VILNIUS UNIVERSITY

Rūta Motiejūnaitė

ROLE OF PERICYTES AND AUTOTAXIN/ LYSOPHOSPHATIDIC ACID SIGNALING IN VASCULAR REGRESSION

Summary of doctoral dissertation

Physical Sciences, Biochemistry (04 P)

Vilnius, 2012

Research for this doctoral thesis was conducted at The Schepens Eye Research Institute (Boston, USA) and the Department of Biochemistry and Biophysics of Vilnius University (Vilnius, Lithuania) in 2007-2012.

Scientific advisor:
Prof. Dr. Andrius Kazlauskas (The Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School; Physical Sciences, Biochemistry – 04 P)

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Address: M.K. Čiurlionio 21/27 – 214, LT-03101, Vilnius, Lithuania.

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Dr. Kęstutis Sužiedėlis (Vilnius University Institute of Oncology, Physical Sciences, Biochemistry – 04 P)

Opponents:

Prof. Dr. Vilmantė Borutaitė (Lithuanian University of Health Sciences, Biomedical Sciences, Biology – 01 B)

Dr. Mindaugas Valius (Vilnius University Institute of Biochemistry, Physical Sciences, Biochemistry – 04 P)

Summary of the doctoral thesis has been distributed on the _____ of August, 2012.

Thesis is deposited at the library of Vilnius University.

VILNIAUS UNIVERSITETAS

Rūta Motiejūnaitė

PERICITŲ IR AUTOTAKSINO / LIZOFOSFATIDO RŪGŠTIES SIGNALINIO KELIO REIKŠMĖS KRAUJAGYSLIŲ REGRESIJAI TYRIMAS

Daktaro disertacijos santrauka

Fiziniai mokslai, biochemija (04 P)

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Mokslinis vadovas:
Prof. dr. Andrius Kazlauskas (The Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School; fiziniai mokslai, biochemija – 04 P)

Disertacija ginama Vilniaus universiteto Biochemijos mokslo krypties taryboje:

Pirmininkas:

Prof. dr. Vida Kirvelienė (Vilniaus universitetas, fiziniai mokslai, biochemija – 04 P)

Nariai:

Dr. Lida Bagdonienė (Vilniaus universitetas, fiziniai mokslai, biochemija – 04 P)

Prof. habil. dr. Dalia Pangonytė (Lietuvos sveikatos mokslų universitetas, biomedicinos mokslai, medicina – 06 B)

Dr. Augustas Pivoriūnas (Valstybinio mokslinių tyrimų instituto Inovatyvios medicinos centras, biomedicinos mokslai, biologija - 01B)

Dr. Kęstutis Sužiedėlis (Vilniaus universiteto Onkologijos institutas, fiziniai mokslai, biochemija – 04 P)

Oponentai:

Prof. dr. Vilmantė Borutaitė (Lietuvos sveikatos mokslų universitetas, biomedicinos mokslai, biologija – 01 B)

Dr. Mindaugas Valius (Vilniaus universiteto Biochemijos institutas, fiziniai mokslai, biochemija – 04 P)

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INTRODUCTION

Neovascularization – growth of new blood vessels - is key to the pathogenesis of solid tumors and blinding eye diseases such as retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR) and neovascular age-related macular degeneration (AMD) (reviewed in [1 - 6]). However, its molecular mechanisms are not fully understood. The discovery that vascular endothelial growth factor A (VEGF-A) plays a major role in the growth of pathological vessels (reviewed in [7 - 10]) led to anti-VEGF agents becoming the first line of treatment for neovascular AMD and their consideration for management of ROP and PDR [9, 10, 11]. Anti-VEGF agents have also been approved for treatment of certain tumors [3, 4]. The success of anti-VEGF agents illustrates how studies of molecular mechanisms of blood vessel development can be translated into innovative therapeutic approaches.

The fact that many neovascular AMD patients do not respond to anti-VEGF treatment or respond only partially [12, 13] highlights our incomplete understanding of the pathogenesis of neovascular eye diseases. Likewise, many tumors are resistant to anti-VEGF therapy, or develop such resistance over time (reviewed in [3, 4]). Given that angiogenic homeostasis is a balance between pro- and anti-angiogenic factors [14, 15], it seems likely that perturbation of vascular regression pathways also contributes to neovascular diseases. However, relatively little attention has been devoted to this intriguing possibility.

Besides preventing the growth and/ or inducing regression of pathological blood vessels in neovascular diseases, an additional clinical challenge is inducing the growth of stable, functional blood vessels in ischemic diseases, where blood supply to tissues is reduced. While growth of new blood vessels can be induced by application of VEGF-A, such vessels are leaky and regress rapidly [16]. Successful revascularization of ischemic tissues will likely require additional agents that would stabilize the new vessels [16, 17, 18]. However, how new vessels become stabilized

is not well understood. The main goal of this study was to learn more about the molecular mechanisms of blood vessel stability and regression. A better understanding of these mechanisms might lead to new therapeutic options for elimination of pathological vessels in neovascular diseases, as well as for revascularization of ischemic tissues.

Newly formed blood vessels become stabilized by their association with pericytes. Pericytes are cells of mesenchymal origin that are found in the walls of smaller blood vessels, typically covering the abluminal surface of the vascular tube. Pericyte covered vessels are less prone to grow or regress, more resistant to changes in growth factor concentration, less leaky and structurally more uniform [19-27]. How pericytes stabilize blood vessels is not well understood. Our goal in the **first part of this study** was to elucidate the molecular mechanism of vessel stabilization by pericytes. Specifically, we aimed to:

1. Develop a simple in vitro model of vessel stabilization by pericytes;

2. Elucidate the molecular mechanism of vessel stabilization by pericytes in this model.

In the **second part of the study**, we focused our attention on molecular mechanisms of vascular regression. We have previously characterized autotaxin (ATX) – a secreted lysophospholipase D – as a vascular destabilization/ regression factor in an in vitro model of vascular regression [28]. We have also shown that regression of hyaloid vessels in the developing eye is accelerated in mice that overexpress ATX [28]. Thus, ATX was sufficient for vascular regression in vivo. However, we did not know if ATX was required for this process. In order to better characterize the role of ATX in regression of blood vessels in vivo, we established a goal to examine the effects of inactivating *Enpp2* (the gene that codes for ATX) on vascular regression in the mouse. Specifically, we aimed to:

3. Knock-out *Enpp2* in endothelial cells in the mouse (endothelial cells are the primary cell type in the vessel wall);

4. Examine the effect of the loss of endothelial ATX expression on regression of hyaloid vessels.

Scientific novelty

Although the fact that pericytes stabilize blood vessels is well established, the molecular mechanisms of this phenomenon are not well understood. In this study, we describe a previously unknown mechanism of vessel stabilization by pericytes. We show that association of vessel-like endothelial cell tubes with pericytes in an in vitro model of vessel formation and regression accelerates metabolism of lysophosphatidic acid (LPA), a vascular regression/ destabilization factor. Reduction in the concentration of LPA in the microenvironment of vascular tubes results in their stability (i.e., lack of regression). We don't yet know how exactly pericyte-associated vascular tubes metabolize LPA, but we show that the concentration of LPA in our model is at least in part regulated by phospholipid phosphatase (LPP)-like enzymes. Our laboratory was the first to show that LPA and autotaxin – an enzyme that catalyzes LPA production – are vascular regression/ destabilization factors, and this study expands our knowledge of molecular mechanisms of vascular stability by showing that at least in vitro pericytes are able to neutralize these factors.

Additionally, this study provides new information on how vascular cells metabolize LPA. Although it has been hypothesized that endothelial cells degrade extracellular (e.g., serum) LPA [29], metabolism of extracellular LPA by endothelial cells has not actually been previously demonstrated.

As part of this study, we have also generated and characterized mice lacking ATX expression in their endothelial cells. We show that these mice are healthy and fertile, and that their blood vessels form and regress normally. Our hypothesis that ATX produced by endothelial cells is required for physiological regression proved to be incorrect. However, these mice can be used to probe the role of endothelial ATX in other vascular functions and processes, such as permeability, thrombosis and lymphocyte homing.

Doctoral thesis contents

The doctoral thesis (in Lithuanian) contains the following parts: Abbreviations, Introduction, Scientific Novelty, Literature Review, Materials and Methods, Results and Discussion, Conclusions, References (309 citations), List of Publications within the area of the thesis (5 publications, 1 conference presentation), Acknowledgements, Figures (34), Tables (4). Total 144 pages.

Materials and Methods

Materials

Reagents were purchased from Sigma-Aldrich, unless indicated otherwise.

Lipids: The form of LPA used in the study was 1-oleoyl-*sn*-glycero-3-phosphate (1-(9Z-octadecenoyl)-*sn*-glycero-3-phosphate). LPC was 1-oleoyl-*sn*-glycero-3-phosphocholine. PA was 1,2-diacyl-*sn*-glycero-3-phosphate prepared from egg yolk lecithin. MAG was 1-oleoyl-*rac*-glycerol.

Inhibitors: LPA receptor inhibitor BrP-LPA was purchased from Echelon Bioscience. GM6001 and the MMP2/9 inhibitor were purchased from Calbiochem.

