Norrin: Molecular and functional properties of an angiogenic and neuroprotective growth factor

Andreas Ohlmann¹, Ernst R. Tamm¹

Institute of Human Anatomy and Embryology, University of Regensburg, Universitätstr. 31, D-93053 Regensburg, Germany

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Norrin is a secreted signaling molecule with structural and functional characteristics of an autocrine and/or paracrine acting growth factor. In the eye, Norrin is constitutively expressed in Müller cells. Norrin specifically binds to Frizzled-4 receptors and activates the canonical Wnt/β-catenin signaling pathway that is profoundly enhanced when Tspan12 is present at the Norrin/Frizzled-4 receptor complex. In the absence of Norrin or Frizzled-4, intraretinal capillaries are not formed during developmental angiogenesis. As a result there is considerable evidence that Norrin and Frizzled-4 are part of an essential signaling system that controls the formation of the retinal vasculature during eye development. Intriguingly, Norrin promotes vessel regrowth and induces the formation of intraretinal capillaries following oxygen-induced retinopathy in mice, an animal model of retinopathy of prematurity. Moreover, Norrin has pronounced neuroprotective properties on retinal ganglion cells (RGC) with the distinct potential to decrease the damaging effects of excitotoxic NMDA-induced RGC injury. The neuroprotective effects of Norrin similarly involve an activation of Wnt/β-catenin signaling and the subsequent induction of neuroprotective growth factor synthesis in Müller cells, such as that of fibroblast growth factor-2 (FGF2) or ciliary neurotrophic factor (CNTF). Overall, Norrin and the molecules involved in its signaling pathway appear to be promising targets to develop strategies that induce intraretinal vessel formation in patients suffering from ischemic retinopathies, or that increase RGC survival in glaucoma.

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References

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1. Introduction

Almost two decades ago, mutations in the NDP gene were found to be causative for Norrie disease, a very rare ocular disorder that typically leads to congenital blindness (Meindl et al., 1992). For a long time, the specific function of Norrin, the encoded gene product of NDP, remained largely unclear. Data obtained in the last several years have provided evidence that Norrin is a secreted signaling molecule with the distinct properties of an autocrine and/or paracrine acting growth factor. In the retina, Norrin is part of an essential signaling system that is critically required for microvascular angiogenesis during development (Xu et al., 2004; Ye et al., 2009). Moreover, the angiogenic properties of Norrin might provide a novel approach to treat retinal microvascular disorders such as retinopathy of prematurity or diabetic retinopathy (Ohlmann et al., 2010). Recent data indicate that Norrin signaling also plays a role in the protection and maintenance of retinal neurons (Seitz et al., 2010). The neuroprotective and angiogenic properties of Norrin derive from similar downstream signaling cascades, which appear to act largely independently from each other. The functional role of Norrin is not restricted to the retina, but is similarly important for maintenance of structure and function in the inner ear and the female reproductive system. Norrin is widely expressed in astrocytes of the brain and in Bergmann glia of the cerebellum (Ye et al., 2011), indicating an important function in brain tissues which remains to be identified. In this review we summarize and discuss the functional properties of Norrin and their implications for retinal diseases. The interested reader is also referred to several other review articles that deal with different aspects of Norrin (Hendrickx and Leyns, 2008; Warden et al., 2007; Ye et al., 2010).

2. Molecular and structural characteristics of Norrin

2.1. Gene and protein structure

Norrin (accession numbers: Nucleotide NM_000266, Protein: NP_000257) is a secreted protein with an N-terminal signaling peptide, and comprises 133 amino acids in humans. The sequence of Norrin shows similarities with proteins containing a cysteine-rich domain (Meindl et al., 1992) and in silico analyses of its potential tertiary structure predict a cystine-knot motif similar to that identified in some growth factors, e.g., that of the TGF-β superfamily (Meintering et al., 1993) (Fig. 1). Norrin is encoded by the NDP gene, which is located on chromosome Xp11.4 in humans. NDP consists of three exons and transcribes a mRNA of 2.1 kb (Berger et al., 1992). Similar to the human gene, murine Ndp contains three exons and is also localized on the X chromosome (accession numbers: Nucleotide NM_010883, Protein: NP_035013). The cDNA of murine Norrin is 6 bp shorter than that of humans resulting in an encoded protein of 131 amino acids (Battinelli et al., 1996). The amino acid sequences of human and murine Norrin are 95% homologous, the homology increases up to 99%, if only the protein sequences of exon 3 with its cystine knot motif are considered (Battinelli et al., 1996). In contrast, no specific signal for Norrin was observed in the rest of the eye. More recently, a knock-in mouse model (Ndp<sup>AP</sup>) that carries the coding sequence of human placental alkaline phosphatase (AP) inserted into the Ndp locus was developed. This knock-in allows more detailed studies on the expression of Norrin. In the retina of adult Ndp<sup>AP</sup> mice, a radial AP-staining from the inner to the outer limiting membrane was detected, strongly indicating a specific expression of Norrin in Müller cells (Ye et al., 2009). During development of the eye, Norrin expression was first observed at embryonic day (E) 15.5 in the region of the optic nerve head. After birth, at postnatal day (P) 4, P7, and in adulthood, a homogeneous AP-staining was seen in Müller cells, but was absent in astrocytes or retinal ganglion cells (Ye et al., 2009). The onset of homogeneous retinal staining for Norrin correlates with the onset of Müller cell differentiation (Young, 1985a,b). Norrin is expressed throughout lifetime and its mRNA is present in the retina in roughly equal amounts in 2 week and 2-year old mice (Lenzner et al., 2002). By Northern blot hybridization, a very high expression of Norrin has been observed in the retina of juvenile monkeys (24–36 months old), and in that of an 80-year old human donor (Bernstein and Wong, 1998). In monkeys, the amounts of Norrin mRNA were analyzed in RNA derived from the fovea or the mid-periphery of the retina, and showed no major differences.

