Impaired angiopoietin/Tie2 signaling compromises Schlemm’s canal integrity and induces glaucoma

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Primary open-angle glaucoma (POAG) is often caused by elevated intraocular pressure (IOP), which arises due to increased resistance to aqueous humor outflow (AHO). Aqueous humor flows through Schlemm’s canal (SC), a lymphatic-like vessel encircling the cornea, and via intercellular spaces of ciliary muscle cells. However, the mechanisms underlying increased AHO resistance are poorly understood. Here, we demonstrate that signaling between angiopoietin (Angpt) and the Angpt receptor Tie2, which is critical for SC formation, is also indispensable for maintaining SC integrity during adulthood. Deletion of Angpt1/Angpt2 or Tie2 in adult mice severely impaired SC integrity and transcytosis, leading to elevated IOP, retinal neuron damage, and impairment of retinal ganglion cell function, all hallmarks of POAG in humans. We found that SC integrity is maintained by interconnected and coordinated functions of Angpt-Tie2 signaling, AHO, and Prox1 activity. These functions diminish in the SC during aging, leading to impaired integrity and transcytosis. Intriguingly, Tie2 reactivation using a Tie2 agonistic antibody rescued the POAG phenotype in Angpt1/Angpt2-deficient mice and rejuvenated the SC in aged mice. These results indicate that the Angpt-Tie2 system is essential for SC integrity. The impairment of this system underlies POAG-associated pathogenesis, supporting the possibility that Tie2 agonists could be a therapeutic option for glaucoma.

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Primary open-angle glaucoma (POAG) is often caused by elevated intraocular pressure (IOP), which arises due to increased resistance to aqueous humor outflow (AHO). Aqueous humor flows through Schlemm’s canal (SC), a lymphatic-like vessel encircling the cornea, and via intercellular spaces of ciliary muscle cells. However, the mechanisms underlying increased AHO resistance are poorly understood. Here, we demonstrate that signaling between angiopoietin (Angpt) and the Angpt receptor Tie2, which is critical for SC formation, is also indispensable for maintaining SC integrity during adulthood. Deletion of Angpt1/Angpt2 or Tie2 in adult mice severely impaired SC integrity and transcytosis, leading to elevated IOP, retinal neuron damage, and impairment of retinal ganglion cell function, all hallmarks of POAG in humans. We found that SC integrity is maintained by interconnected and coordinated functions of Angpt-Tie2 signaling, AHO, and Prox1 activity. These functions diminish in the SC during aging, leading to impaired integrity and transcytosis. Intriguingly, Tie2 reactivation using a Tie2 agonistic antibody rescued the POAG phenotype in Angpt1/Angpt2-deficient mice and rejuvenated the SC in aged mice. These results indicate that the Angpt-Tie2 system is essential for SC integrity. The impairment of this system underlies POAG-associated pathogenesis, supporting the possibility that Tie2 agonists could be a therapeutic option for glaucoma.
The angiopoietin-Tie2 (Angpt-Tie2) pathway controls vascular maturation and stabilization (18–20). Angpt1 acts as an agonist to Tie2, while Angpt2 acts as an agonist and an antagonist to Tie2 in a context-dependent manner (18). Prenatal deletion of Angpt1 in mice leads to impairment in cardiovascular development (21), and prenatal deletion or inhibition of Angpt2 in mice causes defects in lymphatic maturation (22–24). Prenatal deletion of Tie2 also leads to severe impairments in cardiovascular development, including vein specification and venogenesis (18, 25). Regarding the SC, Quaggin and colleagues elegantly showed that the Angpt-Tie2 system is involved in SC formation, as proven with postnatal double Angpt1/Angpt2-deleted or Tie2-deleted mice that exhibited primary congenital glaucoma (26). Moreover, Tie2 mutations have been
identifies in patients with primary congenital glaucoma (27). Considering these exciting findings, we decided to evaluate whether the Angpt-Tie2 system is also critically required for maintenance of SC integrity during adulthood and, if so, what the underlying mechanism is. Here, we show that Angpt-Tie2 signaling is indispensably required for maintaining adult SC integrity by preserving normal AHO and Prox1 expression, as inactivation of this pathway during adulthood causes POAG-like phenotypes. Finally, Tie2 reactivation rescues the phenotypes of POAG in double Angpt1/Angpt2-deleted mice and rejuvenates the SC in aged mice.

Results

Tie2 expression precedes Prox1 expression in the SC. To gain insight about the role of Tie2 signaling in SC formation, we first examined its normal expression in WT C57BL/6J mice. Tie2 and phosphorylated Tie2 at Tyr992 (hereafter named p-Tie2) were expressed in SC progenitors originating from the choroidal veins at P1, and their expression increased further during SC development (Figure 1, A and B). Expression of Prox1 and Klf4, key shear stress-responsive transcription factors (28, 29), became obvious at P7 and continued increasing, and VE-cadherin+ EC junctions started to become distinct (Figure 1, A and B). As the SC differentiated, EC shapes converted from cuboidal to longitudinal (Figure 1, A and B), changes that appeared to correlate with gain of predicted AHO and lymphatic phenotypes (8). Thus, Tie2 expression started prior to that of Prox1 in the ECs of the SC before an acquisition of AHO.