Antibodies: ATX antibodies for Western blotting were a generous gift from dr. Timothy Clair (National Institutes of Health). ATX antibodies for immunohistochemistry were kindly donated by dr. Junken Aoki (Tohoku University). RasGAP and PDGFRβ antibodies were generated in the laboratory. LPP1 antibodies were purchased from Sigma-Aldrich, LPP2 – from Abgent, LPP3 – from Novus Biologicals, collagen IV – from Abcam, NG2 – from Millipore. β-actin antibodies, HRP-conjugated secondary antibodies and non-immune IgG (isotype controls) were bought from Santa Cruz Biotechnology. Fluorescently labeled secondary antibodies were from Jackson Immunoresearch.

Proteins: VEGF used in the study was recombinant human VEGF-A (164 isoform), generously provided by the BRB Preclinical Repository at the National Cancer Institute. Recombinant ATX protein was a gift from dr. Timothy Clair (National Institutes of Health).

Tissue culture

BREC were isolated and cultured as previously described [28]. BRP were a generous gift from Dr. George Davis (University of Missouri – Columbia). They were used at 5-7 passages and cultured on gelatin-coated plates in low-glucose DMEM (Gibco) with 15% FBS (Lonza) and 80 U/mL penicillin – streptomycin solution. C3H 10T1/2 cells were purchased from ATCC. They were used at 15-20 passages and cultured in high-glucose DMEM with 10% FBS and 80 U/mL penicillin – streptomycin solution. HUVEC were purchased from Lonza and cultured in EGM-2 medium (Lonza). They were used between passages 5 and 7. HBVP were bought from ScienCell Research Laboratories. They were used at 5-7 passages and cultured on poly-L-lysine-coated plates in PM medium (ScienCell Research Laboratories). HEK293 cells were cultured in high-glucose DMEM with 10% FBS and 80 U/mL penicillin – streptomycin solution.

Mice

All animal studies were approved by the Institutional Animal Care and Use Committee of the Schepens Eye Research Institute. *Enpp2*$^{F/+}$ mice [30] were a generous gift from dr. Wouter H. Moolenaar (The Netherlands Cancer Institute). Tie2-Cre mice [31] were kindly donated by dr. Yuko Fujiwara (Boston Childrens Hospital).

Tube formation and regression assay

Collagen gel was prepared out of bovine collagen solution (final concentration 2.48 mg/mL, Advanced BioMatrix), 0.02 N NaOH, 20 mM HEPES (Lonza), 2 mg/mL $NaHCO_3$, 0.5

μg/mL fibronectin, 0.5 μg/mL laminin and 10.5 mg/mL RPMI powder (Gibco). The gel was allowed to polymerize for 1 hr at +37°C. Endothelial cells were plated on top of the gel (60000 BREC or 90000 HUVEC for 24-well plates). In the case of co-culture tubes, C3H 10T1/2 cells or pericytes were plated on top of attached endothelial cells 16 hrs later, and the number of endothelial cels was reduced so that the total number of cells plated would be the same under monoculture and co-culture conditions. After 8 more hours, cells were overlayed with a second layer of the collagen gel solution. After polymerization, endothelial cell culture medium with VEGF-A (2.5 ng/mL) was added to induce tube formation. Tube morphogenesis was observed with an inverted phase microscope. Tube formation was assessed at 16 hrs, tube regression – at 24 hrs after addition of VEGF or later. Medium was replaced and fresh VEGF was added every day.
Tube regression was quantified as follows: images of the central area of each well were taken at each time-point. Tube length was measured with ImageJ software (National Institutes of Health). Lengths of all tubes in the image were added up. Tube length at all time-points was normalized to tube length in the same field at 16 hrs. Each experimental condition was assayed in triplicate or quadruplicate. Experiments were repeated at least 3 times.

Measurement of LPA concentration
Prior to measurement of LPA concentration in collagen gels, the gels were digested with collagenase I. The concentration of LPA in medium and collagen gels was measured using a radioenzymatic method [32]. Briefly, lipids were extracted from samples with water-saturated 1-butanol. Extracted lipids were incubated in a reaction mixture containing recombinant LPA acyl transferase and [^{14}C]-oleoyl-CoA (Perkin Elmer). This reaction converts LPA to radioactively labeled PA. Reaction products were resolved by thin layer chromatography. After autoradiography, the amount of radioactively labeled PA was quantified by densitometry. The plasmid encoding LPA acyl transferase was a generous gift from dr. Kazuhiko Kume (Tokyo University) [33].

Measurement of [^{14}C]-LPA metabolism
The rate of LPA metabolism was measured by treating tubes with [oleoyl-1-^{14}C]-LPA (American Radiolabeled Chemicals) and measuring the amount of labeled LPA remaining after 6 hrs of incubation as described in [34]. Briefly, lipids were extracted from medium and collagenase-digested collagen gels and resolved by thin layer chromatography. After autoradiography, the amount of radioactively labeled LPA was quantified by densitometry.

Generation of ATX ECKO mice
Cre-lox technology was used to knock-out *Enpp2* in the endothelial cells in the mouse. Tie2-Cre mice were bred with *Enpp2*$^{F/F}$ mice. *Enpp*2$^{F/+}$ Tie2-Cre^{+} offspring were bred with *Enpp2*$^{F/F}$ mice to generate *Enpp*2$^{F/+}$ Tie2-Cre^{+} (ATX ECKO) mice. Subsequent breedings for colony maintenance and to provide mice for experiments were of *Enpp*2$^{F/F}$ Tie2-Cre^{+} males and *Enpp*2$^{F/F}$ Tie2-Cre^{-} females. Such breedings generated roughly equal numbers of ATX ECKO and control (*Enpp2*$^{F/F}$ Tie2-Cre^{-}) mice. Genotyping was done by PCR of tail snip DNA.

Assessment of hyaloid vessel regression
Eyes of 1, 7, 14 ir 21-day-old mice were fixed for at least 24 hrs in 10% buffered formalin and subsequently washed in PBS. Eyes were embedded in methylmethacrylate and sectioned sagitally to the optic nerve through the center of the eye. Sections were 3 μm thick. Sections were stained with hematoxylin and eosin. 45 sections were prepared per eye. In every 5th section (a total of 10 sections), cell nuclei in hyaloid vessel walls were counted. The numbers of nuclei in the 10 sections were added up.

Assessment of the density and structure of retinal vasculature
Eyes of 3-month-old mice were fixed for 1 hr in 4% paraformaldehyde and washed in PBS. Retinas were dissected. They were incubated for 24 hrs in a solution of fluorescently labeled IB4-GS lectin (Invitrogen) (1:50 in PBS with 1 mM $CaCl_2$ and 0.5% Triton-X100). After washing with PBS (3 washes, 15 min each), 4-5 radial incisions were made to facilitate flatmounting. The retinas were flatmounted in mounting medium (Vector Laboratories). The vasculature was observed and photographed with a fluorescence microscope. The area occupied by fluorescently labeled endothelial cells was measured with ImageJ software and expressed as a percentage of the total area.

Gene silencing
siRNA oligonucleotides directed against LPP1, LPP2, LPP3 and firefly luciferase (non-targeting control) were purchased from Dharmacon (Thermo Scientific, USA). DharmaFECT transfection reagent (Dharmacon) was used for transfection of BREC and BRP. Transfection and gene silencing were based on Dharmacon standard protocols. shRNA sequences for targeting of LPP1-LPP3 and GFP (non-targeting control) were provided by the RNAi Consortium (The Broad Institute, USA). Plasmids with the shRNA sequences were purchased from the Dana Farber Cancer Institute (USA). Lentiviruses with these plasmids were produced and used to infect C3H 10T1/2 cells according to protocols provided by the RNAi Consortium.

Other methods
Immunohistochemistry, SDS-PA gel electrophoresis, Western Blot and immunofluorescence experiments were based on standard protocols. For immunofluorescence analysis of tubes, collagen gels were partially digested with collagenase I prior to analysis.

Statistical analysis
Software used for statistical analysis was GraphPad Prism and Microsoft Excel. Student's t-test was used to compare means of two groups. ANOVA was used to compare means of more than two groups.

RESULTS AND DISCUSSION

I. Development of an in vitro model of vessel stabilization by pericytes

In order to find out how pericytes stabilize blood vessels, we decided to first of all develop a simple in vitro model of vessel stabilization by pericytes and then use this model to study the molecular mechanism of stabilization. We built on an existing in vitro model of blood vessel formation and regression that we have successfully used in the past to study various aspects of blood vessel morphogenesis [28, 35, 36]. In this model, endothelial cells (EC) are plated between two layers of a collagen I gel and treated with vascular endothelial growth factor – A (VEGF). In response to VEGF, EC organize into blood vessel-like tubes. Tube formation takes 12-16 hours. After that, tubes start to spontaneously regress, even though culture medium is replaced and fresh VEGF is added every day. Regression is typically complete by 48 – 72 hours after addition of VEGF.

Pericytes are known to prevent regression of blood vessels in vivo [19, 20, 22, 25] and have been reported to prevent tube regression in in vitro models similar to ours [37, 38]. In order to test if pericytes are able to prevent regression of EC tubes (i.e., stabilize tubes) in our model, we plated mouse C3H 10T1/2 cells (pericyte-like cells [39]) together with bovine retinal endothelial cells (BREC) between two layers of a collagen gel and observed tube formation and regression over time. Cultured in collagen gels together with EC, C3H 10T1/2 cells (hereafter referred to as 10T cells) expressed pericyte markers NG2 and PDGFRβ (data not shown). Just like pericytes, 10T cells attached to the abluminal surface of EC tubes, although generally not with the entire cell body. Importantly, even a relatively small number of 10T cells (1/5 of the EC number) completely blocked tube regression, but did not significantly affect tube formation (data not shown).