Outside the eye, Norrin is expressed in the brain, and in situ hybridization of sections from rabbit, mouse and human brains, mRNA of Norrin was observed in cerebellum, hippocampus, neocortex, and olfactory bulb and epithelium (Berger et al., 1996; Hartzler et al., 1999). Again, somewhat different results were observed using adult Ndp<sup>AP</sup> reporter mice, which showed a distinct AP staining throughout the entire brain. AP staining was specifically localized in astrocytes and radial Bergman glia of the cerebellum, quite comparable to Müller cell labeling in the retina (Ye et al., 2011). During embryonic development of the central nervous system, Norrin appears to be expressed first in neural tube and rhombencephalon (Paxton et al., 2010; Ye et al., 2011). While development proceeds, the expression of Norrin in the spinal cord continuously decreases whereas it is induced in cerebellum, diencephalon, and telencephalon (Ye et al., 2011). In addition, Norrin is expressed in the inner ear. In chicken, Norrin mRNA was detected once the auditory vesicle had been formed. After birth and in adult mice, an inner ear expression of Norrin was found to be localized in the vascularized zone between the organ of Corti and the spiral ganglion as well as in the stria vascularis (Paxton et al., 2010; Ye et al., 2011).

An interesting aspect of the expression pattern of Norrin is the fact that its expression is not restricted to tissues of the central nervous system, but is also observed in reproductive tissues and their precursors. Accordingly, mRNA for Norrin was detected in the mesonephros of chicken by in situ hybridization (Paxton et al., 2010). In tissues from adult mice, mRNA of Norrin was found in kidney, uterus, testis and epididymis by both RT-PCR and Northern blot analyses (Hsieh et al., 2005; Luhmann et al., 2005b). The specific cell populations that express Norrin in these tissues have not been identified so far. In addition, the transcription of Norrin was observed in the human placenta (Luhmann et al., 2005b). To date, no data are available that indicate the specific localization of translated Norrin in those tissues, in which its mRNA expression or promoter activity has been shown. This is probably due to the fact that Norrin is only available in small amounts and/or because of the lack of suitable antibodies.

2.2. Expression and localization of Norrin

Reports of several studies have described the specific expression of Norrin in the retina, although conflicting data have been reported with regards to its specific cellular expression. By in situ hybridization of human, rabbit and mouse eyes, Norrin mRNA was detected in the ganglion cell layer of the retina as well as in the inner and outer nuclear layer (Berger et al., 1996; Hartzler et al., 1999). In contrast, no specific signal for Norrin was observed in the rest of the eye. More recently, a knock-in mouse model (Ndp<sup>AP</sup>) that carries the coding sequence of human placental alkaline phosphatase (AP) inserted into the Ndp locus was developed. This knock-in allows more detailed studies on the expression of Norrin. In the retina of adult Ndp<sup>AP</sup> mice, a radial AP-staining from the inner to the outer limiting membrane was detected, strongly indicating a specific expression of Norrin in Müller cells (Ye et al., 2009). During development of the eye, Norrin expression was first observed at embryonic day (E) 15.5 in the region of the optic nerve head. After birth, at postnatal day (P) 4, P7, and in adulthood, a homogeneous AP-staining was seen in Müller cells, but was absent in astrocytes or retinal ganglion cells (Ye et al., 2009). The onset of homogeneous retinal staining for Norrin correlates with the onset of Müller cell differentiation (Young, 1985a,b). Norrin is expressed throughout lifetime and its mRNA is present in the retina in roughly equal amounts in 2 week and 2-year old mice (Lenzner et al., 2002). By Northern blot hybridization, a very high expression of Norrin has been observed in the retina of juvenile monkeys (24–36 months old), and in that of an 80-year old human donor (Bernstein and Wong, 1998). In monkeys, the amounts of Norrin mRNA were analyzed in RNA derived from the fovea or the mid-periphery of the retina, and showed no major differences.

