Cross-Inhibition of Norrin and TGF-β Signaling Modulates Development of Retinal and Choroidal Vasculature

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Norrin is a highly conserved secreted signaling molecule that forms unique dimers with each monomer adopting a conserved cystine knot fold.1,2 It is an atypical Wnt ligand that binds to frizzled (FZD)4 and leucine-rich repeat-containing G-protein-coupled receptors 4 to activate, in the presence of their co-receptors low-density lipoprotein receptor-related protein (LRP) 5 or 6, the canonical Wnt/β-catenin signaling pathway.3,4 The intensity of Norrin-mediated FZD4 signaling is enhanced in the presence of TSPAN12, which is supposed to induce clustering of the FZD4/LRP5/6 receptor complexes.5

Norrin is essential for the formation of the normal retinal vasculature and its lack in Norrin-deficient mice leads to delayed formation of the superficial retinal vascular plexus on the retinal surface, and to a complete lack of intraretinal capillaries.6–9 The ocular phenotype of Norrin-deficient mice is completely rescued by the additional transgenic overexpression of Norrin in the eye.6 Besides their retinal phenotype, Norrin-deficient mice suffer from an impaired vascularization of the inner ear and cerebellum, strongly indicating a more general role of Norrin for vessel formation in the central nervous system.10–13 The vascular effects of Norrin are not restricted to retinal angiogenesis during development, but also appear to be important for vascular repair and maintenance. During oxygen-induced retinopathy, the mouse model for retinopathy of prematurity, Norrin promotes repair as it induces vessel regrowth into vaso-obliterated areas and the formation of intraretinal capillaries.7–14 As potential downstream mediators of Norrin/β-catenin signaling in microvascular endothelial cells during development and repair, the transcription factor SOX17 and the signaling molecule insulin-like growth factor-1 were identified15,16. Mutations in the encoding Norrie disease protein (NDP) gene cause Norrie disease or familial exudative vitreoretinopathy, conditions that are characterized by retinal hypovascularization. In addition to its role in angiogenesis, Norrin has distinct neuroprotective properties as it saves retinal ganglion cells (RGC) or photoreceptors from apoptosis following excitotoxic or light-induced damage.17–19 Norrin-deficient mice are suffering from a continuous loss of retinal ganglion cells that is rescued upon transgenic Norrin overexpression.6,8

Overall, the effects of Norrin on the retinal vasculature during development and repair after injury are directly opposite to those of TGF-β1 and 2, which are highly expressed in the adult and developing retina.20,21 Mice with an induced deletion of the TGF-β type II receptor, which is essential for TGF-β signaling, show a pronounced neovessel formation of...
retinal capillaries including the formation of microaneurysms and the phenotype of proliferative diabetic retinopathy.\(^2\) In contrast, transgenic mice with TGF-β1 overexpression in the eye do not develop intraretinal vessels and lose their choriocapillaris, the capillary bed beneath the retinal pigment epithelium that is essential for photoreceptor nutrition.\(^23,24\) The effects appear to be mediated via canonical TGF-β1 signaling, which involves the cytoplasmic phosphorylation of SMAD2 and 3, which in turn bind to SMAD4.\(^25,26\) The complexes then translocate into the nucleus to activate specific target genes. Another SMAD molecule, SMAD7, inhibits TGF-β signaling and appears to have an essential role in an autoinhibitory negative feedback regulation of TGF-β signaling.\(^27,28\) In humans, an enhanced expression of TGF-β is associated with various ocular pathologies. In the posterior eye segment, elevated levels of TGF-β were detected in the vitreous humor of patients suffering from retinopathy of prematurity or wet age-related macular degeneration, both conditions associated with retinal hypoxia.\(^29,30\) In contrast, in patients suffering from proliferative diabetic retinopathy, TGF-β levels are decreased.\(^31\) Overall, both experimental and clinical observations suggest a pivotal antiangiogenic role for TGF-β signaling.

Because of the quite distinct and essentially opposing effects of Norrin and TGF-β signaling on the proliferation of ocular capillaries, we were interested to learn if the effects might be mediated by a mutual interaction of their respective signaling pathways. We provide evidence that high amounts of TGF-β1 in the eye cause a dramatic reduction in the activity of canonical Wnt/β-catenin signaling. This effect is inhibited in the presence of high amounts of Norrin, which further induce the expression of SMAD7 to inhibit the activity of TGF-β signaling.

Methods

Animals

All animal procedures performed in this study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the local authorities (Regierung der Oberpfalz, Bavaria, Germany). The generation of transgenic mice with an overexpression of Norrin (βB1-Norrin) or activated TGF-β1 (βB1-TGF-β1) under the control of the lens-specific chicken βB1-crystallin promoter fragment was described previously in detail.\(^8,22\) Mice were bred in the animal facility of the University of Regensburg. For PCR genotyping of transgenic βB1-Norrin mice, primers that span from the sequences of the βB1-crystallin promoter to those of the Norrin cDNA were used. The sequences of the primers were 5‘-ACACTGATGAGGTTGACATTCAAT-3’ and 5‘-TGAGTCTAAGCAGGTACAGAGG-3’. A 768 base pair (bp) DNA fragment was amplified using the thermo cycle profile of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 1 minute for 30 cycles. To screen for potential βB1-TGF-β1 mice, primers were used that span from the βB1-chicken promoter to the SV40 intron of the transgene. The sequences of the primers were 5‘-ACACTGATGAGGTTGACATTCAAT-3’ and 5‘-TGAGTCTAAGCAGGTACAGAGG-3’. The primers were purchased from Life Technologies (Carlsbad, CA, USA). A 506 bp DNA fragment was obtained using the thermal cycle profile of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds for 30 cycles. For all analyses transgenic mice and wild type littermates of either sex were used.

