Induced Attenuation of Scleral TGF-β Signaling in Mutant Mice Increases Susceptibility to IOP-Induced Optic Nerve Damage

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Purpose. Axonal optic nerve (ON) damage in glaucoma is characteristically associated with increased amounts of active transforming growth factor-beta 2 (TGF-β2) in the ON head. Here we investigated the functional role of scleral TGF-β signaling in glaucoma.

Methods. A deficiency of *Tgfbr2*, which encodes for TGF-β receptor type II (TGF-βRII), the essential receptor for canonical TGF-β signaling, was induced in fibroblasts (including those of the sclera) of mutant mice. To this end, 5-week-old mice were treated with tamoxifen eye drops. Experimental glaucoma was induced in 8-week-old mice using a magnetic microbead (MB) model. After 6 weeks of high intraocular pressure (IOP), the ON axons and their somata in the retina were labeled by paraphenylenediamine (PPD) and RNA-binding protein with multiple splicing (RBPMS) immunohistochemistry, respectively, and quantified.

Results. Tamoxifen treatment resulted in a significant decrease of TGF-βRII and its mRNA in the sclera. After 6 weeks of high IOP, reduced numbers of PPD-stained ON axons were seen in MB-injected eyes in comparison with not-injected contralateral eyes. Moreover, MB injection also led to a decrease of retinal ganglion cell (RGC) somata as seen in RBPMS-stained retinal wholemounts. Axon loss and RGC loss were significantly higher in mice with a fibroblast specific deficiency of TGF-βRII in comparison with control animals.

Conclusions. We conclude that the ablation of scleral TGF-β signaling increases the susceptibility to IOP-induced ON damage. Scleral TGF-β signaling in mutant mice appears to be beneficial for ON axon survival in experimentally induced glaucoma.

Keywords: sclera, TGF-β signaling, experimental glaucoma, optic nerve, retinal ganglion cells

Glaucoma, the second leading cause for blindness worldwide,[1,2] is characterized by the continuous loss of optic nerve (ON) axons. Glaucomatous damage occurs at the optic nerve head (ONH), where retinal ganglion cell (RGC) axons exit the eye.[3–5] Data from multiple randomized prospective multicenter studies show that intraocular pressure (IOP) is the main risk factor for the onset and progression of glaucoma.[6–11] The reasons for the degeneration of ON axons in glaucoma are incompletely understood.

Degeneration of ON axons in glaucoma is associated with a continuous rearrangement of the connective tissue elements in the ONH. The changes cause cupping of the ONH, a typical clinical observation in affected patients.[3,12–14] In addition, the peripapillary sclera (PPS) surrounding the ONH changes as its fibrillar extracellular matrix (ECM) rearranges, and its stiffness increases significantly.[15,16]

The structural changes of ONH and PPS correlate with a marked increase of transforming growth factor-beta 1 (TGF-β1) and TGF-β2 in the ONH and aqueous humor of glaucoma patients.[17–19] TGF-β is a signaling molecule with myriad functions. Both TGF-β1 and TGF-β2 are stored in the extracellular matrix in an inactive form and can be released and activated following an increase in tissue strain.[20–22] As a consequence, active TGF-β induces the formation of fibrillar ECM, thereby changing its biomechanical properties.

IOP likely modulates stress and strain in the sclera resulting in increased availability of active TGF-β upon high IOP. This scenario could well explain the high amounts of active TGF-β in the ONH of patients with glaucoma. It appears to be reasonable to assume that higher amounts of active TGF-β in the ONH and PPS contribute to the observed structural and biomechanical changes in glaucoma. Up until now, it is unclear, though, if and how the changes contribute to ON axon loss in glaucoma.

Here, we were interested in learning about the specific function of scleral TGF-β in glaucoma. To this end, we generated mutant mice with an induced deficiency of *Tgfbr2* in fibroblasts. *Tgfbr2* encodes for the TGF-β receptor type II (TGF-βRII) which is the essential receptor for canonical TGF-β signaling. Our results indicate that the reduction of scleral

TGF-β signaling increases the vulnerability of ON axons and RGC somata in experimental glaucoma induced by increased IOP. The molecular changes induced by higher amounts of active TGF-β in the PPS appear to protect ON axons from glaucomatous damage.

Material and Methods

Animals and Animal Husbandry

Mice carrying two floxed *Tgfbr2* alleles (Tgfbr2$^{fl/fl}$) were crossbred with Tgfbr2$^{fl/fl}$/Col1a2-Cre/ERT mice heterozygous for a Cre-recombinase transgene that is specifically activated in fibroblasts upon induction with tamoxifen (TX).[23,24] Tgfbr2$^{fl/fl}$/Col1a2-Cre/ERT animals were used as experimental group and designated as Tgfbr2$^{\Delta Sclera}$; Tgfbr2$^{fl/fl}$ littermates served as controls. The genetic background of Tgfbr2$^{fl/fl}$ mice was 129S1/Sv and that of Col1a2-Cre/ERT animals was C57BL/6J. Breeding resulted in a mixed 129S1/Sv-C57BL/6J background for both experimental and control groups. All animal procedures performed in this study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with institutional guidelines. All experiments were approved by the local authorities (Regierung von Unterfranken, RUF, AZ: 55.2-2532-2-734).

