VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Deletion of Endothelial Transforming Growth Factor—β Signaling Leads to Choroidal Neovascularization

Anja Schlecht,* Sarah V. Leimbeck,* Herbert Jägle,† Annette Feuchtinger,‡ Ernst R. Tamm,* and Barbara M. Braunger*

From the Institute of Human Anatomy and Embryology,* University of Regensburg, Regensburg; the Department of Ophthalmology,† University Clinic Regensburg, Regensburg; and the Research Unit Analytical Pathology,‡ Helmholtz Zentrum Munich, Munich, Germany

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Address correspondence to Barbara M. Braunger, M.D., Ph.D., or Ernst R. Tamm, M.D., Institute of Human Anatomy and Embryology, University of Regensburg, Universitätsstrasse 31, D-93053 Regensburg, Germany. E-mail: barbara.braunger@ur.de or ernst.tamm@ur.de.

The molecular pathogenesis of choroidal neovascularization (CNV), an angiogenic process that critically contributes to vision loss in age-related macular degeneration, is unclear. Herein, we analyzed the role of transforming growth factor (TGF)-β signaling for CNV formation by generating a series of mutant mouse models with induced conditional deletion of TGF-β signaling in the entire eye, the retinal pigment epithelium (RPE), or the vascular endothelium. Deletion of TGF-β signaling in the eye caused CNV, irrespectively if it was ablated in newborn or 3-week-old mice. Areas of CNV showed photoreceptor degeneration, multilayered RPE, basal lamina deposits, and accumulations of monocytes/macrophages. The changes progressed, leading to marked structural and functional alterations of the retina. Although the specific deletion of TGF-β signaling in the RPE caused no obvious changes, specific deletion in vascular endothelial cells caused CNV and a phenotype similar to that observed after the deletion in the entire eye. We conclude that impairment of TGF-β signaling in the vascular endothelium of the eye is sufficient to trigger CNV formation. Our findings highlight the importance of TGF-β signaling as a key player in the development of ocular neovascularization and indicate a fundamental role of TGF-β signaling in the pathogenesis of age-related macular degeneration. (Am J Pathol 2017, 187: 2570–2589; http://dx.doi.org/10.1016/j.ajpath.2017.06.018)

The therapeutic options for treating age-related macular degeneration (AMD), the leading cause of vision loss and blindness in industrialized countries,1–3 are limited.1–3 AMD is a complex disease whose molecular pathogenesis is not well understood.4,5 Vision loss in AMD is caused by choroidal neovascularization (CNV) or geographic atrophy.4,5 In geographic atrophy (late dry AMD), the progressive atrophy of the retinal pigment epithelium (RPE) and of the choriocapillaris are likely causes of photoreceptor degeneration.5 Angiogenic processes that cause immature choroidal vessels to break in and through the RPE into the subretinal space characterize CNV.5 The resulting blood and plasma leakage causes fibrous scarring of the retina (wet AMD). The pathogenic mechanisms underlying the onset of CNV in AMD are unclear. Dysfunction of the RPE and a dysregulation of proangiogenic and immune-stimulating molecular factors in the region of the retinal/choroidal interface are considered as contributing factors.4,5,9,10

The choroidal vasculature forms the choriocapillaris, a vascular bed of highly anastomosed capillaries that is essential for nutrition and oxygen supply of both photoreceptors and RPE.11 The capillaries have a fenestrated endothelial layer and are highly permeable,12 a substantial difference from retinal capillaries. There is evidence from several studies of genetically engineered mouse models indicating that RPE-derived vascular endothelial growth factor (VEGF) is essential for choriocapillaris maintenance. VEGF is expressed in high amounts by RPE cells during embryonic development and in adult life.13 If VEGF expression is compromised, formation of the choriocapillaris in development or its maintenance in adult eyes is severely impaired, leading to choriocapillaris ablation.14–18

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Other growth factors that are secreted by RPE cells in high amounts are transforming growth factor (TGF)-β1 and TGF-β2, causing a concentration of both factors in the RPE/choroid complex that is >10-fold higher than in the retina. A recent study showed that a conditional ocular deletion of TGF-β signaling results in pronounced structural changes of retinal capillaries, including the formation of abundant microaneurysms, leaky capillaries, and retinal hemorrhages. Overall, a phenotype resulted similar to that of diabetic retinopathy in humans. 21

Because TGF-β is present at high concentrations in the region of the RPE/choroid interface, we wondered if TGF-β signaling might not only be required for stability of retinal capillaries, but also for that of the choriocapillaris. To this end, we targeted the TGF-β type II receptor (TβRII), which is essential for TGF-β signaling, and generated a series of mutant mouse strains with an induced conditional deletion in the entire eye, the RPE, or vascular endothelial cells. Herein, we provide evidence that impairment of TGF-β signaling in the vascular endothelium of the eye is sufficient for the development of CNV. Our findings highlight the importance of the TGF-β signaling pathway as a key player in the development of ocular neovascularization and indicate a fundamental role of TGF-β signaling in the pathogenesis of AMD.

Materials and Methods

Mice

All experiments were performed in mice of either sex that were tested negatively for the Rdh8 mutation. 22 Mice with two floxed alleles of Tgbr2 (Tgbr2<sup>fl/fl</sup>) 23 were crossed with heterozygous CAGGCre-ER<sup>T2</sup>, 21 VMD2<sup>rtTA</sup>-Cre, 25 or VECad-Cre-ER<sup>T2</sup> mice. Resulting Tgbr2<sup>fl/fl</sup>,CAGGCre-ER, Tgbr2<sup>fl/fl</sup>,VMD2<sup>rtTA</sup>-Cre, or Tgbr2<sup>fl/fl</sup>,VECad-Cre-ER<sup>T2</sup> mice were used as experimental mice. For simplicity, Tgbr2<sup>fl/fl</sup>,CAGGCre-ER mice will be referred to as Tgbr2<sup>fl</sup> cre , Tgbr2<sup>fl/fl</sup>,VMD2<sup>rtTA</sup>-Cre mice will be referred to as Tgbr2<sup>ΔRPE</sup> cre , and Tgbr2<sup>fl/fl</sup>,VECad-Cre-ER<sup>T2</sup> mice will be referred to as Tgbr2<sup>ΔEC</sup>. Tgbr2<sup>fl/fl</sup> littermates with two unrecombined Tgbr2 alleles served and are referred to as controls. Genetic backgrounds were 129SV (Tgbr2<sup>fl/fl</sup>, VMD2<sup>rtTA</sup>-Cre, and VECad-Cre-ER<sup>T2</sup>) and C57Bl/6 (CAGGCre-ER). All mice were reared in a light/dark cycle of 12 hours (lights on at 7 AM). Genotypes were identified by isolating genomic DNA from tail biopsy specimens, and testing for transgenic Cre sequences and floxed Tgbr2 sequences, as described previously. 21,27