We then assayed primary pericytes for their ability to stabilize EC tubes. As expected, bovine retinal pericytes (BRP) expressed pericyte markers and attached

well to the abluminal surface of BREC tubes (Fig. 1). They also blocked tube regression (Fig. 2). Likewise, human brain microvascular pericytes (HBVP) stabilized tubes organized from human umbilical vein endothelial cells (HUVEC) (data not shown). Of note, neither primary pericytes nor 10T cells were able to form tubes in the abscence of ECs. Importantly, pericyte-unrelated cells (HEK293 cells (data not shown) and primary macrophages (Dr. Magdalena Staniszewska, unpublished observation)) did not stabilize EC tubes.

To sum up, we have developed a simple in vitro model of blood vessel stabilization by pericytes that mimics several important aspects of the interactions between EC and pericytes in vivo. In this model, pericytes attach to the abluminal surface of blood vessel-like tubes and prevent their regression at EC:PC ratios within the range of what has been observed in vivo (100:1 – 1:1 [40]). The stabilization effect appears to be specific to pericytes, as all the pericyte types that we tested were able to stabilize EC tubes, whereas two pericyte-unrelated cell types were not.

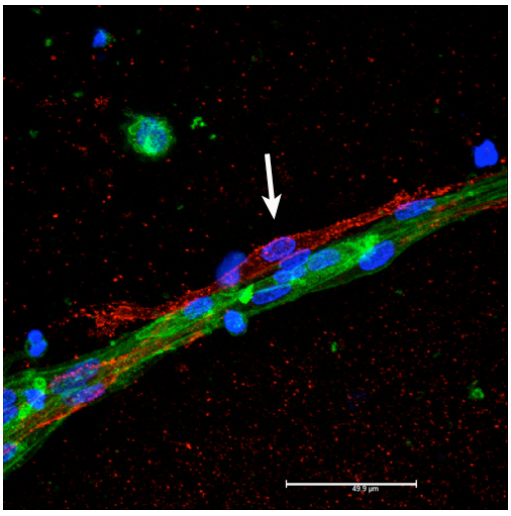


Fig 1. **A fluorescence confocal microscopy image of BREC and BRP tubes.**
Nuclei (blue) were labeled with DAPI. EC (green) were identified by their ability to bind fluorescently labeled lectin IB4-GS. Pericytes (red) were identified by NG2 expression. White arrow points to a pericyte, attached to the abluminal side of an EC tube. Scale bar - 50 μm. Image by dr. Jorge Aranda.

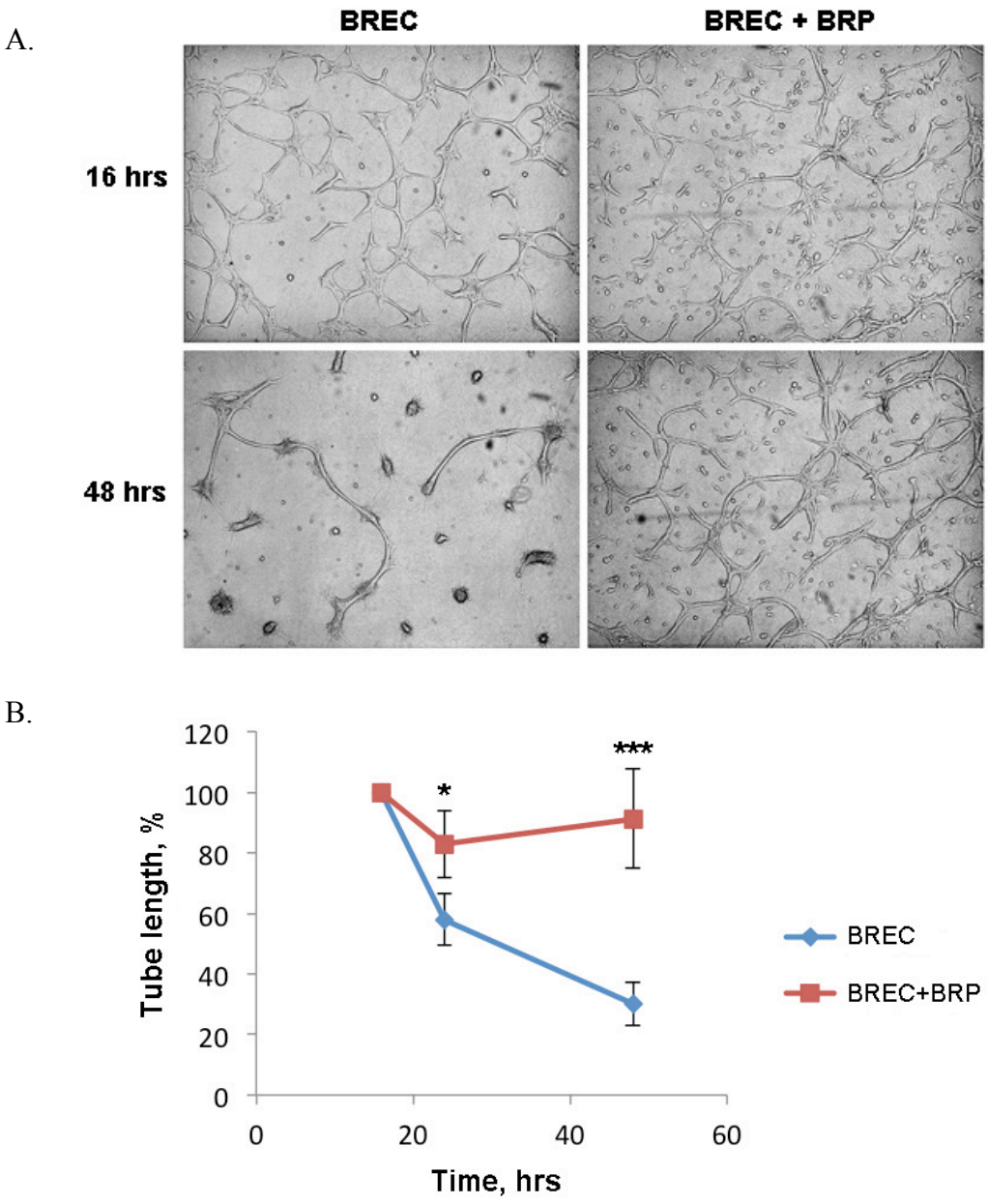


Fig 2. Bovine retinal pericytes blocked regression of vessel-like EC tubes.
Tubes were organized from BREC and BRP cells at a ratio of 4:1. Tube formation was evaluated at 16 hrs after addition of VEGF. Tube regression was evaluated at 24 and 48 hrs after addition of VEGF.
A. Inverted microscopy images of tubes in a representative experiment.
B. Averaged data from 4 independent experiments. Tube length at 16 hrs was set to 100%. Bars show SD. *$p<0.05$; ***$p<0.001$ relative to "BREC+BRP" at the same time-point.

II. Study of the molecular mechanism of vessel stabilization by pericytes

We have previously shown that regression of EC-only (monoculture) tubes was dependent on autotaxin (ATX) – a secreted lysophospholipase D that catalyzes the synthesis of lysophosphatidic acid (LPA) from lysophosphatidylcholine (LPC), abundantly present in serum [28]. LPA binds to its G-protein coupled receptors on the surface of endothelial cells and starts a signaling cascade that leads to regression (Fig. 3). Because LPA was both sufficient and required for regression of monoculture tubes in our model, we hypothesized that tubes organized out of EC and pericytes (co-culture tubes) were stable because pericytes interfered with the ATX – LPA signaling pathway.

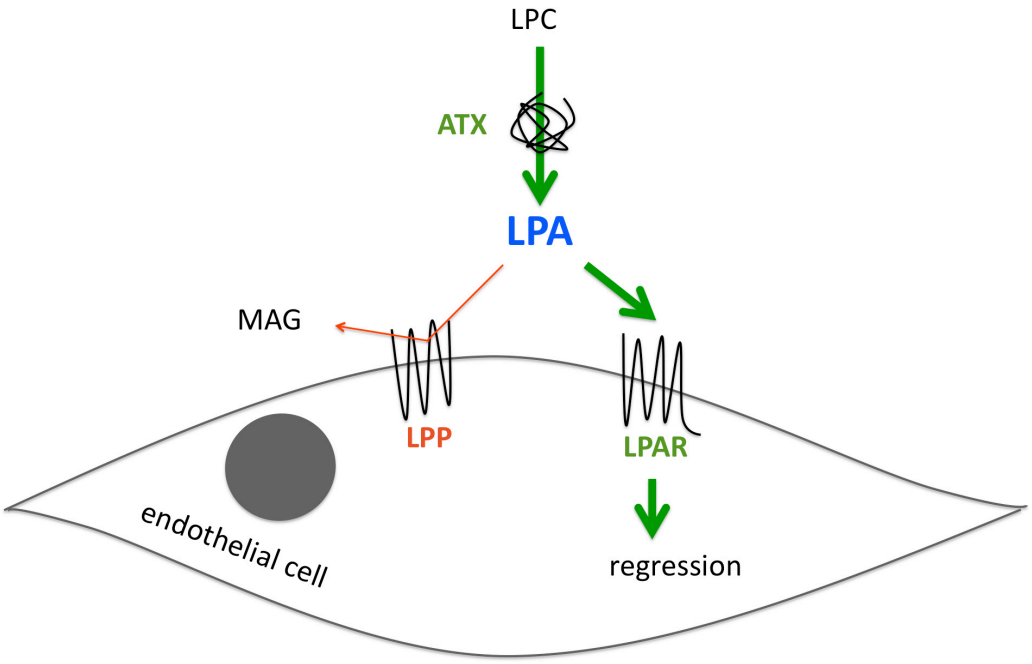


Fig. 3. Molecular mechanism of regression of monoculture tubes.
Synthesis of LPA from LPC is catalyzed by ATX, a secreted lysophospholipase D. LPA binds to its receptors (LPAR) on the membrane of endothelial cells and causes tube regression. Transmembrane phospholipid phosphatases (LPP) can dephosphorylate LPA. The product of dephosphorylation is monoacylglycerol (MAG), which cannot bind to LPA receptors and does not cause regression.

