Flt1and Flk1 mediate regulation of intraocular pressure and their double heterozygosity causes the buphthalmia in mice

Sano, Keigo

(Degree)
博士（医学）

(Date of Degree)
2012-03-25

(Date of Publication)
2013-01-07

(Resource Type)
doctoral thesis

(Report Number)
甲5617

(URL)
https://hdl.handle.net/20.500.14094/D1005617

Flt1 and Flk1 mediate regulation of intraocular pressure and their double heterozygosity causes the buphthalmia in mice

Flt1 と Flk1 は眼圧の制御に関与し、それらの二重ヘテロ欠損はマウスに牛眼を引き起こす

佐野　圭吾, 勝田　修, 白江　哲, 久保田　義顕, 依馬　正次,
須田　年生, 中村　雅胤, 平島　正則

神戸大学大学院医学研究科医科学専攻
血管生物学分野
（指導教員：　平島　正則　准教授）
佐野　圭吾

Key words: Buphthalmia, Development, Endothelial cell, Intraocular pressure, Mouse, VEGF

Flt1 and Flk1 mediate regulation of intraocular pressure and their double heterozygosity causes the buphthalmia in mice

Keigo Sano[a], Osamu Katsuta[b], Satoshi Shirae[b], Yoshiaki Kubota[c], Masatsugu Ema[d], Toshio Suda[e], Masatsugu Nakamura[b] and Masanori Hirashima[a,*]

[a] Division of Vascular Biology, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan

[b] Ophthalmic Research & Development Center, Santen Pharmaceutical Co. Ltd., 8916-16 Takayama-cho, Ikoma-shi, Nara 630-0101, Japan

[c] Center for Integrated Medical Research, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

[d] Department of Anatomy and Embryology, Institute of Basic Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan

[e] The Sakaguchi Laboratory of Developmental Biology, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

* Corresponding author. Address: Division of Vascular Biology, Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan. Phone: +81 78 382 5831. Fax: +81 78 382 5834. E-mail address: mhirashi@med.kobe-u.ac.jp (M. Hirashima).

Abstract

Flt1 and Flk1 are receptor tyrosine kinases for vascular endothelial growth factor-A which play a crucial role in physiological and pathological angiogenesis. To study genetic interaction between the *Flt1* and *Flk1* genes, we crossed between Flt1 and Flk1 heterozygous ($Flt1^{+/-}$ and $Flk1^{+/-}$) mice. We found that Flt1; Flk1 double heterozygous ($Flt1^{+/-}$; $Flk1^{+/-}$) mice showed enlarged eyes similar to the buphthalmia detected in human congenital glaucoma with elevation of intraocular pressure (IOP). Actually, IOP was elevated in $Flt1^{+/-}$; $Flk1^{+/-}$ mice and also in Flt1 or Flk1 single heterozygous mice. However, none of these mutants showed hallmarks of glaucoma such as ganglion cell death and excavation of optic disc. To clarify the pathological causes for enlarged eyes and elevated IOP, we investigate the mice from matings between $Flt1^{+/-}$ and $Flk1^{+/-}$ mice. $Flt1^{+/-}$ mice showed enlarged Schlemm's canal and disordered collagen fibers in the sclera, whereas $Flk1^{+/-}$ mice showed atrophied choriocapillaris in the choroid. These tissues are a part of the main outflow and alternative uveoscleral outflow pathway of the aqueous humor, suggesting that these pathological changes found in $Flt1^{+/-}$ and $Flk1^{+/-}$ mice are associated with the buphthalmia in $Flt1^{+/-}$; $Flk1^{+/-}$ mice.

Keywords

Buphthalmia; Development; Endothelial cell; Intraocular pressure; Mouse; VEGF

1. Introduction

Flt1 and Flk1 are receptor tyrosine kinases for vascular endothelial growth factor-A （VEGF-A） which is a key regulator of physiological and pathological angiogenesis, and of vascular permeability [1]. Gene-targeting studies established a critical but distinct role for these two receptors in vascular development. Flk1 signals are essential for the development of endothelial cells (ECs) and hematopoietic cells during embryogenesis in a cell autonomous manner [2, 3], whereas Flt1 is essential for suppressing the overpopulation of ECs as a decoy receptor for the VEGF-A agonist rather than a signaling receptor in ECs [4, 5]. High levels of VEGF-A expression was especially shown in epithelial tissues adjacent to fenestrated capillary vessels. In the adult eye, soluble isoform of VEGF-A protein secreted by retinal pigment epithelium (RPE) plays an essential role in the maintenance of choriocapillaris (CC) whose ECs are fenestrated [6-8]. On the other hand, VEGF-A is involved in the onset and progression of diabetic retinopathy and exudative age-related macular degeneration (eAMD) by inducing an abnormal neovascularization in the eye [9]. Indeed, VEGF-blocking antibody, ranibizumab has been developed as an effective drug for eAMD [10].

Intraocular pressure (IOP) is an important clinical index since its elevation causes glaucoma or myopia in humans. IOP is regulated by a balance between the production and

excretion of the aqueous humor in the eye. The main outflow pathway of the aqueous humor is composed of chamber angle, trabecular meshwork, and Schlemm's canal, whereas the alternative uveoscleral outflow pathway has also been reported [11]. IOP is thus elevated by an increase in accumulation of extracellular materials in the trabecular and uveoscleral outflow [12]. In the mouse, DBA2/J mouse strain [13, 14], Vav2/Vav3-deficient mice [15], and transgenic mice with ocular overexpression of calcitonin receptor-like receptor [16] have been reported to date as genetic mouse models of the elevation of IOP caused by closure of the angle between the cornea and iris in the anterior chamber. On the other hand, mice with a targeted type I collagen mutation [17] and Myocilin transgenic mice [18] have been reported as those with open angle in which detailed mechanisms of impaired outflow pathway of aqueous humor remain to be elucidated.

In this study, we aimed to analyze genetic interaction between the *Flt1* and *Flk1* genes and investigated ocular phenotypes in Flt1; Flk1 double heterozygous ($Flt1^{+/-}$; $Flk1^{+/-}$) mice, Flt1 or Flk1 single heterozygous ($Flt1^{+/-}$ or $Flk1^{+/-}$) mice, compared with wild-type (WT) mice.

2. Material and Methods

2.1. Mice

A colony of outbred mice heterozygous for a null allele of Flt1 [4] or Flk1 [2, 19] was maintained for these studies. Heterozygous males were crossed to ICR (Japan SLC, Hamamatsu, Japan) random outbred females to generate stock. Mice were genotyped by polymerase chain reaction analysis of ear DNA. Eyes from adult mice were dissected at 3 months of age or older. Animal experiments were approved by the Institutional Animal Care and Use Committee of Kobe University Graduate School of Medicine and Keio University School of Medicine, and carried out in accordance with the animal experimentation guidelines of the Kobe University Graduate School of Medicine and Keio University School of Medicine.

2.2. Measurement of IOP

Every month from 2 to 12 months of age, IOP was measured five times per eye between 1 pm to 3 pm with TonoLab Tonometer (icare Finland) while mice were under sedation within 3-12 min after intraperitoneal injection of ketamine (100 mg/kg) and xylazine (9 mg/kg).

