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Endothelial tyrosine kinase Tie1 is required for normal Schlemm's canal development

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Abstract

Background: Schlemm's canal (SC) is a large vessel residing in the iridocorneal angle and is required to regulate aqueous humor outflow. Normal SC structure and function is indispensable for maintaining normal intraocular pressure (IOP) and elevated IOP is a risk factor for development of glaucoma. Recent reports have identified a key role of the Angiopoietin-Tie2 pathway for SC development and function; however, the role of the orphan receptor Tie1 has not been clarified.

Methods: We used Tie1 knock out (KO) mice to study the function of Tie1 in SC development and function. Realtime quantitative PCR and western blot analyses were used to verify Tie1 deletion. High resolution microscopy of mouse SC whole mount and cross sections were used to study SC morphology. Measurement of IOP in live mice was used to study the impact of Tie1 on SC function.

Results: Tie1 is highly expressed in both human and mouse SC. Tie1 KO mice display hypomorphic SC and elevated IOP as a result of attenuated SC development.

Conclusions: Tie1 is indispensable for SC development and function, supporting it as a novel target for future SC-targeted glaucoma therapies and a candidate gene for glaucoma in humans.

Subject terms:

Basic Science Research; Developmental Biology; Vascular Biology

Intraocular pressure (IOP) is a critical risk factor for glaucoma, and IOP-reducing therapies are central to treatment of this devastating disease. In glaucoma, IOP elevation is due to reduction in the rate of aqueous humor outflow from the ocular anterior chamber. Consequently, there has been considerable research interest in the physiology and molecular regulation of the aqueous humor outflow pathways as a step towards development of novel IOP-lowering drugs.

Schlemm's canal (SC), a large vessel adjacent to the trabecular meshwork, resides in the iridocorneal angle and is responsible for the majority of aqueous humor outflow making it a crucial part of IOP regulation. Described as a 'hybrid vessel', SC originates from the limbal

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blood vessels and subsequently acquires lymphatic phenotypes to perform its specialized function[1].

Recently, we and others have discovered that SC development and function are dependent on the Angiopoietin-Tie2 receptor tyrosine kinase pathway that regulates the development and maintenance of blood and lymphatic vasculature[2]. Dysregulation results in primary congenital glaucoma in mice and humans, and blockade of this signaling pathway in adult mice or non-human primates rapidly leads to IOP elevation[3,4]. However, while other members of this pathway, including Angiopoietin 1, Angiopoietin 2 and their primary receptor Tie2 have been the subject of extensive research, the specific role of the orphan receptor Tie1 in SC has not been explored.

Unlike the primary angiopoietin receptor Tie2, Tie1 does not bind to any of the identified Tie2 ligands. However, Tie1 modulates Tie2 activity in a context dependent manner by forming heterodimers with Tie2[5]. Tie1 is indispensable for microvessel integrity as well as lymphatic and heart valve formation and mouse embryos lacking Tie1 develop severe edema and hemorrhage[6]. However, despite the clear requirement for Tie1 in both blood and lymphatic endothelial function, Tie1's role in the hybrid vessel SC has not been described. Here, we report that Tie1 is required for the development of this unique vessel and that mice lacking Tie1 exhibit a hypomorphic SC insufficient for aqueous humor homeostasis.

Tie1 is highly expressed in both the developing and mature blood and lymphatic vessels. A recent single cell RNA sequencing study has reported Tie1 expression in SC endothelial cells of human, mouse and macaque[7]. Similarly, we observed robust Tie1 protein expression in the endomucin or CD31-labeled SC in both mouse and human eyes (Figure A). To explore the role of Tie1 in SC development, we next generated a new *Tie1* conditional knockout allele in which LoxP sites were inserted around the eighth exon of the *Tie1* gene. Inducible whole-body deletion of Tie1 was achieved using a reverse-tetracycline-controlled transactivator (rtTA) system driven by the ubiquitously expressed Rosa26 promoter. Robust Tie1 deletion was observed at both mRNA level and protein level (Figure B). Importantly, we observed the previously reported lymphatic phenotypes in embryos and pups including edema, chylous ascites and lack of mesenteric lymphatic valves.