How could pericytes interfere with the ATX – LPA signaling pathway? We postulated the following possibilities: pericytes could hamper the response of EC to LPA (for example, by reducing the expression of LPA receptors) and/ or reduce the concentration/ activity of LPA (for example, by reducing ATX expression and therefore LPA production).

We first of all tested whether pericytes were reducing the ability of EC to respond to LPA. We formed tubes out of BREC and 10T cells and treated them with exogenous LPA (1 or 10 μM) or, in a separate set of experiments, with recombinant ATX (1, 2 or 4 μg/mL). Either LPA or ATX induced regression of BREC+10T cell tubes in a dose dependent manner (data not shown). Tubes organized out of HUVEC and HBVP cells were also responsive to LPA (data not shown). These data led us to conclude that pericytes did not interfere with the response of EC to LPA. Thus, if pericytes did, in fact, interfere with the ATX – LPA signaling pathway, they most likely reduced the concentration and/ or activity of LPA.

In order to test this hypothesis, we measured the concentration of LPA in collagen gels with monoculture and co-culture tubes, as well as conditioned media. The concentration of LPA in conditioned medium from monoculture tubes (0.57 μM) was statistically significantly higher than the concentration of LPA in conditioned medium from co-culture tubes (0.47 μM, $p<0.01$), but the difference was small. On the other hand, collagen gels with co-culture tubes had more than 3 times less LPA than collagen gels with monoculture tubes (0.12 μM and 0.41 μM, respectively) (Fig. 4). Because LPA induces a biological response by binding to its receptors on the cell surface, the concentration of LPA in the cellular microenvironment (i.e., the collagen gel) was more relevant to regression than the bulk concentration in the conditioned medium. Of note, collagen gels with BREC+BRP tubes also had less LPA than gels with BREC tubes (data not shown).

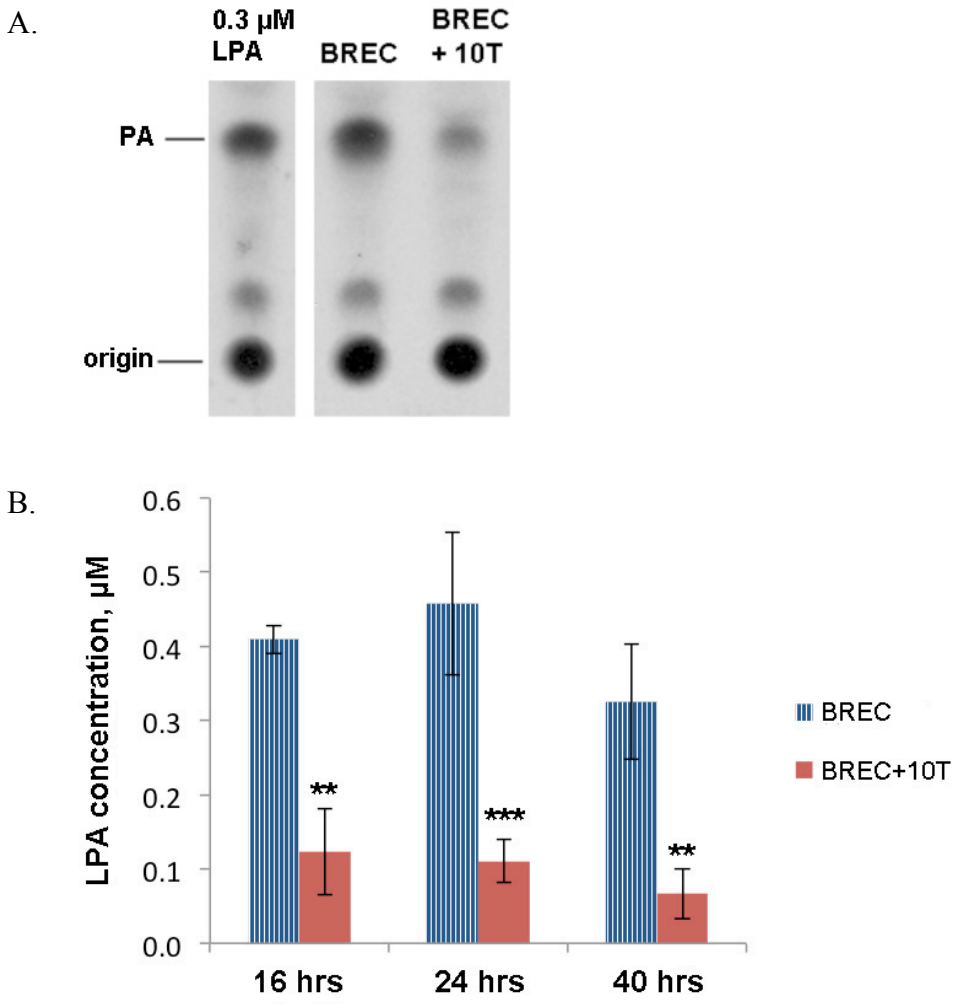


Fig. 4. Collagen gels with BREC+10T cell tubes had less LPA than collagen gels with BREC tubes.

Tubes were formed out of BREC alone or BREC+10T. Collagen gels were collected at 16, 24 and 40 hrs after addition of VEGF. A radioenzymatic method [32] was used to measure the concentration of LPA in collagen gels.

A. LPA concentration in collagen gels at 16 hrs after addition of VEGF. Representative autoradiogram. Intensity of the radioactively labeled phosphatidic acid (PA) signal is dependent on the concentration of LPA in the sample. Leftmost lane – a 0.30 μM LPA standard.

B. LPA concentration in collagen gels at 16, 24 and 40 hrs after addition of VEGF. Averaged data from 3 independent experiments. Bars show SD. **$p<0.01$; ***$p<0.001$ relative to "BREC" at the same time point.

The data described above supported our hypothesis that pericytes stabilized tubes by interfering with the ATX – LPA signaling pathway. Because LPA was necessary for monoculture tube regression [28] and, when added exogenously, was able to induce regression of co-culture tubes, a smaller concentration of LPA in co-culture collagen gels was a good explanation for co-culture tube stability. We concluded that pericytes stabilize tubes by reducing the concentration of LPA in their microenvironment.

How were pericytes reducing the concentration of LPA in collagen gels? We postulated that they were either inhibiting LPA production or enhancing its metabolism. The main enzyme responsible for LPA synthesis in vivo is ATX [30, 41], whereas the main degradative enzymes are phospholipid phosphatases LPP1-3 [29, 34]. Intracellular LPA is an intermediate in the biosynthesis of glycerophospholipids, but conversion of extracellular LPA into higher molecular weight compounds appears to be a rather minor pathway in its metabolism [42, 43]. We therefore focused on LPA production and degradation as the main processes that would change the concentration of LPA in our system.

We knew that ECs secrete ATX in our in vitro model, that an increase in ATX concentration corresponds to the onset of regression, and that ATX is both necessary and sufficient for regression of monoculture tubes [28]. We had also observed that treatment with exogenous ATX induces regression of otherwise stable co-culture tubes (see above). Thus, it seemed likely that co-culture tubes were stable because of a reduction in ATX expression or activity. A shortage of LPC – the substrate for ATX – seemed unlikely, because exogenously added LPC did not induce regression of co-culture tubes (data not shown).

On the other hand, we knew little about the role of LPPs in our system. We had previously shown that overexpression of LPP1 stabilized otherwise spontaneously regressing BREC monoculture tubes [28]. Thus, LPPs were sufficient for tube

stability. In order to test whether they were also necessary for stability, we formed tubes out of BREC and 10T cells and treated them with Na_3VO_4, an phosphatase inhibitor. Na_3VO_4 is not specific for LPPs, but inhibits them well: 100 μM Na_3VO_4 reduces LPP activity by 80-90% [43]. We observed that Na_3VO_4 did, in fact, increase the concentration of LPA in collagen gels with co-culture tubes (Fig. 5). This finding indicated that co-culture tubes had LPA phosphatase activity. Consequently, Na_3VO_4 induced regression of co-culture tubes (Fig. 6). The ability of Na_3VO_4 to induce regression was diminished when we concomitantly treated tubes with BrP-LPA, an inhibitor of a subset of LPA receptors (Fig. 6). Thus, Na_3VO_4 induced regression specifically, through LPA. We obtained similar results when we treated co-culture tubes with $ZnCl_2$, another LPP inhibitor [44] – $ZnCl_2$ induced co-culture tube regression, which was partally blocked by BrP-LPA (data not shown).

