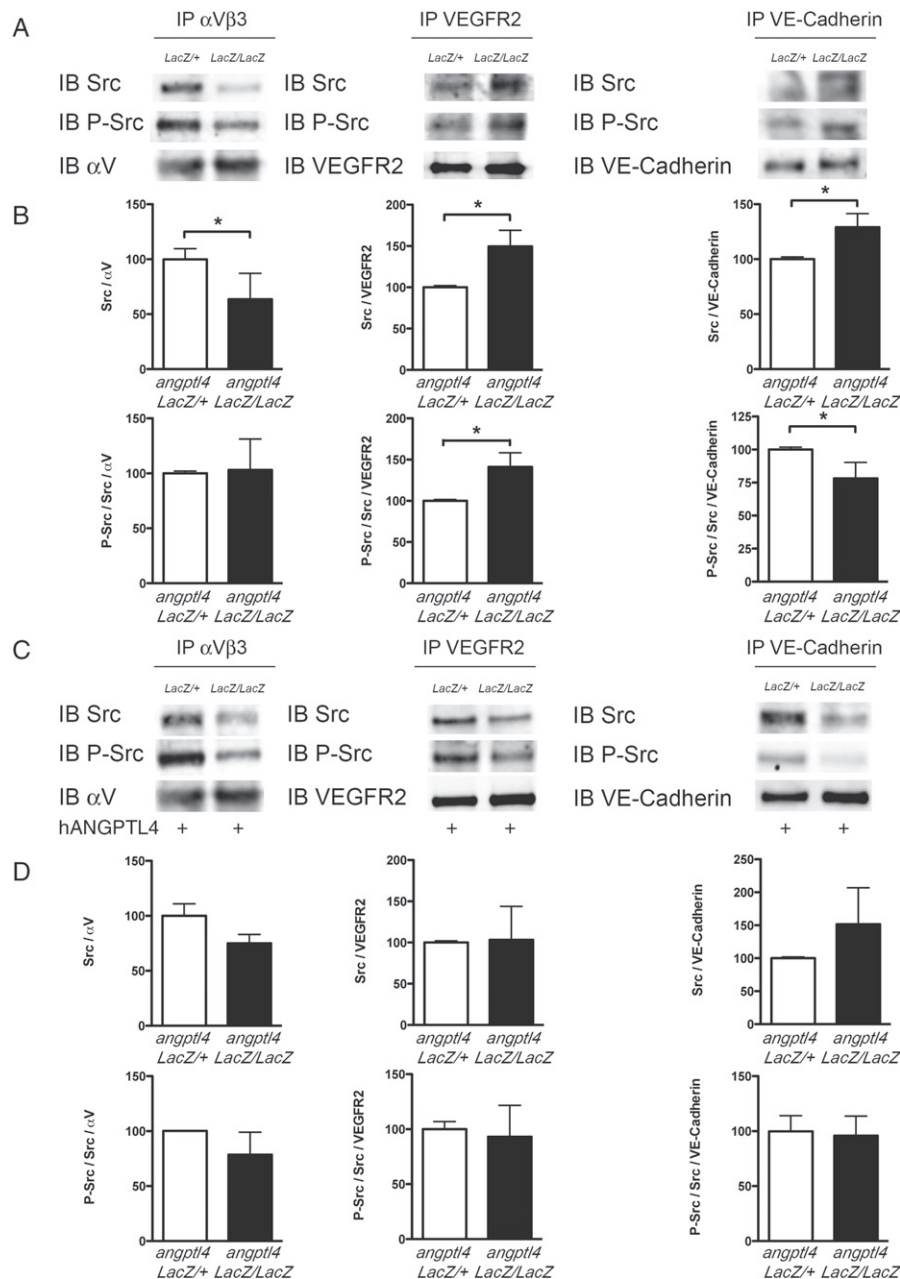


Figure 2. ANGPTL4 modulates Src recruitment and activation at $\alpha V\beta 3$ -VEGFR2 complexes in hypoxia. (A) Immunodetection of Src, phospho-Src (P-Src) and integrin αV (left), VEGFR2 (middle) or VE-cadherin (right) in anti- $\alpha V\beta 3$ (left), anti-VEGFR2 (middle) and anti-VE-cadherin (right) immunoprecipitates from *Angptl4*^{LacZ/+} and *Angptl4*^{LacZ/LacZ} retinas subjected to hypoxia. (B) Bar graphs represent Src normalized to the immunoprecipitated protein (integrin αV , VEGFR2, or VE-cadherin) (upper row) and the ratio of P-Src/Src (normalized to the immunoprecipitated protein) (bottom row). Src recruitment was impaired in integrin $\alpha V\beta 3$ complexes and increased in both VEGFR2 and VE-cadherin complexes in *Angptl4*^{LacZ/LacZ} mice. Src activation is increased in *Angptl4*^{LacZ/LacZ} retinas downstream of VEGFR2 and decreased in VE-cadherin complexes. * $p < 0.05$, $n = 6$ per group. (C) Immunodetection of Src, P-Src and integrin αV (left), VEGFR2 (middle) or VE-cadherin (right) in anti- $\alpha V\beta 3$ (left), anti-VEGFR2 (middle) and anti-VE-cadherin (right) immunoprecipitates from *Angptl4*^{LacZ/+} and *Angptl4*^{LacZ/LacZ} retinas injected