Consistently, Tie2 and Prox1 were also highly detected in adult human SCs (Figure 2A). Cross-section images of the SC in Tie2-GFP and Prox1-GFP mice (30) revealed that Tie2 and Prox1 expression were higher in the ECs located in the inner SC wall (Figure 2B), the principal route of AHO (31). Variable-sized multiple giant vacuoles (GVs), which are outpouchings of SC ECs that bulge into the SC lumen and proportionally reflect AHOc levels (32), and abundant plasmalemma vesicle–associated protein (PLVAP), a marker for endothelial transcytosis and permeability, were detected in the ECs of the inner SC wall (Figure 2C), reflecting active transcellular transport of AH through the SC. Analyses using Angpt1-GFP mice (33) and Angpt2-lacZ mice (22) showed that Angpt1 was expressed in pericytes of SC and Angpt2 in corneal endothelium and TM (black arrowheads) in 2-month-old Angpt1-GFP and Angpt2-lacZ mice. Scale bars: 100 μm. (E) Diagram depicting the sources and distributions of secreted Angpt1 and Angpt2 for the formation and maintenance of Tie2+ SC. CB, ciliary body.

Figure 2. Tie2 in the inner SC wall is constantly exposed to Angpts. (A) Images showing PROX1 and TIE2 in healthy adult human SCs. Scale bars: 100 μm. (B) Cross-section images showing Prox1+ and Tie2+ SC ECs and distribution of collagen IV or PDGFRβ in corneal limbus of 2-month-old Prox1-GFP and Tie2-GFP mice. Dashed arrows indicate direction of AHO. Scale bars: 100 μm. (C) EM images of SC and distribution of PLVAP in the ECs of the inner SC walls in 2-month-old mice. Yellow-lined box is magnified in left lower panel. Blue arrowheads indicate GVs in the inner SC wall, and dashed arrow indicates the direction of AHO. Scale bars: 5 μm (upper panel); 100 μm (right lower panel). (D) Images showing expression of Angpt1 (white arrowheads) in pericytes of SC and Angpt2 in corneal endothelium and TM (black arrowheads) in 2-month-old Angpt1-GFP and Angpt2-lacZ mice. Scale bars: 100 μm. (E) Diagram depicting the sources and distributions of secreted Angpt1 and Angpt2 for the formation and maintenance of Tie2+ SC. CB, ciliary body.

Tie2 is required for SC development and maintenance. To determine the role of Tie2 in SC development, we generated a Tie2ΔEC mouse by crossing the VE-cadherin-Cre-ER^T2 mouse (34) with the Tie2ΔEC mouse (35). We then administered tamoxifen starting at P1 and analyzed the animals 2 months later (Figure 3A).
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ties at the cornea (Supplemental Figure 1, B, C, and E–G). Moreover, Prox1 and Klf4 were markedly reduced in SCs, although their levels were similar in the limbal LVs of $\text{Tie2}^{\Delta \text{EC}}$ mice compared with those of WT mice (Supplemental Figure 1, B and D), indicating that early Tie2 activation is definitely required for SC formation.

To examine the role of Tie2 in maintenance of SC integrity, we deleted Tie2 in the SC ECs by administering tamoxifen into 8-week-old $\text{Tie2}^{\Delta \text{EC}}$ mice and analyzing them 4 weeks later (Figure 5A). Unexpectedly, elevated IOP with a slightly but significantly elongated anterior-to-posterior segment ratio of the eyeball was detected in $\text{Tie2}^{\Delta \text{EC}}$ mice (Figure 5, B–D). Protein levels of Tie2, Prox1, and Klf4 and area and cellularity in SCs were also reduced in $\text{Tie2}^{\Delta \text{EC}}$ mice compared with those of WT mice (Figure 5, C and E–G). EM analysis revealed that the number and diameter of GVs were markedly decreased in SC ECs of $\text{Tie2}^{\Delta \text{EC}}$ mice (Figure 6, A–C), indicating inadequate AHO in their SCs. Moreover, thinned RNFL and markedly attenuated pSTR and PhNR were observed in $\text{Tie2}^{\Delta \text{EC}}$ mice (Figure 6, D–I), implying that an elevated IOP for a month led to retinal neuronal damage and partial visual impairment. In contrast, no apparent changes were detected in the vessels of other organs in $\text{Tie2}^{\Delta \text{EC}}$ mice (Supplemental Figure 2, A–E). Moreover, when we deleted Vegfr2 in 8-week-old $\text{Vegfr2}^{\Delta \text{EC}}$ mice, the mice showed no apparent changes in IOP and SCs 4 weeks later (Supplemental Figure 3, A–F) in spite of the reduced vascularity and number of fenes-

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**Figure 3. Tie2 is critical for SC generation.** (A) Diagram for EC-specific depletion of Tie2 in SC starting at P1 and analyses 8 weeks later using $\text{Tie2}^{\Delta \text{EC}}$ mice. (B–G) Images and comparisons of IOP, anterior (yellow double arrow)/posterior (white double arrow) (ant./post.) segment ratio of the eyeball, relative area, number of Erg+ ECs, and intensities of Tie2, Prox1, and Klf4 immunostaining in CD144+ SC. Dashed lines demarcate the margins of SC. Each yellow-lined image is magnified in the corner. Scale bars: 100 μm. SC area and expression of each molecule in WT mice are normalized to 100%, and their relative levels in Tie2ΔEC mice are presented. n = 4–5 for each group. *P < 0.05 versus WT by Mann-Whitney U test.
To assess the role of Angpt1 or Angpt2 in SC maintenance, we deleted each gene individually by tamoxifen administration into 8-week-old Angpt1iΔ/Δ or Angpt2iΔ/Δ mice and analyzed their SCs 4 weeks later (Supplemental Figure 5, A and G). No changes in IOP were found in either mouse strain (Supplemental Figure 5, B and H). We detected mildly reduced cellularity and levels of Prox1 and Klf4, but not Tie2, in the SCs of both mouse strains, while SC area was slightly reduced only in Angpt2iΔ/Δ mice (Supplemental Figure 5, C–F and I–L). These data imply that Angpt1 and Angpt2 play overlapping but somewhat different roles in the generation and maintenance of SC.