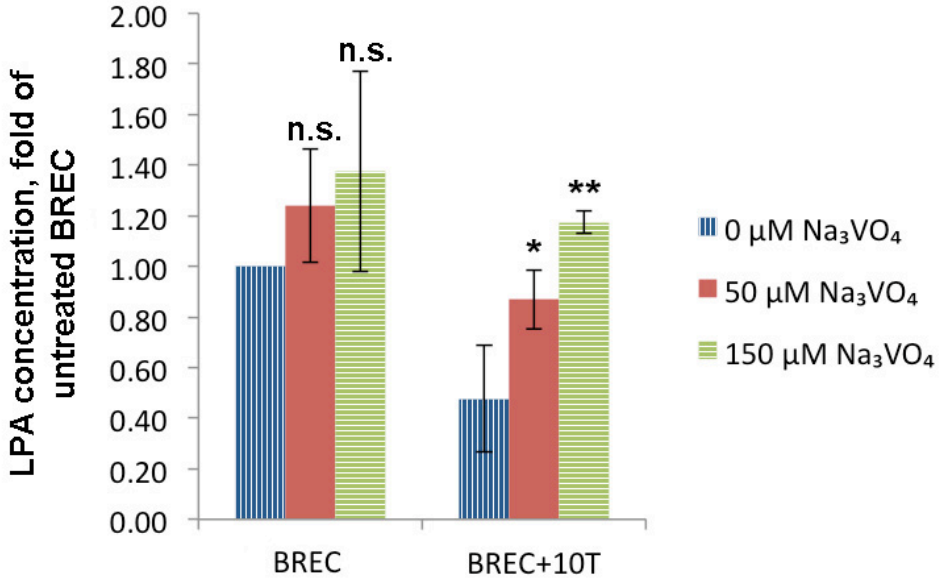


Fig. 5. Na_3VO_4 increased the concentration of LPA in collagen gels with co-culture tubes.
Tubes were formed out of BREC or BREC+10T cells. After tubes formed, they were treated with the indicated concentrations of Na_3VO_4. After 6 hrs, the concentration of LPA in collagen gels was measured. Data were normalized to the concentration of LPA in untreated collagen gels with monoculture tubes (set to 1). Averaged data from 4 independent experiments. Bars show SD. *$p<0.05$; **$p<0.01$; n.s. – not significant ($p>0.05$) (relative to "0 μM Na_3VO_4" of the same type of tubes).

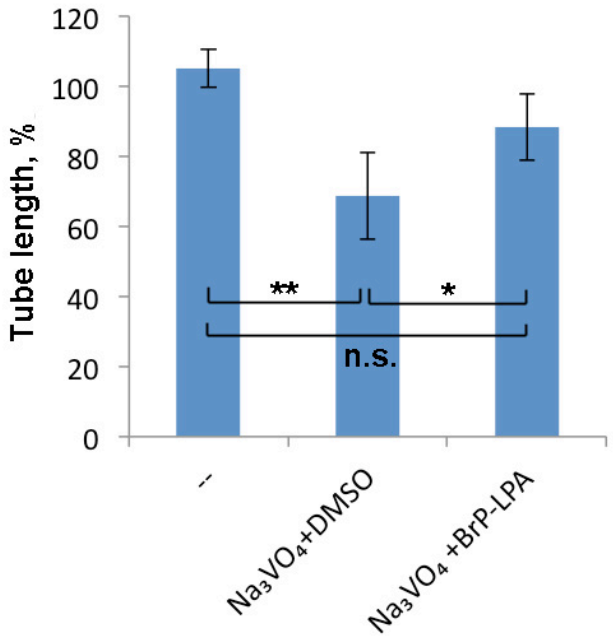


Fig. 6. Na_3VO_4 induced regression of co-culture tubes that was partially inhibited by inhibition of LPA receptors.
Tubes were formed out of BREC and 10T cells. After tubes formed, they were treated with 100 μM Na_3VO_4 and either 30 μM BrP-LPA (in DMSO) or an equal volume of DMSO. Regression was observed after 6 hrs. Tube length right before treatment was set to 100%. Data from one representative experiment. Experiment was repeated 3 times. Columns show means +/- SN (n=4). *p<0.05; **p<0.01; n.s. – not significant (p>0.05).

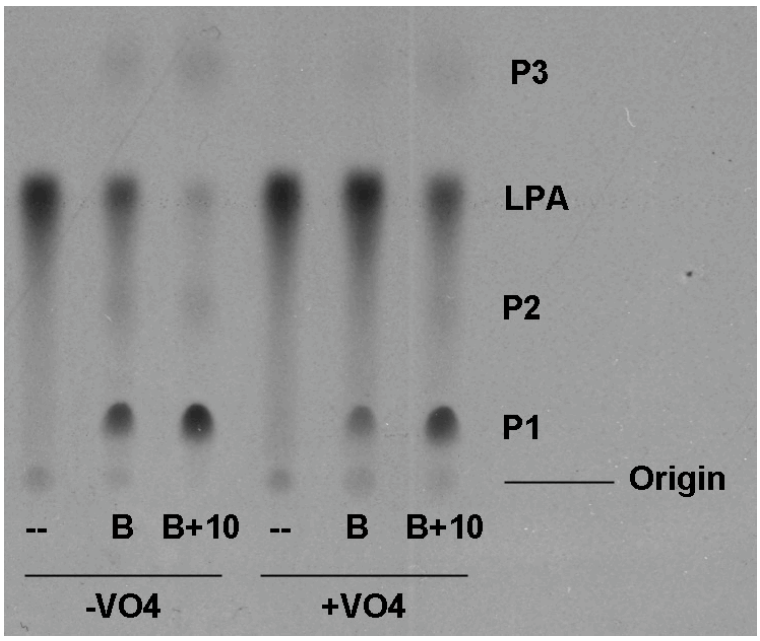
Collectively, these results indicate that co-culture tubes possess LPP-like activity, and that inhibition of this activity inceases the concentration of LPA and induces regression. Thus, LPPs are necessary for tube stability. Besides a decrease in ATX expression/ activity, an increase in LPP expression or activity could therefore also be a reason for the stability of co-culture tubes. Of note, Na_3VO_4 did not significantly increase the concentration of LPA in collagen gels with monoculture tubes (Fig. 5) and did not increase the rate of their regression (data not shown). Thus, either LPP activity of monoculture tubes is low, or there are additional mechanisms that prevent the LPA concentration from rising above a certain level. One such mechanism that has been previously described is inhibition of ATX activity by higher concentrations of LPA [45].

In order to find out if co-culture tubes did, in fact, degrade LPA at a faster rate than monoculture tubes, which would indicate higher LPP activity, we treated BREC and BREC+10T tubes with [oleoyl-1-^{14}C]-LPA. After 6 hrs, we measured how much of the radioactively labeled LPA was still present in the collagen gels. Besides

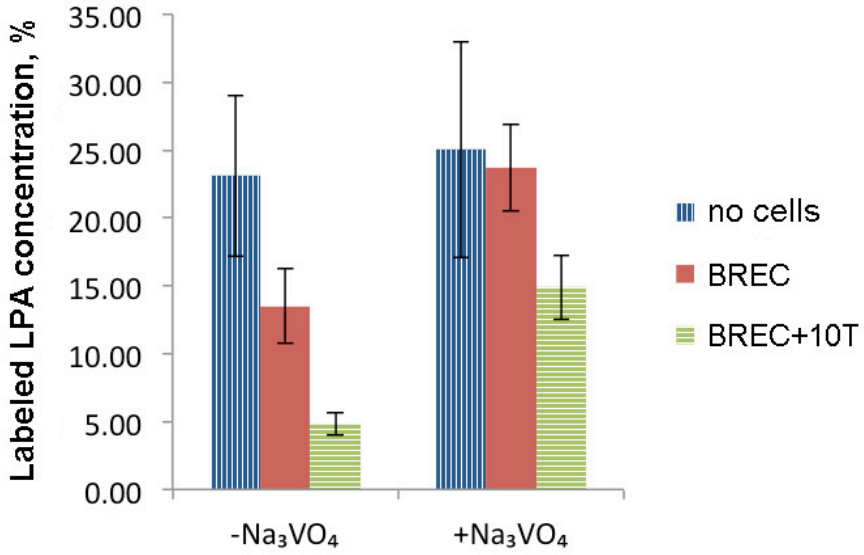
collagen gels with monoculture tubes and co-cultures tubes, we included a control of a collagen gel without any cells. This control allowed us to evaluate how quickly monoculture tubes metabolized LPA. After 6 hrs of incubation, we found 40% less labeled LPA in collagen gels with BREC tubes than in gels without cells (Fig. 7). This indicated that even monoculture tubes metabolized LPA. Gels with co-culture tubes had 80% less LPA than gels without cells, and 67% less than gels with monoculture tubes (Fig. 7). Thus, co-culture tubes metabolized LPA faster than monoculture tubes, which explained the difference in LPA concentration in gels with the two types of tubes.