Maximal and minimal values were excluded, and intermediate three values were used for the data analysis.

2.3. X-gal staining of the eye

Eyes were fixed in 0.1 M sodium phosphate (pH7.3) containing 2% paraformaldehyde (PFA), 0.05% glutaraldehyde, and 2 mM magnesium chloride at room temperature (RT) for 30 min. The eyes were perforated and further fixed in the same fixative at RT for 1 h. Whole-mount eyes were stained with X-gal as described previously [20], sectioned in paraffin, and counterstained with nuclear fast red (Vector).

2.4. Immunohistochemistry

We used rat anti-PECAM-1 (clone Mec13.3, BD Pharmingen), rat anti-endoglin (clone MJ7/18, eBioscience), and rabbit anti-LYVE-1 (RELIATech) primary antibodies, and Alexa Fluor 488-, Cy3-, or Cy5-conjugated secondary antibodies (Invitrogen and Jackson ImmunoResearch).

Eyes were fixed in PBS containing 1% PFA at 4 ˚C for 30 min. The eyes were perforated

and further fixed in the same fixative at 4 ˚C for 2 h. The eye was dissected into three regions; the anterior part consisting of the cornea, iris, and ciliary body, the posterior part consisting of sclera and choroid, and the retina, and further fixed in the same fixative at 4 ˚C for 2 h. The rest of procedure for immunostaining was described previously [21].

2.5. Electron microscopic analysis

Eyes were fixed in 0.1 M phosphate buffer containing 2.5% glutaraldehyde at RT for overnight. The eyes were further fixed in 0.1 M phosphate buffer containing 1% osmium tetroxide at RT for 2 h, dehydrated in ethanol, and embedded in epoxy resin after substituting for n-butyl glycidyl ether. Ultrathin sections were treated with uranyl acetate and lead citrate, and visualized on transmission electron microscopy with Hitachi H-7600 (Hitachi High-Technologies).

3. Results

3.1. Enlarged eyes and elevated IOP in Flt1$^{+/-}$; Flk1$^{+/-}$ mice

To study genetic interaction between the Flt1 and Flk1 genes, we crossed between Flt1$^{+/-}$ and Flk1$^{+/-}$ mice to analyze Flt1$^{+/-}$; Flk1$^{+/-}$ mice. Although none of wild-type (WT), Flt1 or Flk1 single heterozygous (Flt1$^{+/-}$ or Flk1$^{+/-}$) mice showed overt phenotypes (n = 33, n = 32, and n = 39 at 4 months old, respectively), about 24% of Flt1$^{+/-}$; Flk1$^{+/-}$ mice (n = 41, at 4 months old) showed the enlargement of eyes (Fig. 1 A). Since this phenotype is similar to the buphthalmia which is detected in human congenital glaucoma with elevation of IOP, we measured IOP from 2 to 12 months of age for comparison among WT, Flt1$^{+/-}$, Flk1$^{+/-}$ and Flt1$^{+/-}$; Flk1$^{+/-}$ mice. Compared with WT mice, Flt1$^{+/-}$; Flk1$^{+/-}$ mice showed an elevated average IOP by 2.2 mmHg. Flt1$^{+/-}$; Flk1$^{+/-}$ mice with the buphthalmia (n = 3) showed a higher average IOP than those without the enlarged eyes (n = 15) by 3.5 mmHg at 4 months of age. In addition, Flt1$^{+/-}$ and Flk1$^{+/-}$ mice showed elevated average IOPs by 1.8 mmHg and 1.3 mmHg, respectively (Fig. 1 B), although these single heterozygous mutant mice did not exhibit the buphthalmia. Elevation of IOP causes hallmarks of glaucoma such as ganglion cell death and excavation of optic disc.

To investigate whether these pathological changes were detected in these mutant mice, we performed TdT-mediated dUTP Nick-End Labeling (TUNEL) staining in paraffin sections of the retina. We did not detect TUNEL-positive apoptotic cells or excavation of the optic disc in any of analyzed mutant mice (Supplementary Fig. S1). These results indicate that Flt1$^{+/-}$; Flk1$^{+/-}$ mice showed the buphthalmia and elevation of IOP without hallmarks of glaucoma.

3.2. Expression patterns of Flt1 and Flk1 in the adult eye

To perform histopathological analysis in Flt1$^{+/-}$; Flk1$^{+/-}$ mice, we first investigated the expression pattern of Flt1 and Flk1 in the adult eye by detecting a lacZ reporter knocked into the *Flt1* or *Flk1* locus [2, 4]. X-gal staining of heterozygous mice showed that Flt1 and Flk1 both were detected in endothelial cells of the Schlemm's canal, corneal limbus, ciliary body, iris, and choroid (Fig. 2A-D). However, only Flt1 expression was detected in the retinal vasculature (Fig. 2C, D). In addition to endothelial expression, Flk1 was also detected in the stromal cells and endothelial cells in the cornea, and neural cells in the retina (Fig. 2E, F).

3.3. Enlarged Schlemm's canal and disordered collagen fibers of the sclera in Flt1 heterozygous mice

IOP is regulated by a balance between the production and excretion of the aqueous humor in the eye. The main outflow pathway of the aqueous humor is composed of the chamber angle, trabecular meshwork, and Schlemm's canal. In some genetic glaucoma mice, the elevation of IOP results from the maldevelopment of the outflow tissue, including the closure of the chamber angle, thickened trabecular meshwork, and absence of Schlemm's canal [13-16]. We first performed histological analysis of this main outflow pathway. In sections stained with hematoxylin and eosin or transmission electron micrograms, we did not find an occlusion of chamber angle or an abnormal deposition of extracellular matrix in the trabecular meshwork of enlarged $Flt1^{+/-}$; $Flk1^{+/-}$ eyes (Supplementary Fig. S2). We next investigate the vascular network of the outflow pathway including the Schlemm's canal and corneal limbal blood vessels by whole-mount confocal microscopy of the cornea, iris, and ciliary body with antibodies to a pan-endothelial marker PECAM-1 and a lymphatic marker LYVE-1. Compared with WT and $Flk1^{+/-}$ mice, we found that the Schlemm's canal was enlarged in $Flt1^{+/-}$ and $Flt1^{+/-}$; $Flk1^{+/-}$ mice (Fig. 3A). In different optical sections, we found that collector channels connecting with the corneal limbal blood vessels were comparable among WT, $Flt1^{+/-}$, $Flk1^{+/-}$, and $Flt1^{+/-}$; $Flk1^{+/-}$ mice (Fig. 3B, arrowheads). Vascular pattern of blood vessels and lymphatic vessels in the corneal limbus were also comparable (Fig. 3C). Although

our previous study showed that soluble Flk1 plays an important role in inhibiting lymphatic vessel growth into the cornea [22], we did not detect abnormal distribution of lymphatic vessels in the cornea of $Flk1^{+/-}$ mice, indicating that half amount of Flk1 protein is enough for this inhibition of lymphatic growth. These results indicated that Flt1 heterozygosity is associated with enlarged Schlemm's canal.