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2.3. Signaling pathways of Norrin

The characteristic phenotype of Norrin-deficient (Ndp<sup>−/−</sup>) mice involves an impaired outgrowth of capillaries from the superficial vascular plexus in the retinal surface resulting in a complete lack of
intraretinal capillaries (Luhmann et al., 2005a; Ohlmann et al., 2005; Rehm et al., 2002; Richter et al., 1998). In addition, the hyaloid vasculature, which is transiently formed within the vitreous cavity during eye development, persists in Ndp^{−/−} mice. Strikingly similar retinal phenotypes were observed in Frizzled-4-deficient (Fzd4^{−/−}) (Xu et al., 2004) and in Lrp5-deficient mice (Lrp5^{−/−}) (Kato et al., 2002; Xia et al., 2008). Both in Fzd4^{−/−} and Lrp5^{−/−} mice the hyaloid vasculature persists. Intraretinal capillaries lack in Fzd4^{−/−} animals and are considerably reduced in number in Lrp5^{−/−} mice. The molecular reasons for the similar phenotypes were identified in very elegant studies by Xu and coworkers (Xu et al., 2004), who discovered an essential signaling system that controls the formation of retinal capillaries during development.

Frizzled-4 receptor belongs to the family of frizzled receptors that upon ligand binding, signal through the canonical Wnt/β-catenin pathway after interaction with its co-receptors Lrp5 or 6 (He et al., 2004). During embryonic development and in the adult organism, Wnt signaling is required for a multitude of very fundamental processes such as cell proliferation, establishment of cell polarity and cell differentiation (Logan and Nusse, 2004). In mammalian organisms, Wnt signaling is mediated via 19 different cysteine-rich Wnt glycolipoproteins that can activate 10 different frizzled receptors (MacDonald et al., 2009). Activation of the canonical Wnt/β-catenin signaling pathway requires the engagement of a frizzled receptor and a co-receptor such as Lrp5 or 6 (He et al., 2004). Signaling downstream of the receptors is accomplished by stabilization of the transcription factor β-catenin. In the un-induced state, β-catenin binds to axin to form a degradation complex that includes casein kinase 1α, glycogen synthase kinase 3β, and adenomatous polyposis coli (APC) gene product. In this complex β-catenin is phosphorylated, then ubiquitinated by the E3 ubiquitin ligase β-Trcp, and subsequently is degraded (Fig. 2A) (MacDonald et al., 2009). When Wnt ligands bind to the frizzled/Lrp receptor complex, the β-catenin degradation complex becomes inactivated, resulting in stabilization of β-catenin. Finally, β-catenin translocates into the nucleus and induces the expression of Wnt-specific target genes after interaction with Lef/Tcf transcription factors (Fig. 2B) (MacDonald et al., 2009).

To demonstrate that Norrin is a non-Wnt ligand that is able to bind Frizzled-4 and activate the canonical Wnt/β-catenin signaling pathway, Xu and co-workers (Xu et al., 2004) used a HEK-293 Wnt/β-catenin reporter cell line (TOPflash). TOPflash cells express luciferase under the control of β-catenin/Lef/Tcf response elements. After transfection with Norrin, Frizzled-4, Lrp5 or 6 there was substantial activation of the Wnt/β-catenin signaling pathway (Xu

Fig. 1. A. Genomic structure of the NDP gene. B. Protein sequence of Norrin. The three putative evolutionarily conserved disulfide bonds are indicated that are predicted to form the cystine knot motif. The putative signal peptide of Norrin is displayed in gray characters. C. Structure of human Norrin based upon its primary amino acid sequence. Ribbon plot from predicted atomic coordinates generated by the I-TASSER server. The carboxy-terminal cysteine residues of the cysteine-rich domain that is expected to adopt a cysteine knot motif are shown by a yellow stick model. From (Ohlmann et al., 2011).
To analyze if Norrin can bind other frizzled receptors, HEK-293, TOPflash and COS cells were transfected with Norrin, Lrp5 and several frizzled receptors other than Frizzled-4. Intriguingly, Norrin did only bind to Frizzled-4 (Smallwood et al., 2007; Xu et al., 2004), strongly indicating that Norrin mediates its function specifically via this type of frizzled receptor. In addition, it was shown that Norrin binds with high affinity to the cysteine-rich domain of Frizzled-4, and not to frizzled co-receptors Lrp5 and 6 (Xu et al., 2004). It is of interest that both Lrp5 and 6 were able to mediate the Norrin-induced activation of Wnt/β-catenin signaling. Lrp5-deficient mice have a more benign phenotype than Ndp−/− mice and show a reduced number of capillaries in the retina, but not a complete absence (Xia et al., 2008). Very likely, Lrp 6 is capable to compensate, at least partially, for the loss of Lrp5.