To study effect of Tie1 on SC, we induced excision at embryonic day 15.5, a time point before SC development. At adult age, we observed dramatic attenuation of SC size in Tie1 KO eyes compared to control littermates. Increased convolutions including focal narrowing or twists were also observed in the SC of Tie1 KO mice (Figure C). In addition to SC, the conventional outflow pathway contains distal outflow structures including connector channels which drain SC and the limbal vasculature to which they connect. These structures were simplified in Tie1 knockout mice, with disorganized patterning and attenuation of capillary arcades apparent around the entire circumference of the eye (Figure D). Although these structures are not believed to strongly contribute to aqueous humor outflow resistance, SC develops by sprouting angiogenesis from this vessel network and this phenotype may provide a mechanism for the hypomorphic SC observed in KO mice.

To study the functional impact of the morphological defect observed in SC, we measured IOP at 3.5 months of age under non-sedated conditions using a rebound tonometer (Figure E). While control animals were within the normal range, the IOP of mutant littermates was significantly elevated (Control average IOP = 12.1, Mutant average IOP = 16.83, p = 0.0129).

To confirm that Tie1 is required during SC development, we analyzed SC at postnatal day 5. At this stage, the control SC has formed a continuous tube-like morphology with very few gaps along the wall of SC. However, in KO group, the endothelial cells fail to form a continuous structure. Instead, the canal appeared disorganized with gaps and apparent endothelial sprouting, resulting in the hypomorphic SC observed in adults (Figure F).

Together, this study highlights a previously undescribed role of Tie1 in SC and the aqueous humor outflow pathway and identifies a novel target for future SC-targeted glaucoma therapies and a candidate gene for glaucoma in humans.

Methods:

Mouse and breeding

To generate Tie1fl/fl mice, a floxed conditional allele of Tie1 was created using a targeted ES clone (EPD0735_3_C06) where tandem bacterial lacZ (β-galactosidase) and neomycin-resistance selection (neo^r) cassettes were incorporated between exons 7 and 8 of the mouse Tie1 locus, with additional loxP (Cre recombinase recognition) sites flanking exons 8 and 9, obtained from the Knockout Mouse Project Repository (KOMP https://www.komp.org). Correctly targeted ES clones were used for blastocyst injection and generation of mouse chimera. Subsequent mouse offspring harboring the targeted allele ($Tie1^{neo}$) were evaluated for gross phenotypes. A conditional floxed Tie1 allele ($Tie1^{flx}$) was generated by breeding $Tie1^{neo}$ mice with Flpe recombinase transgenic mice resulting in the removal of the neo^r expression cassettes. Whole-body targeted mice were derived from crosses between $Tie1^{flx}$ mice with the driver strain Rosa-rtTA:tetOCre mice[8]. Whole-body timed deletion of Tie1 was achieved by giving doxycycline-containing drinking water (0.5% wt/vol with addition of 5% sucrose to improve palatability) to the Tie1RosartTA:tetOCre mice for indicated time periods.

Verification of Tie1 deletion

Lung tissue was used for western blot and qPCR to verify Tie1 deletion. For qPCR, lung tissue from whole-body KO mice was collected, and total RNA was isolated using Trizol reagent (Life Technologies). cDNA was then generated using the iScript kit (Bio-Rad Laboratories), and real-time PCR was performed on an ABI 7500 thermocycler (Applied Biosystems) using iTaq SYBR Green master mix (Bio-Rad Laboratories) and the following primers: Gapdh, forward 5′-AAGGTCATCCCAGAGCTGAA-3′, reverse 5′-CTGCTTCACCACCTTCTTGA-3′; Tie1, forward: 5′-GCATGAAACTTCGCAAGGCCA-3, reverse: 5′-GGAGTCGAGGTGCAGTCAA-3′.

For western blot, lung tissue was homogenized in RIPA buffer supplied with proteinase inhibitor (Sigma P8340). The lung lysate was centrifuged at 10,000 × g for 20 minutes at 4°C to pellet cell debris, and then the supernatant was transferred to a fresh microfuge tube without disturbing the pellet. The samples were mixed with SDS sample buffer (reducing agent TCEP was added to a final concentration of 40 mM) and heated to 95°C for 3 minutes before loading onto an SDS-PAGE gel. Samples were separated by SDS-PAGE and transferred to PVDF membranes using standard procedures. Tie1 was detected by rabbit anti mouse Tie1 antibody (Thermo Fisher PA5-27903).