Induction of Cre Recombinase

To activate Cre recombinase, which leads to the excision of the floxed Tgfbr2 allele in Tgfbr2$^{\Delta Sclera}$ animals, experimental animals and their respective control littermates were treated with 10 µL per eye of TX-containing eye drops (5 mg/mL) at an age of 5 weeks, three times a day for 5 consecutive days, as described previously.[25]

Microbead Injection and IOP Measurements

Intracameral injection of magnetic microbeads was performed as published previously.[26] Mice were anesthetized by an intraperitoneal injection of ketamine–xylazine. After the induction of pupil dilatation using tropicamid eyedrops (Mydriaticum Stulln; Pharma Stulln, Stulln, Germany), 2.4×10^6 beads (Invitrogen Dynabeads M-450 Epoxy; Thermo Fisher Scientific, Waltham, MA, USA) were injected into the anterior chamber of the right eye in a final volume of 3 µL. The left eye served as an intra-animal control. IOP was measured non-invasively under inhalation anesthesia by isoflurane. Measurements were conducted at the same time of day (8 AM–9 AM) using a TonoLab tonometer (Icare, Vantaa, Finland) and following the manufacturer's recommendations.[27] Six measurements were taken and automatically processed by the instrument's software. The average of 4 measurements, excluding the highest and lowest result, was given as final result. Measurements were repeated if SD was not $\leq$2.5. IOP was measured directly before microbead injection, 3 d after injection and then in weekly intervals up to 6 wks.

RNA Analysis

Sclerae of experimental and control mice were dissected 2 weeks after treatment with TX. Total RNA of sclerae was extracted with pegGOLD TriFast (VWR, Radnor, PA, USA) according to the manufacturer's recommendations. First-strand cDNA was prepared from total RNA using the qScript cDNA Synthesis Kit (Quantabio, Gaithersburg, MD, USA) according to the manufacturer's instructions. Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed on an iQ5 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) using the following temperature profile: 40 cycles of 10 seconds melting at 95°C and 40 seconds of annealing and extension at 60°C. Primer pairs (Table 1) were purchased from Invitrogen and extended over exon–intron boundaries. RNA that was not reversely transcribed served as negative control for real-time RT-PCR. Receptor for activated C kinase 1 (RACK1) was used as the housekeeping gene for relative quantification of the real-time RT-PCR experiments. Quantification was performed with iQ5 Standard Edition (Version 2.0.148.60623) software (Bio-Rad Laboratories).

Western Blot Analysis

Protein of sclerae was extracted with pegGOLD TriFast according to manufacturer's recommendations, and protein content was measured with the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. Western blot analysis was performed with specific antibodies as described previously.[28] Antibodies were used as follows: rabbit anti-TGF-βRII (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-connective tissue growth factor (CTGF, 1:500; R&D Systems, Minneapolis, MN, USA), Rabbit Anti-Alpha-Tubulin (1:1000; Rockland Immunochemicals, Pottstown, PA, USA), and Goat anti-Rabbit HRP and Goat anti-Rabbit Alkaline Phosphatase (1:2000; Cell Signaling Technology). The intensity of the bands detected by western blot analysis was determined using appropriate software (AIDA Image Analyzer, Elysia Raytest, Straubenhardt, Germany; ImageLab, Bio-Rad Laboratories).

Light Microscopy and Quantification of Optic Nerve Axons

Eyes were enucleated and fixed in Karnovsky's solution (2.5% glutaraldehyde and 2.5% paraformaldehyde [PFA] in 0.1-M cacodylate buffer) for 24 hours. After rinsing in

Table 1. Sequences of Primer Pairs Used for RT-PCR

Primer	Species	Orientation	Sequence 5′ to 3′	Position
TGF-βRII	*Mus musculus*	Forward	agaagccgcatgaagtctg	634–652
		Reverse	ggcaaaccgtctccagagta	686–705
CCN2/CTGF	*Mus musculus*	Forward	tgacctggaggaaaacattaaga	1013–1035
		Reverse	agccctgtatgtcttcacactg	1124–1103
RACK1	*Mus musculus*	Forward	tctgcaagtacacggtccag	514–533
		Reverse	gagacgatgatagggttgctg	604–584