Tspan12 is a highly conserved protein of the tetraspanin family with a predicted polypeptide binding site and four transmembrane domains. Phenotype analyses of Tspan12-deficient mice (Tspan12−/−) revealed retinal vascular changes that were strikingly similar to those observed in Ndp−/− and Fzd4−/− mice (Junge et al., 2009). Tspan12−/− mice show a complete absence of intraretinal capillaries and a delayed regression of hyaloid vessels. Accordingly, Junge and coworkers (Junge et al., 2009) examined the possibility that Tspan12 could be involved in Norrin/Frizzled-4 signaling as well. To test this hypothesis, Junge et al. cotransfected TOPflash HEK-293 cells with Frizzled-4, Lrp5 and Tspan12. This combination let to threefold increase in Norrin-mediated activation of the Wnt/β-catenin reporter, when compared with cells without Tspan12 expression (Junge et al., 2009). Binding studies indicated that Tspan12 associates with the Norrin/Frizzled-4/Lrp5 receptor complex to enhance receptor clustering (Junge et al., 2009). Intriguingly, the amplification of Wnt/β-catenin signaling by Tspan12 was not observed when the
cells were treated with Wnt3a (Junge et al., 2009), strongly indicating that Tspan12 selectively enhances Norrin-mediated Wnt/b-catenin signaling.

Canonical Wnt/b-catenin signaling induces the expression of specific target genes. To identify potential downstream mediators of Norrin signaling, Ye and colleagues performed microarray analyses with microvascular endothelial cells from retina, yolk sac and brain of Ndp+/−, Lrp5−/−, Fzd4−/− or wild-type mice after treatment with Norrin (Ye et al., 2009). In the course of these studies, the extracellular matrix-associated protein SparcL1/Hevin/SC1Mast9 and the transcription factor Sox17, a member of the HMG-box family of transcription factors, had the strongest correlation with Norrin/Frizzled-4 signaling (Ye et al., 2009). To test the hypothesis that Sox17 is a critical factor in mediating the effects of Norrin/Frizzled-4 signaling, Sox17 was overexpressed in endothelial cells prepared from Fzd4−/− mice. Endothelial cells lacking Frizzled-4 did not form tubes in culture. In contrast, overexpressing of Sox17 in Frizzled-4-deficient endothelial cells rescued cell–cell contacts and promoted formation of capillary-like structures (Ye et al., 2009). This observation suggested that Sox17 is a potent downstream mediator of Norrin/Frizzled-4 signaling.

We analyzed in a candidate approach the influence of Norrin on the expression of several angiogenic growth factors and their receptors in human retinal microvascular endothelial cells (HRMEC). The expression of angiopoietin-2 (Ang-2) was modulated by Norrin signaling. Since the vascular phenotype in retinae of Ang-2-deficient mice shares similarities with that of Ndp+/− mice (Gale et al., 2002; Hackett et al., 2002), we wondered whether Norrin might mediate its angiogenic functions via Ang-2. To test our hypothesis, HRMEC were treated with Norrin, Ang-2 and/or inhibitory anti-Ang-2 antibodies. The presence of antibodies that blocked the action of Ang-2 reduced the Norrin-mediated increase in cell proliferation by more than 35% (Ohlmann et al., 2010), strongly indicating that the effects of Norrin on microvascular endothelial cells are, at least partially, mediated via Ang-2 signaling. Quite similar to Norrin, Ang-2 is essential for the development of intraretinal capillaries which are largely lacking in Ang-2-deficient mice (Gale et al., 2002; Hackett et al., 2002). In addition, Ang-2-signaling appears to play a role for maintenance of vascular integrity in the adult retina. Experimentally induced high amounts of Ang-2 cause pericyte loss of retinal capillaries both in normal and diabetic rats and mice, and overall enhance vascular pathology in the diabetic retina (Hammes et al., 2004; Pfister et al., 2010). In contrast, reduced Ang-2 expression in heterozygous Ang-2-deficient mice completely prevents diabetes-induced pericyte loss of retinal capillaries (Pfister et al., 2010). So far, no data are available that indicate if and how Norrin is involved in the modulation of Ang-2 signaling in the adult retina.

2.4. Biochemical characteristics of Norrin

Norrin is a secreted protein with high affinity to extracellular matrix. In modified COS-7 cells that overexpress Norrin, the secreted protein could be isolated from the extracellular matrix of the cultured cells, but was not detected in the culture medium (Perez-Vilar and Hill, 1997). This finding appears to indicate that Norrin acts locally in an autocrine or paracrine manner. To follow up on this hypothesis, Xu and colleagues studied Norrin-overexpressing HEK-293 and Wnt/b-catenin reporter (TOPFlash) cells in culture (Xu et al., 2004). Cells were grown either spatially separated, but sharing the same cell culture medium, or alternatively in mixed co-culture. In mixed co-culture the Norrin-induced activation of Wnt/b-catenin signaling was approximately sevenfold higher when compared to that observed in spatially separated cell cultures (Xu et al., 2004), strongly suggesting an autocrine or paracrine signaling function of Norrin. More specifically, Norrin shows high affinity to heparin and heparin-binding enhances the binding of Norrin to Frizzled-4 in a dose dependent manner (Ohlmann et al., 2010; Smallwood et al., 2007).