We wanted to know if LPPs were responsible for the disappearance of labeled LPA from collagen gels. Because LPPs dephosphorylate LPA to monoacylglycerol (MAG), we expected to find labeled MAG among the products of the reaction. However, even though we detected three different product bands (Fig. 7), none of them migrated the same distance as an unlabeled MAG standard (data not shown). It's possible that we didn't detect MAG because even though it was formed, it was then quickly metabolized. Another approach we took to probe the role of LPPs was to treat tubes with Na_3VO_4 alongside labeled LPA. This phosphatase inhibitor completely blocked LPA metabolism by monoculture tubes (Fig. 7), but only partially inhibited LPA metabolism by co-culture tubes. Thus, phosphatases were most likely responsible for the degradation of extracellular LPA by monoculture tubes. Phosphatases were also important for the degradation of LPA by co-culture tubes, but those tubes also most likely utilized other mechanisms of extracellular LPA degradation.

A.



B.



C.

Conditions compared		p value
No cells	BREC	**
No cells	BREC+10T	***
BREC	BREC+10T	*
No cells, + Na_3VO_4	BREC, + Na_3VO_4	n.s.
No cells, + Na_3VO_4	BREC+10T, + Na_3VO_4	**
BREC, + Na_3VO_4	BREC+10T, + Na_3VO_4	*
No cells	No cells, + Na_3VO_4	n.s.
BREC	BREC, + Na_3VO_4	***
BREC+10T	BREC+10T, + Na_3VO_4	***

Fig. 7. Co-culture tubes metabolized LPA faster than monoculture tubes.
Collagen gels without cells, with BREC tubes or with BREC+10T tubes were treated with [oleoyl-1-^{14}C]-LPA (40 nCi/mL). Some wells also received 100 μM Na_3VO_4. After 6 hrs, collagen gels were collected; lipids were isolated and resolved by TLC. Radioactive species were detected by autoradiography.
A. Representative autoradiogram. "--" – gel without cells, "B" – gel with BREC tubes, "B+10" – gel with BREC+10T tubes, "-VO4" – 0 μM Na_3VO_4, "+VO4" – 100 μM Na_3VO_4, "P1-3" – putative products of LPA metabolism.
B. Averaged data from 3 independent experiments. Spot intensity was densitometrically quantified and normalized to the intensity of the spot of added labeled LPA at 0 hrs (set to 100%). Bars show SD.
C. Table of p values. *$p<0.05$; **$p<0.01$; ***$p<0.001$; n.s. – not significant ($p>0.05$).

The experiments described above showed that co-culture tubes metabolized LPA faster, but it was still unclear what specific mechanisms and enzymes were involved. We were unable to find any literature data on mechanisms of extracellular LPA metabolism other than dephosphorylation. LPP1-3 are the best known phosphatases that dephosphorylate LPA, and therefore we focused on the role of those enzymes. We first of all compared the expression of LPP1, LPP2 and LPP3 in BREC tubes and BREC+10T tubes. We also compared ATX expression, because even though we knew that co-culture tubes metabolize LPA faster, the possibility remained that they also produce less LPA.

We detected all the four enzymes – LPP1, LPP2, LPP3 and ATX – by Western blot in both types of tubes (data not shown). Protein levels of ATX and LPP1 were similar in monoculture and co-culture tubes. On the other hand, co-culture tubes had about 1.6 times more LPP2 than monoculture tubes, and this difference was statistically significant ($p<0.05$). Expression of LPP3 varied greatly experiment to experiment, and thus a similar fold difference in expression of this protein between co-culture and monoculture tubes was not statistically significant. To sum up, stability of co-culture tubes could not be explained by changes in ATX or LPP1 expression, but the observed increased expression of LPP2 in co-culture tubes could be the cause of accelerated LPA degradation, reduced concentration of LPA in the

collagen gel and lack of tube regression. Importantly, although we eliminated changes in ATX and LPP1 expression as causes of co-culture tube stability, these enzymes might still be important in prevention of regression through changes in their activity.

If co-culture tubes are stable because of increased expression of LPP2, reducing expression of this protein should induce their regression. In order to test if the correlation beween LPP2 expression and tube stability represented a causative relationship, we silenced LPP2 in BREC and BRP with a specific siRNA. We then formed tubes out of BREC with reduced LPP2 expression and non-transfected BRP, or out of non-transfected BREC and BRP with reduced LPP2 expression. Unexpectedly, silencing LPP2 did not induce tube regression in either case (data not shown). We additionally silenced LPP2 in 10T cells with a specific shRNA. Tubes formed out of these 10T cells and non-transfected BRECs did not regress, either. Therefore, we concluded that LPP2 was not required for stability of co-culture tubes. Expression of LPP2 in endothelial cells is lower than that of LPP1 and LPP3 [46, 47], and thus it is conceivable that even though expression of LPP2 is higher in co-culture tubes, this doesn't appreciably affect the concentration of LPA in the collagen gel. In order to probe the role of the other two LPPs, LPP1 and LPP3, we attempted to silence them in BREC, BRP and 10T cells, but unfortunately were unsuccessful in significantly reducing the protein levels of these enzymes. To summarize, although we showed that co-culture tubes metabolize LPA faster, we were unable to answer the question of whether this was due to LPPs (and if yes, which ones).

Up to this point, we have investigated how pericytes stabilize EC tubes through interference with the ATX – LPA signaling pathway. Other groups have described other mechanisms of vessel stabilization by pericytes. For example, ANG-1, an MMP (matrix metalloproteinase) inhibitor TIMP3, VEGF-A, TGFβ1 and certain other factors produced by pericytes promote EC survival and/ or regulate

vascular stability [37, 38, 48, 49, 50]. We wanted to find out if these factors play a role in tube stability in our model as well, and if so, if they are connected to the ATX – LPA signaling pathway. To that effect, we did a series of simple experiments wherein we formed BREC tubes, treated them with the various survival/ stabilization factors individually and observed whether they were able to stop spontaneous tube regression. Neither ANG-1, nor TGFβ1, nor a higher concentration of VEGF-A than we normally use to induce tube formation (50 ng/mL instead of 2.5 ng/mL) slowed regression down (data not shown). Although we couldn't conlude that these agents weren't important for tube stability in our model, these data showed that neither of them was individually sufficient.

In order to probe the role of MMP and TIMP, we treated BREC tubes with GM6001, a general MMP inhibitor. This inhibitor increased tube formation (data not shown) and blocked tube regression (Fig. 8). We obtained similar data with a more specific inhibitor of MMP2 and MMP9 (data not shown). Thus, MMP, just like LPA, were required for tube regression in our model. These data fit well with published studies that showed that pericytes produce an MMP inhibitor TIMP3, which blocks tube regression in in vitro models similar to ours, as well as blood vessel regression in vivo [37, 38]. It is therefore likely that in our model pericytes stabilize tubes in part by producing MMP inhibitors such as TIMP. Because this mechanism has already been described, we did not further this line of investigation.

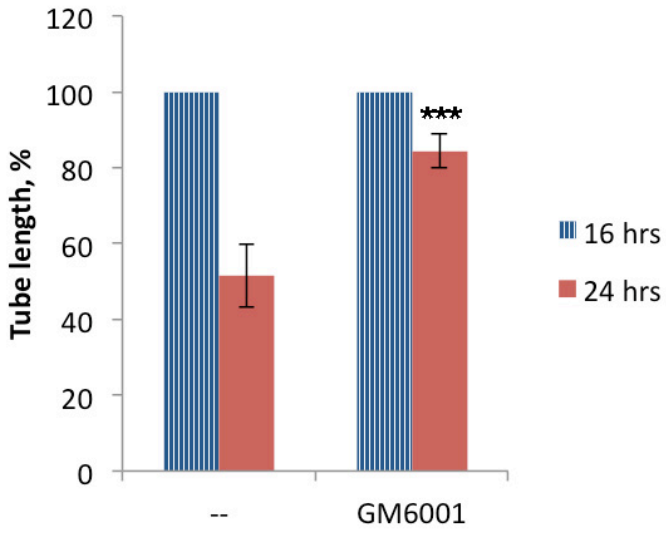


Fig. 8. MMP inhibition inhibited tube regression. BREC tubes were formed in the presence of 10 μM GM6001 where indicated. Regression was observed at 24 hrs. Data were normalized to tube length at 16 hrs, which was set to 100%. One representative experiment in shown. Experiment was repeated 3 times. Data are means +/- SD (n=3). ***p<0.001.

Summarizing the data presented in this study, we propose the following model for tube stabilization by pericytes: pericytes stabilize tubes by accelerating the metabolism of LPA, thereby reducing the concentration of this regression factor in the microenvironment of the tubes (Fig. 9).