These findings led us to generate double Angpt1/ Angpt2-deficient (A1:A2Δ/Δ) mice. Compared with WT mice, SC formation was severely impaired together with markedly reduced Prox1, Klf4, Tie2, and p-Tie2 at P7 in the A1:A2Δ/Δ mice (Supplemental Figure 5, C–F and I–L). These data imply that Angpt1 and Angpt2 play overlapping but somewhat different roles in the generation and maintenance of SC.

Figure 4. Inadequate SC development by Tie2 depletion impairs AHO and retinal ganglion cell function. (A and B) EM images and comparison of the number of GVs (blue and black arrowheads) in the inner SC wall. Right panels depict illustrative views of GVs. Scale bars: 10 μm. n = 4 for each group. *P < 0.05 versus WT by Mann-Whitney U test. (C–E) Images and comparison of thickness (red arrowheads) and Tubb3 distribution of RNFL. Black-lined box is magnified in middle panel. INL, inner nuclear layer; ONL, outer nuclear layer; GCL, ganglion cell layer. Scale bars: 200 μm. (F–H) Representative wave responses of electroretinogram and comparisons of pSTR amplitude and PhNR/b-wave amplitude ratio. n = 4 for each group. *P < 0.05 versus WT by Mann-Whitney U test.
SCs compared with those of WT (Figure 7, A–G). Moreover, the A1:A2i Δ/Δ mice had scant GVs, thinned RNFL, and markedly attenuated pSTR and PhNR (Figure 8, A–H), results similar to those of a previous report (26). Of special note, when we deleted both Angpt1 and Angpt2 in 8-week-old A1:A2i Δ/Δ mice, the mice surprisingly had elevated IOP with a significantly elongated anterior-to-posterior segment ratio of the eyeball, markedly reduced area, cellularity, and protein levels of Prox1, Klf4, Tie2, and p-Tie2, and increased αSMA+ cells in SC compared with those of WT mice 4 weeks later (Figure 9, A–G). In addition, increased caspase-3+ apoptotic ECs were found in SCs of the A1:A2i Δ/Δ mice 1 week after tamoxifen administration (Supplemental Figure 7, A–C), suggesting that SC regression in A1:A2i Δ/Δ mice could be mainly attributed to increased apoptosis of ECs. Moreover, the A1:A2i Δ/Δ mice had a reduced number and diameter of GVs, thinned RNFL, and markedly attenuated pSTR and PhNR (Figure 10, A–I). Together, these findings show that deletion of both Angpt1 and Angpt2 from adulthood induces severe SC regression and phenotypes similar to those in acquired adult-onset POAG.

Throughout the experiments, we detected no noticeable alterations and differences in SCs of the Tie2ΔEC mouse or A1:A2i Δ/Δ mice prior to tamoxifen administration, with each compared with their respective control WT mice. 

**Prox1 is also required for adequate SC development and maintenance.** We and others recently reported that Prox1 is expressed in SC ECs (7, 10) and that its expression is changed by AHO and VEGFR3 signaling (8, 9). To determine the role of Prox1 in SC development, we generated a Prox1ΔEC mouse by crossing the VE-cadherin–Cre-ERT2 mouse (34) with the Prox1 fl/fl mouse (39), administered tamoxifen starting at P5, and analyzed them at P7 (Figure 11A). Reduced SC area and diminished Prox1, Klf4, Tie2, and p-Tie2 in SCs were detected in Prox1ΔEC mice compared with those of WT mice (Figure 11, B–D). To evaluate the role of Prox1 in SC maintenance, we deleted Prox1 in SCs starting at 8 weeks old and analyzed them 2 weeks later (Figure 12A) because most of the mice died within 3 weeks, presumably due to severe intestinal edema and hemorrhage. At 2 weeks later, SCs of Prox1ΔEC mice had slightly reduced cellularity and diminished Klf4, Tie2, and p-Tie2 and reduced and disrupted VE-cadherin+ EC junctions compared with those of WT mice, although there were no differences in IOP, SC area, or number and diameter of GVs between the 2 groups (Figure 12, B–I). Thus, Prox1 plays a substantial role in SC formation, while it plays a minor role in SC maintenance.
**PROX1** is regulated by TIE2 in LECs. The aforementioned findings led us to investigate whether a certain molecular cooperation exists among Tie2, AHO, and Prox1 in the formation and maintenance of SC. To gain insight into this question, we performed RNA-sequencing (RNA-seq) analysis in the primarily cultured human dermal LECs (hDLECs) that were incubated with a control siRNA construct (siControl), siTIE2, or siPROX1 (see Methods). Although the transcriptomes of the siTIE2 and siPROX1 gene sets were more similar to each other than to those of the siControl gene set (Supplemental Figure 8A), they displayed more differentially expressed genes (DEGs) than the overlapping genes (Supplemental Figure 8B). Further gene ontology (GO) analysis revealed that the siTIE2 signature was related to defects in the maturation and tubulation of ECs and eNOS signaling, an indicator of flow-induced shear stress (40) (Supplemental Figure 8C and Supplemental Table 3). Meanwhile, the signature of the siPROX1 gene set included defects in the integrity of ECs (Supplemental Figure 8D and Supplemental Table 4). In addition, changes in mRNA and protein expression of hDLECs transfected with siTIE2 (siTIE2-LECs) or siControl (siControl-LECs) were validated. In comparison with those of siControl-LECs, siTIE2-LECs displayed reduced mRNA and protein expression of KLF4, VE-cadherin, and PROX1 (Figure 13, A–D). Moreover, when hDLECs were stimulated with a TIE2 agonistic antibody, ANGPT2-binding and TIE2-activating antibody (ABTAA) (41), TIE2 downstream signals such as AKT and ERK were activated (Figure 13, E and F). Furthermore, ABTAA attenuated IFN-γ-induced reduction of PROX1 levels in hDLECs, and its effect was abolished by ERK inhibitor, but not AKT inhibitor, indicating that TIE2 activation contributed to upregulating or maintaining PROX1 levels through activation of ERK signaling (Figure 13, G and H). Together, these results implied that interconnected and coordinated roles of TIE2 and PROX1 in SC ECs are required for the formation and maintenance of SC; TIE2 is essential for promoting tubulation and lumen formation for initiation of AHO, which subsequently upregulates PROX1 during SC formation, while it is also indispensable for maintaining SC integrity by keeping adequate AHO and PROX1 activity.