The sclera contains a plenty amount of collagen fibers and elastic fibers which mechanically support the construction of eyes [23]. To investigate these mechanical fibers in enlarged eyes of $Flt1^{+/-}$; $Flk1^{+/-}$ mice, we performed histological analysis of the sclera. Transmission electron microscopy showed that collagen fibers in the sclera of WT mice and $Flk1^{+/-}$ mice were uniform in size, whereas various sizes and higher density of collagen fibers were detected in the sclera of Flt1 heterozygous mice (Fig. 3D). These results indicated that Flt1 heterozygosity is associated with disordered collagen fibers in the sclera.

3.4. Atrophied CC in Flk1 heterozygous mice

VEGF-A derived from the RPE plays a crucial role in the maintenance of the CC [7] and its receptors, Flt1 and Flk1 both are strongly expressed in CC (Fig. 2C, D). To investigate the vascular structure of CC, we performed whole-mount confocal microscopy of the choroid with

antibody to endoglin which labels the CC. In WT and $Flt1^{+/-}$ mice, a typical dense honeycomb vascular network of the CC was detected, whereas the CC was atrophied with more avascular areas in $Flk1^{+/-}$ and $Flt1^{+/-}$; $Flk1^{+/-}$ mice (Fig. 4). The reduced vascular density was observed in both the central and the peripheral choroidal vasculature. To investigate whether the atrophy of CC affect the RPE, we used Phalloidin to label the cortical actin of RPE. Despite abnormal CC patterns in $Flk1^{+/-}$ and $Flt1^{+/-}$; $Flk1^{+/-}$ mice, the organization of the RPE was intact (Supplementary Fig. S3). The vasculature in the retina and iris appeared unaffected in these mutant mice (Supplementary Fig. S4). These results indicated that Flk1 heterozygosity is associated with atrophied CC.

4. Discussion

In this study, we showed enlarged eyes in Flt1$^{+/-}$; Flk1$^{+/-}$ mice. Compared with WT mice, IOP was elevated in Flt1$^{+/-}$, Flk1$^{+/-}$, and Flt1$^{+/-}$; Flk1$^{+/-}$ mice. However, none of them showed hallmarks of glaucoma such as ganglion cell death and excavation of optic disc. Flt1 heterozygosity is associated with enlarged Schlemm's canal and disordered collagen fibers in the sclera, whereas Flk1 heterozygosity is associated with atrophied CC.

Enlarged eyes in Flt1$^{+/-}$; Flk1$^{+/-}$ mice were similar to the buphthalmia which is detected in human congenital glaucoma with elevation of IOP. Indeed, Flt1$^{+/-}$; Flk1$^{+/-}$ mice showed the elevation of IOP, but they did not show hallmarks of glaucoma in the retina. Of note is that average IOPs in Flt1$^{+/-}$; Flk1$^{+/-}$ mice went up and down throughout adulthood although they were constantly higher than those in WT mice by about 1.5 mmHg (Fig. 1B). Previous studies regarding mice with eye projection and hallmarks of glaucoma showed that IOP was more severely elevated [15, 16]. It is thus likely that gentle increase in IOP did not induce pathological changes in retinal neural cells. We also detected the elevation of IOP in Flt1$^{+/-}$, and Flk1$^{+/-}$ mice, although they did not exhibit the buphthalmia. Taken together, ocular phenotypes in Flt1$^{+/-}$; Flk1$^{+/-}$ mice were caused by combined effects of Flt1 and Flk1 heterozygosity.

In Flt1 heterozygous mice, we found the enlargement of the Schlemm's canal, suggesting obstruction of the main out flow pathway of the aqueous humor. However, corneal limbal blood vessels and their collector channels with the Schlemm's canal appeared unaffected. Endothelial cells in the Schlemm's canal express many blood endothelial markers [24] and our expression analysis also detected the expression of Flt1 and Flk1. Since Flt1 works as a negative regulator for endothelial cell growth, Schlemm's canal may be enlarged directly by excess VEGF-A protein in Flt1 heterozygous mice. We also found the disorder of collagen fibers in Flt1 heterozygous sclera. Although it remains to be elucidated how Flt1 heterozygosity affects the organization of collagen fibers in the sclera, this is probably a direct result from reduced function of Flt1 rather than from mechanical stress induced by elevated IOP since $Flk1^{+/-}$ mice with elevated IOP show a normal arrangement of collagen fibers in the sclera. In mice with a targeted type I collagen mutation leading to age-dependent collagen accumulation, the age-related elevation of IOP with open anterior chamber angle was reported [17]. Since the sclera is a part of alternative uveoscleral outflow pathway of the aqueous humor, the quality and quantity of collagen fibers in the sclera may affect IOP. Taken together, disordered collagen fibers in Flt1 heterozygous sclera may result in the elevation of IOP.

In Flk1 heterozygous mice, we found the atrophy of the CC, suggesting a change of the

blood flow in the choroid and optical blood vessels. This is probably a direct result from reduced function of Flk1 since VEGF-A/Flk1 signals play an important role in maintenance of the CC [7]. A previous study using a fluorescent tracer suggested that the CC is a part of uveoscleral outflow pathway of the aqueous humor [11], suggesting that atrophied CC in Flk1 heterozygous choroid results in the elevation of IOP.

Our histopathological analysis still left some open questions regarding the relationship between vascular phenotypes, eye ball morphology, and regulation of IOP. It is tempting to speculate that, in $Flt1^{+/-}$; $Flk1^{+/-}$ mice, the eye projection by getting collagen fibers loosen in the sclera compensates for increased IOP by vascular defects to keep intact neural tissues in the retina. This study is the first report that $Flt1^{+/-}$; $Flk1^{+/-}$ mice showed the buphthalmia in mice.

Acknowledgments

We thank Dr. Janet Rossant for mutant mice for the *Flt1* and *Flk1* gene; Drs. Akiyoshi Uemura, Yoko Fukushima, Kazuichi Maruyama, and Yoichi Kurebayashi for helpful discussion; and Drs. Noriko Odani, Katsumi Oomachi, Tohru Shibata, and Takashi Yamanouchi for technical supports. This study was supported in part by Grant-in-Aid for JSPS Fellows (K.S.), Grant-in-Aid for Scientific Research (C) (M.H.), the Uehara Memorial Foundation (M.H.), and the Global COE Program "Global Center for Education and Research in Integrative Membrane Biology" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Figure Legends

Fig. 1. Appearance of eyes and measurement of IOP. (A) Appearance of eyes is shown from 9-month-old mice whose genotypes are indicated on the top. Enlarged eyes similar to the buphthalmia were detected only in $Flt1^{+/-}$; $Flk1^{+/-}$ mice. (B) IOP was measured from 2 to 12 months of age. $Flt1^{+/-}$, $Flk1^{+/-}$ and $Flt1^{+/-}$; $Flk1^{+/-}$ mice constantly showed elevated average IOPs, compared with WT mice. Data represent the mean ± S. E. M. of 5 - 20 eyes. Significant difference, * $P < 0.05$, ** $P < 0.01$ versus WT by Student's t-test.