Induction of Cre Recombinase

Cre recombinase in CAGGCre-ER and VECad-Cre-ER<sup>T2</sup> mice is tamoxifen dependent. In CAGGCre-ER mice, the Cre-ER fusion protein is ubiquitously expressed. Cre recombinase is restricted to the cytoplasm and will only access the nucleus after binding to tamoxifen. 24,28 VECad-Cre-ER<sup>T2</sup> mice carry the Cre-ERT2 expression cassette under regulatory control of the mouse vascular endothelial-cadherin gene promoter region that specifically drives gene expression in the vascular endothelium. 26 To activate Cre recombinase in Tgbr2<sup>ΔEY</sup> and Tgbr2<sup>ΔEC</sup> mice, the conditional knockout animals and their respective control littermates were equally treated with tamoxifen containing eye drops from postnatal day (P) 4 to P8, as described previously. 29 A second stock of Tgbr2<sup>ΔEY</sup> and control mice was treated from P21 to P25, a time when the development of the retinal vasculature is complete. For simplicity, Tgbr2<sup>ΔEY</sup> mice that were tamoxifen treated from P4 to P8 will be referred to as early induced, and Tgbr2<sup>ΔEY</sup> mice that were tamoxifen treated from P21 to P25 will be referred to as late induced. Cre recombinase in VMD2<sup>rtTA</sup>-Cre;Tgbr2<sup>ΔEY</sup> is doxycycline dependent and under the control of the RPE-specific bestrophin promoter. 20 Doxycycline (Appli-Chem, Darmstadt, Germany) was diluted in phosphate-buffered saline (PBS) to a final concentration of 0.1 g/mL, and doxycycline eye drops (10 µL) were pipetted onto the eyes of Tgbr2<sup>ΔRPE</sup> mice and their control littermates from P21 to P25 three times per day. A detailed list of the individual treatment time points and subsequently performed analyses of the eyes is presented in Table 1.

mT/mG and R26R Reporter Mice

The efficiency of the Cre recombinase activation in VeCad-Cre-ER<sup>T2</sup> mice was confirmed using mT/mG reporter mice, 30 which express a membrane-targeted green fluorescent protein after Cre-mediated excision. Heterozygous VeCad-Cre-ER<sup>T2</sup> mice were crossed with homozygous mT/mG mice. The resulting offspring (VeCad-Cre-ER<sup>T2+/-</sup>;mT/mG<sup>+/−</sup> and wildtype;mT/mG<sup>+/−</sup>) were treated with tamoxifen eye drops from P4 to P8, according to previously published protocols. 21,28 Mice were euthanized at P10. After enucleation, the eyes were fixed in 4% paraformaldehyde (PFA) for 4 hours, washed extensively in phosphate buffer (PB; 0.1 mol/L, pH 7.4), incubated in 10%, 20%, and 30% sucrose overnight, and shock frozen in tissue-mounting medium (O.C.T. Compound; DiTec, Bamberg, Germany). Frozen sections were washed three times in PB (5 minutes each), and cell nuclei were counterstained with DAPI (Vectorshield; Vector Laboratories, Burlington, CA) 1:10 diluted in fluorescent mounting medium (Serva, Heidelberg, Germany). Successful activation of the Cre recombinase in CAGGCre-ER mice was confirmed using Rosa26 reporter (R26R) reporter mice. 31 Heterozygous CAGGCre-ER mice were crossed with homozygous R26R mice. The resulting offspring were treated with tamoxifen eye drops from P21 to P25, according to previously published protocols. 27,29 Mice were euthanized at the age of 4 weeks, and the eyes were enucleated and processed for LacZ staining, which was performed as described previously. 29,22
Paraffin sections were analyzed by light microscopy (Carl Zeiss, Jena, Germany).

**Tgfbr2 Deletion PCR**

*Tgfbr2* deletion PCR screens its successful genomic deletion after Cre-mediated recombination. DNA samples of the sensory retina and the choroid (including cells of the RPE) served as templates. Primer pairs and protocols are described elsewhere.29,33 Actin was used as the loading control.

**Morphology and Microscopy**

Eyes were enucleated, fixed for 24 hours in 2% PFA/2.5% glutaraldehyde,34 and embedded in Epon (Serva), as described elsewhere.35,36 Semithin meridional sections (1 μm thick) were cut through the eyes and stained, according to the method of Richardson et al.57 The sections were analyzed on an Axio Imager Z1 microscope (Carl Zeiss) using Axiovision software version 4.8 (Carl Zeiss). Ultrathin sections were processed according to protocols published previously,38,39 stained with uranyl acetate and lead citrate, and analyzed on a transmission electron microscope (Libra; Carl Zeiss).

**Immunohistochemistry**

Before TβRII, collagen IV, and VEGF-A staining, eyes were fixed for 4 hours in 4% PFA, washed extensively in PB (0.1 mol/L, pH 7.4), and embedded in paraffin, according to standard protocols. Paraffin sections (6 μm thick) were deparaffinized and washed in water. For detection of TβRII, sections were treated with boiling citrate buffer (1 x 10 minutes, pH 6), washed again in water, and incubated in PB. For detection of collagen IV and VEGF-A, sections were pretreated with 0.05 mol/L Tris-HCl (5 minutes) and covered with Proteinase K [100 μL of Proteinase K in 57 mL of Tris-HCl (0.05 mol/L), 5 minutes], washed in water, incubated in 2N HCl (20 minutes), and washed again in water. Sections were incubated in PB for 5 minutes and then blocked with 2% bovine serum albumin, 0.2% cold water fish gelatine, and 0.1% Triton X (TβRII), 2% bovine serum albumin and 0.1% Triton X (collagen IV), or 5% nonfat dry milk (VEGF-A) at room temperature for 60 minutes. Primary antibodies (Table 2) were diluted in a 1:10 dilution of blocking solution in PB. Before F4/80 and plasmalemma vesicle-associated protein (PLVAP) staining, eyes were fixed for 4 hours in 4% PFA, washed extensively in PB, incubated in 10%, 20%, and 30% sucrose/PBS overnight at 4°C, and shock frozen in tissue-mounting medium. For immunohistochemistry, frozen sections were washed three times in PB/PBS (F4/80) for 5 minutes each and blocked at room temperature with 2% bovine serum albumin, 0.02% NaN3, and 0.01% Triton X in PBS), and incubated at 4°C overnight. After three washes in PB/PBS (5 minutes each), biotinylated antibodies were applied for 1 hour and diluted in a 1:10 dilution of the blocking solution. Then, appropriate secondary antibodies (Table 2), diluted in a 1:10 dilution of the blocking solution, were applied on the sections for 1 hour. Sections were washed again three times in PB/PBS, and cell nuclei were counterstained with DAPI (Vectashield) diluted (1:10) in.

<table>
<thead>
<tr>
<th>Mouse model</th>
<th>Treatment</th>
<th>Performed analyses</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tgfbr2</em>^−/−^ and controls</td>
<td>Tamoxifen P4-P8</td>
<td>Morphology</td>
<td>2 and 3 months</td>
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<td></td>
<td></td>
<td>FITC-dextran</td>
<td>6 weeks</td>
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<td>3D imaging</td>
<td>6 weeks</td>
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<td></td>
<td></td>
<td>Electron microscopy</td>
<td>2.5 months</td>
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<tr>
<td></td>
<td></td>
<td>RNA analyses</td>
<td>6 weeks</td>
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<tr>
<td></td>
<td></td>
<td>Immunohistochemistry</td>
<td>4 and 6 weeks</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Morphology</td>
<td>3 and 6 months</td>
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<tr>
<td></td>
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<td>FITC-dextran</td>
<td>2 months</td>
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<td></td>
<td></td>
<td>Angiography</td>
<td>3 and 6 months</td>
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<td></td>
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<td>ERG</td>
<td>3 and 6 months</td>
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<tr>
<td></td>
<td></td>
<td>Electron microscopy</td>
<td>3 months</td>
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<td></td>
<td></td>
<td>RNA analyses</td>
<td>5 weeks</td>
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<tr>
<td></td>
<td></td>
<td>Immunohistochemistry</td>
<td>3 months</td>
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<tr>
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<td>Morphology</td>
<td>6 weeks</td>
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<td>Electron microscopy</td>
<td>4 and 6 weeks</td>
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<td>Morphology</td>
<td>6 months</td>
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<tr>
<td></td>
<td></td>
<td>Angiography</td>
<td>6 months</td>
</tr>
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3D, three dimensional; ERG, electroretinography; FITC, fluorescein isothiocyanate; P, postnatal day.