with ANGPTL4 (350 pm) and subjected to hypoxia. (D) Bar graphs represent Src normalized to the immunoprecipitated protein (integrin αV , VEGFR2, or VE-cadherin) (upper row) and the P-Src/Src ratio (normalized to the immunoprecipitated protein) (bottom row). Intravitreal delivery of human ANGPTL4 (hANGPTL4) (350 pm) rescued the *Angptl4*^{LacZ/LacZ} phenotype. IB, immunoblotting.

that ANGPTL4 has two main roles under hypoxia: (1) it recruits Src kinase downstream of integrin $\alpha V\beta 3$; and (2) it inhibits Src signalling downstream of VEGFR2. Because integrin $\alpha V\beta 3$ has been reported to localize at focal adhesions, we next sought to investigate whether integrin $\alpha V\beta 3$ complexes could also be found at the same subcellular location as VEGFR2 complexes. Our results showed that integrin $\alpha V\beta 3$ co-immunoprecipitated with

VEGFR2 complexes *in vivo* (supplementary material, Figure S1), thus opening the possibility of a direct role of the interaction of ANGPTL4 and integrin $\alpha V\beta 3$ complexes in VEGFR2 signalling. However, these studies do not allow complete discrimination between a direct or an indirect role of integrin $\alpha V\beta 3$ downstream signalling on VEGFR2 complexes.