To analyze retinal vasculature, mice were deeply anesthetized with ketamine (120 mg/kg body weight), intramuscularly (IM) and xylazine (8 mg/kg body weight) IM at the indicated time points, and then perfused through the left ventricle with 1 mL PBS containing 50 mg high molecular weight (MW = 2,000,000) FITC-dextran (TdB Consultancy, Uppsala, Sweden). The eyes were enucleated and placed in 4% paraformaldehyde (PFA; Carl Roth, Karlsruhe, Germany) for 4 hours. After washing in PBS overnight, eyes were embedded in Tissue Tek (Sakura Finetek, Torrance, CA, USA) according to standard protocols and subjected to cryosectioning.

Expression and Purification of Human Recombinant Norrin

The protocol for generation of the expression vector, and synthesis and purification of human recombinant (hr) Norrin has been reported in detail previously.\(^7\) Briefly, for expression of hr Norrin, further referred to as Norrin, stably transfected 293 EBNA cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal calf serum (FCS), 20 μg/mL gentamycin, 250 μg/mL geneticin (G418), and 300 μg/mL hygromycin (all from Life Technologies). To harvest Norrin-conditioned cell culture medium, cells were cultured in medium without FCS for 3 days. Protein purification of recombinant Norrin was performed by affinity chromatography using heparinagarose (Sigma-Aldrich Corp., St. Louis, MO, USA). After washing and equilibration in PBS, heparin agarose (Sigma-Aldrich Corp.) was incubated with conditioned cell culture medium for 1 hour at 4°C and loaded on a fresh chromatography column (Bio-Rad Laboratories, Hercules, CA, USA). After washing three times with PBS, bound proteins were eluted from agarose using 1 to 2 M NaCl in PBS. Eluted fractions were analyzed by Western blot analyses using antibodies against human Norrin (R&D Systems, Minneapolis, MN, USA) and the HisTag epitope (Life Technologies). The purity of Norrin-containing fractions was examined by silver staining of an SDS polyacrylamide gel. After dialyzing against PBS, protein content was measured by semiquantitative SDS polyacrylamide gel and visualized by silver staining according to standard protocol.

Cell Culture

Human retinal microvascular endothelial cells (HRMEC; Cell Systems, Kirkland, WA, USA) were cultured in supplemented microvascular endothelial cell growth medium (PromoCell, Heidelberg, Germany) containing 100 U/mL penicillin (Life Technologies) and 100 μg/mL streptomycin (Life Technologies) at 37°C and 5% CO₂. Transfected mink lung epithelial cells (MLECs) were cultured in DMEM supplemented with 10% FCS and 250 μg/mL geneticin (G418) at 37°C and 5% CO₂, as described previously.\(^33\) All experiments were performed in unsupplemented or serum-free cell culture medium.

Measurement of TGF-β Signaling Activity by Luciferase Assay

The activity of TGF-β1 signaling was measured in MLECs that were stably transfected with a luciferase reporter under the control of a TGF-β responsive truncated plasminogen activator inhibitor (PA) promoter fragment (Serpinet).\(^33\) Then, 2 x 10⁴ MLECs per well were seeded on a 96-well tissue culture plate, allowed to attach, and starved overnight in serum-free cell culture medium. After incubation of cells with 100 ng/mL Dickkopf (Dkk)-1 (R&D Systems), 1 ng/mL TGF-β1 (R&D Systems) and/or 40 ng/mL Norrin for 20 hours, MLEC were lysed and luciferase activity was measured as described previously with an Autolumat LB953 (Berthold Technologies, Oak Ridge, TN, USA).\(^34\) Luciferase activity was indicated as
For relative quantification, the housekeeping genes intron boundaries and were purchased from Life Technologies. PCR. All PCR primers (Table) were designed to span exon.

2.1). were analyzed using the iQ5 optical system software (version

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2000c (Peqlab) was used to measure the RNA concentration and the OD260/OD280 ratio. Only total RNA with a 260/280 ratio between 1.6 and 2.0 was used for first-strand cDNA synthesis with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). HotStar Taq DNA polymerase (Qiagen, Hilden, Germany) was used for PCR according to the manufacturer’s instructions. A NanoDrop spectrophotometer ND-2000c (Peqlab) was used to measure the RNA concentration and the OD260/OD280 ratio. Only total RNA with a 260/280 ratio between 1.6 and 2.0 was used for first-strand cDNA synthesis with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories). Real-time RT-PCR analyses were performed on a Bio-Rad iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories). HotStar Taq DNA polymerase (Qiagen, Hilden, Germany) was used for PCR according to the manufacturer’s protocol. PCR was performed in a volume of 15 μL, consisting of 1.5 μL 10X PCR buffer (Qiagen), 1.5 μL 25 mM MgCl2 (Qiagen), 0.3 μL dNTPs (10 mM each, Qiagen), 0.3 μL HotStart Taq (5 U/μL), and 1.5 μL of 1X SYBR green I solutions (Sigma-Aldrich Corp.). The following temperature profile was used: 40 cycles with 10 seconds denaturation at 95°C, 40 seconds of annealing and extension at 60°C. RNA that had not been reverse-transcribed served as negative control for real-time RT-PCR. All PCR primers (Table) were designed to span exon.

introns boundaries and were purchased from Life Technologies. For relative quantification, the housekeeping genes GNB2L1 for HRMEC and LaminA for mouse retinae were used. Results were analyzed using the iQ5 optical system software (version 2.1).