Fig. 2. Expression patterns of Flt1 and Flk1 in the adult eye. Expression analysis was performed by detecting a lacZ reporter knocked into the *Flt1* or *Flk1* locus. (A-D) Flt1 and Flk1 both were expressed in endothelial cells of the Schlemm's canal (arrowhead), corneal limbus, ciliary body (cb), iris (ir), and choroid (ch). (C, D) Flt1 but not Flk1 was expressed in the retinal vasculature. Flk1 is expressed by retinal neural cells in inner nuclear layer (inl) and outer nuclear layer (onl). (E, F) Flk1 but not Flt1 was expressed in the corneal stroma (cs) and corneal endothelium (cen). cep, corneal epithelium; co, cornea; gcl, ganglion cell layer; rpe, retinal pigment epithelium; sc, sclera. Scale bars, 250 μm.

Fig. 3. Enlarged Schlemm's canal and disordered collagen fibers of the sclera in Flt1 heterozygous mice. (A-C) Whole-mount confocal microscopy of the cornea, iris, and ciliary body was performed with antibodies to a pan-endothelial marker PECAM-1 (green) and a lymphatic marker LYVE-1 (red). (A) Enlarged Schlemm's canal (asterisks) was detected in $Flt1^{+/-}$ and $Flt1^{+/-}$; $Flk1^{+/-}$ mice. (B) In different optical sections, collector channels (arrowheads) connecting with the corneal limbal blood vessels were comparable among WT, $Flt1^{+/-}$, $Flk1^{+/-}$, and $Flt1^{+/-}$; $Flk1^{+/-}$ mice. (C) Vascular pattern of blood vessels and $LYVE\text{-}1^{+}$ lymphatic vessels in the corneal limbus were also comparable. Scale bars, 250μm (D) Transmission electron microscopy of the sclera shows that collagen fibers in the sclera of WT mice and $Flk1^{+/-}$ mice were uniform in size, whereas various sizes and higher density of collagen fibers were detected in the sclera of $Flt1^{+/-}$ and $Flt1^{+/-}$; $Flk1^{+/-}$ mice. Scale bars, 2μm.

Fig. 4. Atrophied CC in Flk1 heterozygous mice. Whole-mount confocal microscopy of the choroid was performed with antibody to endoglin. A typical dense honeycomb vascular network of the CC was detected in WT and $Flt1^{+/-}$ mice, whereas the CC was atrophied with more avascular areas in both the central and the peripheral choroidal vasculature of $Flk1^{+/-}$ and $Flt1^{+/-}$; $Flk1^{+/-}$ mice. Scale bars, 100μm.

References

[1] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, Nat. Med. 9 (2003) 669-676.

[2] F. Shalaby, J. Ho, W.L. Stanford, K.D. Fischer, A.C. Schuh, L. Schwartz, A. Bernstein, J. Rossant, A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis, Cell 89 (1997) 981-990.

[3] Y. Sakurai, K. Ohgimoto, Y. Kataoka, N. Yoshida, M. Shibuya, Essential role of Flk-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 1076-1081.

[4] G.H. Fong, J. Rossant, M. Gertsenstein, M.L. Breitman, Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium, Nature 376 (1995) 66-70.

[5] S. Hiratsuka, O. Minowa, J. Kuno, T. Noda, M. Shibuya, Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 9349-9354.

[6] A.S. Maharaj, M. Saint-Geniez, A.E. Maldonado, P.A. D'Amore, Vascular endothelial growth factor localization in the adult, Am. J. Pathol. 168 (2006) 639-648.

[7] M. Saint-Geniez, T. Kurihara, E. Sekiyama, A.E. Maldonado, P.A. D'Amore, An essential role for RPE-derived soluble VEGF in the maintenance of the choriocapillaris, Proc. Natl. Acad. Sci. U.S.A. 106 (2009) 18751-18756.

[8] M. Saint-Geniez, A.E. Maldonado, P.A. D'Amore, VEGF expression and receptor activation in the choroid during development and in the adult, Invest. Ophthalmol. Vis. Sci. 47 (2006) 3135-3142.

[9] A.N. Witmer, G.F. Vrensen, C.J. Van Noorden, R.O. Schlingemann, Vascular endothelial growth factors and angiogenesis in eye disease, Prog. Retin. Eye Res. 22 (2003) 1-29.

[10] G.D. Yancopoulos, Clinical application of therapies targeting VEGF, Cell 143 (2010) 13-16.

[11] J.D. Lindsey, R.N. Weinreb, Identification of the mouse uveoscleral outflow pathway using fluorescent dextran, Invest. Ophthalmol. Vis. Sci. 43 (2002) 2201-2205.

[12] B.T. Gabelt, P.L. Kaufman, Changes in aqueous humor dynamics with age and glaucoma, Prog. Retin. Eye Res. 24 (2005) 612-637.

[13] S.W. John, R.S. Smith, O.V. Savinova, N.L. Hawes, B. Chang, D. Turnbull, M. Davisson, T.H. Roderick, J.R. Heckenlively, Essential iris atrophy, pigment dispersion, and glaucoma in DBA/2J mice, Invest. Ophthalmol. Vis. Sci. 39 (1998) 951-962.

[14] M. Scholz, T. Buder, S. Seeber, E. Adamek, C.M. Becker, E. Lutjen-Drecoll, Dependency

of intraocular pressure elevation and glaucomatous changes in DBA/2J and DBA/2J-Rj mice, Invest. Ophthalmol. Vis. Sci. 49 (2008) 613-621.

[15] K. Fujikawa, T. Iwata, K. Inoue, M. Akahori, H. Kadotani, M. Fukaya, M. Watanabe, Q. Chang, E.M. Barnett, W. Swat, VAV2 and VAV3 as candidate disease genes for spontaneous glaucoma in mice and humans, PloS one 5 (2010) e9050.

[16] L.M. Ittner, K. Schwerdtfeger, T.H. Kunz, R. Muff, K. Husmann, C. Grimm, F. Hafezi, K.S. Lang, M.O. Kurrer, J. Gotz, W. Born, J.A. Fischer, Transgenic mice with ocular overexpression of an adrenomedullin receptor reflect human acute angle-closure glaucoma, Clin. Sci. 114 (2008) 49-58.

[17] F. Mabuchi, J.D. Lindsey, M. Aihara, M.R. Mackey, R.N. Weinreb, Optic nerve damage in mice with a targeted type I collagen mutation, Invest. Ophthalmol. Vis. Sci. 45 (2004) 1841-1845.

[18] V. Senatorov, I. Malyukova, R. Fariss, E.F. Wawrousek, S. Swaminathan, S.K. Sharan, S. Tomarev, Expression of mutated mouse myocilin induces open-angle glaucoma in transgenic mice, J. Neurosci. 26 (2006) 11903-11914.

[19] M. Ema, S. Takahashi, J. Rossant, Deletion of the selection cassette, but not cis-acting elements, in targeted Flk1-lacZ allele reveals Flk1 expression in multipotent mesodermal progenitors, Blood 107 (2006) 111-117.