Reduced Angpts and Tie2, cellularity, cell-cell junction, and transectosis in aged SC. We next investigated the molecular and cellular changes of aged SC because aging is a major risk factor for adult-onset POAG (42). In particular, AHO resistance between SC and TM increases with age in humans (4), while AHO and lym-
The most intriguing finding of this study is the identification of a critical role of the Angpt-Tie2 system in maintaining SC integrity (Figure 17). Impairment of this system could be involved in adult-onset POAG, which is the most common type of glaucoma in patients. Of importance, Tie2 is highly present in SC ECs in adults and supposedly decreases with aging, which is an important risk factor for POAG. Worthy of special note, Tie2 activation can recover the phenotypes of POAG in double Angpt1/Angpt2-deleted mice and can rejuvenate aged SC.

**Discussion**

The most intriguing finding of this study is the identification of a critical role of the Angpt-Tie2 system in maintaining SC integrity (Figure 17). Impairment of this system could be involved in adult-onset POAG, which is the most common type of glaucoma in patients. Of importance, Tie2 is highly present in SC ECs in adults and supposedly decreases with aging, which is an important risk factor for POAG. Worthy of special note, Tie2 activation can recover the phenotypes of POAG in double Angpt1/Angpt2-deleted mice and can rejuvenate aged SC.
Abnormally high resistance against AHOc through SC is a major causative factor in the pathogenesis of glaucoma, especially for POAG (5). When SC is impaired, AH drainage is inhibited and IOP is increased, ultimately leading to glaucoma. In parallel, when there is an impairment in peripheral LVs, drainage of interstitial fluid is inhibited, ultimately leading to lymphedema. In this regard, glaucoma induced by impaired SC could be considered as a sort of “eye lymphedema.” Our findings highlight SC as a functional reservoir of AHO as well as a main structure for glaucomagenesis when severely impaired. Although it has been long debated (43–45), recent studies indicate that AHOc, rather than AHOu, is a primary route for AHO in C57BL/6J mice (46, 47), similar to that in humans. We note that a more than 50% reduction in SC area and a marked reduction in transcytotic activity in SC ECs are prerequisites for the significant elevation of IOP, retinal neuronal damage, and impairment of retinal ganglion cell function. This phenomenon could be explainable based on a previous report (48) showing SC elasticity in tolerating increased resistance to AHOc to a certain degree. In fact, patients with POAG have markedly reduced SC dimensions, which are correlated with increased outflow resistance (49–52), emphasizing a significance for SC in POAG pathogenesis. Moreover, GVs, which are formed in response to a pressure gradient between IOP and episcleral venous pressure and transport AH, are rarely found in the ECs of the inner SC wall of POAG patients (53), reflecting severely reduced flow through AHOc. In this regard, pathogenesis of POAG is similar to pathogenesis in adult-onset glaucomatous phenotypes of Tie2 ΔEC or A1Δi/A2iΔmice.

This study demonstrates that the pericyte-derived Angpt1 and the endothelium- and TM-derived Angpt2 play overlapping and profound roles through Tie2 signaling not only in the generation, but also in the maintenance of SC. In this regard, transcriptional regulation of Angpt1 and Angpt2 during SC generation and maintenance needs to be further elucidated. While Tie2 deletion and double Angpt1/Angpt2 deletion induced severe glaucomatous phenotypes with severe regression of SC, deletion of either Angpt1 or Angpt2 did not result in elevated IOP despite mild SC regression. In this regard, Angpt2 seems to play an agonistic rather than antagonistic role for Tie2 in the generation and maintenance of SC. Intriguingly, we found a significant downregulation of Angpt2 in the TM of 24 individuals with POAG compared with that of 26 control subjects in the profiled human metadata (data from NCBI’s Gene
Expression Omnibus; GEO GSE27276, GSE4316 and GSE27058), supporting our hypothesis. Our detailed analyses indicate that Tie2 activation is definitely required for formation of the canal tube and lumen by differentiation and maturation of SC progenitor cells for initiation of AHO, which subsequently upregulates expression of Prox1 and Klf4 during SC development. In addition, transcriptional activity of Prox1 is also required for differentiation and maturation of SC and upregulation of Tie2 and Klf4 expression during SC development. However, while continuous Angpt-Tie2 signaling and AHO are definitely required to maintain Tie2 activation and SC integrity (8), the transcriptional activity of Prox1 seems somewhat redundant. Based on these findings, an interconnected pathway of Tie2-AHO-Prox1 could be proposed as an essential loop for SC formation and maturation, while only a biconnected pathway between Tie2 and AHO could be proposed as a critical loop for the maintenance of SC integrity (Figure 17). Indeed, reduced Angpt-Tie2 signaling not only leads to defective SC development, but also impedes maintenance of SC integrity, leading to compromised AH drainage. In this respect, Tie2 activation could be a potential therapeutic approach for ameliorating glaucoma progression through recovery of SC integrity and drainage function and subsequent IOP reduction (Figure 17). Indeed, we demonstrate that Tie2 activation through intraocular administration of ABTAA reduces the elevated IOP and recovers SC regression in double Angpt1/Angpt2-deleted mice. This finding was in parallel with a previous report about the substantial role of VEGFC-VEGFR3 signaling in rejuvenating aged SC (9). In addition, considering that VEGFC-VEGFR3 signaling plays an essential role in SC formation (8, 9) and that Angpt-Tie2 signaling shares this role, it is supposed that there is a close cross-talk between those signaling pathways in SC.