Western Blot Analyses and pSMAD3 ELISA

Murine retinal proteins were isolated in radioimmunoprecipitation assay (RIPA) buffer as described previously.12 Protein concentration was measured by the BCA (Interchim, San Diego, CA, USA) method and up to 25 μg total proteins were subjected to 10% SDS-PAGE. Proteins subsequently were transferred on a polyvinylidene fluoride (PVDF) membrane (Roche, Basel, Switzerland) by semidry blotting. After blocking with 5% BSA (β-catenin) or 5% low fat milk (SMAD7) in PBS-T, membranes were incubated overnight with a rabbit-anti-β-catenin (1:1000; Cell Signaling Technology, Danvers, MA, USA) and documented with the LAS 3000 Image Visualizer work station (Fujifilm, Tokyo, Japan). As a loading control, an HRP-conjugated antibody was used (Cell Signaling Technology). Antibody hybridization was visualized using the Immobilon HRP substrate (Millipore, Billerica, MA, USA) and documented with the LAS 3000 Imager work station (Fujifilm, Tokyo, Japan). As a loading control, an HRP-conjugated anti-GAPDH antibody was used (Cell Signaling Technology). The densitometry of Western blot analyses was performed with the Aida Image Analyzer v.4.06 software (Raytest, Angleur, Belgium). Relative protein concentrations for pSMAD3 were analyzed by ELISA following manufacturer’s recommendations (Cell Signaling Technology). In brief, protein concentrations of retinal samples were measured by the BCA method and 10 μg total protein was subjected to pSMAD3 ELISA. For read out, the absorbance at 450 nm was measured with an ELISA plate reader (Tecan, Männedorf, Switzerland).

Light Microscopy

Eyes were enucleated and fixed in cacodylate buffer with 2.5% PFA (Carl Roth) and 2.5% glutaraldehyde (Serva, Heidelberg, Germany) for 4 hours. After washing overnight in cacodylate buffer, eyes were postfixed with OsO4, dehydrated, and embedded in Epon according to standard protocols (Carl Roth). Semithin sagittal sections (1 μm) of eyes were stained

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with fuchsin-methylene blue and analyzed by light microscopy on an Axiovision microscope (Carl Zeiss).

**Immunohistochemistry and TUNEL Labeling**

For immunohistochemistry and TUNEL labeling (Promega, Madison, WI, USA), eyes were fixed with 4% PFA for 4 hours and embedded in paraffin according to standard procedures. For pSMAD3 staining, sections were incubated in 0.1 M sodium citrate (pH 7.4) at 100°C for 5 minutes, washed with 1X tris-buffered saline (TBS), and at room temperature for 5 minutes and blocked with 2% BSA, 0.2% cold water fish gelatin (Aurion, Wageningen, The Netherlands) and 0.1% Triton X-100 in 1X TBS for 60 minutes. After three washes (10 minutes each) with 1X TBS, sections were incubated with rabbit anti-pSMAD3 antibodies (1:100, Cell Signaling Technology) in 0.2% BSA, 0.02% cold water fish gelatin, and 0.01% Triton X-100 in 1X TBS overnight at 4°C. For detection of collagen type IV, sections were pretreated with proteinase K (100 μl proteinase K [Sigma-Aldrich Corp.] in 57 mL Tris-HCl [0.05 mol/L]) for 5 minutes, washed in water, and incubated in 2N HCl for 30 minutes. After washing with phosphate buffer (0.1 M) 3 times for 5 minutes, sections were blocked with 2% BSA for 60 minutes before incubation with rabbit anti-collagen type IV antibodies (1:100; Chemicon International, Temecula, CA, USA) in 0.2% BSA overnight at 4°C. Before adding biotinylated anti-rabbit antibodies (1:50; Vector Laboratories, Burlingame, CA, USA) for 60 minutes, slices were washed three times (10 minutes each) with 1X TBS for pSMAD3 and with 0.1 M phosphate buffer for collagen type IV staining. Following an additional three washes (10 minutes each) with 1X TBS or 0.1 M phosphate buffer, samples were incubated with Alexa-488 coupled streptavidin (1:1000; Molecular Probes, Eugene, OR, USA) for 60 minutes. After final incubation, all specimens were washed again three times, mounted in fluorescent mounting medium containing 1:50 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories), and analyzed on an Axiovision fluorescence microscope (Carl Zeiss, Jena, Germany).

Apoptotic retinal cells were detected by TUNEL assay using the Deadend Fluorometric TUNEL system (Promega). TUNEL-labeling of paraffin sections was performed according to the manufacturer’s instructions. For quantification, the total number of TUNEL-positive cells were quantified in the entire retina of sagittal sections and calculated as the number of TUNEL-positive cells per 10,000 μm² retina.