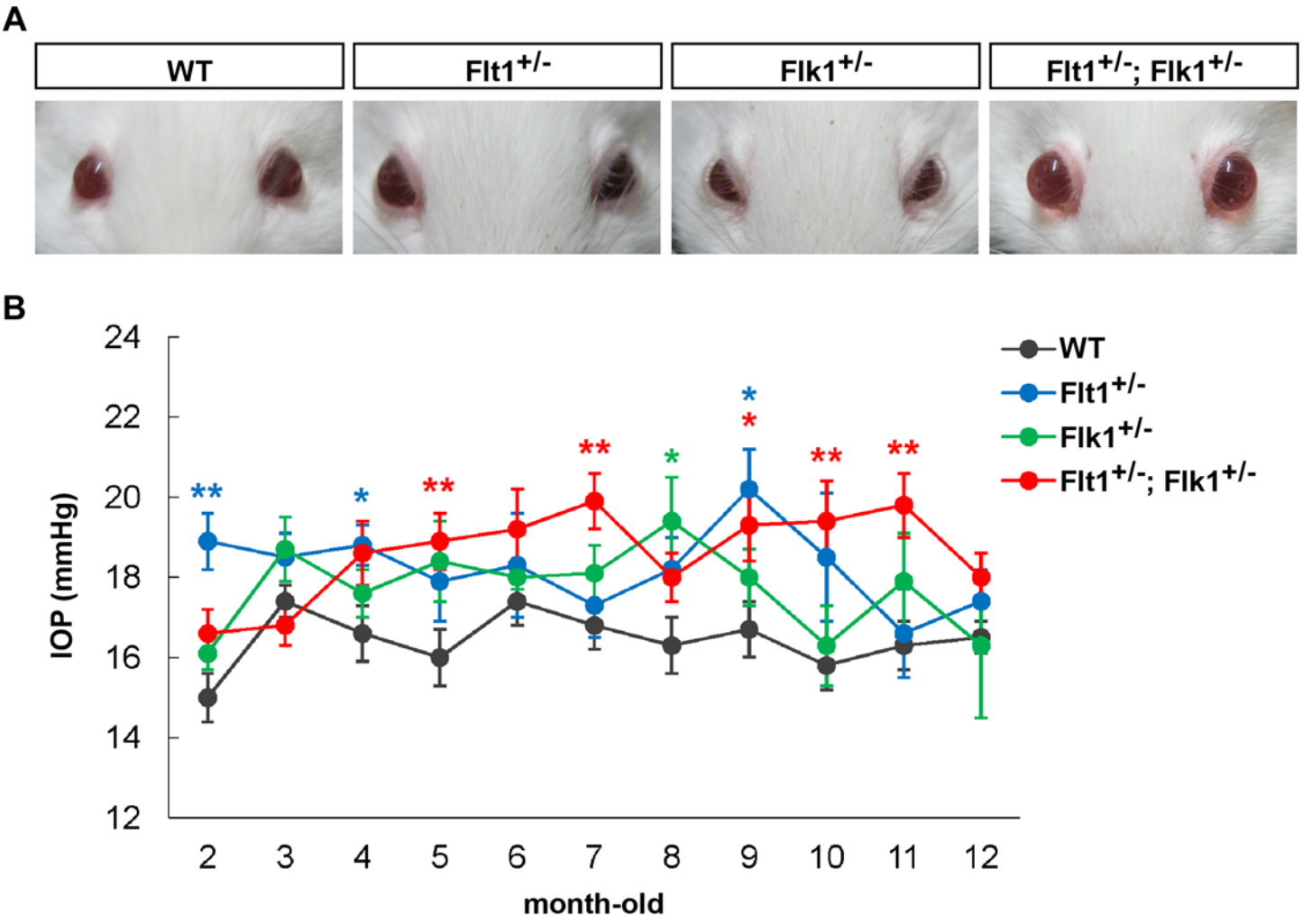
[20] M. Hirashima, Y. Lu, L. Byers, J. Rossant, Trophoblast expression of fms-like tyrosine kinase 1 is not required for the establishment of the maternal-fetal interface in the mouse placenta, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 15637-15642.

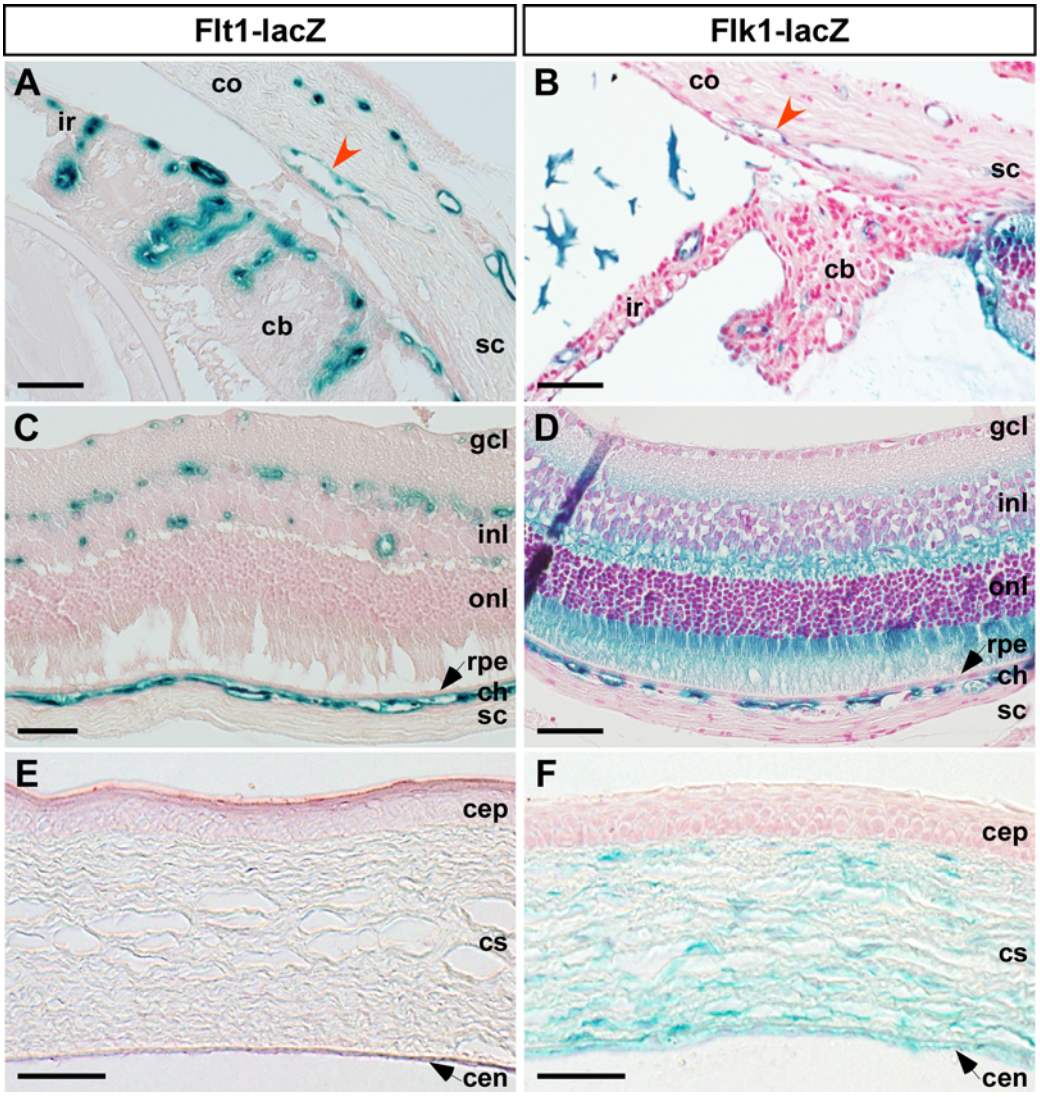
[21] M. Hirashima, K. Sano, T. Morisada, K. Murakami, J. Rossant, T. Suda, Lymphatic vessel assembly is impaired in Aspp1-deficient mouse embryos, Dev. Biol. 316 (2008) 149-159.