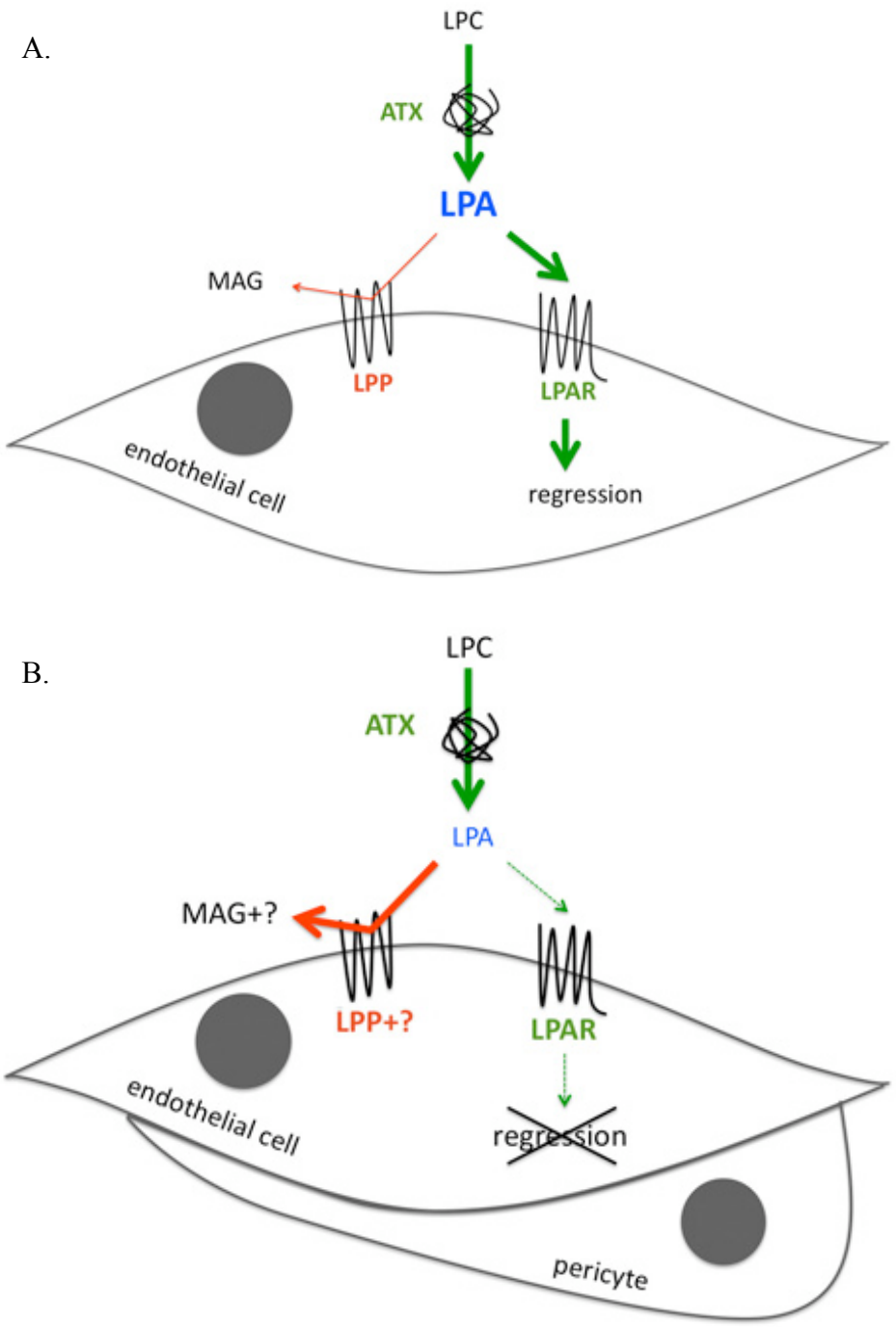


Fig. 9. Molecular mechanism of co-culture tube stability.
Tubes formed out of EC and pericytes (B) metabolize extracellular LPA faster than tubes formed out of just EC (A). This might be caused by increased LPP activity, but other enzymes capable of metabolizing LPA may also play a role. When there is less LPA in the cellular microenvironment, less LPA can bind to its receptors on the cell surface and send a regression signal. "?" denotes unidentified enzymes that might metabolize LPA and the unidentified products of the reactions they catalyze.

III. Effect of endothelial *Enpp2* inactivation on blood vessel formation and regression

As mentioned above, ATX, a secreted lysophospholipase D, catalyzes the production of LPA, a factor that is both necessary and sufficient for EC tube regression in our in vitro model [28]. We wanted to know if ATX controls regression in vivo as well. To this end, we studied regression of hyaloid vessels in transgenic mice that overexpress ATX [51]. Hyaloid vessels are a vascular bed in the eye (more specifically, in the vitreous) that, in mice, regresses within the first 2-3 weeks of life [52]. These vessels regressed faster in ATX transgenic mice [28]. Thus, ATX was able to accelerate regression in vivo. However, we still didn't know if ATX was necessary for in vivo regression. The most straightforward way to test that would be to knock-out the gene that codes for ATX in the mouse (*Enpp2*) and compare regression in these mice to that in wild-type mice. Unfortunately, *Enpp2* knock-out mice die during embryonic development [30, 41] and thus do not reach an age where we could observe regression.

In our in vitro experiments, ATX made by EC was important for tube regression. Silencing of this gene inhibited regression [28]. We therefore decided to generate mice with the *Enpp2* gene knocked-out specifically in EC. Because several different cell types make ATX, we hoped that inactivation of the gene in endothelial cells only would not cause embryonic lethality and would allow for the study of the importance of ATX in in vivo regression. Hereafter we will refer to ATX endothelial knock-out mice as ATX ECKO mice.

We derived ATX ECKO mice by breeding transgenic Tie2-Cre mice that express the Cre recombinase in endothelial cells, heart cells of endothelial origin and a subset of blood cells [31] with mice that had *loxP* sequences (recognized by Cre) inserted into the *Enpp2* gene so that they flank the sequence of the catalytic site of the enzyme ($Enpp2^{F/F}$ mice [30]). After a few generations of breeding, we had

generated mice that had both genetic modifications: expressed Cre in their endothelial cells and had *loxP* sequences in the *Enpp2* gene (*Enpp2*$^{F/F}$ Tie2-Cre^{+} mice). In Cre-expressing cells of these mice (i.e., mainly EC), Cre should have recognized its substrate sequences and excized the sequence between them, thereby inactivating *Enpp2*. These were the ATX ECKO mice.

ATX ECKO mice did not die during embryonic development. In fact, they survived to adulthood, were fertile and appeared healthy. Immunohistochemistry of tissue sections with ATX-specific antibodies confirmed a lack of ATX expression in the blood vessels of ATX ECKO mice (data not shown).

We proceeded to measure the rate of hyaloid vessel regression in ATX ECKO mice and control mice (which had the *loxP* sequences in the *Enpp2* gene, but did not have the Cre-encoding transgene). To that effect, we collected eyes of mice that were 1, 7, 15 or 21 days old, prepared thin sections of those eyes, stained them with hematoxylin and eosin and counted the number of lumen-surrounding nuclei in the vitreous (i.e., the number of cells in hyaloid vessels) in 10 sections. We found that neither the rate nor the extent of hyaloid vessel regression was changed in ATX ECKO mice (Fig. 10). Thus, endothelial cell ATX was not required for hyaloid vessel regression.

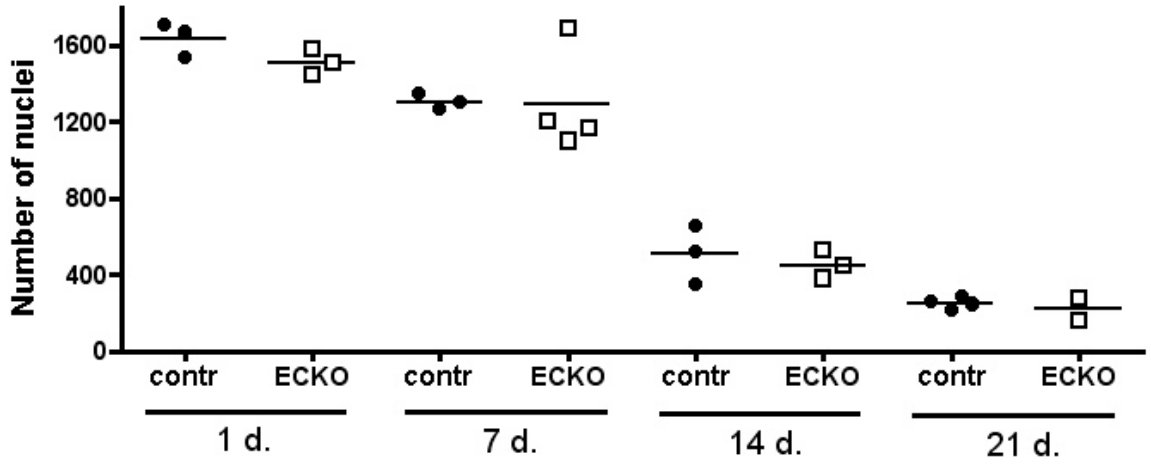


Fig. 10. Regression of hyaloid vessels was unaffected in ATX ECKO mice.
Number of nuclei in hyaloid vessels was counted in eye sections of ATX ECKO (□) and control (●) littermates of indicated ages (in days). Each symbol represents an individual mouse. Horizontal lines show means.

Another vascular bed where regression of blood vessels occurs postnatally and is easy to follow is the retinal vasculature. Mice are born with an avascular retina, which becomes vascularized within the first 3 weeks of life, just as hyaloid vessels regress. First, a dense, undifferentiated vascular plexus is formed, which subsequently matures as excess vessels are pruned (regress) and the remaining vessels differentiate into arteries, veins and capillaries [53, 54].