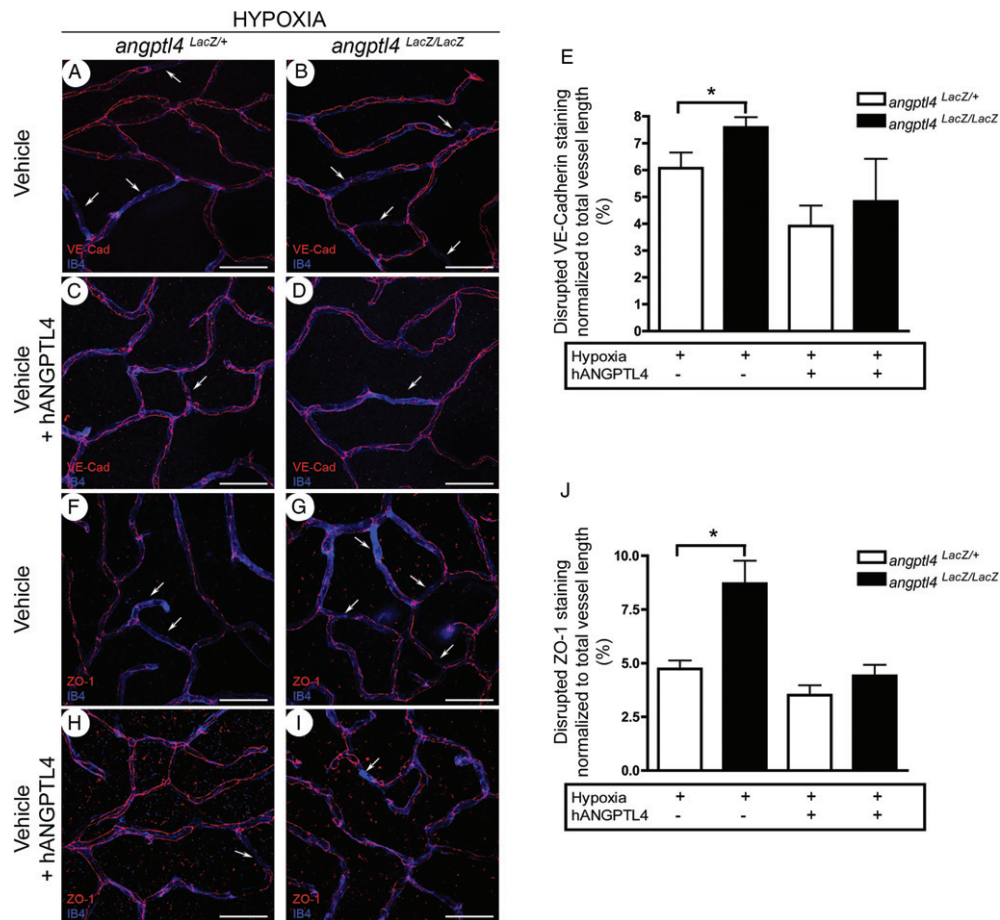


Figure 3. ANGPTL4 regulates endothelial junction organization in hypoxic retinas *in vivo*. (A–D, F–I) Flat wholemount adult retinas subjected to hypoxia were stained with isolectin B4 (blue) and with anti-VE-cadherin (red, A–D) and anti-ZO-1 (red, F–I) antibodies. White arrows show disrupted VE-cadherin or ZO-1 staining. (E, J) Bar graphs represent the ratio between the length of disrupted junction staining (VE-cadherin and ZO-1) and the total vessel length. (C–E, H–J). Under hypoxia, *Angptl4^{LacZ/LacZ}* mice show increased disruption of VE-cadherin and ZO-1 staining, which was restored by intravitreal delivery of human ANGPTL4 (hANGPTL4), * $p < 0.05$ for *Angptl4^{LacZ/LacZ}* versus *Angptl4^{LacZ/+}* control mice; $n = 3$ per group. Scale bar: 25 μ m.

ANGPTL4 regulates endothelial junction organization in hypoxic retinas *in vivo*

Tight endothelial adherens junction organization is a critical feature of the control of vascular integrity, and loss of junction organization triggers increased permeability [34]. VE-cadherin is the major component of endothelial adherens junctions, and plays a key role in regulating tight junctions [35]. We therefore quantified junction organization and disruption by using VE-cadherin and ZO-1 immunostaining (Fig. 3A–D, F–I), with methods published previously [12]. As expected, disrupted junctions were observed in hypoxia-exposed mice. Enhanced loss of vascular integrity in *Angptl4^{LacZ/LacZ}* mice was shown by quantifying the increase in disrupted adherens junctions (VE-cadherin) and tight junctions (ZO-1) (Fig. 3E, J). Intravitreal delivery of ANGPTL4 prevented the disorganization of both types of endothelial cell junction in both groups ($p < 0.05$; Figure 3C–E, H–J). These results show that ANGPTL4 protects endothelial adherens and tight junctions from hypoxia-induced disruption.

ANGPTL4 counteracts VEGF signalling and dissociation of VEGFR2 complexes with integrin α v β 3 and VE-cadherin

VEGF plays a key role in inducing vascular permeability, and VEGF-induced Src activity results in both loss of vascular integrity and increased vascular leakage [36]. Moreover, Src kinase also governs the association of VEGFR2 and integrin α v β 3 [37]. We therefore analysed whether the effect of ANGPTL4 on VEGF-induced Src signalling might also counteract the dissociation of VE-cadherin and integrin α v β 3 complexes with VEGFR2 *in vitro* in primary human endothelial cells (HUVECs). IP of VEGFR2 (Figure 4A) showed that ANGPTL4 prevented VEGF-induced Src activation at VEGFR2 complexes (phospho-Src/Src ratio, normalized to immunoprecipitated VEGFR2; Figure 4B, right panel) and VEGF-induced dissociation of: (1) VE-cadherin–VEGFR2; and (2) α v–VEGFR2 (Figure 4B, left and middle panels, respectively).