Extracellular Norrin is reported to form oligomers with a molecular weight of 25–200 kDa, a scenario that appears to involve disulfide bonds generated by the cysteine at position 95 of the human Norrin sequence (Perez-Vilar and Hill, 1997; Shasray and Trese, 2003). There are conflicting data regarding the functional role of the half-cystine residue 95 for dimerization of Norrin. By site-specific mutagenesis, C95A and C95R mutant Norrin was generated and dimer formation was analyzed. In C95A mutant Norrin, dimerization was observed (Perez-Vilar and Hill, 1997), whereas in C95R mutant Norrin, monomers were predominantly detected (Junge et al., 2009). Interestingly, C95R mutant Norrin still binds to Frizzled-4 receptors, but is unable to activate the Wnt/b-catenin signaling pathway, suggesting that dimer formation is important for Norrin’s biochemical function (Junge et al., 2009).

Norrin contains at its C-terminus a highly conserved cysteine-rich domain that is reported to adopt a cystine knot motif (Meindl et al., 1992). In addition to the three evolutionarily conserved disulfide bonds that form the cystine knot motif, an additional cystine residue has been supposed to be formed by cysteine 93 and 131 of human Norrin. To characterize the cysteine-rich domain and the predicted cystine knot motif of Norrin in more detail, Smallwood and colleagues replaced several C-terminal cysteines by alanine residues (Smallwood et al., 2007). Norrin mutations that disrupted the predicted cystine knot motif substantially decreased its affinity to Frizzled-4 and its capacity to activate Wnt/b-catenin signaling. However, substitution of cysteine residues 93 and 131 had only minor effects on Norrin’s function (Smallwood et al., 2007). While structural analyses that verify the presence of a cystine knot have yet to be done, the available biochemical data strongly suggest the existence of a cystine knot and its important role for the bioactivity of Norrin.

3. Functional properties of Norrin

3.1. The angiogenic functions of Norrin

In the mouse eye, the retinal vasculature is comprised of three distinct capillary networks, which develop after birth. During the second postnatal day in mice, retinal vessels first appear from the hyaloid artery near the optic nerve and grow into the retina underneath the inner limiting membrane of the retina. Over the next 5 days the retinal vessels spread radially from the optic nerve to the ora serrata forming the superficial vascular layer. Beginning in the second week and starting near the optic nerve, vessels sprout from the superficial vascular plexus into the inner retina to form the capillary plexus in the outer and inner plexiform layers. In the third postnatal week the retinal vasculature undergoes substantial remodeling and maturation. Development of the retinal vasculature in the mouse eye is completed at around P20 (for review see (Dorrell and Friedlander, 2006; Fruttiger, 2002)).

During the initial analyses of Ndp+/− mice, intravitreal fibrous masses and distinct structural changes in the retina with a general disorganization of the ganglion cell layer were described (Berger et al., 1996). In further studies, a persistent hyaloid vasculature and defects in the retinal vasculature of Ndp+/− mice were identified (Richter et al., 1998). The results of these studies suggested that the absence of Norrin disrupted normal retinal vascular development. In the normal mouse eye, hyaloid vessels form a well-organized temporary vascular network at birth, which starts to regress at P4 and completely disappears by P16 (Ito and Yoshioka, 1999). In Ndp−/− mice, the regression of hyaloid vessels is delayed as is the
spreading of capillaries at the inner retinal surface, which are stunted and fail to reach the ora serrata (Ohlmann et al., 2004; Richter et al., 1998). In addition, a complete lack of intraretinal capillaries is observed (Rehm et al., 2002; Richter et al., 1998). The capillaries that do form are abnormal in structure, have fenestrated capsule endothelial cells (Richter et al., 1998), an incomplete covering with mural cells (Ye et al., 2009), are leaky and do not form a blood brain barrier (Luhmann et al., 2005a). A very characteristic finding in the eyes of Ndp<sup>+/−</sup> mice is the formation of focal strands and aggregates of cells (Luhmann et al., 2005a; Ohlmann et al., 2005), which resemble angiogenic sprouts and extend from the vessels at the inner retinal surface to the inner plexiform layer and the outer surface of the inner nuclear layer (Fig. 3). The angiogenic sprouts appear to be arrested in their development and do not form a vascular lumen (Fig. 3). Available data strongly indicate that the structural changes of retinal capillaries are essentially phenocopied in the eyes of Fzd4<sup>−/−</sup> and Tspan12<sup>−/−</sup> mice (Junge et al., 2009; Xu et al., 2004; Ye et al., 2009).