[22] R.J. Albuquerque, T. Hayashi, W.G. Cho, M.E. Kleinman, S. Dridi, A. Takeda, J.Z. Baffi, K. Yamada, H. Kaneko, M.G. Green, J. Chappell, J. Wilting, H.A. Weich, S. Yamagami, S. Amano, N. Mizuki, J.S. Alexander, M.L. Peterson, R.A. Brekken, M. Hirashima, S. Capoor, T. Usui, B.K. Ambati, J. Ambati, Alternatively spliced vascular endothelial growth factor receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth, Nat. Med. 15 (2009) 1023-1030.

[23] P.G. Watson, R.D. Young, Scleral structure, organisation and disease. A review, Exp. Eye Res. 78 (2004) 609-623.

[24] K.M. Perkumas, W.D. Stamer, Protein markers and differentiation in culture for Schlemm's canal endothelial cells, Exp. Eye Res. 96 (2012) 82-87.


A
WT
Flt1+/-
Flk1+/-
Flt1+/-; Flk1+/-
B
IOP (mmHg)
24
22
20
18
16
14
12
2
3
4
5
6
7
8
9
10
11
12
month-old
WT
Flt1+/-
Flk1+/-
Flt1+/-; Flk1+/-


Flt1-lacZ
Flk1-lacZ
A
co
ir
cb
sc
B
co
sc
cb
ir
C
gcl
inl
onl
rpe
ch
sc
D
gcl
inl
onl
rpe
ch
sc
E
cep
cs
cen
F
cep
cs
cen

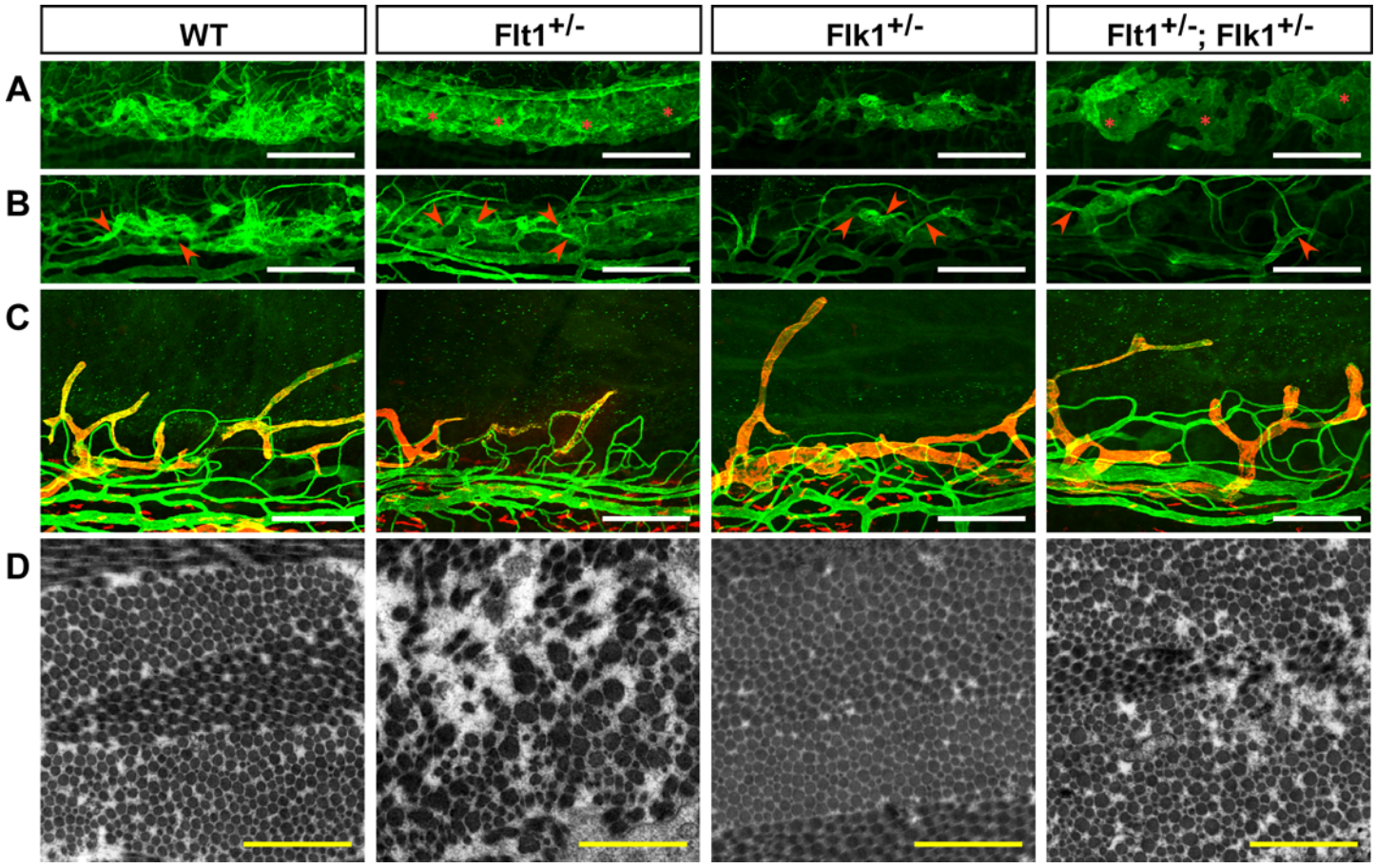
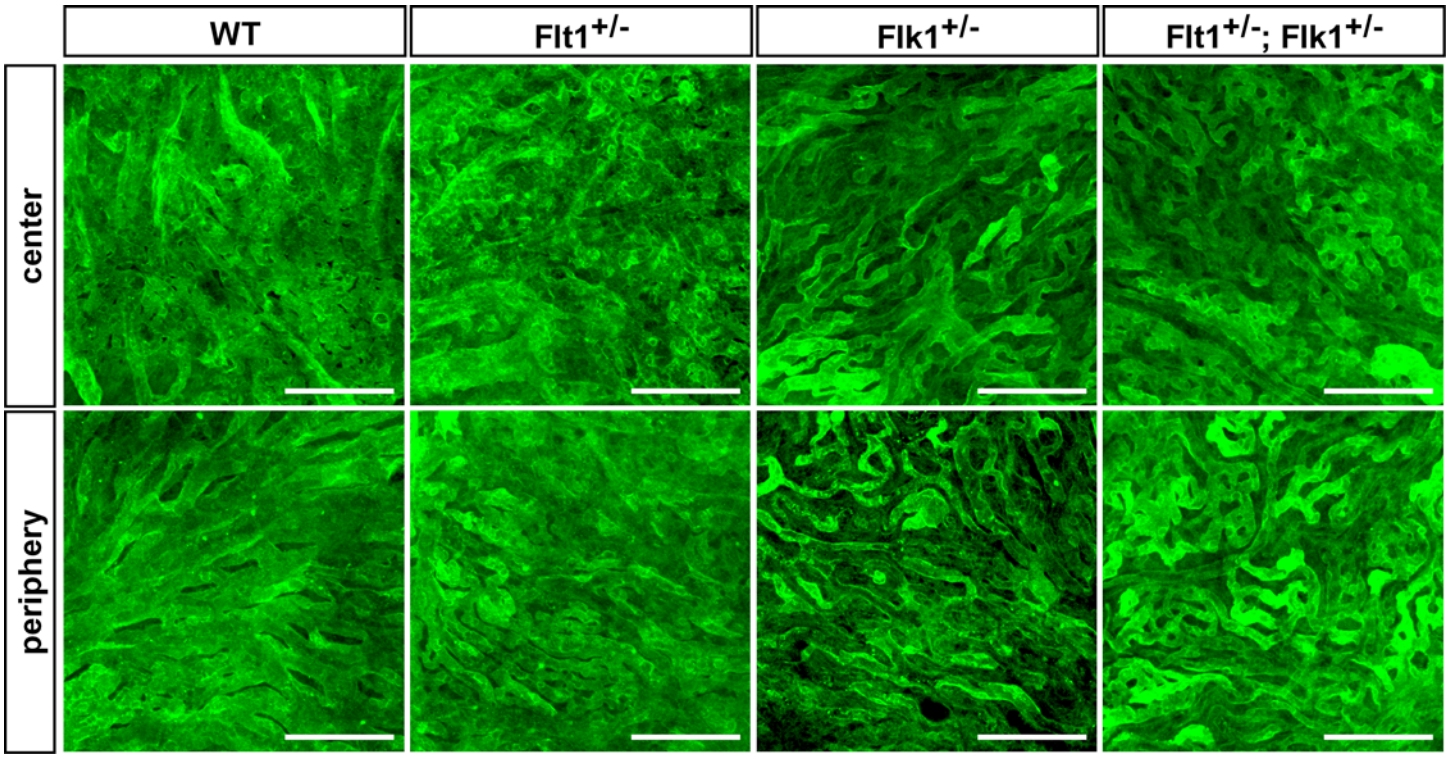