Cellular senescence of SC is associated with accumulation of harmful stimuli and reactive oxygen species, which may cause dysfunctional drainage in humans and mice (39, 54). Along with the previous report demonstrating decreased AHO and reduced expression of lymphatic markers such as Prox1 and VEGFR3 in aged SC (8), we reveal here that aged SC ECs exhibit attenuated intercellular junctions and decreased cellularity and transcytosis together with an attenuated Angpt-Tie2 system. However, there was no significant difference in IOP between young adult and aged mice. We postulate that this is because, as with human eyes, AH formation rate is decreased by about 40% in aged mice compared with young adult mice (46); thus, resistance to AHOc is not proportionally elevated with regressing SC in aged animals. Nevertheless, given that Tie2 activation apparently rejuvenates aged SC, intra-ocular administration of Tie2 agonist could be one of therapeutic options for treating patients with age-dependent POAG.
**Methods**

*Mice.* Specific pathogen-free (SPF) C57BL/6J mice (catalog 000664), Tic2-GFP mice (catalog 003658), and UBC-Cre-ER<sup>T2</sup> mice (catalog 007001) were purchased from the Jackson Laboratory. Angpt1<sup>fl/fl</sup> mice were a gift from Yoshikazu Nakaoka (Osaka University, Osaka, Japan), Angpt1-GFP mice were a gift from Sean J. Morrison (University of Texas Southwestern, Dallas, Texas, USA), Angpt2-lacZ mice were a gift from Nicholas Gale (Regeneron Pharmaceuticals), and Vegfr2<sup>fl/fl</sup> mice were a gift from Masanori Hirashima (Kobe University, Kobe, Japan). Prox1-GFP (30), Angpt1-GFP (33), Prox1<sup>fl/fl</sup> (39), Tic2<sup>fl/fl</sup> (35), Angpt1<sup>fl/fl</sup> (36, 37), Angpt2<sup>fl/fl</sup> (38), Vegfr2<sup>fl/fl</sup> (55), and Angpt2-lacZ (22) mice were transferred and bred in our SPF animal facility at KAIST. To deplete Prox1, Tic2, or Vegfr2 genes specifically in ECs, VE-cadherin–Cre-ERT<sup>2</sup> mice (34) were mated with either Prox1<sup>fl/fl</sup>, Tic2<sup>fl/fl</sup>, or Vegfr2<sup>fl/fl</sup> mice to obtain EC-specific Prox1<sup>−/−</sup>, Tic2<sup>−/−</sup>, or Vegfr2<sup>−/−</sup> mice, respectively, in a tamoxifen-dependent manner. To delete Angpt1 or Angpt2 globally, Angpt1<sup>−/−</sup> or Angpt2<sup>−/−</sup> mice were crossed with the UBC-Cre-ER<sup>T2</sup> mouse. Tamoxifen (2 mg, T5648, Sigma-Aldrich) was injected intraperitoneally a total of 3 times every 2 days into the 8-week-old genetically modified mice.

**Figure 10.** SC regression in A1:A2<sup>−/−</sup> mice leads to adult-onset glaucoma. (A–C) EM images and comparisons of the number and diameter of GVs (blue arrowheads) in the inner SC wall. Blue-lined box is magnified in right panel. Red double arrows indicate diameters of GV. Scale bars: 10 μm (left panels); 2 μm (right panels). n = 4 for each group. *P < 0.05 versus WT by Mann-Whitney U test. (D–F) Images and comparisons of thickness (red arrowheads) and Tubb3 distribution of RNFL. Black-lined box is magnified in middle panel. Scale bars: 200 μm. (G–I) Representative wave responses of electroretinogram and comparisons of pSTR amplitude and PhNR/b-wave amplitude ratio. n = 4 for each group. *P < 0.05 versus WT by Mann-Whitney U test.
mice. For postnatal mice, except Prox1fl/fl mice, 50 μg 4-hydroxytamoxifen (H7904) was injected subcutaneously every 2 days from P1 to P5. Prox1fl/fl mice intercrossed with VE-cadherin-Cre-ER T2 mice were injected with 4-hydroxytamoxifen daily from P5 to P6 due to early lethality. All mice were fed with free access to a standard diet (PMI LabDiet) and water. Mice were anesthetized with an intraperitoneal injection of 40 mg/kg ketamine and 5 mg/kg xylazine before any procedures.

Sampling of human corneas. Human corneal samples were obtained from the peripheral corneoscleral rim of healthy cadaveric donor tissue (Lions Eye Bank, Portland, Oregon, USA) left over from penetrating keratoplasty at the Samsung Medical Center. These were exempted from the formal review of the IRB at Samsung Medical Center due to the lack of patient identifiers and the intention of being otherwise discarded. We examined the protein expression of TIE2 and PROX1 in SC of these samples using immunofluorescence (IF) staining.