Importantly, the vascular phenotype of Ndp<sup>+/−</sup> mice is completely rescued (Fig. 3) when Norrin is ectopically overexpressed in the lens (Ohlmann et al., 2005), a finding which strongly suggests that Norrin acts on top of a signaling cascade to direct the specific morphogenesis of these capillaries within the retina. Norrin may simply serve as a chemoattractant which is secreted from retinal cells to generate a gradient, and angiogenic sprouts may respond to this gradient to form the capillary network of the retina. The observation though that ectopic overexpression of Norrin from the lens rescues the retinal phenotype of Ndp<sup>+/−</sup> mice strongly argues against the hypothesis of a Norrin-gradient driving capillary growth in the developing retina (Ohlmann et al., 2005). Moreover, the finding that Norrin expression is predominately seen in Müller cells, which radially stretch through the entire retina, indicates a more homogenous secretion of Norrin throughout all layers of the retina. It is rather likely that the overall presence of Norrin in the extracellular space of the developing retina is specifically required to initiate the distinct biological processes in retinal microvascular endothelial cells that are required for capillary formation in this tissue, an assumption that is supported by in vitro data. Accordingly, Norrin induced proliferation, migration and tube formation as well as an increased survival of both human retinal and dermal microvascular endothelial cells, effects that could be blocked when Dickkopf (DKK)-1, an inhibitor of canonical Wnt/β-catenin signaling, was added (Ohlmann et al., 2010). The observations strongly indicate that Norrin mediates its angiogenic properties by directly acting on microvascular endothelial cells via activation of the canonical Wnt/β-catenin signaling pathway.

The lack of intraretinal capillaries results in retinal hypoxia as indicated by the accumulation of the transcription factor hypoxia-inducible factor (Hif-1α) in the retina of two week-old Ndp<sup>+/−</sup> mice (Luhmann et al., 2005a) and by characteristic functional changes that are seen by electroretinography (ERG) (Ohlmann et al., 2005). Retinal hypoxia and increasing amounts of Hif-1α are very likely the cause for the increased expression of vascular endothelial growth factor-A (VEGF-A) that has been observed in retinas of Ndp<sup>+/−</sup> mice at P10 and later on (Luhmann et al., 2005a). VEGF-A is a characteristic target gene of Hif-1α (Fong, 2009) and may well prevent or delay regression of the hyaloid vasculature in Ndp<sup>+/−</sup> mice. Alternatively, Norrin may be involved in the signaling processes that normally lead to programmed cell death of the endothelium of hyaloid vessels and to their subsequent regression. There is evidence that the canonical Wnt/β-catenin signaling pathway is involved here and that macrophage-derived Wnt7b is acting as a short paracrine signal that binds to Fizzled-4 on endothelial cells of hyaloid vessels and induces their programmed cell death (Lobov et al., 2005). The persistence of the hyaloid vasculature in Lrp5<sup>−/−</sup> and Fzd4<sup>−/−</sup> mice supports this assumption (Xia et al., 2008; Xu et al., 2004). There is the possibility that, similar to Wnt7b, binding of Norrin to Fizzled-4 may be required to induce the regression of the hyaloid vasculature during development, although data from Norrin-overexpressing mice strongly argue against this assumption. When Norrin is ectopically overexpressed in the mouse eye via a strong lens-specific promoter and available in high amounts in the vitreous body, the hyaloid vasculature persists (Ohlmann et al., 2005). The vessel density of the tunica vasculosa lentis, the specific part of the hyaloid vasculature that surrounds the lens, increases considerably, and the newly formed capillaries show striking similarities with those of the retina such as lack of fenestrae and the presence of numerous pericytes. Apparently, Norrin induces capillary growth also when ectopically expressed as long as Fizzled-4 is present as receptor on microvascular endothelial cells.

Prior to the development of the superficial vascular plexus in the mouse, a network of astrocytes is formed on the retinal surface, a process that begins at around embryonic day (E) 19 and continues until birth. The astrocyte network serves as scaffolding for the growth of the superficial retinal vessels (Fruttiger, 2002). After establishment of the primary superficial retinal vasculature, the endothelial tubes are covered by pericytes for stabilization of retinal capillaries and to induce further differentiation processes (Dorrell and Friedlander, 2006). In Ndp<sup>+/−</sup> mice, rarefaction of the superficial vascular plexus and a reduced number of branching points of retinal capillaries were detected (Luhmann et al., 2005a; Ye et al., 2009). Still, the structure of the astrocyte network on the retinal surface appeared to be normal in newborn Ndp<sup>+/−</sup> mice, and it was not until P21 that damage to the network was detected (Luhmann et al., 2005a), suggesting that the vascular defects of Ndp<sup>+/−</sup> mice are not associated with primary defects in astrocyte development. In contrast, a reduced number of pericytes and an incomplete pericyte sheath around retinal capillaries and veins was observed in Ndp<sup>+/−</sup> mice, but not around the arteries of the animals (Ye et al., 2009). Since essentially similar observations were found in Fzd4<sup>−/−</sup> mice (Ye et al., 2009), the data strongly indicate that pericyte recruitment in the retina involves a Norrin/β-catenin-mediated crosstalk between Müller cells and endothelial cells. Interestingly, overactivation of this signaling pathway inhibits recruitment of smooth muscle cells in the mouse aorta during development (Ye et al., 2009).