Table 1. Treatment Time Points and Performed Analyses

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TGF-β and Choroidal Neovascularization

Table 2 Antibodies Used for Immunohistochemistry

<table>
<thead>
<tr>
<th>Primary antibody (manufacturer)</th>
<th>Secondary antibody (manufacturer)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TJRII-L21 (Santa Cruz Biotechnology, Dallas, TX), 1:20</td>
<td>Anti-rabbit, biotinylated (Vector Laboratories, Burlington, CA), 1:500; streptavidin Alexa 488 (Invitrogen, Carlsbad, CA), 1:1000</td>
</tr>
<tr>
<td>Collagen IV (Rockland Immunochemicals Inc., Limerick, PA), 1:100</td>
<td>Anti-rabbit Cy-3 conjugated (Jackson Immuno Research Labs, West Grove, PA), 1:2000</td>
</tr>
<tr>
<td>VEGF-A (R&amp;D Systems, Minneapolis, MN), 1:50</td>
<td>Anti-goat, biotinylated (Vector Laboratories), 1:500; streptavidin Alexa 546 (Invitrogen), 1:1000</td>
</tr>
<tr>
<td>F4/80 (Acris Antibodies GmbBH, Herford, Germany), 1:600</td>
<td>Anti-rat Cy-3 conjugated (Jackson Immuno Research Labs), 1:2000, in PBS</td>
</tr>
<tr>
<td>PLVAP (Santa Cruz Biotechnology), 1:50</td>
<td>Anti-rat Cy-3 conjugated (Jackson Immuno Research Labs), 1:2000</td>
</tr>
</tbody>
</table>

In all cases, fixation was performed with 4% paraformaldehyde. PBS, phosphate-buffered saline; PLVAP, plasmalemma vesicle-associated protein; TJRII, transforming growth factor-β type II receptor.

fluorescent mounting medium (Serva). Sections were investigated on an Axio Imager Z1 fluorescent microscope using appropriate Axiovision software.

Quantification of F4/80-Positive Cells on Immunohistochemical Sections

Experimenters (A.S. and B.M.B.) were blinded regarding the genotype. Antibody incubation times were strictly monitored, and microscope settings (eg, the illumination time and intensity) were identical between individual sections. Only sections in midsagittal orientation were analyzed that stretched through the optic nerve to ensure comparable situations between the individual sections. F4/80-positive cells were counted per hemisphere in the retina and in the choroid/choriocapillaris.

Dextran Perfusion

Before dextran perfusion, mice were deeply anesthetized with ketamine [120 mg/kg body weight (bw)] and xylazine (8 mg/kg bw). Afterward, mice were perfused through the left ventricle with 1 mL of PBS containing 50 mg fluorescein isothiocyanate (FITC)—dextran (mol. wt., 2000 kDa; TdB Consultancy, Uppsala, Sweden). The eyes were enucleated and fixed in 4% PFA for 2 hours (flat mounts)/4 hours (sections) and washed in PB. Eyes were cut into sections, placed on glass slides, and counterstained with DAPI (Vectashield) 1:10 diluted in fluorescent mounting medium. FITC-dextran perfused sections were investigated on an Axio Imager Z1 fluorescent microscope.

Quantification of CNV

After dextran perfusion, flat mounts of the posterior eye segment (containing the retina, RPE, choroid, and sclera) were dissected. Similar to the preparation of retinal flat mounts, the posterior eye segment was flat mounted on glass slides using fluorescent mounting medium. The entire flat mount was analyzed using an Axio Imager Z1 fluorescent microscope. Focusing on the retinal pigment epithelium allowed the quantification of the CNV penetrating through the RPE. To visualize the CNV, Z-stacks were generated that ranged from the superficial retinal plexus to the optical barrier of the pigmented RPE.

ERG Data

Mice were dark adapted for at least 12 hours before the experiments and anesthetized by s.c. injection of ketamine (65 mg/kg bw) and xylazine (13 mg/kg bw). Pupils were dilated with tropicamide eye drops (Mydriaticum Stulln; Pharma Stulln GmbH, Stulln, Germany). Silver needle electrodes served as reference (forehead) and ground (tail), and gold wire ring electrodes served as active electrodes. Electroretinograms (ERGs) were recorded with a Ganzfeld bowl (Ganzfeld QC450 SCX; Roland Consult, Brandenburg, Germany) and an amplifier/recording unit (RETI-Port; Roland Consult), band pass filtered (1 to 300 Hz), and averaged. Single-flash scotopic (dark-adapted) responses to a series of 10 light-emitting diode flash intensities (range, −3.5 to 1.0 log cd ∙ second/m²) were recorded. After 10 minutes of adaption to a white background illumination (20 cd/m²), single-flash photopic (light-adapted) responses to three xenon-flash intensities (1, 1.5, and 2 log cd ∙ second/m²) were recorded. All analyses and plotting were performed with R version 3.2.1 (The R Foundation for Statistical Computing, Vienna, Austria) and ggplot2 version 2.1.0.10

Fundus Imaging and Angiography

Imaging of the retinal vasculature was performed with a commercially available imaging system (Micron III; Phoenix Research Laboratories, Pleasanton, CA). Light source and imaging path filters (low and high pass at 500 nm) were used for fluorescein angiography. Mice were anesthetized by s.c. injection of ketamine (65 mg/kg bw) and xylazine (13 mg/kg bw), and their pupils were dilated with tropicamide eye drops before image acquisition. Fluorescein angiography was performed with s.c. injection of 75 mg/kg bw fluorescein-sodium (Alcon, Hünenberg, Switzerland).
RNA Analysis

Total RNA from retinas was extracted with TriFast (Peqlab Biotechnologie GmbH, Erlangen, Germany), and first-strand cDNA synthesis was performed with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA), according to the manufacturer’s guidelines. Real-time quantitative RT-PCR analyses were performed with the iQ5 Realtime PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA), according to the manufacturer's guidelines. Real-time RT-PCR amplification was performed to confirm that none of the housekeeping genes were regulated and then the geometric mean of the crossing threshold of all three housekeeping genes was used for relative quantification. Quantification was performed using BioRad iQ5 Standard-Edition software version 2.1 (Bio-Rad Laboratories GmbH, Munich, Germany) and the ΔΔCT method in Excel (Microsoft Corp., Redmond, WA).41