In order to examine the role of ATX in vascular regression of a vascular bed other than the hyaloid vessels, we compared the retinal vasculature of 3-month-old ATX ECKO and control mice. We collected retinas of these mice, stained them with fluorescently labeled lectin IB4-GS that binds to EC and compared the structure and density of the vascular plexi. We did not see any major differences between the vascular plexi of the two genotypes. In both cases all the three layers of the retinal vasculature were present. The vascular trees were similar in their architecture, with arteries and veins clearly distinguishable (Fig. 11). We quantified the density of the

vasculature with image processing software and determined that it was identical in ATX ECKO and control mice (data not shown).

These data show that endothelial cell ATX is not required for formation of mature, differentiated retinal vasculature. It must be noted that we didn't directly study regression of retinal vessels. It is possible that loss of ATX in EC inhibited regression of retinal vessels, but also inhibited initial vascular sprouting, with the end result being unchanged vascular density at 3 months of age. However, in combination with hyaloid vessel regression data, these data are more consistent with an interpretation that EC ATX is not required for physiological formation and regression of blood vessels.

How can these data be reconciled with our in vitro data, as well as the in vivo observation that hyaloid vessels regress faster in ATX overexpressing mice? One explanation is that ATX overexpression can induce regression, but normal, physiological regression in not dependent on ATX. Alternatively, ATX is important for regression, but the endothelium is not an important source of this enzyme. ATX produced by endothelial cells was necessary for regression in our in vitro model, but this model is a simplified reflection of how regression proceeds in vivo. Recently, ATX inhibitors suitable for in vivo use have been developed [55]. We plan to inject such an inhibitor into the bloodstream and/ or the vitreous of mice undergoing morphogenesis of ocular vessels and thereby test if ATX, regardless of its source, is important for blood vessel regression. Finally, ATX might be important for pathological, but not physiological regression of blood vessels. We plan to test this hypothesis in mouse models of pathological angiogenesis and regression such as the mouse model of oxygen-induced retinopathy. We hope that these experiments will unequivocally resolve the role of ATX in in vivo regression of blood vessels.

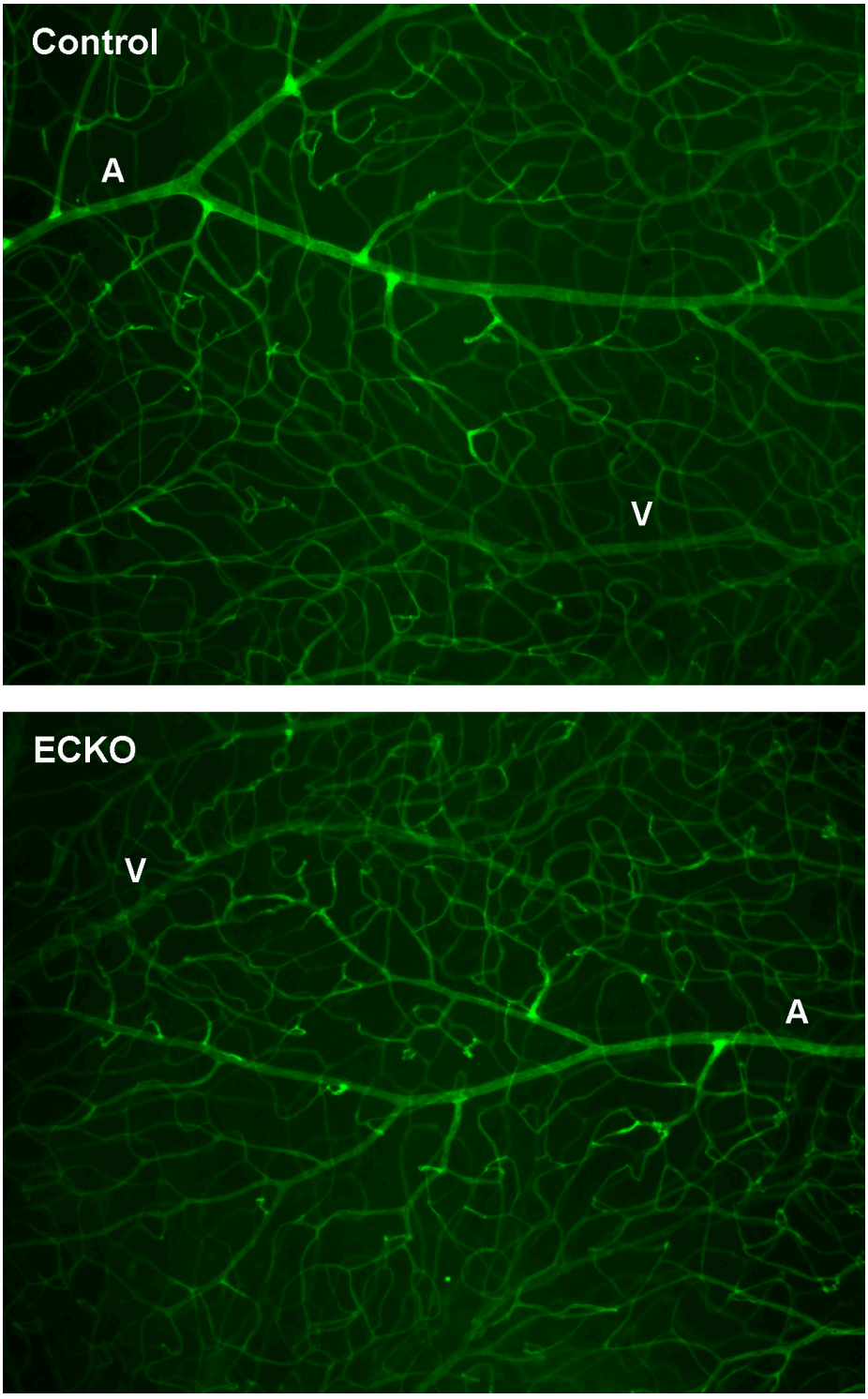


Fig. 11. Retinal vasculature of ATX ECKO mice was unchanged.
Retinas of 3-month-old control mice (top panel) and ATX ECKO mice (bottom panel) were stained with fluorescently labeled IB4-GS, an EC-selective lectin. A – artery, V – vein.

CONCLUSIONS

1. In an in vitro model of blood vessel-like tube regression, pericytes stabilize tubes by enhancing metabolism of LPA, a vascular regression/ destabilization factor;
2. *Enpp2* endothelial cell knock-out mice are viable and fertile;
3. Endothelial cell *Enpp2* is not required for hyaloid vessel regression nor formation of mature retinal vasculature.

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Santrauka

Kraujagyslių augimo, stabilumo ir regresijos sutrikimai siejami su vėžiu, išeminėmis ligomis, akių ligomis ir kt. Nors kraujagyslių augimo molekuliniai mechanizmai neblogai ištirti, apie kraujagyslių stabilumą ir regresiją žinoma kur kas mažiau. Naujas kraujagysles stabilizuoja pericitai – mezenchimos kilmės pagalbinės kraujagyslės sienelės ląstelės. Pirmojoje šio darbo dalyje tyrėme kraujagyslių stabilizavimo pericitais molekulinį mechanizmą. Išvystėme paprastą kraujagyslių stabilizavimo pericitais *in vitro* modelį. Šiame modelyje iš endotelio ląstelių suformuotų kraujagysles primenančių vamzdelių regresiją sustabdė pericitų pirmtakai ir pirminiai pericitai, bet ne pericitams negimininingos ląstelės. Nustatėme, kad vamzdelių padengimas pericitais *in vitro* paskatino regresijos veiksnio lizofosfatido rūgšties (LFR) metabolizmą. Sumažėjusi LFR koncentracija lėmė vamzdelių stabilumą. Už LFR metabolizmą bent iš dalies buvo atsakingi savo savybėmis fosfolipidų fosfatazes LPP primenantys fermentai. Antrojoje šio darbo dalyje tyrėme, ar LFR sintezuojantis fermentas autotaksinas (ATX) svarbus kraujagyslių regresijai *in vivo*. Išvedėme peles, kurių endotelio ląstelėse buvo atrankiai išveiklintas ATX koduojantis genas *Enpp2*. Nustatėmė, kad ATX išveiklinimas pelės endotelio ląstelėse neturėjo įtakos nei stiklakūnio kraujagyslių regresijai, nei brandaus tinklainės kraujagyslių tinklo susiformavimui.

Curriculum vitae

Name: Rūta Motiejūnaitė
Address: The Schepens Eye Research Institute, 20 Staniford St., Boston, MA 02114, USA
Telephone: +1-617-912-2524
E-mail: ruta_motiejunaite@meei.harvard.edu

Education

2007- PhD student, Biochemistry. Vilnius University, Vilnius, Lithuania
2006 MS (magna cum laude), Biochemistry. Vilnius University.
2004 BS (cum laude), Biochemistry. Vilnius University

Professional experience

2006- Pre-doctoral research fellow. The Schepens Eye Research Institute, Massachusetts Eye and Ear Infirmary, Harvard Medical School, Boston, USA.
2005-2006 Specialist. Molecular Biology Laboratory, Department of Biochemistry and Biophysics, Vilnius University.
2005 Laboratory technician, Department of Biochemistry and Biophysics, Vilnius University

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