Histological analyses. IF staining for whole-mounted and sectioned corneas and retinas were performed as previously described (8). Briefly, enucleated eyes were first fixed with 4% paraformaldehyde (PFA) in PBS at room temperature (RT) for 20 minutes (for adult mice) or 2% PFA in PBS at 4°C for 30 minutes (for postnatal mice). For IF staining of sectioned corneas, fixed eyeballs were then dehydrated in 20% sucrose solution overnight, embedded in tissue-freezing medium (Leica), and cut into 14-μm sections. For IF staining of whole-mounted retina, after isolating the retina from eyeball, the retinas were additionally fixed with 1% PFA in PBS at RT for 1 hour. Samples were blocked with 5% donkey or goat serum in PBST (0.3% Triton X-100 in PBS) and then incubated in blocking solution with one or more of the following antibodies at 4°C overnight: anti-CD31 (hamster monoclonal, clone 2H8, catalog MBA1398Z, Millipore); anti-collagen type IV (rabbit polyclonal, catalog LSL-LB-1407, Cosmo Bio); Cy3-conjugated anti-F-actin (catalog A12379, Thermo Fisher Scientific); anti-Prox1 (rabbit polyclonal, catalog 102-PA32AG, ReliaTech); anti-Prox1 (goat polyclonal, catalog AF2727, R&D systems); anti–VE-cadherin (CD144) (rat monoclonal, clone 11D4.1, catalog 550548, BD Biosciences); anti-Tie2 (goat polyclonal, catalog AF762, R&D Systems); anti–p-Tie2 (rabbit polyclonal, catalog AF2720, R&D Systems); anti-Klf4 (goat polyclonal, catalog AF3158, R&D Systems); anti-Erg (rabbit polyclonal, catalog SC-353, Santa Cruz Biotechnology Inc.); FITC- or Cy3-conjugated anti–α-SMA (mouse monoclonal, clone 1A4, catalog F3777/catalog C6198, Sigma-Aldrich); anti–LYVE-1 (rabbit polyclonal, catalog 11-034, AngioBio); anti-VEGFR3 (goat polyclonal, catalog AF743, R&D Systems); anti-VEGFR2 (goat polyclonal, catalog AF644, R&D Systems); anti-ki67 (rabbit monoclonal, clone SP6, catalog ab16667, Abcam); anti-Angpt2 (human monoclonal, clone 4H10) (41); anti-PDGFRβ (rat monoclonal, clone APB5, catalog NC0091961, eBioscience); anti-Tubb3 (rabbit polyclonal, catalog PRB-435P, BioLegend); and anti–cleaved caspase-3 (rabbit polyclonal, catalog 9661, Cell Signaling Technology). Following several washes, the samples were incubated at RT for 4 hours with the following secondary antibodies: FITC-, Cy3- or Cy5-conjugated anti-hamster IgG, anti-rabbit IgG, anti-rat IgG, anti-goat IgG, and anti-human IgG antibody (Jackson ImmunoResearch). For whole-mounted corneas or retinas, the samples were cut radially and then mounted in fluorescent mounting medium (Vector or Dako).

To evaluate β-gal activity, the corneas were incubated with a staining solution (5 mM potassium ferricyanide, 2 mM magnesium chloride, 5 mM potassium ferrocyanide, and 1 mg/ml 4-chloro-5-bromo-
V16, Carl Zeiss) was performed to measure RNFL thickness, and retinal sections were photographed at both the left and right points approximately 500 μm away from the center of the optic nerve head. Morphometric analyses of SC, cornea, and retina were performed using ImageJ software (NIH) or ZEN 2012 software (Carl Zeiss). The relative area of SC was calculated as a percentage of the CD144+ or CD31+ area divided by its control area. To determine the area of limbal LVs and BVs, Prox1+ areas and CD144+ areas were measured. To quantify the relative expression of Prox1 and Klf4, intensities were measured in the nucleus region of CD144+ or CD31+ SC. To quantify the expression of Tie2, p-Tie2, VEGFR2, and VEGFR3, intensities were measured in the CD144+ or CD31+ SC area.

3-indolyl-β-D-galactopyranoside [X-gal] in PBS) at 37°C for 12 hours. SA-β-gal activity was examined with the Senescence Detection Kit (Cell Signaling Technology) according to the manufacturer’s instructions. All immunofluorescent imaging procedures were performed using a Zeiss LSM 780 or 880 confocal microscope equipped with argon and helium-neon lasers (Carl Zeiss). The images of β-gal activity were acquired with a microscope equipped with a CCD camera (Carl Zeiss). For H&E staining, retinas were fixed overnight in 4% PFA. After tissue processing using standard procedures, samples were embedded in paraffin and cut into 5-μm sections through the center of the eye (determined by the presence of the optic disc and optic nerve), followed by H&E staining. Standard light microscopy (Axio Zoom.

Figure 12. Prox1 is required for SC maintenance. (A) Diagram for EC-specific depletion of Prox1 in SCs in 8-week-old mice and analyses 2 weeks later using Prox1ΔEC mice. (B-F) Images and comparisons of IOP, relative area, number of Erg+ ECs, and intensities of Prox1, Klf4, Tie2, and p-Tie2 immunostaining in CD144+ SC. Dashed lines demarcate the margins of SC, and each area marked by a yellow box is magnified in the top corner. Scale bars: 100 μm. SC area and expression of each molecule in WT group are normalized to 100%, and their relative levels in Prox1ΔEC mice are presented. n = 4 for each group. *P < 0.05 versus WT by Mann-Whitney U test. (G-I) EM images and comparisons of the number and diameter of GVs (blue arrowheads) in the inner SC wall. n = 4 for each group. Scale bar: 10 μm (G).
2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, and dehydrated with a series of increasing ethanol concentrations followed by resin embedding. Ultrathin sections (70 nm) were obtained with an ultramicrotome (UltraCut-UCT, Leica) and were then collected on copper grids. After staining with 2% uranyl acetate and lead citrate, samples were examined by transmission EM (Tecnai G2 Spirit Twin, FEI) at 120 kV.

Electroretinogram. To assess the functionality of inner retina at indicated time points, a full-field electroretinogram was recorded in the indicated mice using the Phoenix Micron IV System (Phoenix Research Labs). Mice were dark or light adapted for 12 hours prior to electroretinogram monitoring, then were anesthetized and placed on a heating pad to maintain body temperature. After pupil dilatation by 1-time topical application of 0.5% tropicamide/0.5% phenylephrine mixed eye drop (Mydrin-P, Santen), the cornea was located by gold-plated objective lens, and silver-embedded needle electrodes were located at the forehead (reference) and tail (ground). Using LabScribeERG software.