Table 3 Primers Used for Real-Time PCR Amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence forward</th>
<th>Sequence reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-TGTCCGTCGGATCTTGAC-3'</td>
<td>5'-CCTGTCTACACCTTCTTG-3'</td>
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<tr>
<td>GNB2L</td>
<td>5'-TCTCAGTACACGTTACAG-3'</td>
<td>5'-ACAGTAGTGATTGCTG-3'</td>
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<tr>
<td>RPL32</td>
<td>5'-GCTGGCTATCGTGGTGAG-3'</td>
<td>5'-TGACTGGTCTGATGACT-3'</td>
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<tr>
<td>Tgfbr2</td>
<td>5'-AGAGCCGAATGCTACCGT-3'</td>
<td>5'-CGGCAAACCTCCACAGTA-3'</td>
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<tr>
<td>Ccl2</td>
<td>5'-CATCCACAGTGTGCTGCA-3'</td>
<td>5'-GATCTACTTTGTTGATAG-3'</td>
</tr>
<tr>
<td>Cd68</td>
<td>5'-CTCTTCATAAGCCGAGGCT-3'</td>
<td>5'-TCAAGGTTGAAAGAAACA-3'</td>
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<td>Fgap</td>
<td>5'-TCGAGAAAAATCCGAC-3'</td>
<td>5'-GTCCTGACCAAGAGTTGACT-3'</td>
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<td>Il-6</td>
<td>5'-GCTACCAAACTGGAATACTGAGA-3'</td>
<td>5'-CCAGGCTATGTGATCCAGAA-3'</td>
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<tr>
<td>iNos</td>
<td>5'-GGCGTGTACGAGATATCAGA-3'</td>
<td>5'-CCATGATGGGTACATTCTCAG-3'</td>
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<td>Tnf-α</td>
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<td>Vegf-a-120</td>
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<td>Vegf-a-164</td>
<td>5'-GAACAAAGGAGAATCTGAG-3'</td>
<td>5'-CGAGTCTGGTTTTTGCAAGGAC-3'</td>
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<td>Fgf2</td>
<td>5'-CGGCTCTACTGCAAGAACG-3'</td>
<td>5'-TGCTTGGAGTTGATGTGACG-3'</td>
</tr>
</tbody>
</table>

Deep Tissue Imaging by Three-Dimensional Light-Sheet Fluorescence Microscopy

Mice were deeply anesthetized with ketamine (120 mg/kg bw) and xylazine (8 mg/kg bw), and 130 μL of Tomato lectin (Perkin Elmer, Inc., Waltham, MA) was slowly injected intravenously. The eyes were euthanized 15 minutes after injection by cervical dislocation. The eyes were enucleated and fixed using Paxgene Tissue Containers (Qiagen, Hilden, Germany), according to the manufacturer’s recommendations, and thereafter underwent a chemical procedure of optical clearing, as previously described.42 Cleared transparent whole mouse eyes were imaged on a light-sheet fluorescence microscope (UltraMicroscope II; LaVision BioTec, Bielefeld, Germany), equipped with an sCMOS camera (Andor Neo, Concord, MA) and a 2×/0.5 numerical aperture objective lens (MVPLAPO 2×; Olympus, Hamburg, Germany). The specimens were two sided, illuminated by a planar light sheet using a white light laser (SuperK EXTREME EXW-9; NKT Photonics, Birkerød, Denmark). To visualize the specific T.lectinSence 680 signals for vascularization, a bandpass filter set with an excitation range of 640/30 nm and an emission range of 690/50 nm was used in combination with an additional filter set (excitation, 531/40 nm; emission, 593/40 nm) for detection of autofluorescence (morphology). By moving the specimen chamber vertically stepwise (step size, 4 μm) through the laser light sheet, optical sections were obtained. To ensure standardized imaging regions for each eye, the scan always covered 600 μm above to 600 μm below the optical nerve. Maximum intensity projections were performed by InspectorPro software version 5.0 (LaVision BioTec, Bielefeld, Germany).

Statistical Analysis

All results are expressed as means ± SEM. Comparisons between the mean variables of two groups were made by a two-tailed t-test. Significance of the ERG analyses was evaluated using a one-way analysis of variance test, followed by a Tukey honest significant difference post hoc test. P ≤ 0.05 was considered statistically significant.

Study Approval

All procedures conformed to the tenets of the NIH’s Guide for the Care and Use of Laboratory Animals,43 European Union Directive 2010/63/E, and institutional guidelines;
all procedures were approved by the local authority (Regierung der Oberpfalz, AZ 54-2532.1-44/12 and DMS 2532-2-85).

**Results**

Deletion of Ocular TGF-β Signaling in Mouse Pups Leads to Choroidal Neovascularization

To investigate the role of the TGF-β signaling pathway for the structure of retinal and choroidal vessels, the essential TβRII receptor in the eyes was removed via tamoxifen-induced conditional deletion. To this end, Tgfb2/fl;CAGGCre-ER mice were generated and treated from P4 to P8 with tamoxifen. For simplicity, mice were generated and treated from P4 to P8 with tamoxifen receptor in the eyes was removed via tamoxifen-induced continuous ongoing. To learn if TGF-β signaling is also important for maintenance of the adult choriocapillaris when retinal vascular development is completed, we deleted TGF-β signaling by an essentially comparable approach in 3-week—old mice with completely developed retinal vasculature. Mice were treated with tamoxifen eye drops from P21 to P25 and are further referred to as late-induced Tgfb2 Δtfr mice. To confirm successful tamoxifen-induced recombination, we crossed the mice with Cre reporter mice carrying the R26R allele. Tamoxifen-treated eyes were stained for β-galactosidase, and an intense staining was seen

RPE, choroid, and sclera) of 4- to 6-week—old animals had 2.86 ± 0.59 CNVs per eye (n = 7) (Supplemental Figure S1B). To confirm this observation by an independent method, three-dimensional imaging of lectin-perfused, optical-cleared, and transparent whole eyes was performed (Figure 2B). The choriocapillaris did not resolve into single capillaries but rather appeared as an intense fluorescent line covering the outer surface of the retina, a fact attributed to the immense density and blood flow of this capillary bed.44 In control eyes, the three plexuses of the retinal vasculature could be completely visualized and were essentially normal. In contrast, in early-induced Tgfb2 Δtfr mice, vessels that originated from the choriocapillaris entered the retina and continued into the retinal vascular bed, forming obvious anastomoses with retinal vessels (Figure 2B). We now characterized the vascular wall of the newly formed vessels by transmission electron microscopy. As expected, in control mice, the endothelium surrounding retinal vessels was continuous (Figure 2C), whereas that around the choriocapillaris was fenestrated. When the newly formed vessels in the subretinal space of early-induced Tgfb2 Δtfr mice were analyzed, distinct fenestrations covered by a typical diaphragm were regularly observed (Figure 2C). To further support this finding, immunohistochemical staining was performed for PLVAP, a vascular protein, an intrinsic component of the diaphragm of vascular fenestrae, and a marker for fenestrated endothelia.45–48 As expected, in control and early-induced Tgfb2 Δtfr animals, the continuous endothelium of the retinal vasculature did not show any PLVAP immunoreactivity (Supplemental Figure S1A), whereas the fenestrated endothelium of the choriocapillaris showed an intense PLVAP signal (Figure 2D). In early-induced Tgfb2 Δtfr mice, intense PLVAP immunoreactivity was observed in the areas of neovascularization in the subretinal space (Figure 2D). Overall, our results strongly indicated that loss of TGF-β signaling in early-induced Tgfb2 Δtfr mice caused CNV.