**Statistics**

All values are expressed as mean ± SEM. For statistical analyses, a 1-way ANOVA was performed to compare the mean variables, followed by a least significant difference (LSD) post hoc test for data that meet the criteria of the assumption of homogeneity of variances and a Games Howell post hoc test for data that did not meet the criteria of homogeneity of variances. P values less than 0.05 were considered statistically significant.

**Results**

**Norrin Inhibits Anti-Angiogenic Effects of TGF-β on Retinal Vasculature and Choriocapillaris**

In a recent study, we showed that transgenic βB1-TGF-β1 mice with ocular overexpression of active TGF-β1 completely lack intraretinal capillaries and the layer of the choriocapillaris that is required for oxygen supply and nutrition of photoreceptors.23 We now crossed transgenic βB1-TGF-β1 mice with βB1-Norrin animals that overexpress Norrin in the eye25 to generate double transgenic βB1-TGF-β1/βB1-Norrin mice. After perfusion with FITC-coupled dextran, well-structured superficial and deep vascular plexuses were observed in retinai of wild type and transgenic βB1-Norrin mice at P16 (Fig. 1A, 1B). In addition, in the inner plexiform layer, a few vessels were seen that began to form the intermediate vascular plexus (Figs. 1A, 1B). In contrast, as expected, in transgenic βB1-TGF-β1 mice no intraretinal capillaries were observed (Fig. 1C). However, in retinai of double transgenic βB1-TGF-β1/βB1-Norrin mice, well established superficial as well as deep vascular plexuses were detected (Fig. 1D). Moreover, in meridional sections of retinae from βB1-Norrin mice and wild type littermates, vessels of the choriocapillaris were observed adjacent to the RPE (Figs. 1E, 1F), suggesting an intact and dense choriocapillaris network, which was not detectable in transgenic βB1-TGF-β1 mice (Fig. 1G). The loss of the choriocapillaris was rescued in double transgenic mice, in which vessels that lie close to the RPE were readily detected (Fig. 1H). To confirm our observations in an independent assay, immunostaining for collagen type IV was performed to visualize the basal lamina of microvascular endothelial cells in the retina.22 In the central inner retina of wild type mice, a dense capillary network was observed below the inner limiting membrane, which also was labeled (Fig. 1I). As expected, at P8 the vessels of the superficial vascular plexus spread from the retinal center to its periphery (Fig. 1J). In contrast, retinae of βB1-TGF-β1 transgenic mice completely lacked the retinal vasculature (Fig. 1K). Only a thickened inner limiting membrane was seen covering the retinal surface (Fig. 1K).

The absence of retinal vessels in βB1-TGF-β1 mice was rescued in double transgenic littermates in which numerous vessels were detected in all regions of the inner retina (Fig. 1L). Overall, our observations strongly indicated that Norrin inhibits the anti-angiogenic effects of TGF-β on retinal capillaries and choriocapillaris.

**Norrin Prevents Neuronal Apoptosis in the Retina of βB1-TGF-β1 Mice**

We previously reported that the combined lack of retinal capillaries and choriocapillaris in βB1-TGF-β1 mice is associated with pronounced apoptosis of retinal neurons.24 Therefore, we wanted to know if retinal apoptosis is attenuated in double transgenic βB1-TGF-β1/βB1-Norrin animals. By light microscopy, we observed in the retina of transgenic βB1-TGF-β1 mice a substantial number of pyknotic nuclei compared to βB1-Norrin or wild type littermates (Figs. 2A–C). In contrast to βB1-TGF-β1 mice, in double transgenic βB1-TGF-β1/βB1-Norrin animals the number of pyknotic nuclei was obviously lower (Fig. 2D). To further confirm our observation by an independent assay, apoptotic retinal cells were stained by TUNEL. On retinal meridional sections from βB1-Norrin mice and wild type littermates only very few apoptotic cells in the inner retina were observed (Figs. 2E, 2F). In contrast, in transgenic βB1-TGF-β1 animals, a substantial number of TUNEL-positive cells were detected in the inner (INL) and outer (ONL) nuclear layers of the retina (Fig. 2G). Quite remarkably, the increase in TUNEL-positive cells in the retina was substantially attenuated in double transgenic βB1-TGF-β1/βB1-Norrin mice (Fig. 2H), an observation that we confirmed by quantification. In βB1-Norrin and wild type littermates, only 1.6 ± 0.1 and 1.5 ± 0.2 TUNEL-positive cells per 10,000 μm² retina were detected, respectively (Fig. 2I). In contrast, in transgenic βB1-TGF-β1 mice the number of apoptotic retinal cells was increased significantly by 2.5-fold compared to wild type littermates (3.9 ± 1.6; P < 0.001; Fig. 2I). However, in double transgenic mice, the number of TUNEL-positive cells was substantially reduced by...
more than 35% compared to B1-TGF-β1 mice (2.4 ± 0.4; P = 0.01; Fig. 2I), strongly suggesting that the Norrin-induced rescue of retinal and choroidal capillaries inhibits, at least partially, the neuronal apoptosis in retinæ of B1-TGF-β1 mice. As reported previously, the vitreous cavity fails to develop in contrast, in transgenic B1-Norrin mice (Fig. 3A), B1-TGF-β1 (G), B1-TGF-β1/B1-Norrin animals (H), and wild type littermates (E) after perfusion with FITC-labeled dextran at P16. SC, sclera. Blue, DAPI staining. Scale bars: (A–D) 100 μm. (E–H) Representative sagittal sections through the chorioretinal interface from transgenic B1-Norrin (F), B1-TGF-β1 (G), B1-TGF-β1/B1-Norrin animals (H), and wild type littermates (E) after perfusion with FITC-labeled dextran at P16. Scale bars: (E–H) 20 μm. (I–L) Representative collagen type IV staining (red) on meridional sections through the retina and higher magnifications from the peripheral (top and middle) and central (lower) retina from transgenic B1-TGF-β1 (K), B1-TGF-β1/B1-Norrin animals (L), and wild type littermates (I) at P8. Blue, DAPI staining. Scale bars: (I–L) 200 μm.