The number of Erg+, Ki-67+, and caspase-3+ ECs were counted within the CD144+ or CD31+ SC area of the random 0.0558 or 0.0803 mm² area. The expression of α-SMA on the outer wall of the SC was calculated as a percentage of positive area divided by CD144+ or CD31+ SC area. In order to measure the expression of Tubb3 in retinal nerve fiber, intensities were calculated in the Tubb3 RNFL and divided by retinal area. All measurements of SC were performed in all quarters of the anterior segment in each eye, excluding the portion of the bifurcation of LPCAs, unless indicated otherwise. For statistical analysis, the values from all quarters in each eye were averaged. For comparison of staining intensities, the values were normalized by the background signals in nonvascularized regions, and their ratios were normalized by control and presented as percentages.

EM. To capture ultrastructure EM images of SC, eyeballs were sectioned after perfusion-fixation with 4% PFA and 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Samples were then fixed overnight in 2.5% glutaraldehyde, post-fixed with 1% osmium tetroxide, and dehydrated with a series of increasing ethanol concentrations followed by resin embedding. Ultrathin sections (70 nm) were obtained with an ultramicrotome (UltraCut-UCT, Leica) and were then collected on copper grids. After staining with 2% uranyl acetate and lead citrate, samples were examined by transmission EM (Tecnai G2 Spirit Twin, FEI) at 120 kV.

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IOP was measured by placing the tip of the pressure sensor approximately 1/8 inch from the central cornea immediately after anesthetizing the mice. The digital readouts of 5 consecutive IOP measurements were acquired from the tonometer.

Intraocular administration. Using a glass capillary pipette-fitted Nanoliter 2000 Microinjector (World Precision Instruments), approximately 1 μl PBS containing the premixed Angpt2 (0.01 μg) and ABTAA (5 μg) was injected intravitreally into one eye and approximately 1 μl PBS containing anti-human Fc (5 μg) was injected intravitreally into the contralateral eye of the same animal.

Cell culture and siRNA transfection. hDLECs were isolated and cultured as previously described (57) with the approval of the University of California, San Diego (Version 3, Phoenix Research Labs), the stimulus and recording of electrotetinogram were performed according to the manufacturer’s instructions. To obtain PhNR and the b-wave of the photopic electretinogram (light-adapted conditions), a digital bandpass filter ranging from 2 to 200 Hz and stimuli ranging from 0.4 to 2.2 log (cd·sec/m²) with 1.3 log (cd·sec/m²) background were used. To yield pSTR of the scotopic electretinogram (dark-adapted condition), filters ranging from 0.05 to 300 Hz and stimuli ranging from –5.5 to –3.0 log (cd·sec/m²) were used. After averaging the signals, the amplitude was presented by LabScribeERG software and used for analyses.

IOP measurement. IOP measurements were performed with a rebound tonometer (TonoLab, Tiolat) as previously described (56).
using Lipofectamine RNAiMAX (Invitrogen) at a final concentration of 40 nM for 24 to 48 hours according to the manufacturer’s protocol. Then siRNA was washed out and cells were harvested 72 hours after the start of transfection.

RNA extraction and quantitative real-time RT-PCR. Total RNA of lung, limbus, or central portion of the cornea was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. For extraction of total RNA from the cultured hDLECs, RiboEx (GeneAll) was used. RNA (2 μg) was reverse transcribed into cDNA using GoScript Reverse Transcription Kit (Promega). Then quantitative real-time PCR
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and gene sets are indicated in Supplemental Tables 3 and 4. RNA-seq gene expression heatmap was generated using Cluster and TreeView from the Eisen laboratory (60). We used Multiple Experiment Viewer (MeV) from The Institute of Genomic Research (TIGR) to plot gene expression heatmap of genes encoding biological functional terms (http://mev.tm4.org/#/welcome).

IF staining of cultured hDLECs. Indicated hDLECs plated in an 8-well chamber (Lab-Tek, Thermo Fisher Scientific) were fixed with 1% PFA in PBS at RT for 10 minutes, permeabilized with PBST (0.5% Triton X-100 in PBS) at RT for 5 minutes, blocked with 5% donkey serum in PBST (0.1% Triton X-100 in PBS) at RT for 30 minutes, and incubated with the following primary antibodies at 4°C overnight: anti-VE-cadherin (CD144) (mouse monoclonal, clone BV6, catalog MABT134, Millipore) and anti-PROX1 (goat polyclonal, catalog AF2727, R&D systems). The cells were then incubated with secondary antibodies (Jackson ImmunoResearch) in the dark at RT for 1 hour and mounted with DAPI-containing mounting medium (DAKO). Images were taken with a confocal laser scanning microscope (LSM 780, Carl Zeiss).

Immunoblot analysis. To evaluate the changes in PROX1 expression in hDLECs, cells were incubated with control siRNA or siTIE2 for 48 hours and harvested at 72 hours after the start of transfection. For immunoblot detection of TIE2 downstream signaling proteins, ERK, p-ERK (T202/Y204, p-ERK), AKT, and p-AKT (S473, p-AKT) hDLECs were incubated with a premixed solution containing 2 μg/ml human ANGPT2 (R&D systems) and 10 μg/ml ABTAA for 30 minutes without serum starvation as previously described (41). To examine changes in PROX1 expression by ABTAA treatment, hDLECs were treated with premix solutions containing IFN-γ (Peprotech) – as an inhibitor of PROX1 – and 2 μg/

was performed using FastStart SYBR Green Master Mix (Roche) and S1000 Thermocycler (Bio-Rad) with the indicated primers (Supplemental Table 2). The real-time PCR data were analyzed with Bio-Rad CFX Manager Software (Bio-Rad). GAPDH was used as a reference gene, and the results were presented as relative expression to control.