TGF-β Signaling Is Required to Prevent CNV in Late-Induced Tgfb2 Δtfr Mice

Next, we wondered whether the formation of CNV after TβRII deletion might be supported by the facts that retinal vascular development is not completed in mouse pups and that signaling processes that promote angiogenesis are continuously ongoing. To learn if TGF-β signaling is also important for maintenance of the adult choriocapillaris when retinal vascular development is completed, we deleted TGF-β signaling by an essentially comparable approach in 3-week—old mice with completely developed retinal vasculature. Mice were treated with tamoxifen eye drops from P21 to P25 and are further referred to as late-induced Tgfb2 Δtfr mice. To confirm successful tamoxifen-induced recombination, we crossed the mice with Cre reporter mice carrying the R26R allele. Tamoxifen-treated eyes were stained for β-galactosidase, and an intense staining was seen

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throughout the eye of CAGGCre-ER:R26R mice, whereas ocular tissues of R26R littermates, which did not carry the CAGGCre-ER transgene, were completely unstained (Supplemental Figure S2A). Furthermore, the significant deletion of retinal Tgfb2 was confirmed by real-time RT-PCR analyses (Supplemental Figure S2B). Next, we analyzed the ocular morphology of late-induced Tgfb2<sup>Δ<sub>eye</sub></sup> mice and controls at the age of 3 (Figure 3, A and B) and 6 (Figure 4, A and C) months. Retinal structure was essentially normal in control animals (Figure 3, A and B, and Figure 4, A and C), and no obvious changes were observed in the inner retina of 3-month—old late-induced Tgfb2<sup>Δ<sub>eye</sub></sup> animals (Figure 3B). However, similar to our observations in early-induced Tgfb2<sup>Δ<sub>eye</sub></sup> animals, all of the late-induced Tgfb2<sup>Δ<sub>eye</sub></sup> mice showed structural changes in the subretinal space that were essentially comparable to that observed in early-induced Tgfb2<sup>Δ<sub>eye</sub></sup> mice (Figure 3, A and D, and Figure 4, A—C). In focal areas in which photoreceptor outer segments were shortened or completely missing, vessels were observed in the subretinal space. By transmission electron microscopy, a fenestrated endothelial layer was identified surrounding the vessels (Figure 5B). The RPE in those areas was multilayered and frequently contained cystic and amorphous inclusions. In 6-month—old late-induced Tgfb2<sup>Δ<sub>eye</sub></sup> mice, the choroid was thickened and photoreceptor outer segments were completely missing (Figure 4, A—C). The RPE was reduced to a flat layer or formed focal accumulations in those areas in which subretinal vessels...
were present. In vivo imaging and fluorescein in vivo angiography of 3-month-old animals showed a regular fundus and retinal vasculature in both control and late-induced Tgfbr2<sup>−/−</sup> mice (Figure 3C). To investigate the retinal vasculature in more detail, we additionally perfused 2-month-old, late-induced Tgfbr2<sup>−/−</sup> mice with high-mol. wt. FITC-dextran and analyzed the vasculature on meridional sections. The retinal vasculature had a regular

**Figure 2**
Subretinal neovascularization with fenestrated endothelium (fE) after deletion of transforming growth factor-β (TGF-β) signaling in the eyes of early-induced Tgfbr2<sup>−/−</sup> mice. A: Fluorescein isothiocyanate–dextran (green)–perfused retinal meridional section of a control and Tgfbr2<sup>−/−</sup> mouse at 6 weeks of age. White arrows point toward choroidal vessels breaking through the Bruch membrane (BM) and retinal pigment epithelium (RPE; nuclei of the RPE marked by white arrowheads) into the subretinal space. Nuclei are DAPI stained (blue). B: Light-sheet fluorescence microscopy of transparent eyes of 6-week-old lectin-injected Tgfbr2<sup>−/−</sup> mice and a control littermate. The control mouse shows an essentially regular arborized retinal vasculature. The Tgfbr2<sup>−/−</sup> mice have an irregular arrangement of the retinal plexus and form anastomoses between retinal and choroidal vessels (arrows). C: Transmission electron microscopy of an intraretinal vessel outlined with a continuous endothelium (cE) in a 2.5-month-old control animal. In contrast, the subretinal neovascularization in the Tgfbr2<sup>−/−</sup> littermate has a fE (black arrows, right panel). The right panel shows the boxed region of the middle panel in higher magnification. D: Immunoreactivity for plasmalemma vesicle-associated protein (PLVAP; red) in the retina at 4 weeks of age. The control and the Tgfbr2<sup>−/−</sup> animal show a thin, one-layered PLVAP signal in the choriocapillaris (CC; white arrowheads). In addition, the Tgfbr2<sup>−/−</sup> mouse displays PLVAP-positive signals (white arrows) in the RPE and subretinal space. Nuclei are DAPI stained (blue). Scale bars: 50 μm (A and D); 120 μm (B); 1000 nm (C, left and right panels); 5000 nm (C, middle panel). 3D, three dimensional; C, choroid; ONL, outer nuclear layer.
appearance, and the vessels were in their anatomically correct localization. Furthermore, we did not observe FITC-dextran or fluorescein leaking into the vitreous, the subretinal space, or the surrounding tissue (Figure 3, C and E). As we had observed a significant reduction of the pericyte marker neural/glial antigen 2 in the retina of early-induced Tgfbr2$$^{Δe\text{ye}}$$ animals, we now focused on the coating of the choroidal vessels with neural/glial antigen 2-positive pericytes in 4-week-old early-induced and 6-week-old late-induced Tgfbr2$$^{Δe\text{ye}}$$ animals. We did not observe a difference in pericyte coverage compared to controls (data not shown). When the eyes at the age of 6 months were analyzed by in vivo fundus imaging and fluorescein angiography, control mice showed no pathological changes, in neither the fundus nor the retinal vasculature (Figure 4D). In contrast, in late-induced Tgfbr2$$^{Δe\text{ye}}$$ mice, the fundus was hyperpigmented, indicating RPE proliferation or dedifferentiation; in some animals, the retina had detached (Figure 4D). In parallel to the structural changes, marked functional changes by ERG that reflected the observed gradual degeneration of the retina from 3 to 6 months of age in late-induced Tgfbr2$$^{Δe\text{ye}}$$ mice were detected (Supplemental Figure S3, A–G). Although, in 3-month-old Tgfbr2$$^{Δe\text{ye}}$$ mice, the scotopic responses showed a similar waveform but slightly lower amplitudes compared to control mice, the photopic waveform was unchanged in amplitude and implicit time. However, the difference was not statistically significant. In 6-month-old Tgfbr2$$^{Δe\text{ye}}$$ mice, both the scotopic and photopic responses could not be distinguished from noise in almost all eyes.
Expression of Angiogenic Factors and Immune Modulating Cytokines in Tgfbr2<sup>eye</sup> Mice

The immunovascular axis, a cross talk of the RPE with immune and vascular cells, is considered a potent driver of CNV formation. To clarify the mechanisms behind CNV induced by TβRII deletion, we consequently analyzed the mRNA expression levels of immune-modulating cytokines and markers for the reactivity of Müller glia and microglia cells, respectively. Early-induced Tgfbr2<sup>eye</sup> mice showed a significant increase in retinal mRNA expression levels of glial fibrillary acidic protein, Cd68, inducible nitric oxide synthase, Il-6, tumor necrosis factor-α, and monocyte chemotactant protein-1 (chemokine (C-C motif) ligand 2) at the age of 6 weeks compared to control littermates (Figure 6A). However, in 3-month—old late-induced Tgfbr2<sup>eye</sup> mice, the retinal mRNA expression levels were not significantly enhanced compared to controls; in fact, glial fibrillary acidic protein was even significantly down-regulated (Figure 6B). Next, we used an antibody against F4/80 to label macrophages and microglia cells. The number of F4/80-positive cells was significantly increased in the choroid of early-induced Tgfbr2<sup>eye</sup> animals compared to controls (Figure 6C). Similarly, 3-month—old late-induced Tgfbr2<sup>eye</sup> animals also presented a distinct accumulation of F4/80-positive cells in the choroid when compared to controls (Figure 6D). Furthermore, the number of F4/80-positive cells in the retina was significantly increased in early-induced Tgfbr2<sup>eye</sup> animals compared to controls (Figure 6C). In vivo funduscopy and fluorescein angiography of the control animal are normal. Tgfbr2<sup>eye</sup> mice show a hyperpigmented fundus (middle panels) or retinal detachment (right panels). Diameters of the image sections refer to 2 mm retina. Scale bars: 500 μm (A); 20 μm (B); 50 μm (C). INL, inner nuclear layer; IPL, inner plexiform layer; PS, photoreceptor segment; RGC, retinal ganglion cell.