**Norrin Restores TGF-β–Induced Suppression of β-Catenin**

Since our data indicated that Norrin attenuates TGF-β–induced effects, we wondered which underlying mechanism mediates the effects. Following this, we first analyzed β-catenin protein levels in retinæ of double transgenic animals. In wild type mice a specific band for β-catenin was detected at P8, an observation that was more pronounced in transgenic B1-Norrin mice (Fig. 3A). However, in B1-TGF-β1 mice, the levels for β-catenin in the retina were dramatically reduced compared to wild type littermates. This effect was restored in double transgenic B1-TGF-β1/B1-Norrin mice (Fig. 3A). Quantification of the protein levels from transgenic B1-TGF-β1 mice by densitometry detected a significant reduction of β-catenin by approximately 60% compared to wild type controls (0.41 ± 0.16; P < 0.001; Fig. 3B). In retinæ of double transgenic B1-TGF-β1/B1-Norrin mice, the diminished protein levels for β-catenin in B1-TGF-β1 mice were restored to that of wild type littermates (0.94 ± 0.14), a difference that was statistically significant (B1-TGF-β1 versus B1-TGF-β1/B1-Norrin P = 0.02; Fig. 3B). To further confirm that Norrin not only increases β-catenin protein levels but also enhances Wnt/β-catenin signaling, mRNA levels for Axin2 were analyzed. Following activation of Wnt/β-catenin signaling, the mRNA expression of Axin2, an inhibitor of Wnt/β-catenin signaling,36,37 is induced, which is why Axin2 is a common marker for detecting the activity of Wnt/β-catenin signaling. In retinæ of B1-Norrin mice, only a moderate increase in Axin2 mRNA level was detected compared to wild type controls (Fig. 3C). In contrast, in transgenic B1-TGF-β1 mice a marked reduction of Axin2 mRNA expression of more than 30% was observed compared to wild type controls (0.68 ± 0.12, P = 0.049; Fig. 3C). This effect was completely rescued in double transgenic mice (1.12 ± 0.13, P = 0.016; Fig. 3C). In summary, our data strongly suggested that TGF-β mediates a substantial decrease in the amounts of retinal β-catenin and canonical Wnt/β-catenin signaling, an effect that is completely antagonized by Norrin.

**Norrin Decreases the Phosphorylation of SMAD3 in Inner Retina**

Since we found that Norrin restores the TGF-β–mediated reduction of β-catenin levels, we wondered whether Norrin might influence the expression of components of the TGF-β or Wnt/β-catenin pathway. To this end, we first analyzed if Norrin
reduces the expression of TGF-β1 and -β2 or of TGF-β–receptor (TGFBR)-1, -2 and -3 mRNA or enhances the expression of FZD4 and LRP5 mRNA in the retinae of double transgenic βB1-TGF-β1/βB1-Norrin mice. In retinal mRNA from βB1-Norrin mice, the expression of Axin2 mRNA was quantified by real-time RT-PCR and compared to wild-type controls. (*p < 0.05; **p < 0.01; ***p < 0.001; n ≥ 6 mice per genotype; mean ± SEM).

**Figure 2.** Norrin inhibits TGF-β–mediated effects on the vitreoretinal interface and thereby TGF-β–induced apoptosis. (A–D) Representative meridional semithin sections through the retina from transgenic βB1-Norrin (B), βB1-TGF-β1 (C), βB1-TGF-β1/βB1-Norrin animals (D), and wild type littermates (A) at P5. In double transgenic mice, a small vitreous cavity (VC) formed and the number of pyknotic nuclei (arrows) was lower (D) in contrast to transgenic βB1-TGF-β1 animals (C). Scale bars: (A–D) 40 μm. (E–H) Representative TUNEL staining (green, arrows) on sagittal sections of retinae from transgenic βB1-Norrin (F), βB1-TGF-β1 (G), βB1-TGF-β1/βB1-Norrin mice (H), and wild type littermates (E) at P5. Blue, DAPI staining. Scale bars: (E–H) 50 μm. (I) For quantification, the number of TUNEL-positive cells was calculated and plotted as apoptotic cells per 10,000 μm² retina (*p < 0.05; **p < 0.01; n ≥ 7 mice per genotype; mean ± SEM).