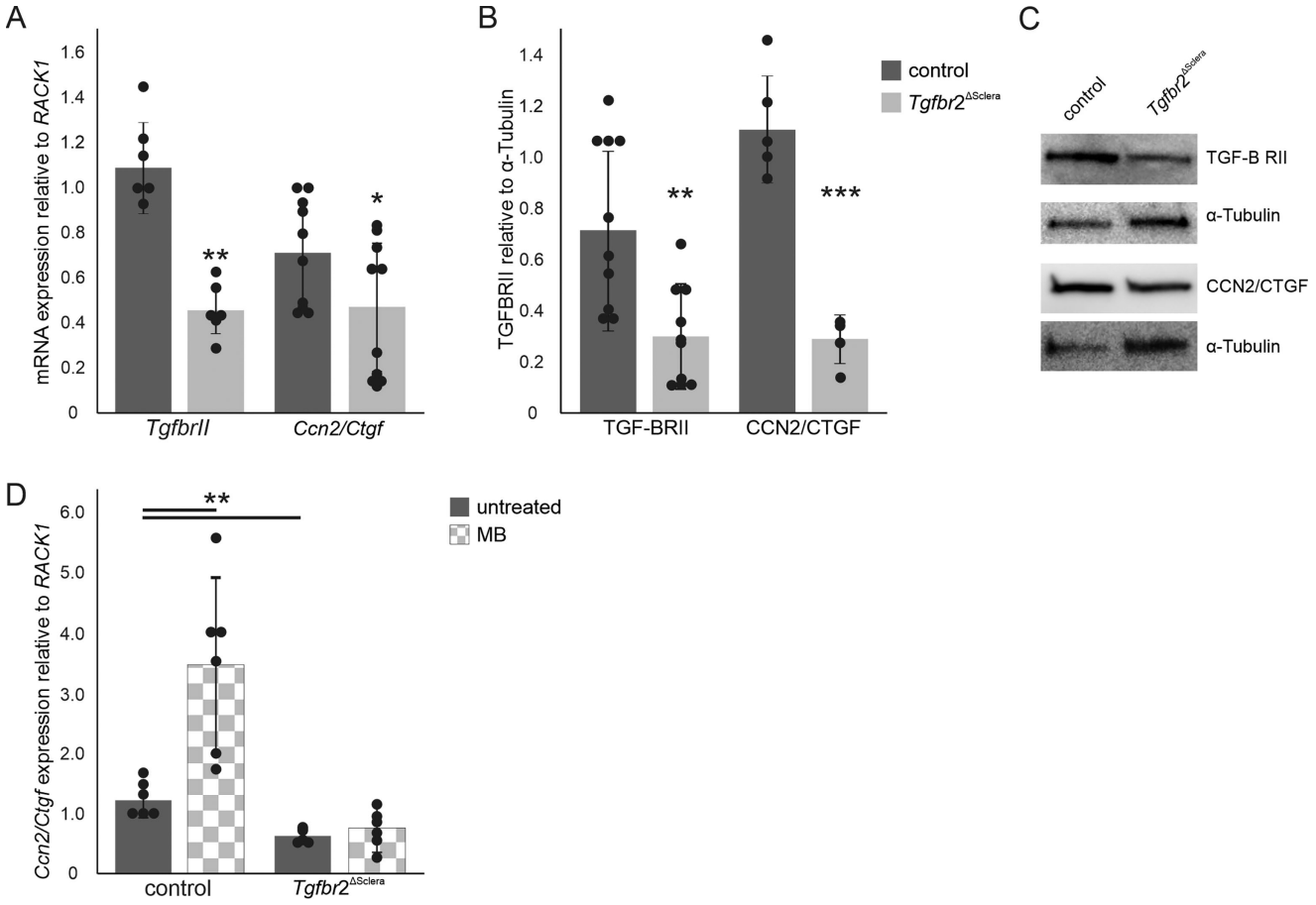

Figure 1. TGF-βRII and CCN2/CTGF were reduced in the sclera of Tgfbr2$^{\Delta Sclera}$ animals after treatment with TX. (**A**) Real-time RT-PCR of scleral mRNA. Two weeks after the treatment with tamoxifen the expression of *TgfbrII* mRNA and that of its downstream target *Ccn2/Ctgf* was significantly reduced in Tgfbr2$^{\Delta Sclera}$ animals compared to control littermates (*TgfbrII:* control, 1.08 $\pm$ 0.21; Tgfbr2$^{\Delta Sclera}$, 0.45 $\pm$ 0.12; $P = 0.0001$; $n = 6$; *Ccn2/Ctgf:* control, 0.71 $\pm$ 0.24; Tgfbr2$^{\Delta Sclera}$, 0.44 $\pm$ 0.31; $P = 0.044$; $n = 10$). (**B**) Densitometry of western blot analysis for the amounts of TGF-βRII and CCN2/CTGF in scleral proteins. Both were significantly lowered by TX treatment (TGF-βRII: control, 0.72 $\pm$ 0.32; Tgfbr2$^{\Delta Sclera}$, 0.30 $\pm$ 0.19; $P = 0.002$; $n = 10$; CCN2/CTGF: control, 1.13 $\pm$ 0.21; Tgfbr2$^{\Delta Sclera}$, 0.28 $\pm$ 0.11; $P = 0.0002$; $n = 5/4$). (**C**) Representative western blot for TGF-βRII and CCN2/CTGF in scleral proteins of control and Tgfbr2$^{\Delta Sclera}$ animals. For normalization α-tubulin was used. (**D**) Six weeks after injection of MB *Ccn2/Ctgf* mRNA was significantly increased in the sclera of control animals when compared to the sclera of the untreated control eye (control, 1.22 $\pm$ 0.26; control MB, 3,46 $\pm$ 1.47; $P = 0.01$; $n = 6$). In contrast, *Ccn2/Ctgf* mRNA was not altered after MB injection in the experimental group (Tgfbr2$^{\Delta Sclera}$, 0.64 $\pm$ 0.11; Tgfbr2$^{\Delta Sclera}$ MB, 0.77 $\pm$ 0.37; $P = 0.42$; $n = 6$). In addition, 6 weeks after TX treatment, *Ccn2/Ctgf* was significantly reduced in the sclera of untreated eyes of Tgfbr2$^{\Delta Sclera}$ animals in comparison with untreated controls (control, 1.23 $\pm$ 0.26; Tgfbr2$^{\Delta Sclera}$, 0.65 $\pm$ 0.11; $P = 0.004$; $n = 6$).

0.1-M cacodylate buffer, post-fixation was accomplished in a mixture of 1% osmium tetroxide (OsO_4) and 0.8% potassium ferrocyanide in 0.1-M cacodylate buffer for 2 hours at 48°C. Eyes were then dehydrated in a graded series of ethanol and embedded in EPON (Serva Electrophoresis, Heidelberg, Germany). Myelinated optic nerve axons were visualized by paraphenylenediamine (PPD; Carl Roth, Karlsruhe, Germany) staining of EPON-embedded semithin sections. PPD-stained cross-sections were visualized by brightfield microscopy using a 100× oil immersion objective for highest resolution. The area of the optic nerve was measured with AxioVision Viewer 3.0 (Carl Zeiss Microscopy, Oberkochen, Germany).

Optic nerve axons were quantified using AxonCounter, a precise and unbiased stereological sampling scheme implemented as an ImageJ (National Institutes of Health, Bethesda, MD, USA) plugin that was recently developed by us and described previously.[29] In short, after calibrating the image with the scale bar, the area of the optic nerve was measured in square micrometers using ImageJ, and myelinated axons in five fields (50 × 50 µm each) were counted. The mean density (axons/µm^2) in each of the five measured regions was multiplied by the total area of the optic nerve to give the estimated total number of axons. Axons were quantified in a blinded manner by two individual observers (MG, KK). Mean values of both counts were used for statistical analysis.