SC immunofluorescent confocal microscopy

Mouse eye wholemount staining procedure was performed as described elsewhere[9]. In brief, enucleated mouse eyes were fixed in 2% PFA. After fixation, the eye was trimmed to remove conjunctiva and any remaining connective tissue. A razor blade was used to make an incision from the optic nerve to the center of the cornea. Scissors were used to cut the bottom third of the sclera followed by removal of crystalline lens and retina. The eyes were then blocked and permeabilized in buffer containing 5% donkey serum, 1% BSA and 0.3% Triton X-100. The eyes were then stained with CD31(BD#553370), Prox1(R&D #AF2727), Tie1(R&D #AF619) and Endomucin (Abcam #106100), followed by staining with fluorchrome-conjugated secondary antibodies. After staining, series of small cuts were made using scissors on both the sclera and cornea side toward the limbal region to flatten the SC area. Eyes were mounted so that the out surface of the eye faced the cover slip.

For cross sections of human and mouse eye, eyes were fixed as before and embedded in paraffin for sectioning. 8-um sections were collected on slides and processed for immunofluorescent staining. Stained eyes were imaged by a confocal microscope (Nikon A1). For whole mount eyes, Z stacks across the SC were taken. ImageJ Fiji software[10] was used to process and analyze the images.

IOP measurement

IOP was measured using a Tonolab rebound tonometer (iCare). Briefly, mouse was held firmly by one hand while the other hand held the tonometer. A very light weight probe from the tonometer was used to make momentary contact with the cornea in a rebound manner and give a reading of IOP. A series of measurements were taken until the readings become relatively consistent. Consistent readings were recorded and the mean value of those readings was used as the IOP of that eye. The technician/investigator was blinded to genotype of the mouse for measurements.

Statistics

Statistical analysis was performed using Prism 8 software (GraphPad). Throughout the text, values are reported as means ± SEM. Indicated P values were obtained using Mann-Whitney U test. * indicates $p < 0.05$, ** $p < 0.01$.

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Disclosure:

Susan E. Quaggin holds patents related to therapeutic targeting of the ANGPT-TEK pathway in ocular hypertension and glaucoma and receives research support, owns stock in and is a director of Mannin Research. S. Quaggin also receives consulting fees from AstraZeneca, Janssen, the Lowy Medical Research Foundation, and Roche/Genentech; is Chair of the External Scientific Advisory Board for AstraZeneca; and is a scientific advisor or member of AstraZeneca, Genentech/Roche, the Karolinska CVRM Institute, the Lowy Medical Research Institute, Mannin, Novartis, and Pfizer. Benjamin R Thomson has applied for a patent related to therapeutic targeting of the ANGPT-TEK pathway. Unrelated to this research, Benjamin R Thomson receives research funding from Bayer. The other authors declare no competing financial interests.

Nonstandard abbreviations and acronyms:

SC	Schlemm's canal
KO	Knock out
IOP	Intraocular pressure

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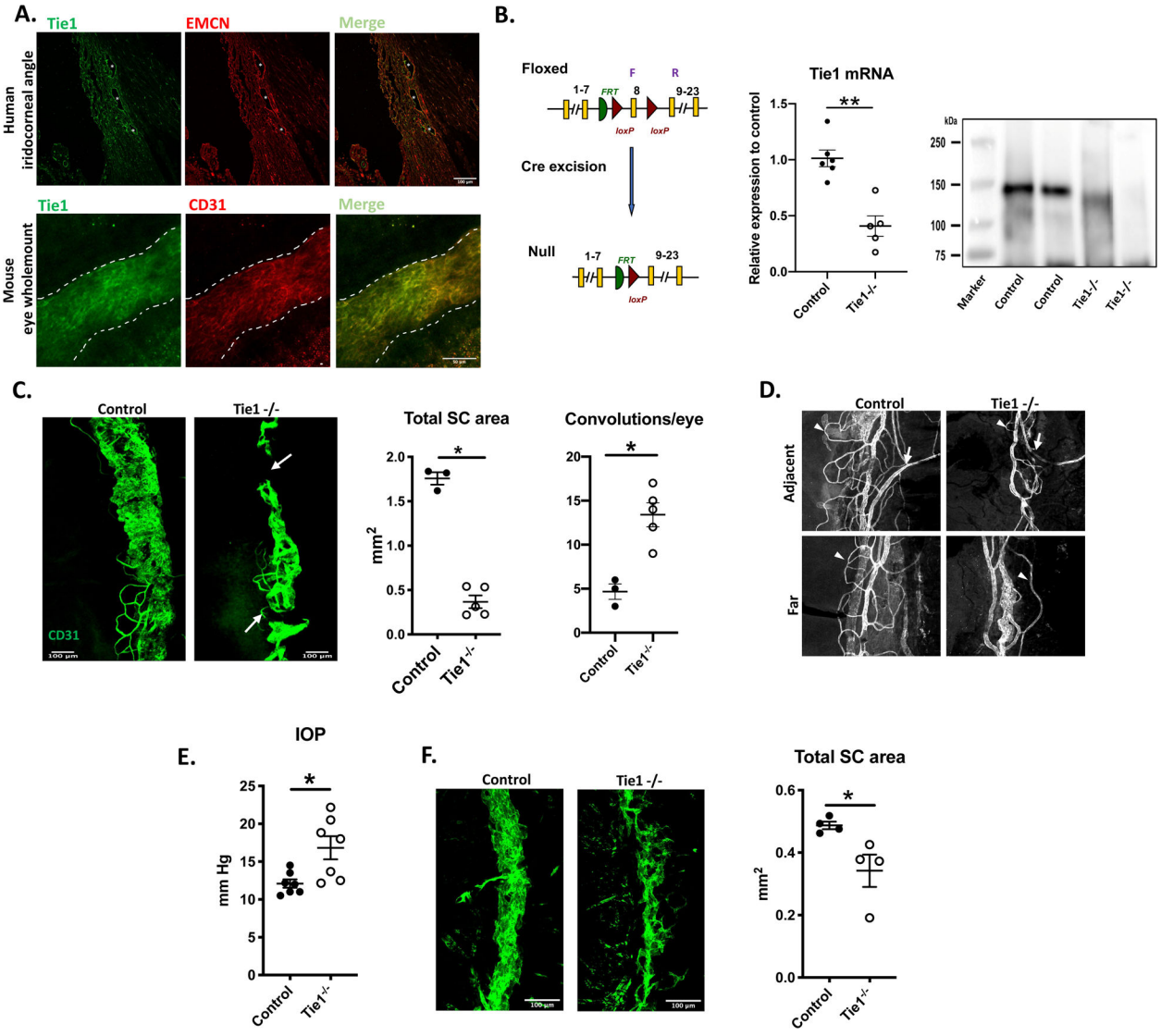
Highlights:

- A new Tie1 KO mouse model was generated
- A role of Tie1 in SC development and function was uncovered
- A novel target for future SC-targeted glaucoma therapies and a candidate gene for glaucoma in humans was identified



A. Upper panel: Cross section of the human iridocorneal angle stained with Tie1 (R&D #AF619) and EMCN antibodies (abcam 106110). SC (*) was identified by its proximity with iridocorneal angle and diameter. Lower panel: Mouse eye whole mount stained with Tie1(R&D #AF619) and CD31 antibodies (BD Pharmingen 553370) illustrated SC (indicated by dashed line).

B. Left: schematic representation of targeted allele and null allele. Primers for qPCR were designed to cross the 8th and 10th exon. Middle: quantitative PCR of Tie1 gene normalized to CD31. Right: western blot from whole adult lung for Tie1 protein. For the qPCR experiment, Tie1 excision was induced at E17.5 and lung tissue was harvested at P5. For the western blot experiment, Tie1 excision was induced at E13.5 and lung tissue was harvested from adult mice. For qPCR, Mann Whitney test was performed. P=0.0043.

C. Confocal microscopy of whole mount eyes from 12-week-old mice stained with CD31 showing the morphology of SC in control and Tie1−/− mice. Images were collected as Z stacks so that several planes across the whole SC depth were captured and are shown

as maximum intensity projections. Areas of whole SC per eye were measured by Fiji on stitched whole SC images. Convolution is indicated by arrow. Number of convolutions in each eye were counted manually. Mann Whitney test was performed. P=0.0357 for SC area. P=0.0357 for convolution numbers.

D. The limbal region was examined by confocal microscopy. At the regions both adjacent to and far from the episcleral vein (arrow), the complexity of blood vessels was decreased, highlighted by significantly decreased capillary arcades (arrowhead) and disorganized patterning.

E. Elevated intraocular pressure (IOP) was observed in Tie1−/− mice at 12 weeks of age when measured by rebound tonometry. Mann Whitney test was performed. P=0.0169.

F. Confocal microscopy of whole mount mouse eye from 5-day-old pups stained with CD31 showing the morphology of SC in control and Tie1−/− mice. Images were collected as in Figure C. Areas of whole SCs were measured by Fiji. Mann Whitney test was performed. P=0.0286.

In each experiment, similar number of female and male animals were analyzed. In all panels, data were represented as mean with SEM. * indicates $p < 0.05$, ** $p < 0.01$.