**Figure 3.** Norrin restores TGF-β–induced suppression of β-catenin signaling in retina. Representative Western blot analysis (A) and densitometry (B) for β-catenin of retinal proteins from βB1-Norrin, βB1-TGF-β1, βB1-Norrin/βB1-TGF-β1 mice, and wild type littermates at P8 (*p < 0.05; ***p < 0.001; n ≥ 11 mice per genotype; mean ± SEM). (C) Quantitative real-time RT-PCR for Axin2 in retinal mRNA from βB1-Norrin, βB1-TGF-β1, βB1-Norrin/βB1-TGF-β1 mice, and wild type controls at P8 (*p < 0.05; n ≥ 6 mice per genotype; mean ± SEM).
mice at P8, no significant changes in the expression of any of those molecules were detected compared to wild type littermates (Fig. 4). The same was the case for βB1-TGF-β1 and double transgenic βB1-TGF-β1/βB1-Norrin mice, with the notable exception of Tgfbr3, which was substantially decreased in its expression in βB1-TGF-β1 and double transgenic βB1-TGF-β1/βB1-Norrin mice compared to wild type controls (Fig. 4). No differences in the retinal expression of any of the molecules was observed when comparing βB1-TGF-β1 mice and double transgenic βB1-TGF-β1/βB1-Norrin animals (Fig. 4).

Canonical TGF-β signaling is mediated via the phosphorylation of SMAD2 and -3 by TGF-β receptors, which in turn translocate into the nucleus to induce the expression of specific target genes. Since we could not identify a changed expression of Tgf-β1 and -β2 and their receptors in retinae of double transgenic mice, we wondered if Norrin diminishes canonical TGF-β signaling. To this end, immunohistochemical staining for phosphorylated SMAD3 (pSMAD3), a key mediator molecule of the canonical TGF-β pathway, was performed. In retinae of wild type controls, only a weak staining for pSMAD3 was observed (Fig. 5A). As expected, in retinae of transgenic βB1-TGF-β1 mice (Fig. 5B), an intense signal for pSMAD3 was detected in nuclei of the RGC layer and all parts of the INL (Fig. 5B). In contrast, only a weak immunoreactivity for pSMAD3 was seen in the ONL of retinae from mice with an overexpression of TGF-β1 (Fig. 5A). However, in βB1-TGF-β1/βB1-Norrin mice no specific staining for pSMAD3 was observed neither in the RGC layer nor in the INL (Fig. 5C), strongly suggesting that Norrin inhibits SMAD3 phosphorylation in retinal neurons and/or neuroglia of the inner retina.

To further confirm our histological observations, an ELISA for phosphorylated SMAD3 was performed. In retinal proteins from βB1-Norrin mice at P8 no change in pSMAD3 levels were observed compared to wild type controls (Fig. 5D). In contrast, in retinae from transgenic βB1-TGF-β1 mice an 80% increase in pSMAD3 was detected (1.83 ± 0.32; Fig. 5D). The enhanced phosphorylation of SMAD3 in βB1-TGF-β1 mice was completely rescued in animals with an additional overexpression of Norrin (0.84 ± 0.17; P = 0.029; Fig. 5D). In summary, our data indicated that the Norrin-induced inhibition of retinal TGF-β signaling correlates with reduced activity of intracellular SMAD3 and likely involves the canonical signaling pathways.
Norrin Inhibits TGF-β Signaling via the Induction of SMAD7

Since SMAD7 has the distinct potential to inhibit TGF-β signaling via several mechanisms, we analyzed whether Norrin modulates TGF-β signaling via SMAD7. In retinal mRNA from 8-day-old βB1-TGF-β1 mice, a significant reduction of *Smad7* levels was detected compared to wild type littermates (0.47 ± 0.06; *P* = 0.02; Fig. 6A). In contrast, in double transgenic βB1-TGF-β1/βB1-Norrin mice, the decrease of retinal SMAD7 mRNA observed in βB1-TGF-β1 animals was completely normalized compared to wild type controls (1.04 ± 0.11; *P* = 0.002 [βB1-TGF-β1 vs. βB1-Norrin/βB1-TGF-β1; Fig. 6A). To analyze whether the normalization of SMAD7
mRNA in double transgenic mice is followed by an increase in its translation. Western blot analyses were performed. In wild type controls, specific bands for SMAD7 were detected in retinal proteins, which were similarly intensive to that from βB1-Norrin, βB1-TGF-β1, βB1-Norrin/βB1-TGF-β1 animals, and wild type controls at P8 (P < 0.05; **P < 0.01; n ≥ 7 mice per genotype; mean ± SEM). Western blot analysis (B) and densitometry (C) for SMAD7 on retinal proteins from βB1-Norrin, βB1-TGF-β1, βB1-Norrin/βB1-TGF-β1 mice and wild type controls at P8 (*P < 0.05; n ≥ 11 mice per genotype; mean ± SEM).

FIGURE 6. Norrin inhibits TGF-β signaling via an induction of SMAD7. (A) Quantitative real-time RT-PCR for Smad7 on retinal RNA from βB1-Norrin, βB1-TGF-β1, βB1-Norrin/βB1-TGF-β1 animals, and wild type controls at P8 (P < 0.05; **P < 0.01; n ≥ 7 mice per genotype; mean ± SEM). Western blot analysis (B) and densitometry (C) for SMAD7 on retinal proteins from βB1-Norrin, βB1-TGF-β1, βB1-Norrin/βB1-TGF-β1 mice and wild type controls at P8 (*P < 0.05; n ≥ 11 mice per genotype; mean ± SEM).