Staining and Quantification of Retinal Ganglion Cells

Eyes were enucleated and fixed in 4% (w/v) PFA in phosphate-buffered saline (PBS) for 15 minutes. Dissected retinae were stored in 0.1-M PBS overnight at 4°C. The next day the retinae were fixed in 100% methanol

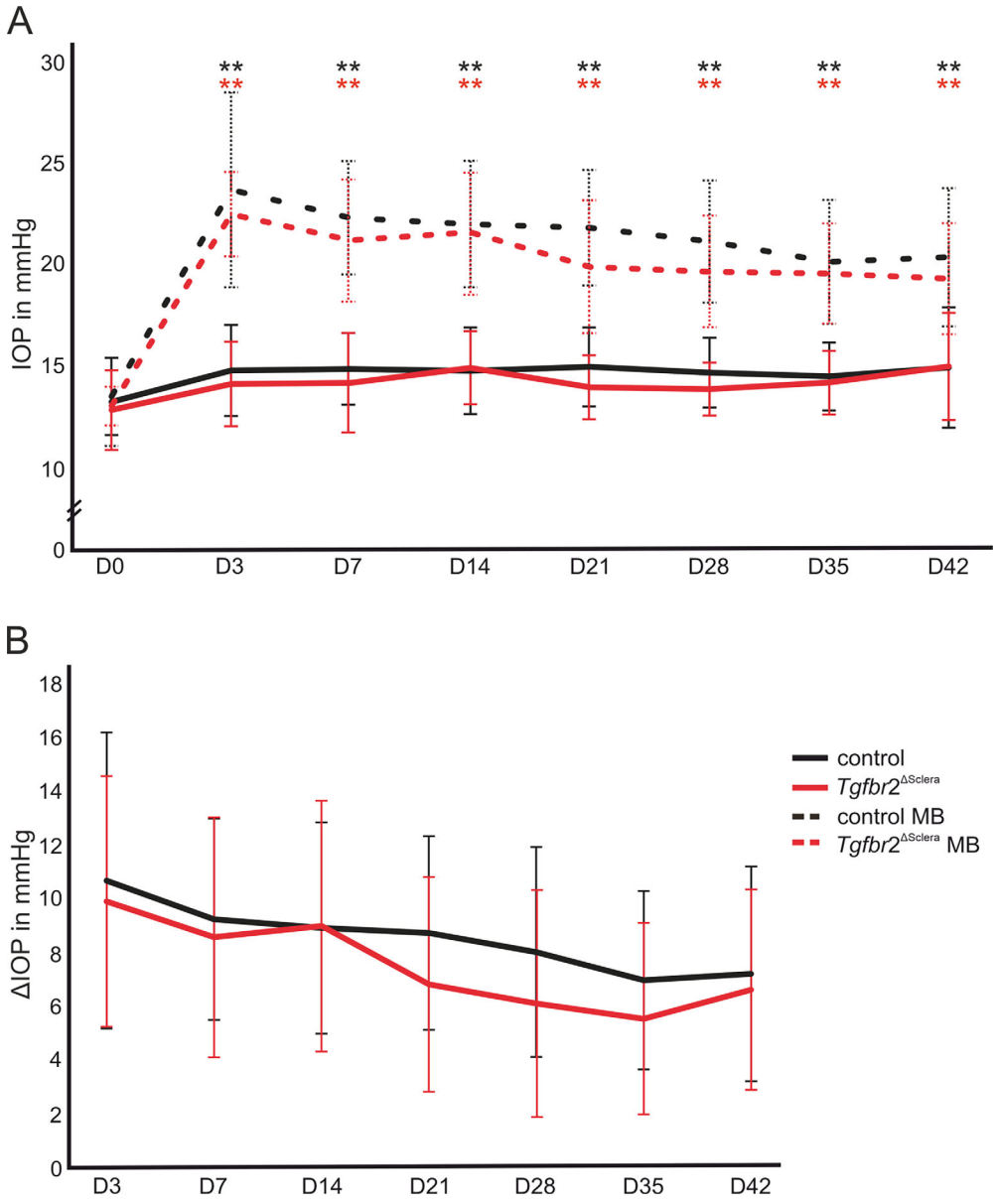

Figure 2. IOP was significantly increased after the injection of MBs in control and Tgfbr2$^{\Delta Sclera}$ animals. (**A**) IOP did not differ between control and Tgfbr2$^{\Delta Sclera}$ animals before injections (Tgfbr2$^{\Delta Sclera}$, 12.03 ± 2.06; control, 12.56 ± 2.27; $P = 0.39$; $n = 29$ each). After injection, IOP was significantly elevated for the duration of 6 weeks (see Table 2; $n = 29$). (**B**) ΔIOP was not different between control and Tgfbr2$^{\Delta Sclera}$ mice at any time point measured ($n = 29$).