WT
Flt1+/-
Flk1+/-
Flt1+/-; Flk1+/-
A
B
C
D

Fig. 4



Supplementary Data for the manuscript

Flt1 and Flk1 mediate regulation of intraocular pressure and their double heterozygosity causes the buphthalmia in mice

Keigo Sano, Osamu Katsuta, Satoshi Shirae, Yoshiaki Kubota, Masatsugu Ema, Toshio Suda, Masatsugu Nakamura and Masanori Hirashima

Supplementary Material and Methods

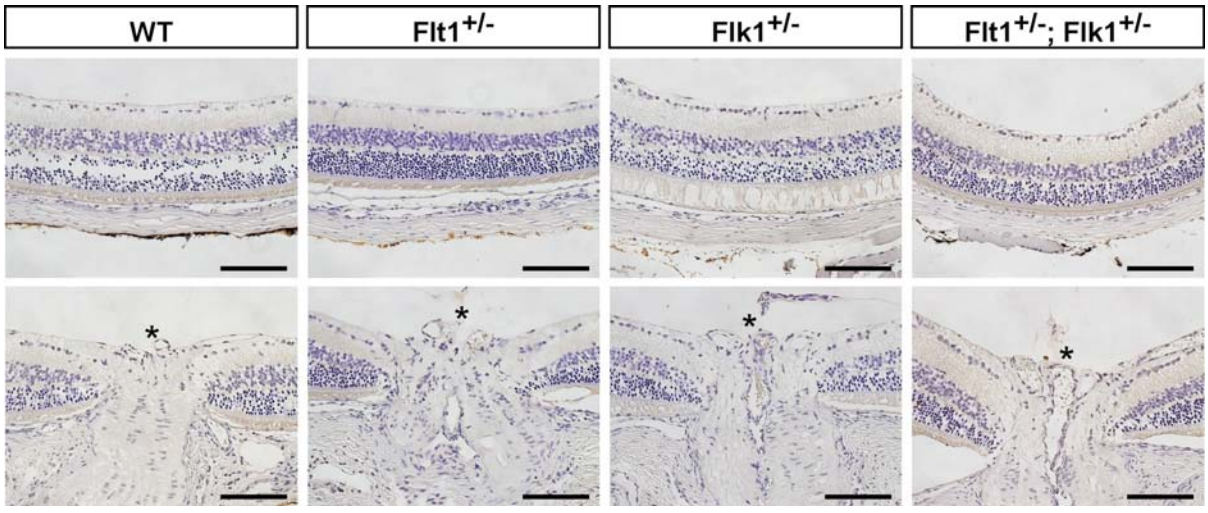
Apoptosis assay

Eyes were fixed in PBS containing 3.7% formaldehyde at 4˚C for overnight. The eyes were dehydrated in ethanol, and embedded in paraffin after substituting for xylene. TdT-mediated dUTP Nick-End Labeling (TUNEL) staining of paraffin sections was performed with In Situ Cell Death Detection Kit POD (Roche Applied Science) and 3,3'-diaminobenzidine (Dojindo) as a substrate, according to the manufacturer's instructions.

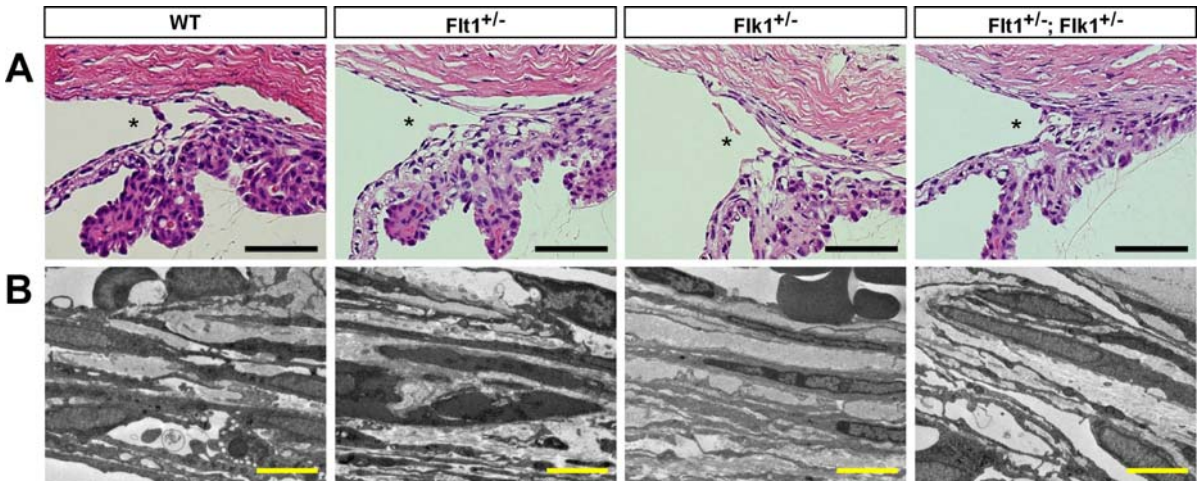
Immunohistochemistry

We used rat anti-PECAM-1 (clone Mec13.3, BD Pharmingen), rat anti-endoglin (clone MJ7/18, eBioscience), Cy3-conjugated mouse anti-α-smooth muscle actin (clone 1A4, Sigma), Alexa Fluor 488-conjugated secondary antibodies (Invitrogen), and Alexa Fluor 546-conjugated Phalloidin (Invitrogen). The rest of procedure for dissection and immunostaining is described in the manuscript.