Norrin Inhibits TGF-β Signaling In Vitro

To test if Norrin interferes with the expression of typical target proteins of TGF-β signaling, the expression of PAI-1 mRNA, a known SMAD-mediated TGF-β1 responsive protein, was analyzed in HRMEC by real-time RT-PCR after incubation with TGF-β1 and/or Norrin. Incubation of HRMEC with Norrin for 16 hours did not change PAI-1 mRNA expression, whereas the treatment of cells with TGF-β1 induced an up to 3.6-fold increase in PAI-1 mRNA (3.6 ± 0.4; **P < 0.001; Fig. 7A). The TGF-β1–mediated effect on PAI-1 mRNA expression was significantly blocked by approximately 50% when HRMEC were additionally incubated with Norrin (1.9 ± 0.3; **P < 0.002; Fig. 7A). To confirm our results by an independent assay, MLEC that express luciferase under the control of a truncated TGF-β–sensitive PAI-1 promoter were incubated with TGF-β1 and/or Norrin. After treatment for 20 hours, no difference in luciferase activity was seen between Norrin-exposed MLEC and untreated control cells (Fig. 7B). In contrast, in TGF-β1-incubated cells, a significant increase in luciferase activity (7.0 ± 0.3; **P < 0.001) was observed compared to untreated cells (Fig. 7B). The TGF-β1–mediated effect was significantly reduced by more than 20% when MLEC were additionally incubated with Norrin (5.5 ± 0.3; **P < 0.001; Fig. 7B).

To analyze whether the inhibitory effect of Norrin on TGF-β signaling involves the activation of the Wnt/β-catenin signaling pathway, MLEC were incubated with TGF-β1, Norrin, and/or...
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DKK-1, an inhibitor of the Wnt/β-catenin signaling pathway. After incubation of the cells with DKK-1, no change in luciferase activity was measured (Fig. 7B). However, after an additional treatment of the cells with DKK-1, the Norrin-mediated reduction in TGF-β1-induced luciferase activity was completely abolished (7.0 ± 0.4; P < 0.001; Fig. 7B), strongly suggesting that Norrin inhibits TGF-β activity via the activation of the Wnt/β-catenin signaling pathway.

**DISCUSSION**

We concluded that TGF-β signaling attenuates canonical Wnt/β-catenin signaling in the retina by reducing the amounts of β-catenin. This effect is antagonized by Norrin, which increases the amounts of β-catenin, induces the expression of Smad7, an inhibitor of canonical TGF-β signaling, and rescues the effects of TGF-β signaling on the phenotype of the retinal and choroidal vasculature. Our conclusions rest upon: (1) the observation that Norrin antagonizes the TGF-β-induced lack of retinal and choroidal vasculature, and consequently neuronal apoptosis, (2) the finding that Norrin normalizes TGF-β-mediated suppression of retinal β-catenin protein levels, (3) the observation that Norrin inhibits TGF-β-mediated pSmad3 signaling, (4) the capability of Norrin to induce Smad7 expression in the presence of high retinal amounts of TGF-β, (5) the potential of Norrin to reduce TGF-β-mediated induction of target genes, and finally (6) the finding that DKK-1 blocks Norrin-mediated effects.

Crosstalk between TGF-β and Wnt/β-catenin signaling is an extensively studied and common theme during early embryonic development and morphogenesis, as well as for tumor progression in the adult organism. During early development the mutual regulation of TGF-β and Wnt/β-catenin signaling is required for tissue patterning, a scenario that involves opposing effects on the developing mesenchyme. While BMP2 and 4, both members of the TGF-β family, induce the expression of Wnt-8 in frog mesenchymal cells, BMP2 downregulates Wnt-7 and β-catenin in mesenchymal cells from chicken. In contrast, synergistic signaling of the TGF-β and Wnt/β-catenin pathway has been reported for neuronal development, gastric cancer, or mesenchymal stem cell self-renewal. Quite intriguingly, the crosstalk between TGF-β and β-catenin signaling is mediated by several transcription factors of both pathways, SMADs and components of the β-catenin destruction complex, molecules that also similarly are involved in the crosstalk between TGF-β and Norrin observed in our study. However, there is substantial evidence that both signaling pathways exert essentially opposite effects during fetal growth of retinal and choroidal capillaries, as well as for their maintenance in the adult mammalian eye. For development of retinal vasculature in mice, capillaries grow from the optic nerve head along the inner retina toward its periphery to form the primary inner retinal vascularplexus between P0 and P8. The primary vascularplexus than transitions to a mature vasculature, which requires TGF-β signaling for pericyte recruitment, maintenance of the blood-retinal barrier, and microvascular vascular homeostasis. After the superficialplexus has been established, vessels grow into the retina to the outer and inner edges of the ONL where they form the deep and intermediate vascularplexuses. For development of the intraretinal vasculature and for its repair after injury, Norrin-induced canonical Wnt/β-catenin signaling is essential, while TGF-β has pronounced antiangiogenic effects on retinal and choroidal vessels. Consequently, deficiency in TGF-β signaling causes retinal neovascularization.

Development of retinal vasculature involves various interactions between neurons, glia cells, and microvascular endothelial cells, and is well characterized for the formation of hypoxia inducible factor (HIF)-1α mediated retinal angiogenesis. During development of the retinal vasculature, hypoxia increases HIF-1α expression in retinal neurons, which in turn leads to an induction of several angiogenic factors and subsequently to vessel formation toward the hypoxic retina. Intriguingly, in a previous study we observed a substantial decrease of HIF-1α in the inner retina of βB1-TGF-β1 mice, which correlates well with our observation of an enhanced pSmad3 signaling in cells of the inner retina. Since Norrin blocks pSmad3 phosphorylation in inner retina of double transgenic mice, it is most likely that Norrin mediates its inhibitory effects on TGF-β signaling via acting on neurons/neuroglia in the inner retina. This explanation is supported by reports of neuronal Fzd4 expression in the retinal ganglion cell layer as well as the INL and ONL.