Table 2. IOP and ΔIOP Values for Control and Tgfbr2$^{\Delta Sclera}$ Mice Before and After Injection of MBs

	Mean ± SD					
Day	**Control**	**Control MB**	**Tgfbr2$^{\Delta Sclera}$**	**Tgfbr2$^{\Delta Sclera}$ MB**	**ΔIOP Control**	**ΔIOP Tgfbr2$^{\Delta Sclera}$**
D0	12.56 ± 2.27	12.83 ± 1.99	12.13 ± 2.06	12.34 ± 2.11	0.00 ± 0.00	0.00 ± 0.00
D3	14.18 ± 2.35	23.50 ± 5.03	13.48 ± 2.18	22.23 ± 3.67	10.67 ± 5.51	9.89 ± 4.66
D7	14.38 ± 2.35	20.97 ± 2.89	13.80 ± 3.44	21.18 ± 4.94	8.75 ± 3.62	8.27 ± 4.61
D14	14.37 ± 2.04	21.73 ± 3.26	14.32 ± 2.56	21.31 ± 3.94	8.90 ± 3.93	8.97 ± 4.67
D21	14.72 ± 2.41	21.15 ± 3.45	13.18 ± 2.13	19.51 ± 2.69	8.71 ± 3.61	7.14 ± 4.00
D28	14.07 ± 1.80	20.83 ± 3.16	13.21 ± 1.37	19.27 ± 2.87	8.00 ± 3.91	6.95 ± 4.23
D35	13.87 ± 1.76	19.79 ± 3.20	13.55 ± 1.64	19.19 ± 2.30	6.95 ± 3.32	6.87 ± 3.56
D42	14.50 ± 3.18	20.02 ± 3.57	14.64 ± 2.90	18.92 ± 2.47	7.19 ± 4.00	6.60 ± 3.73

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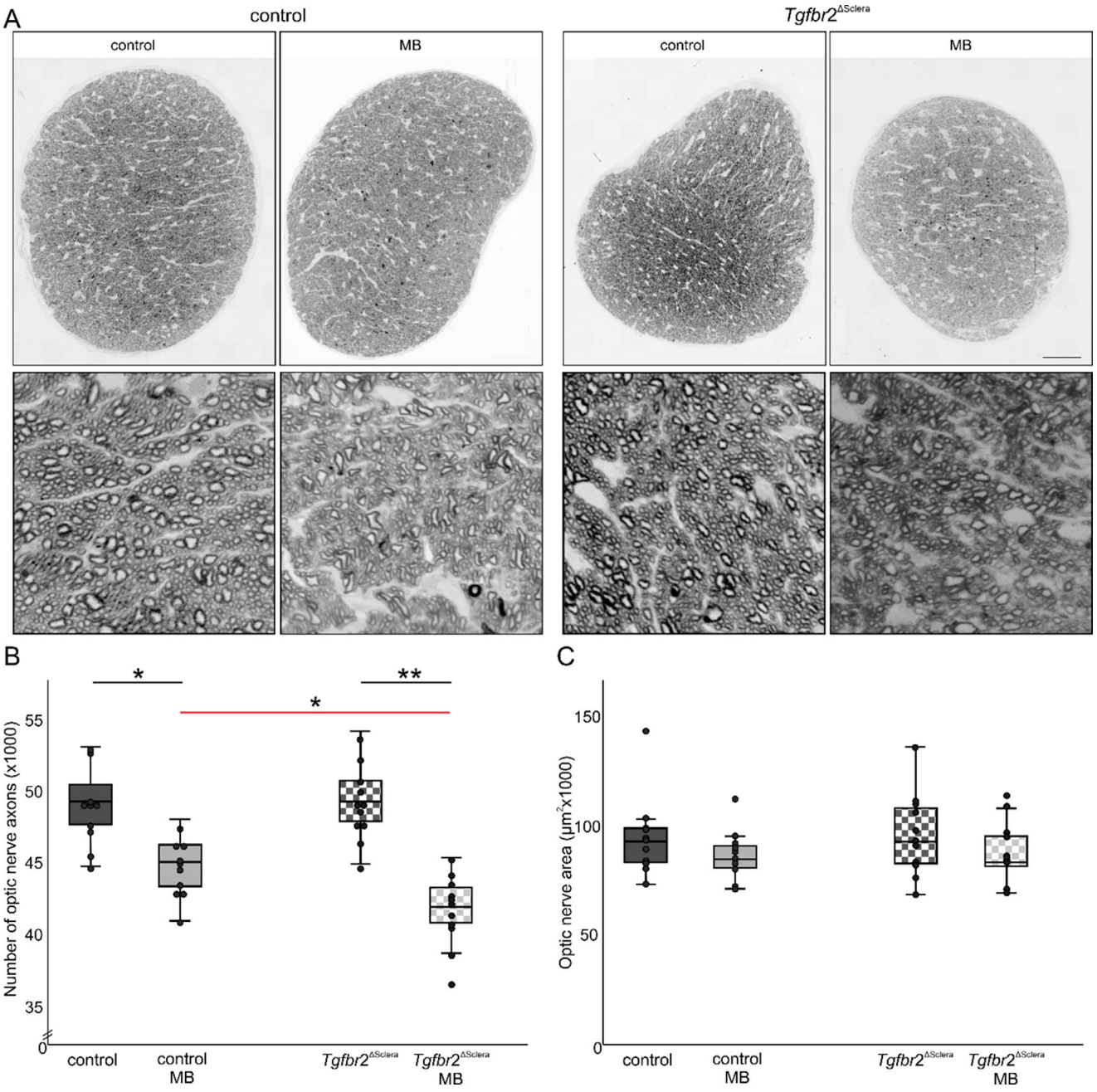

Figure 3. Axon loss was significantly increased in Tgfbr2$^{\Delta Sclera}$ mice but optic nerve size was not affected by genotype or high IOP. (**A**) PPD-stained cross-sections of optic nerves used for axon quantification showed no gross morphological differences between the groups. Larger areas devoid of axons and increased numbers of swollen axons were observed in MB eyes of both groups. *Scale bars*: 50 µm. Higher magnifications show an area of 50 × 50 µm. (**B**) Axon numbers of not-injected eyes did not differ between control and Tgfbr2$^{\Delta Sclera}$ mice (control, 48,686 ± 2644; Tgfbr2$^{\Delta Sclera}$, 49,133 ± 2543; $P = 1.00$; $n = 10/11$), but axon numbers of Tgfbr2$^{\Delta Sclera}$ mice were significantly lower after 6 weeks of high IOP than those of control mice (control ocular hypertension, 44,450 ± 4954; Tgfbr2$^{\Delta Sclera}$ ocular hypertension, 41,636 ± 2460; $P = 0.028$; $n = 10/11$). (**C**) ON area was not altered by the ablation of scleral TGF-β signaling or the injection of magnetic MBs (control, 94,498 ± 18,273 µm^2; Tgfbr2$^{\Delta Sclera}$, 95,767 ± 19,143 µm^2; control MB, 86,149 ± 11,330 µm^2; Tgfbr2$^{\Delta Sclera}$ MB, 86,914 ± 15,252 µm^2; $n = 10/11$).