Figure 4 Subretinal neovascularization and retinal degeneration after deletion of transforming growth factor—β (TGF-β) signaling in the eyes of 6-month—old late-induced Tgfbr2<sup>eye</sup> mice. A: Stained semithin sections (1 μm thick) of the retinal hemispheres of 6-month—old animals, according to the method of Richardson et al. The control animal shows regular retinal morphology. In contrast, in the Tgfbr2<sup>eye</sup> littermate, photoreceptor outer segments are completely degenerated. B: Detailed magnification of stained semithin sections of the outer retina and the choroid (C) of 6-month—old Tgfbr2<sup>eye</sup> mice and a control littermate, according to the method of Richardson et al. The morphology of the control mouse is normal. In contrast, the Tgfbr2<sup>eye</sup> mouse has erythrocyte-filled vessels in the retinal pigment epithelium (RPE; Tgfbr2<sup>eye</sup> top panel, arrowheads), areas with a thickened (Tgfbr2<sup>eye</sup> middle panel) and thinned (Tgfbr2<sup>eye</sup> bottom panel) RPE, and an accumulation of pigmented cells in the sensory retina (Tgfbr2<sup>eye</sup> top, middle, and bottom panels, arrows). C: Detailed magnification of the retina. In the Tgfbr2<sup>eye</sup> mouse, the retina is degenerated, with a complete loss of photoreceptors. The RPE is disorganized, pigmented cells accumulate in the sensory retina, and the choroid is thickened. D: In vivo funduscopy and fluorescein angiography of the control animal are normal. Tgfbr2<sup>eye</sup> mice show a hyperpigmented fundus (middle panels) or retinal detachment (right panels). Diameters of the image sections refer to 2 mm retina. Scale bars: 500 μm (A); 20 μm (B); 50 μm (C). INL, inner nuclear layer; IPL, inner plexiform layer; PS, photoreceptor segment; RGC, retinal ganglion cell.
4-week—old Tgfbr2<sup>−/−</sup> mice had significantly elevated mRNA expression levels for the angiogenic factors Vegf-a 120, Vegf-a 164, fibroblast growth factor-2, insulin growth factor-1, angiopoietin 2, and platelet-derived growth factor-b compared to control littermates. We now performed immunohistochemistry for VEGF-A to localize the increased levels in the retina of early-induced Tgfbr2<sup>−/−</sup> mice (Figure 6E). Although VEGF-A immunoreactivity was not detectable in control eyes, there was distinct and intense staining of the ganglion cell layer of early-induced Tgfbr2<sup>−/−</sup> mice. When we screened the retinas of late-induced Tgfbr2<sup>−/−</sup> mice and their control littermates for the expression levels of the Vegf-a isoforms 120 and 164, the expression of Vegf-a 120 was not altered, whereas the expression of Vegf-a 164 was significantly reduced in 5-week—old late-induced Tgfbr2<sup>−/−</sup> mice compared to controls (Figure 6F). However, at the age of 6 months, the retinal expression levels of both Vegf-a 120 and 164 were significantly higher in late-induced Tgfbr2<sup>−/−</sup> mice compared to controls. Overall, angiogenic and immune reactivities were much higher in early-induced than in late-induced Tgfbr2<sup>−/−</sup> mice.

Formation of Basal Lamina Deposits in Tgfbr2<sup>−/−</sup> Mice

Because CNVs need to find their way across BM and through the RPE barrier, we used transmission electron microscopy to analyze the structure of both. Control mice showed the regular, five-layered architecture of the BM (Figure 5, A and B). In early- and late-induced Tgfbr2<sup>−/−</sup> mice, the BM had thickened as the result of an accumulation of collagen fibers and fine fibrillar extracellular material was detected between the basal lamina of the choriocapillaris and the elastic layer of the BM (Figure 5, C and D). In the control, the basal laminae of RPE and choriocapillaris endothelium are labeled. In places, the RPE basal lamina is interrupted (arrow) and electron-dense nodules arise to extend between the basolateral RPE infoldings. Higher magnification is given (bottom right panel). Immunoreactivity for collagen IV (red) in the retinal/choroidal interface in early-induced, 4-week—old Tgfbr2<sup>−/−</sup> mice. In the control, the basal laminae of RPE and choriocapillaris endothelium are labeled. In Tgfbr2<sup>−/−</sup> mice, staining is seen in the basal lamina of the choriocapillaris. Staining is irregular and patchy in the region of the RPE. Nuclei are DAPI stained (blue). RPE nuclei are marked by arrowheads. In Tgfbr2<sup>−/−</sup> mice, labeling is seen in the basal lamina surrounding choriocapillaris vessels, but is incomplete underneath the RPE. In places, the vascular basal lamina continues between RPE cells (arrows), indicating areas of chorioidal neovascularization. Nuclei are DAPI stained (blue). RPE nuclei are marked by arrowheads. Scale bars: 500 nm (A and B, top panels); 250 nm (B, bottom panels); 50 µm (C and D). ONL, outer nuclear layer.
Moreover, in some areas, the RPE basal lamina had been replaced by polymorphous electron-dense material that was localized between the elastic layer of the BM and the RPE basal infoldings (Figure 5A). In other areas of the same eye, irregular nodules arising from the RPE basal lamina and with comparable electron density were found between the basal infoldings of the RPE (Figure 5A). In addition, the basal lamina was frequently found to be interrupted where nodules arised (Figure 5, A and B). Nodules and RPE basal lamina interruptions were found frequently in early-induced mice and more rarely in late-induced mice. The changes were essentially similar in structure to basal lamina deposits typically found in CNV in humans patients with AMD. In addition, we found interruptions of the RPE basal lamina with an associated accumulation of electron-dense material in the adjacent RPE infoldings (Figure 5A). Next, we labeled the basal laminae of RPE and choriocapillaris by collagen type IV immunohistochemistry. In control mice, the basal laminae of both RPE and choriocapillaris endothelium were continuously labeled (Figure 5, C and D). In early-induced Tgfbr2\textsuperscript{D\textsubscript{E}} eye mice, continuous staining was only seen in the basal lamina of the choriocapillaris. Staining was irregular and patchy in the region of the RPE (Figure 5C). In late-induced Tgfbr2\textsuperscript{D\textsubscript{E}} eye mice, continuous labeling was seen in the basal lamina surrounding choriocapillaris vessels, but was incomplete underneath the RPE.
(Figure 5D). In places, the vascular basal lamina reached between RPE cells, indicating areas of CNV formation.

**Cell-Specific Conditional Deletion of Tgfbr2 in the RPE and Vascular Endothelium**

Next, we aimed at identifying the specific cell type that is responsible for CNV in Tgfbr2 

Deletion of transforming growth factor–β (TGF-β) signaling in the retinal pigment epithelium (RPE). Stained semithin sections, according to the method of Richardson et al. A: Retinal hemispheres of 6-month–old animals. The control mouse and the Tgfbr2ΔRPE mouse do not obviously differ in structure. B: Detailed magnification of retina/choroid in a Tgfbr2ΔRPE mouse and its control littermate, showing an essentially normal morphology. C: In vivo funduscopy and fluorescein angiography show no obvious alterations in the Tgfbr2ΔRPE mouse compared to the control. Diameters of the image sections refer to 2 mm retina. D: Detailed magnifications of the interface of the outer retina, RPE, and choroid, again showing a regular morphology in the Tgfbr2ΔRPE mice and controls. Scale bars: 500 μm (A); 50 μm (B); 20 μm (D). INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cell.