The critical molecule for canonical Wnt/β-catenin signaling is cytosolic β-catenin, whose levels are controlled by the β-catenin destruction complex. In the absence of activating ligands such as Wnt molecules or Norrin, the complex reduces the levels of cytosolic β-catenin by promoting its polyubiquitination and degradation, and thereby keeping the canonical Wnt/β-catenin signaling inactive. In contrast, Wnt ligands induce stabilization of β-catenin, and favor its cytosolic accumulation and translocation into the nucleus to finally activate canonical Wnt/β-catenin signaling. The marked reduction in the amounts of β-catenin and Axin2 that we observed in animals with transgenic TGF-β1 expression is consistent with retinal inactivity of canonical Wnt/β-catenin signaling. A comparable TGF-β2-induced and β-catenin-dependent inhibition of canonical Wnt/β-catenin was recently reported for cells of the trabecular meshwork in the anterior eye. In addition, a quite comparable TGF-β-induced reduction of cellular β-catenin levels was observed in mesenchymal stem cells or in cultured human Müller glia with stem cell characteristics. Central molecules for the TGF-β-mediated suppression of Wnt/β-catenin signaling are Smad7 and Smad4. Beside other molecules, Smad7 can interact with axin, the scaffold protein of the β-catenin destruction complex, which in turn leads to a β-catenin accumulation. Since mice with an overexpression of TGF-β have a substantial decrease in Smad7 expression, this effect could lead to an increased activity of the β-catenin destruction complex and subsequently to reduced β-catenin levels. Vice versa, Smad4, which is required for translocation of phosphorylated Smad2/3 into the nucleus, is essential for a TGF-β-mediated degradation of β-catenin in cells from the mammary gland. An additional mechanism to decrease β-catenin levels was observed in glioblastoma cells, in which activation of TGF-β signaling induces an enhanced expression of the secreted frizzled-related protein sFrp1, a competitive inhibitor of Wnt/β-catenin signaling through binding at the cysteine-rich domain of frizzled receptors. Even though the exact mechanism of how TGF-β suppresses β-catenin signaling in the eye remains unclear, it is reasonable to assume that the well characterized function of Norrin as an atypical Wnt ligand and its capability to activate the canonical Wnt/β-catenin signaling pathway causes or contributes to the normalization of β-catenin in mice with combined Norrin/TGF-β1 overexpression.

Another mechanism to induce cross-inhibition of both pathways during crosstalk of TGF-β and Wnt signaling is TGF-β/Wnt-controlled transcription, which may result in the antagonistic expression of specific target genes. Such a mechanism might significantly contribute to the effects of Norrin on TGF-β signaling observed in our study, as Norrin overexpression caused an increase in the retinal amounts of
SMAD7 and its mRNA in transgenic mice, which were substantially reduced when only TGF-β1 was overexpressed. Transcriptional regulation of SMAD7 expression is versatile and involves various transcription factors, including pSMAD2/3.57 While pSMAD2/3 are mediators of TGF-β signaling and enhance the expression of SMAD7, it is tempting to speculate that Norrin can induce SMAD7 expression via an activation of Wnt/β-catenin signaling. SMAD7 belongs to the group of inhibitory SMADs that can block downstream signaling of TGF-β via various mechanisms. Most prominent are inhibitory interactions of SMAD7 with TGF-β type I receptors and activated SMAD2 or 3, leading to their degradation via ubiquitilation.27 In addition, a lesser-known mechanism was reported in cortical neurons following inhibition of GSK3β through LiCl treatment that leads to an increase in pCREB/p300 signaling, which in turn inhibits pSMAD3 signaling.59 Accordingly, a SMAD7-induced lack of SMAD2/3 activation is a likely explanation for the decrease in the amounts of phosphorylated SMAD3 that we observed in eyes with combined Norrin/TGF-β1 overexpression. Quite intriguingly, in epithelial cell lines SMAD7 is capable to stabilize β-catenin by initiating molecular interactions that prevent its phosphorylation and degradation.54 It is tempting to speculate that a similar mechanism contributes to the increase in the Norrin-induced amounts of β-catenin despite the presence of active TGF-β1.

TGF-β and Wnt signaling pathways also are known to reciprocally regulate their ligand production, an effect that is used, for example, to establish extracellular gradients of morphogens during embryonic development. Since we could neither observe substantial effects of Norrin on the expression of TGF-β1 and 2, or on their critical receptors TGFβRI and TGFβRII, nor an effect of TGF-β on the Wnt receptors LRPS or FZD4, we regard such a scenario as less relevant during vascular development and maintenance in the mammalian retina. We did observe though a Norrin-mediated inhibition of the expression of PAI-1, which is a very characteristic target gene of TGF-β signaling. Our observation that DKK-1, an inhibitor of the frizzled-4 co-receptor LRPS/6,50 blocked the effect argues for the involvement of canonical Wnt/β-catenin signaling.

Overall, an extensive cross-inhibition of canonical TGF-β and Norrin-induced Wnt/β-catenin signaling appears to be an important element of the signaling cascades that are required during development of retinal and choroidal capillaries, their maintenance in the adult eye, and their repair after injury.

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