The endothelial cells lining the capillaries on the retinal surface of Ndp<sup>+/−</sup> mice have fenestrae with a typical diaphragm (Richter et al., 1998), quite unlike normal retinal or hyaloid capillaries that form the blood-retinal barrier (Cuthbertson and Mandel, 1986; Ito and Yoshioka, 1999). Accordingly, vascular leakage during fluorescence angiography and an increased expression of plasmalemma vesicle-associated protein (Pvlp1), a marker for fenestrated endothelial cells, were reported in Ndp<sup>+/−</sup> mice (Luhmann et al., 2005a; Schafer et al., 2009), strongly suggesting impaired formation of the blood-retinal barrier. It is intriguing to speculate that a Norrin-mediated activation of the canonical Wnt/β-catenin signaling to establish the blood-retinal barrier is a more widespread mechanism, since the blood-brain barrier of brain capillaries is similarly under the influence of Wnt/β-catenin signaling (Liebner et al., 2008). In some regions of the brain, the expression of Norrin in astrocytes may be an important and more general factor to facilitate the critical role of astrocytes for stabilization and maintenance of the blood-brain barrier (Abbott, 2002; Haseloff et al., 2005). Such a function appears not to be essential, as no obvious defects in the blood brain barrier of cerebral or cerebellar capillaries have been observed in Norrin-deficient mice.

Vascular changes in Ndp<sup>+/−</sup> mice are not restricted to the retina, but are observed in the inner ear as well. During initial
Fig. 3. Ectopic transgenic Norrin overexpression from the lens in mouse line NDP-29 restores normal angiogenesis in Ndp<sup>−/−</sup> mutant mice. A–F, light (A–D) and electron microscopy (E–F) of Ndp<sup>−/−</sup> mutant mice (B,D,F) and Ndp<sup>−/−</sup> mutant mice with ectopic expression of norrin (Nor-29/Ndp<sup>−/−</sup>; A,C,E) at P21. A, C, E. In Nor-29/Ndp<sup>−/−</sup> mice, capillaries are seen in both inner (A, arrows) and outer (C, arrows) plexiform layer. By electron microscopy (E), the capillaries express the typical ultrastructural characteristics of retinal capillaries, and are covered by pericytes (Pe) and surrounded by a complete basal lamina (arrows). B, D, F. In Ndp<sup>−/−</sup> mutant mice, the surface of the retina is covered by a dense vascular membrane (arrows in B), while no capillaries are observed in deeper layers of the retina. Angiogenic sprouts extend from the vascular membranes at the inner retinal surface to the inner plexiform layer (arrows, D). By electron microscopy, no vascular lumen is observed in the angiogenic sprouts (F). G, H, staining of vascular endothelial cells in Nor-29/Ndp<sup>−/−</sup> (G) and Ndp<sup>−/−</sup> (H) mice with biotinylated *griffonia* (*bundeira*) *simplicifolia* lectin I. In Nor-29/Ndp<sup>−/−</sup> eyes, capillaries are positively labeled on the retinal surface, and in both inner and outer plexiform layer (arrows); gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer. Scale bars: 20 μm in A, B, G, H; 8 μm in C,D; 2 μm in E,F. From (Ohlmann et al., 2005).
mediated canonical Wnt/Frizzled-4 in inner ear hair cells is unclear. The organ of Corti, and vestibular hair cells in the maculae and stria vascularis develop an elevated auditory brainstem response threshold consistent with a defect in the peripheral auditory system (Xu et al., 2004). In 11-month-old animals, most of the capillaries in the stria vascularis are lost along with a near complete loss of inner and outer hair cells. Overall, the findings in the stria vascularis of Ndp\(^{-/-}\) and Fzd4\(^{-/-}\) mice strongly suggest that the properties of Norrin on microvascular endothelial cells in the inner ear are mediated via Norrin/Frizzled-4 signaling as well. It is of interest that the strongest expression of Frizzled-4 in the inner ear is not seen in endothelial cells of stria vascularis capillaries, but in inner hair cells in the organ of Corti, and vestibular hair cells in the maculae and cristae (Wang et al., 2001). So far, the specific functional role of Frizzled-4 in inner hair cells is unclear.

Intriguingly, recent studies demonstrated that Wnt7a- and 7b-mediated canonical Wnt/\beta\text{-}catenin signaling induces vascularization in the brain (Daneman et al., 2009; Stenman et al., 2008). Different ligands appear to use the canonical Wnt/\beta\text{-}catenin signaling pathway in brain, retina and inner ear as a more general mechanism to induce vascular development in the central nervous system and its associated sensory organs.