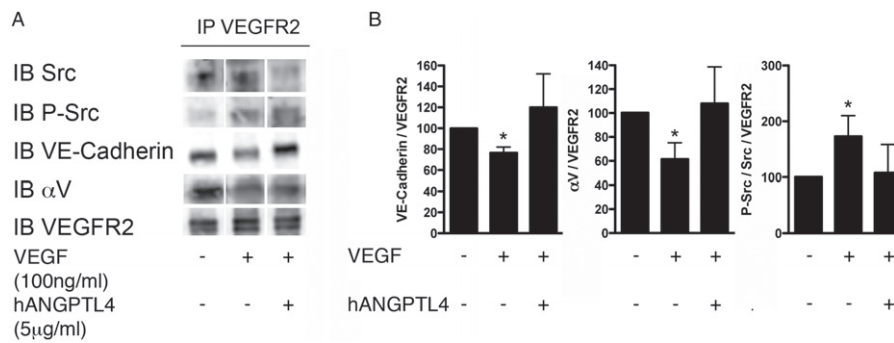


Figure 4. ANGPTL4 blocks VEGF signalling and dissociation of VE-cadherin-VEGFR2 and αv -VEGFR2 complexes. (A) Immunodetection of Src, phospho-Src (P-Src), VE-cadherin, integrin αv and VEGFR2 in anti-VEGFR2 immunoprecipitates from confluent HUVECs stimulated for 5 min with VEGF (100 ng/ml), ANGPTL4 (5 μ g/ml), or both. (B) The bar graph represents ratios of VE-cadherin and VEGFR2 (left), of integrin αv and VEGFR2 (middle) and of P-Src and Src normalized to VEGFR2 (right), and correspond to three independent experiments. Stimulation with VEGF alone induces dissociation of VE-cadherin-VEGFR2 and αv -VEGFR2 complexes, and human ANGPTL4 (hANGPTL4) restores basal levels of IP of VE-cadherin and VEGFR2. IB, immunoblotting.

Vascular leakage is increased in *Angptl4*^{LacZ/LacZ} mice during choroidal neovascularization

We next sought to investigate whether ANGPTL4-driven vascular protection was relevant during laser-induced choroidal neovascularization, a mouse model for AMD. Angiogenesis and vascular leakage were quantified in *Angptl4*^{LacZ/LacZ} mice and *Angptl4*^{LacZ/+} control mice after laser injury and choroidal flat mounts. We found that *Angptl4*^{LacZ/LacZ} mice showed impaired neovascularization at 14 days (Figure 5A–C) and 21 days (Figure 5D–F). FITC-dextran was found within perfused blood vessels (where it co-localized with endothelial ICAM2 immunostaining) but also extravasated, i.e. outside blood vessels (Fig. 5G, H). To distinguish extravasated from intravascular FITC-dextran, we quantified the total FITC-dextran-positive surface and normalized it to the ICAM2-positive surface. This analysis of vascular leakage demonstrated increased extravasation of FITC-dextran in *Angptl4*^{LacZ/LacZ} mice as compared with controls (+52%; Figure 5I).

Discussion

Here, we provide mechanistic insights into the control of hypoxia-induced VEGF-mediated vascular permeability, because we have demonstrated that the ANGPTL4- $\alpha v\beta 3$ axis: (1) recruits Src downstream of integrin $\alpha v\beta 3$; (2) attenuates pro-permeability phospho-Src signalling downstream of VEGFR2; and (3) protects inter-endothelial cell junction integrity, thereby protecting the vascular barrier against hypoxia-induced vascular leakage.