ERT2 mice were used with endothelial-specific tamoxifen-inducible Cre expression. Specific recombination in retinal and choroidal vessels was confirmed by green fluorescent protein immunoreactivity in VeCad-Cre-ERT2 crossed with mTmG reporter mice (Supplemental Figure S2E). When analyzing the structure of 6-week–old Tgfbr2ΔEC mice, the pronounced structural changes that we had reported21 for early-induced Tgfbr2Δeye mice, such as retinal and vitreal neovascularization, retinal detachment, and vitreal hemorrhages, were completely absent (Figure 8, A and B). Still, similar to our results seen in early-induced Tgfbr2Δeye mice,21 dilated vessels were frequently observed in the inner nuclear layer surrounded by electron-dense, extravascular material (Figure 8, C and E). Intriguingly, at the retinal/choroidal interface, focal areas were frequently observed in which photoreceptor outer segments were degenerated or absent, the RPE was multilayered, and vascular endothelial cells, together with extravasated erythrocytes, had accumulated in the subretinal space (Figure 8D). FITC-dextran–perfused ocular sections of Tgfbr2ΔEC mice showed tracer leakage from the choiopapillaris toward the RPE and choroidal vessels penetrating the RPE, indicating a breakdown of the outer blood-retinal barrier and the formation of CNV (Figure 9A). In 4-week–old Tgfbr2ΔEC animals and controls, the choroidal vessels were covered by neural/glial antigen 2–positive pericytes (data not shown). In contrast to
our data from Tgfbr2\textsuperscript{D\textsubscript{EC}} mice, we did not observe an accumulation of F4/80-positive cells in the choroid of Tgfbr2\textsuperscript{D\textsubscript{EC}} mice (Figure 9B) (control: 95.0 ± 8.40, \( n = 4 \); Tgfbr2\textsuperscript{D\textsubscript{EC}}: 111.67 ± 10.41, \( n = 3 \); \( P \geq 0.05 \)). In contrast, F4/80-positive cells were increased in the retina (control: 22.5 ± 1.71, \( n = 4 \); Tgfbr2\textsuperscript{D\textsubscript{EC}}: 108.0 ± 3.51, \( n = 3 \); \( P \leq 0.001 \)). In addition, immunoreactivity for collagen IV in the RPE/BM region was seen in basal laminae of cho- riocapillaris and RPE and did not markedly differ between Tgfbr2\textsuperscript{D\textsubscript{EC}} mice and controls (Figure 9C). Transmission electron microscopy confirmed the findings seen by light microscopy and the presence of degenerated photoreceptor outer segments, multilayered RPE, and extravasated erythrocytes (Figure 9D). Moreover, plasma-derived electron-dense material did not pass the RPE tight junctions in controls but accumulated between RPE and photoreceptor outer segments in Tgfbr2\textsuperscript{D\textsubscript{EC}} mice, indicating breakdown of the RPE barrier (Figure 9E). Overall, our data obtained in Tgfbr2\textsuperscript{D\textsubscript{EC}} mice strongly support the conclusion that deletion of TβRII in vascular endothelial cells alone is sufficient to promote the formation of CNV.

**Discussion**

We conclude that the deletion of TGF-β signaling in the ocular microenvironment is sufficient to induce CNV and other phenotypic characteristics of AMD in humans. Lack of endothelial TGF-β signaling is sufficient to trigger the onset of CNV, whereas its lack in the RPE is not relevant in this context. This conclusion is based on the following: i) the generation of mice with TβRII deficiency in the entire microenvironment of the eye, in vascular endothelial cells, or in the RPE; ii) the frequent detection of capillaries with fenestrated, PLVAP-positive endothelium that originate from the choriocapillaris and traverse the RPE to...
anastomose with retinal capillaries; iii) the presence of basal lamina-like deposits around the RPE basal infoldings and of areas with multilayered RPE; iv) the degeneration of photoreceptor outer segments; and v) the finding of distinct accumulations of F4/80-positive macrophages in the choroid.

TGF-β Functions at the Retinal/Choroidal Interface to Prevent CNV

The results of our study clearly support the concept that a major function of TGF-β signaling in choroid and retina is the stabilization of the choroidal and retinal vascular beds that are each essential for neuronal integrity in the sensory retina. This function includes the prevention of neovascularization processes in the two capillary beds that would otherwise cause neuronal dysfunction and death in the retina. It is of interest that the sensitivity to the induced lack of TGF-β signaling differs between the two vascular beds, as the formation of microaneurysms, leaky capillaries, and hemorrhages of the retinal vasculature is only seen when TGF-β signaling is deleted in the ocular microenvironment shortly after birth. In contrast, the formation of CNV is seen regardless if TGF-β signaling is interfered with in newborn or 3- to 4-week-old animals. A likely explanation is the fact that the retinal vasculature of the mouse eye forms and differentiates in the first 2 weeks after birth and may be more vulnerable to lack of TGF-β signaling during that period. A critical contributing factor may be the failure of pericyte differentiation around retinal capillaries that results from deficiency of TGF-β signaling during that period. In contrast, the formation of CNV is completed in late embryonic life, and the choriocapillaris is mature at birth.

The coating of the choroidal vessels with neural/glial antigen 2-positive pericytes in early- and late-induced Tgfbr2Δeye and Tgfbr2ΔEC animals was comparable to controls, which appears to indicate that lack of pericyte presence or differentiation is not a requirement for CNV. Data from genetically modified mice with a specific

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**Figure 9** Structural changes of the retinal/choroidal interface in Tgfbr2ΔEC mice. A: Fluorescein isothiocyanate (FITC)–dextran–perfused retinal meridional sections of a 6-week–old Tgfbr2ΔEC mouse and its control littermate. White arrows point toward tracer leakage in the retinal pigment epithelium (RPE; middle panel) and choroidal vessels (right panel) invading the RPE. Nuclei are DAPI stained (blue). B: FITC-dextran (green)–perfused and F4/80 (red)–immunostained sections. The control and the Tgfbr2ΔEC littermate show immunoreactivity for F4/80 (red)–positive cells in the choroid, which are not higher in number in the mutant. Nuclei are DAPI stained (blue). C: Immunoreactivity for collagen IV (red) in the RPE/Bruch membrane (BM) region of 6-week–old mice. Basal laminae of choriocapillaris and RPE are regularly labeled in control and mutant. Nuclei are DAPI stained (blue), and RPE nuclei are marked by white arrowheads. D and E: Transmission electron microscopy of RPE/BM in 4- to 6-week–old mice. D: In the control, photoreceptor outer segments, RPE, and BM are of normal structure. In the Tgfbr2ΔEC mouse, areas of degenerated photoreceptor outer segments, multilayered RPE (black arrowheads point toward nuclei of RPE cells), and extravasated erythrocyte (Er) are present. E: Plasma-derived electron-dense material does not pass the RPE tight junctions in controls (black arrowheads). In contrast, in the mutant, it accumulates between RPE and photoreceptor outer segments (black arrowheads). Scale bars: 50 μm (A); 20 μm (B and C); 1000 nm (D); 500 nm (E). ONL, outer nuclear layer.
deficiency of platelet-derived growth factor-B in endothelial cells further challenge the role of pericytes in the formation of CNV as these mice show a varying degree of pericyte loss without developing CNV.54,55 The higher sensitivity of the choriocapillaris to lack of TGF-β signaling, as opposed to the mature retinal vasculature, may also be caused by the presence of the high amounts of VEGF at the retinal/choroidal interface that are continuously secreted by the RPE, the only source of VEGF in the back of the adult eye.35 Secretion of VEGF occurs in a polarized manner to the RPE basolateral side facing the choriocapillaris56,57 and is expected to convey a proliferative signal to endothelial cells of the choriocapillaris that are no more under the influence of constitutive TGF-β signaling in animals with a deletion of TβRII.