(MeOH) for 15 minutes at −25°C and then washed three times in 0.1-M PBS for 15 minutes. For antigen retrieval, retinae were incubated in 0.05-M NH_4Cl for 45 minutes followed by three washing steps in 0.1-M PBS. After blocking with 3% bovine serum albumin (BSA) in tris-buffered saline with 0.1% Triton X-100 for 1 hour at room temperature, RGCs were stained using rabbit anti-RNA-binding protein with multiple splicing (RBPMS, 1:1000; GeneTex, Irvine, CA, USA) for 2 hours at room temperature (RT). Afterward, the retinae were washed three times with 0.1-M PBS followed by incubation for 1 hour at room temperature with Cy3 Goat Anti-Rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA, USA). Negative controls were carried out using only secondary antibodies.

Table 3. ON Size, Axon Density, and Axon Numbers for Control and Tgfbr2$^{\Delta Sclera}$ Mice Before and After Injection of MBs

	ON Size (µm^2)	Axons per µm^2	Axon Number	RGC per mm^2
		Mean ± SD		
Control	94,498 ± 18,273	0.559 ± 0.05	48,686 ± 2644	2206 ± 175
Control MB	86,149 ± 11,330	0.501 ± 0.07	44,450 ± 1954	1954 ± 212
Tgfbr2$^{\Delta Sclera}$	95,767 ± 19,143	0.560 ± 0.08	49,133 ± 2543	2258 ± 225
Tgfbr2$^{\Delta sclera}$ MB	86,914 ± 15,252	0.495 ± 0.09	41,636 ± 2460	1727 ± 258
		P		
Control vs. Tgfbr2$^{\Delta sclera}$	1.000	1.000	1.000	1.000
Control MB vs. Tgfbr2$^{\Delta sclera}$ MB	1.000	1.000	**0.028**	**0.043**
Control vs. control MB	1.000	0.439	**0.002**	**0.025**
Tgfbr2$^{\Delta sclera}$ vs. Tgfbr2$^{\Delta sclera}$ MB	1.000	0.257	**0.000**	**0.000**

Bold numbers represent statistically significant *P* values.

After washing three times with 0.1-M PBS, whole retinae were flatmounted onto glass slides using Cytomation Fluorescent Mounting Medium (Dako, Glostrup, Denmark).

To count the total number of RGCs, the area of the whole retina was measured with AxioVision Viewer 3.0. After calibrating the image with the scale bar, the area of the whole retina was again measured in square micrometers using ImageJ, and the stained RGCs of the whole retina were counted. At least 75% of the retina was analyzed for each sample. RGCs were quantified in a blinded manner by two individual observers (MG, KK), and mean RGC numbers from both quantifications were used for the statistical analysis. RGC numbers were calculated as RGCs/mm^2.

Statistical Analysis

Data are expressed as mean ± SD. Statistical evaluation was performed using SPSS Statistics 26 (IBM, Chicago, IL, USA). Normal distribution of data was ensured using the Kolmogorow–Smirnow test with Lilliefors correction. Student's *t*-test for unpaired data and one-way analysis of variance (ANOVA) were used to evaluate statistical significance. Levels of statistical significance and the numbers for each experiment are indicated in the figure legends.

Results

Induced Deletion of Scleral TGF-βRII and Reduced TGF-β Signaling

To confirm the induced deletion of TGF-βRII, we analyzed its expression in the sclera 2 weeks after TX treatment. *TgfbrII* was significantly reduced in Tgfbr2$^{\Delta Sclera}$ animals in comparison with control littermates (Tgfbr2$^{\Delta Sclera}$, 0.45 ± 0.12; control, 1.08 ± 0.21; $P \leq 0.01$; $n = 6$) (Fig. 1A). Regarding TGF-βRII, western blot analysis showed a 59% reduction in Tgfbr2$^{\Delta Sclera}$ mice 2 weeks after induction, corroborating the mRNA results (Tgfbr2$^{\Delta Sclera}$, 0.30 ± 0.19; control, 0.72 ± 0.32; $P \leq 0.01$; $n = 10$) (Fig. 1B). To analyze if the 55% reduction in TGF-βRII resulted in decreased activity of the TGF-β pathway, we quantified the relative expression of *Ccn2/Ctgf*, a typical downstream target of TGF-β signaling. *Ccn2/Ctgf* was significantly reduced by 50% in experimental animals (Tgfbr2$^{\Delta Sclera}$, 0.44 ± 0.31; control, 0.71 ± 0.24; $P \leq 0.05$; $n = 9$) (Fig. 1A). CCN2/CTGF protein was also significantly reduced after TX treatment (Tgfbr2$^{\Delta Sclera}$, 0.28 ± 0.11; control, 1.13 ± 0.21; $P \leq 0.001$; $n = 5/4$) (Fig. 1B). After microbead (MB) injection, the expression of *Ccn2/Ctgf* significantly increased in control animals. In contrast, no increase was observed in Tgfbr2$^{\Delta Sclera}$ animals (control, 1.22 ± 0.26; control MB, 3.46 ± 1.47, $P \leq 0.01$; Tgfbr2$^{\Delta Sclera}$, 0.64 ± 0.11; Tgfbr2$^{\Delta Sclera}$ MB, 0.77 ± 0.37, $P = 0.42$; $n = 6$) (Fig. 1D).