ANGPTL4 has been implicated in vascular permeability; however, its precise role remains debatable in experimental systems. Previous results identified ANGPTL4 as an inhibitor of endothelial permeability [6,8,13,14], or as paradoxically stimulating permeability [9,10]. This might depend on its

interaction with its various binding partners. In keratinocytes, ANGPTL4-bound integrin $\beta 1$ activated the FAK-Src-PAK1 signalling pathway, which is important for cell migration [16]. In addition, ANGPTL4 produced by wound keratinocytes also interacts directly with vitronectin and fibronectin in the wound bed. Endothelial cell-specific deletion of genes encoding either integrin $\alpha 5$ or integrin αv do not result in developmental vascular defects [38,39], whereas the absence of both leads to deficient fibronectin assembly and embryonic lethality associated with defective vascular remodelling [39]. It has been proposed that compensation between these integrins, and perhaps others, could account for these observations [39]. Because phenotypes of *Angptl4*-deficient mice do not overlap with those of integrin αv -deficient and integrin $\beta 3$ -deficient mice, we assume that the interaction between ANGPTL4 and these integrins is not unique.

Src signalling is complex, and is active at different subcellular compartments, such as focal adhesions and endothelial adherens junctions, which can result in different effects on cell-matrix and cell-cell adhesion, respectively. For example, Src activation downstream of integrin $\alpha v\beta 3$ complexes in carcinoma cells promotes anchorage-independent tumour growth *in vitro* [40,41]. On the other hand, Src associates directly with VE-cadherin, and Src blockade stabilizes VEGFR2-VE-cadherin and limits loss of vascular integrity during injury [36,42]. In the retina, integrin $\alpha v\beta 3$ can be found co-immunoprecipitating with VEGFR2 at adherens junctions (supplementary material, Figure S1). This is in accordance with other studies demonstrating that integrin $\beta 3$ co-localizes with JAM-1 in adherens junctions of HUVECS [43]. Thus, further studies will be required to investigate whether Src activation downstream of integrin $\alpha v\beta 3$ binding to ANGPTL4 occurs at the adherens junction compartment and has a direct effect on VEGFR2 and VE-cadherin signalling. An alternative hypothesis would be that the ANGPTL4- $\alpha v\beta 3$ integrin axis recruits Src signalling at