TGF-β and VEGF as Part of a Homeostatic System to Maintain Integrity of the Choriocapillaris

Not only VEGF but also TGF-β1 and TGF-β2 are present at high amounts at the retinal/choroidal interface.20 It is tempting to speculate that both factors are critical parts of a homeostatic system designed to maintain structure and function of the choriocapillaris. In this system, VEGF would be required to maintain the extreme high density of the choriocapillaris and its fenestrations,57,58 whereas TGF-βs antagonize any proliferative properties of VEGF signaling on the vascular endothelium. Failure in the balance of this homeostatic system would cause ablation of the choriocapillaris,14,59,60 whereas TGF-βs induce the transcription of one another in multiple cell types61–66 and may well do so in the RPE, establishing an autoregulatory feedback system designed to maintain structure and function of the choriocapillaris. We realize that this concept needs to be validated in further studies that should also aim at a complete identification of the signaling network that drives endothelial proliferation and CNV formation downstream of TGF-β signaling deficiency.

Potential Neuroprotective Effects of TGF-β

Late-induced Tgfr2<sup>Δexon</sup> mice showed a dramatic deterioration of the retina. The prolonged presence and formation of CNV, and its detrimental effects on structure and function of the retina, may explain this finding. It may also indicate a participation of TGF-β signaling in a neuroprotective pathway independent of the formation of CNV. We recently showed that TGF-β signaling protects retinal neurons from developmental programmed cell death.52 It is well possible that it also contributes to maintenance of adult neurons by protecting them from apoptosis. In support of such a scenario are findings reported by Walshe et al,67 who neutralized TGF-β in the adult mouse eye via expression of soluble endoglin, a TGF-β inhibitor. Apoptosis of retinal ganglion cells was observed, as were functional deficits detected by ERG. Neuroprotective activities of TGF-βs were reported for multiple types of neurons throughout the central nervous system, such as in the striatum,68 spinal cord,69 substantia nigra,70 or hippocampus.71 Similarly, there is increasing evidence that VEGF signaling is important for the trophic maintenance of neurons and their survival after injury, effects that appear to be mediated via the VEGF receptor-2.72–76 Mice with a constitutive high expression of VEGF in retinal ganglion cells are protected from retinal ganglion cell degeneration after axotomy via VEGF receptor-2 and downstream activation of extracellular signal-regulated kinase 1/2 and Akt pathways.77 Although TGF-β/VEGF are antagonists in their actions at the retinal/choroidal interface and the choriocapillaris, they maintain Integrity of the Choriocapillaris

For the initiation of CNV, lack of TGF-β activity appears to be more relevant than sole increase of VEGF activity, as mice with overexpression of VEGF in the RPE develop an intrachoroidal neovascularization, but no CNV.59,60 VEGF and TGF-βs induce the transcription of one another in multiple cell types61–66 and may well do so in the RPE, establishing an autoregulatory feedback system designed to maintain structure and function of the choriocapillaris. We realize that this concept needs to be validated in further studies that should also aim at a complete identification of the signaling network that drives endothelial proliferation and CNV formation downstream of TGF-β signaling deficiency.

Figure 10  Schematic of signaling events at the retinal-choroidal interface. Left panel: Normally, the retinal pigment epithelium (RPE) secretes high amounts of both transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) that both ensure an appropriate physiological microenvironment for the choriocapillaris, including its VEGF-mediated maintenance, and TGF-β-mediated inhibition of proliferation. Right panel: The deletion of Tgfbr2 in endothelial cells results in the specific disability of TGF-β to act on endothelial cells (red cross). This results in an imbalance of the effects of VEGF/TGF-β, a scenario that promotes the proliferation of the vascular endothelium of the choriocapillaris in the direction of the RPE and, finally, results in choroidal neovascularization.
may cooperate in their neuroprotective activities. Both functions would serve the ultimate goal to maintain structure and function of the retina.

The Formation of BlamD-Like Material Is Not Required for CNV

Although CNV was consistently observed in the eyes of both early- and late-induced Tgfb2<sup>Δevy</sup> mice, and in those of Tgfb2<sup>ΔEC</sup> mice, other findings were predominant only in the eyes of early-induced Tgfb2<sup>Δevy</sup> mice. This includes the formation of homogeneous extracellular material between the basal infoldings of the RPE and internal to the RPE basal lamina. The material was similar in electron density and structure to that of basal lamina deposits (BlamDs), a characteristic finding in patients with early age-related macular degeneration. BlamD-like changes were commonly observed in early-induced Tgfb2<sup>Δevy</sup> mice, only rarely observed in late-induced Tgfb2<sup>Δevy</sup> mice, and not observed in Tgfb2<sup>ΔEC</sup> mice, suggesting that they are not a requirement for CNV and breakdown of the RPE barrier. Our immunohistochemical data indicate that the homogeneous extracellular BlamD-like material contains collagen type IV, an observation that correlates with the observation that basal lamina proteins (eg, collagen IV and laminin) are present in BlamDs in human patients with AMD. BlamD formation in AMD is likely caused by RPE dysfunction. A comparable dysfunction might more easily be observed in patients with early CNV. In late-induced mice that are absent in late-induced mice. We attribute this observation to the marked changes in the inner retina of early-induced mice that are absent in late-induced mice. Still, those molecules might well be elevated in the microenvironment of the choroidal/retinal interface, where macrophages accumulate in mice with CNV.

TGF-β Signaling and Blood-Retinal Barrier

Leakage of high-mol. wt. FITC-dextran and erythrocytes into the retina and subretinal space, after induced TGF-β signaling deficiency, implicates disruption of the inner and outer blood retinal barriers. Deficiency of the TGF-β signaling pathway in endothelial cells is likely the major contributor in this scenario. Comparable, mice with an endothelial-specific deficiency of SMAD4, an intracellular downstream mediator of TGF-β signaling, or mice with a deletion of TβRII specifically in endothelial cells in the brain show perinatal intracerebral hemorrhages and a breakdown of the blood-brain barrier. Furthermore, the virus-driven expression of soluble endoglin results in an inhibition of TGF-β1 signaling in the murine retina, a situation that also causes breakdown of the blood-retinal barrier, most likely mediated through a decreased association of the tight junction proteins occludin and zona occludens-1.

TGF-β Signaling in Human Patients with AMD

Several independent case-control genome-wide association studies detected an association of two synonymous polymorphisms in exon 1 of the high-temperature requirement A1 (HTRA1) gene, with a high risk to develop AMD. The gene product HTRA1 appears to play a causative role in CNV. Intriguingly, a recent study provided evidence that the two synonymous HTRA1 variants influence their protein interaction with TGF-β1, leading to an impaired regulation of TGF-β signaling. Moreover, a collaborative genome-wide association study identified TGFBR1, the gene encoding for the TGF-β1 type I receptor, as a new susceptibility gene for AMD, further highlighting the importance of the TGF-β signaling pathway in the context of AMD.

Conclusion

Our findings emphasize the importance of TGF-β signaling as a key player in the development of ocular neovascularization and implicate a fundamental role of TGF-β signaling in the pathogenesis of AMD. A more thorough understanding of this role at the retinal/choroidal interface has the distinct potential to lead to the development of novel...
treatments strategies preventing CNV in patients experiencing AMD.

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Supplemental Data

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