IOP Increase in MB-Injected Mice

Prior to bead injection, IOP did not differ between control and Tgfbr2$^{\Delta Sclera}$ mice (Tgfbr2$^{\Delta Sclera}$, 12.03 ± 2.06; control, 12.56 ± 2.27; $P = 0.39$; $n = 29$) (Fig. 2A). IOP was significantly increased from 3 to 42 days after injection of MBs. No difference in IOP was detected between Tgfbr2$^{\Delta Sclera}$ and control animals at any time point (for IOP values, see Table 2). The increase in IOP (ΔIOP = IOP injected eye – IOP not-injected eye) did not differ between the experimental group and the control group at any time point analyzed (Fig. 2B, Table 2).

Reduced Scleral TGF-β Signaling Increases Loss of Axons and RGCs

High IOP causes loss of RGC axons and somata in experimental glaucoma. Therefore, we quantified PPD-stained RGC axons in optic nerve cross-sections and RGC somata in RBPMS-stained retinal wholemounts of control and Tgfbr2$^{\Delta Sclera}$ animals 6 weeks after the injection of magnetic MBs. In control animals, 6 weeks of high IOP led to loss of axons (control, 48,686 ± 2644; control MB, 44,450 ± 1954; $P \leq 0.001$; $n = 10$), and in Tgfbr2$^{\Delta Sclera}$ animals the observed loss (Tgfbr2$^{\Delta Sclera}$, 49,133 ± 2543; Tgfbr2$^{\Delta Sclera}$ MB, 41,636 ± 2460; $P \leq 0.001$; $n = 11$) was significantly increased ($P \leq 0.05$) (Fig. 3B, Table 3). High IOP had no effect on either ON size or on axon density (axons per µm^2) in control or Tgfbr2$^{\Delta Sclera}$ mice (Fig. 3C, Table 3).

Staining against RBPMS indicated a decrease of RGCs 6 weeks after MB injection, especially in the periphery (Fig. 4A). Quantification of RBPMS-positive cells showed that, for control animals, the average RGC density (RGCs/mm^2) was 2206 ± 175, which decreased to 1954 ± 212 (86% ± 9.61%) after 6 weeks of high IOP ($P \leq 0.001$; $n = 14$). In Tgfbr2$^{\Delta Sclera}$ animals, RGC density decreased from 2258 ± 225 to 1727 ± 258 (74% ± 11.43%; $P \leq 0.001$; $n = 13$). This decrease was significantly higher than in control animals ($P \leq 0.05$) (Fig. 4B).