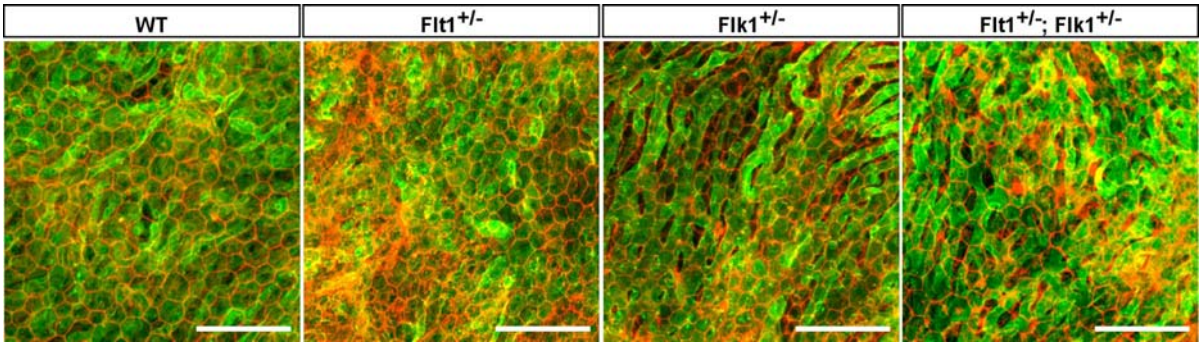
Supplementary Results



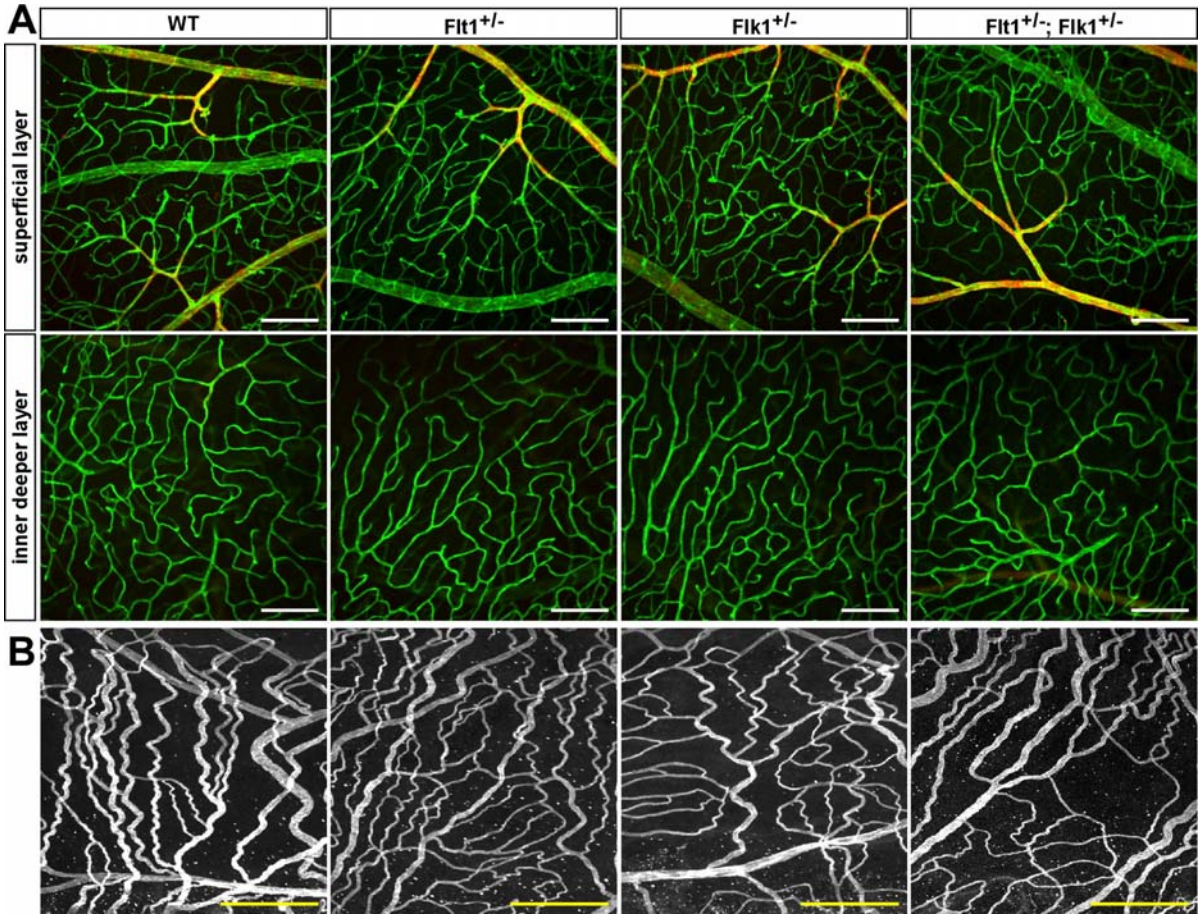
Supplementary Fig. S1. TUNEL staining in the retina. Apoptosis assay was performed in sections by TUNEL staining, followed by counterstaining with hematoxylin. Increased TUNEL-positive apoptotic cells were not detected in any mice whose genotypes are indicated on the top. Excavation of the optic disc (asterisk) was not, either. Scale bars, 500μm.



Supplementary Fig. S2. Histological analysis of the main outflow pathway of the aqueous humor. (A) In sections stained with hematoxylin and eosin, the chamber angle (asterisk) is open. Scale bars, 250μm. (B) In transmission electron micrographs, an abnormal deposition of extracellular matrix was not detected in the trabecular meshwork of enlarged $Flt1^{+/-}$; $Flk1^{+/-}$ eyes. Scale bars, 4μm.



Supplementary Fig. S3. Histological analysis of the CC and RPE. Confocal microscopy of flat-mount tissues composed of the choroid and RPE with antibody to endoglin (green) and phalloidin (red) which label the CC and the cortical actin of RPE, respectively. The organization of the RPE was not affected. Scale bars, 100μm.



Supplementary Fig. S4. Vascular analysis in the retina and iris. (A) Whole-mount confocal microscopy of the retina was performed with antibodies to PECAM-1 (green) and α-smooth muscle actin (red). (B) Whole-mount confocal microscopy of the iris was performed with antibody to PECAM-1. Blood vessels in these tissues were comparable among WT, $Flt1^{+/-}$, $Flk1^{+/-}$, and $Flt1^{+/-}$; $Flk1^{+/-}$ mice. Scale bars, 250μm.