RNA-seq. The cultured hDLECs were incubated with siPROX1, siTIE2, or siControl according to the aforementioned protocol, and RNA-seq of the incubated cells was performed as previously described (59). In brief, 2 μg total RNA was isolated and incubated with magnetic beads coated with oligo-dT. Other RNAs except mRNA were removed by washing solution. Library production was started by random hybridization of starter/stopper heterodimers to the poly(A) RNA still connected to the magnetic beads. Illumina-compatible linker sequences were contained in the starter/stopper heterodimers. By a single-tube reverse transcription and ligation reaction, the starter was extended to the next hybridized heterodimer, where the newly synthesized cDNA insert was linked to the stopper. Second-strand synthesis was carried out to liberate the library from the beads, and then the library was amplified. We barcoded the amplified library. High-throughput sequencing was conducted as paired-end 100 sequencing using HiSeq 2000 (Illumina). Mapping of RNA-seq reads was performed using the TopHat software tool to acquire the alignment file, which was used to bring together transcripts, assess their exuberances, and identify DEGs or isoforms using cufflinks. The Ingenuity Pathway Analysis tool (QIAGEN) was used to interpret data in the context of canonical pathways, biological processes, and networks. Significance of the canonical pathways and biological function and networks were tested by the Benjamini-Hochberg procedure, which adjusts the P value to correct for multiple comparisons, and their activation or inhibition was determined with reference to activation z-scores. The activation scores and gene sets are indicated in Supplemental Tables 3 and 4. RNA-seq gene expression heatmap was generated using Cluster and TreeView from the Eisen laboratory (60). We used Multiple Experiment Viewer (MeV) from The Institute of Genomic Research (TIGR) to plot gene expression heatmap of genes encoding biological functional terms (http://mev.tm4.org/#/welcome).

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Figure 16. Tie2 activation rescues regressed SC of A1:A2Δ/Δ mice. (A) Diagram depicting the experiment schedule in WT and A1:A2Δ/Δ mice for administration of tamoxifen, intraocular ABTAA (~5 μg, left eye), and Fc (~5 μg, right eye), periodic measurements of IOP, and analyses of their SCs. (B–E) Images and comparisons of IOP relative area, and intensities of Prox1 and Tie2 immunostaining in CD144+ SC. Dashed lines demarcate SC. Scale bars: 100 μm. SC area and expression of each molecule in WT mice treated with Fc are normalized to 100%, and relative levels of other groups are presented. n = 5 for each group. *P < 0.05 by Kruskal-Wallis test followed by Tukey’s HSD test with ranks.
ml human ANGPT2 and 10 μg/ml ABTAA with or without either 50 μM of ERK inhibitor U0126 (Sigma-Aldrich) and 50 μM of PI3 kinase inhibitor LY294002 (Sigma-Aldrich) in 0.5% DMSO-containing media for 12 hours. hDLECs cultured in 0.5% DMSO media without aforementioned agents were used as control. The cells were rinsed once with cold PBS and lysed in cold Complete Lysis-M Buffer (Roche) containing protease and phosphatase inhibitors (Roche). The lysates were separated by SDS-PAGE gel and transferred to nitrocellulose membranes. After blocking with 4% skim milk, the membranes were incubated with the following primary antibodies at 4°C overnight: anti-ERK, anti–p-ERK, anti-β-actin (rabbit monoclonal, clone C4, catalog 9272, and catalog 9271, all from Cell Signaling Technology); anti-β-actin (rabbit monoclonal, clone C4, catalog SC-47778, Santa Cruz Biotechnology Inc.); and anti-PROX1 (goat polyclonal, catalog AF2727, Santa Cruz Biotechnology Inc.). Membranes were then incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) at RT for 1 hour. Chemiluminescent signals were detected with a luminescent image analyzer (LAS-1000 mini, Fujifilm), and band intensities of indicated proteins normalized to β-actin in each lane were compared with ImageJ software (NIH).

**Data availability.** All original microarray data were deposited in the NCBI’s GEO (GSE83299).

**Statistics.** No statistical method was used to predetermine the sample size. The investigators were blind to the genotypes of animals during experiments. Animals or samples were not randomized during experiments and were not excluded from analyses. All parameters of genetically modified mice were compared with those of littermate controls. Male and female mice were not distinguished in neonatal mice, while only male mice were used in adult experiments. Values were presented as mean ± SD. Statistical significance was determined by the 2-sided Mann-Whitney U test between 2 groups or ANOVA followed by Tukey’s honest significant difference (HSD) test with ranks for multiple-group comparison. Statistical analysis was performed with PASW statistics 18 (SPSS). Statistical significance was set at P < 0.05.

**Study approval.** Animal care and experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee (no. KA2015-15) of KAIST, and mice were handled in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research (http://www.arvo.org/About_ARVO/Policies/Statement_for_the_Use_of_Animals_in_Ophthalmic_and_Visual_Research/).

**Author contributions**
JK, Dae-Young Park, HB, Do Young Park, DK, CKL, SS, and GYK designed and performed the experiments and analyzed the data. TYC and DHL provided the human samples and critical comments on this study. YK, YKH, YH, HGA, and GO provided the mice and critical comments on this study. JK, Dae-Young Park, HB, Do Young Park, DK, CKL, SS, and GYK designed and performed the experiments and analyzed the data. TYC and DHL provided the human samples and critical comments on this study. JK, Dae-Young Park, and GYK generated the figures and wrote and edited the manuscript. GYK directed and supervised the project.

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