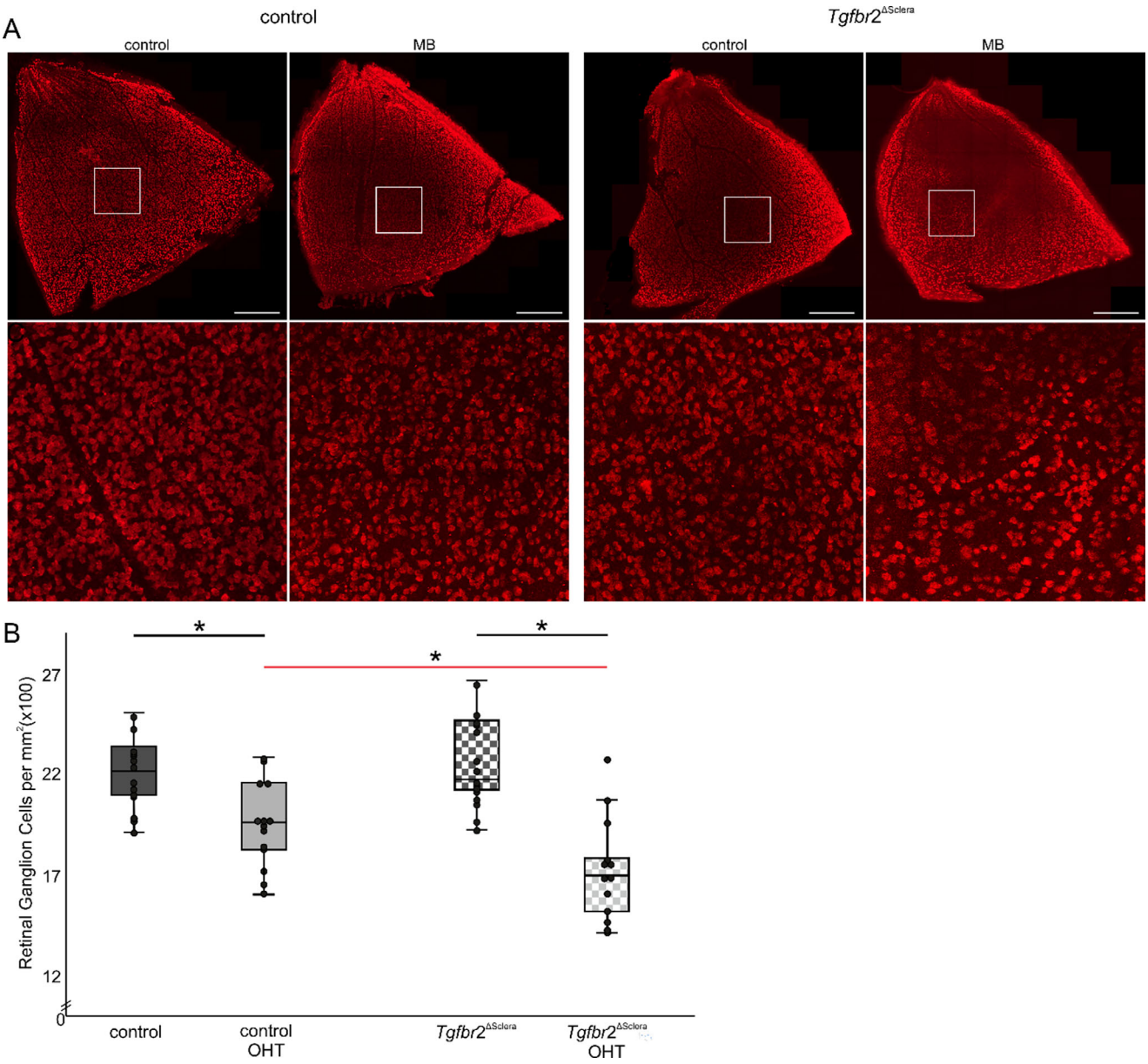

Figure 4. RGC loss was elevated in Tgfbr2$^{\Delta Sclera}$ mice following MB injection when compared to control littermates. (**A**) Representative images of RBPMS-stained retinae. *Scale bars*: 500 µm. Higher magnifications show an area of 500 × 500 µm. (**B**) RGC numbers for not-injected eyes did not differ between control and Tgfbr2$^{\Delta Sclera}$ mice (control, 2206 ± 175; Tgfbr2$^{\Delta Sclera}$, 2258 ± 225; $P = 1.00$; $n = 13$), but RGC numbers for Tgfbr2$^{\Delta Sclera}$ mice were significantly lower 6 weeks after MB injection than those of control mice (control MB, 1954 ± 212; Tgfbr2$^{\Delta Sclera}$ MB, 1727 ± 258; $P = 0.035$; $n = 13$).

Discussion

We conclude that scleral TGF-β signaling protects RGC somata and their axons in the optic nerve from damage caused by high IOP in experimental glaucoma. This conclusion rests on (1) the induced reduction of TGF-βRII and its mRNA in the sclera of Tgfbr2$^{\Delta Sclera}$ mice, (2) the generation of high IOP following MB injection, and (3) the significant increase in RGC and axon loss in mice with reduced TGF-β signaling in the sclera.

Currently, the molecular mechanisms behind this protective effect are unclear. Our approach supposedly causes a reduction in canonical TGF-β signaling in all cells with high activity of the Col1a2 promoter throughout the body. Commonly, high activity of this promoter is seen in fibroblasts. It appears reasonable to assume that the protective effect against IOP-induced damage in the eye is due to specific functions in scleral fibroblasts that are close to the ONH such as the PPS. As in other dense connective tissues, TGF-β signaling in the sclera induces collagen formation and contractility of fibroblasts. Both scenarios should lead to greater stiffness of the sclera and may be the cause for the greater scleral stiffness that has been observed in glaucoma. Our results appear to indicate that higher scleral stiffness protects ON axons from damage in glaucoma. In this context, it is of interest to note that scleral TGF-β decreases in myopia, leading to reduced ECM production and scleral thickness,[30–32] and that

patients with myopia have a higher risk for ON damage in glaucoma.[33–35]

The function of TGF-β in the eye is not restricted, though, to the sclera. In contrast, TGF-β is almost ubiquitously expressed in ocular tissues[18,36–38] and appears to have a multitude of tissue-specific functions. For example, TGF-β signaling is required for maintenance of retinal and choroidal vessels in the adult or developing eye and attenuates apoptosis of retinal neurons during development.[39–41] Accordingly, although scleral TGF-β might protect from degeneration in glaucoma, high activity of TGF-β signaling in other ocular cell types might rather promote it. Such effects might explain the observations that treatment of mice with losartan, an inhibitor of the angiotensin 1 (AT1R) receptor, protects from ON degeneration in MB-induced experimental glaucoma.[42] Losartan suppresses Smad2 phosphorylation and TGF-β expression in its canonical pathway,[43,44] thereby reducing TGF-β signaling activity. On the other hand, the effects may also be related to other signaling pathways that act downstream of AT1R receptors. In a rat glaucoma-model, a similarly protective effect could be shown for candesartan, a related AT1R inhibitor.[45]

Several potential caveats of this study should be considered: First, it is unclear if the induced deficiency of TGF-β signaling in mutant mice directly affected IOP levels following MB-induced ocular hypertension; if so, was it different than that in the control eyes? Our weekly IOP measurements strongly argue against such a scenario but naturally give no information on the complete IOP history during each of the 42 days following bead injection. Second, rebound tonometry as used in the present study to measure IOP is influenced by the biomechanical properties of the cornea. The induced deficiency of TGF-β signaling may influence those properties. We regard such an effect as unlikely, as in a previous study using the mouse as model[42] neither normal IOP nor experimentally induced high IOP (measured by TonoLab and invasively by cannulation) was affected by losartan, a compound that induces inhibition of TGF-β signaling. Third, axon loss was significant yet small in both groups (9% in the control group vs. 15% in the experimental group) and did not result in significant differences in axon density. Increasing the duration of elevated IOP would likely cause an increase in axon loss and a significant decrease in axon density. Finally, it remains to be shown if the observed effects in murine eyes with an astrocyte-based glial lamina are also relevant for the primate eye with a lamina cribrosa containing numerous connective tissue strands. Nevertheless, identification of the molecular mechanisms that are induced by scleral TGF-β signaling to protect ON axons from damage in murine experimental glaucoma is likely to provide novel insights into the pathogenesis of glaucoma.

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