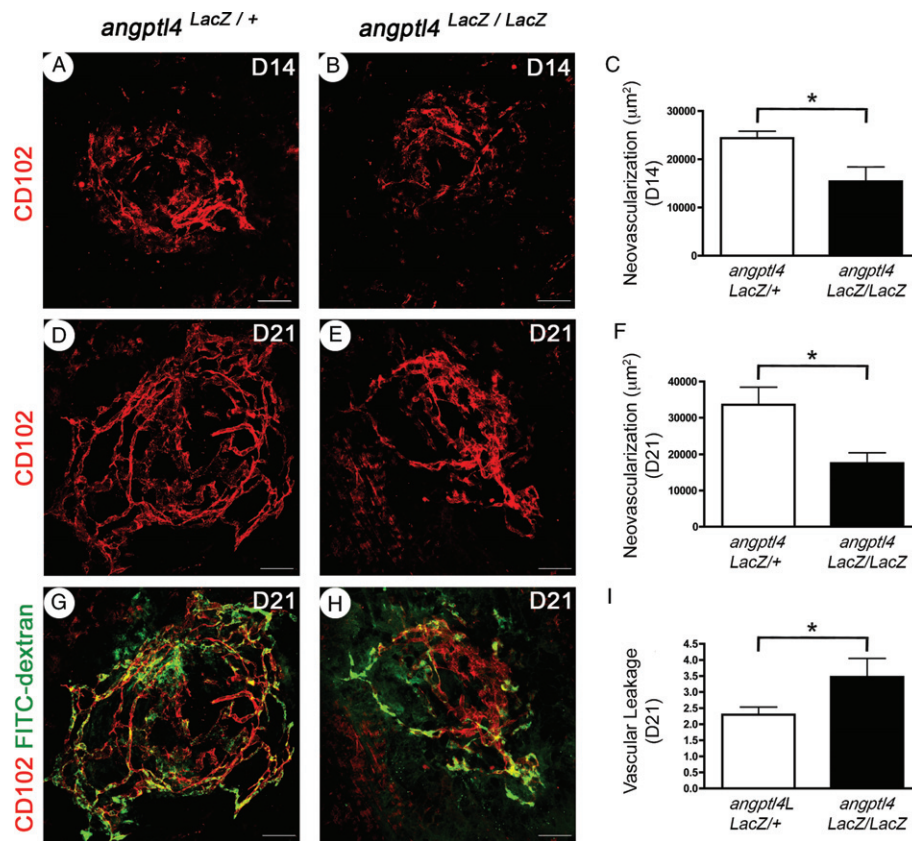


Figure 5. Vascular permeability is increased in *Angptl4*^{LacZ/LacZ} mice during choroidal neovascularization. (A, B, D, E, G, H) Confocal images of whole-mount *Angptl4*^{LacZ/+} and *Angptl4*^{LacZ/LacZ} choroids at 14 days [D14, (A, B)] and 21 days [D21 (D, E, G, H)] after laser insult stained with CD102 (red). (G, H) Prior to euthanasia, FITC-dextran 70S (green) was injected intravenously. Scale bar: 50 μm . (C, F) Bar graphs represent mean neovascularization area (CD102+ area, μm^2) \pm standard error of the mean (SEM). (I) Bar graphs represent mean vascular leakage, calculated as the ratio between FITC-dextran-positive and ICAM2-positive surfaces \pm SEM. * $p < 0.05$; $n = 4$ mice per group.

focal adhesions and has an indirect effect on junctional signalling.

In addition, we reported previously that ANGPTL4 also binds to heparan sulphate [29], and Stenzel *et al.* demonstrated that fibronectin modulates VEGFR2 and phosphoinositide 3-kinase-AKT signalling in an integrin-dependent and integrin-independent manner in retinal angiogenesis [44]. It might therefore be postulated that both the abundance of matrix molecules and the expression of integrins on its target cells might control the bioavailability and activity of ANGPTL4, as previously shown for VEGF [44–46]. This phenomenon might thereby be responsible for these tissue-specific opposing responses, i.e. context-dependent effects of various forms of ANGPTL4. Indeed, ANGPTL4 is a 55-kDa glycoprotein that is processed into an N-terminal 20-kDa form and a C-terminal 35-kDa form. The full-length protein and the N-terminal 20-kDa form interact with the extracellular matrix, whereas the C-terminal form is soluble. Huang *et al.* previously reported that the C-terminal 35-kDa form associates with VE-cadherin and claudin-5, resulting in their internalization, translocation of β -catenin to the nucleus, and a leaky endothelium [10]. It should be noted that no specific protein convertase has been identified in this cleavage, and that the mechanisms controlling it *in vivo*

are not yet elucidated. Moreover, whereas blockade of ANGPTL4 cleavage reduces its inhibitory effects on lipoprotein lipase activity and decreases its ability to raise plasma triglyceride levels [47], the biological importance of this cleavage event is not fully understood with respect to its effect on vascular integrity. In our study, full-length ANGPTL4 protected against hypoxia-driven vascular disorganization. Therefore, the context-dependent effect of ANGPTL4 on vascular integrity might depend on which form, matrix-bound or soluble, is present in the environment, such as the human eye, as recently reported in [7,48], and should therefore be taken into account for developing therapeutic strategies using ANGPTL4, aimed at controlling vascular permeability in ischaemic and/or ocular diseases.

In summary, the data reported here identify a novel mechanism by which ANGPTL4 counteracts hypoxia-driven vascular permeability through integrin α v β 3 binding, modulation of VEGFR2-Src kinase signalling, and endothelial junction stabilization, thereby showing that recombinant ANGPTL4 may be a relevant candidate for protecting endothelial cell barrier integrity and inhibiting vascular leakage in pathological conditions.

Author contributions statement

The authors contributed in the following way: EGP, ALF, MD, AG, CM, SG: designed and performed research, analysed and interpreted data, performed statistical analysis, and wrote the manuscript; MP, MS, SA, BMR: provided expert assistance; SRB, CF: designed and performed SPR experiments, and interpreted SPR data; DMV, AJM, GPY, GT: provided relevant material.

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SUPPLEMENTARY INFORMATION ONLINE

Figure S1. Integrin α v β 3 localizes in VEGFR2 complexes.

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