### 3.1.1. The role of Norrin in vascular pathology

Since Norrin has pronounced angiogenic properties in the developing retina and mutations in NDP are associated with a more severe course of retinopathy of prematurity (Shastry et al., 1997), we wondered if Norrin can positively influence repair mechanisms of the retinal vasculature under pathological conditions. To analyze this potential role of Norrin, we employed the oxygen-induced retinopathy (OIR), murine model of retinopathy of prematurity (Smith et al., 1994), using transgenic mice that had ectopic overexpression of Norrin either in the lens (\beta1-Crystallin-Norrin) or in the retinal pigment epithelium (Rpe65-Norrin). Both mouse lines show no obvious alterations of retinal vasculature under physiological conditions (Ohlmann et al., 2010). In \beta1-Crystallin-Norrin mouse, but not in Rpe65-Norrin mice, the hyaloid vasculature persists (Ohlmann et al., 2005, 2010). To induce OIR, mice were exposed to an atmosphere of 75% oxygen for 5 days starting at P7, and returned back to room air at P12 (Smith et al., 1994). In the course of this treatment, a central vaso-obliteration of the retinal superficial vascular plexus happens, while further vascular development in the retina is inhibited. After oxygen exposure and during relative retinal hypoxia, vessel regrowth into vaso-obiterated areas and preretinal tuft formation occurs (Smith et al., 1994). In wild-type mice, the expression of Norrin was found to be substantially suppressed during high oxygen exposure (Ohlmann et al., 2010), already indicating that lack of Norrin may be involved in the development of OIR. In contrast, overexpression of Norrin in the eye markedly reduced retinal vaso-obliteration during oxygen treatment (Ohlmann et al., 2010). Beside its protective effects on retinal vessels, Norrin also enhanced vessel regrowth into vaso-obiterated areas following an OIR. In comparison with wild-type controls, the vaso-obiterated area in transgenic mice was only half the size at P14, an effect that could be blocked by Dickkopf (Dkk)-1, an inhibitor of the canonical Wnt/\beta\text{-}catenin signaling pathway (Ohlmann et al., 2010). In the murine OIR model, oxygen treatment also inhibits development of intraretinal vasculature, which normally begins at around P8 (Dorrell and Friedlander, 2006). Accordingly, the development of intraretinal capillaries in wild-type mice remained delayed, whereas in transgenic mice with an ocular overexpression of Norrin a continuous repair and increase in intraretinal capillarization was observed (Fig. 4) (Ohlmann et al., 2010). Finally, the improved retinal vascularization of mice with a transgenic Norrin-overexpression caused a substantial (more than 80%) reduction in the formation of preretinal tufts when compared with wild-type littermates (Fig. 5) (Ohlmann et al., 2010).

Fig. 4. Norrin promotes formation of intraretinal capillaries following oxygen-induced retinopathy. A. The area covered by deep retinal capillaries was quantified at P12, P14 and P16 for \beta1-Crystallin-Norrin mice and controls (mean ± SEM; n > 10) and plotted as percentage of total retinal area. B–E. Representative retinal whole mounts (B,C) of \beta1-Crystallin-Norrin mice (C,E) and wild-type controls (B,D) at P16 following induction of oxygen-induced retinopathy. In wild-type animals, predominantly vessels of the superficial plexus are detectable (D, arrowheads) whereas in \beta1-Crystallin-Norrin mice deep retinal capillaries are abundant (E, arrows). Magnification bars: 500 μm (B,C); 100 μm (D,E). From (Ohlmann et al., 2010).
The results obtained in transgenic mice with Norrin overexpression are supported by those from a study in which mice were treated with inhibitory anti-Frizzled-4 antibodies (Paes et al., 2011). Following OIR, vessel regrowth into vaso-obliterated areas was significantly reduced when Norrin/Frizzled-4 signaling was blocked by anti-Frizzled-4 antibodies (Paes et al., 2011).

Overall, the data available strongly indicate that a Norrin/Frizzled-4-mediated activation of the canonical Wnt/\(\beta\)-catenin signaling pathway can substantially enhance vessel regrowth after retinal damage. Norrin overexpression not only prevented the OIR-induced inhibition of intraretinal capillaries, but also inhibited the development of a pathological preretinal neovascularization. The critical problems in retinopathy of prematurity result from the incomplete repair of the avascular areas, and the fact that the resulting hypoxia induces an irregular preretinal neovascularization. Similar processes of ischemia-induced neovascularization are observed in terminal stages of diabetic retinopathy. Clearly, the application of a factor like Norrin that specializes in the formation of regularly structured retinal capillaries appears to be a promising therapeutic tool to treat ischemic retinopathies, and other vascular disorders of the retina.

3.2. The neuroprotective function of Norrin

In addition to their pronounced vascular retinal phenotype, \(Ndp^{yl}\) mice show structural and functional changes of retinal neurons. The changes involve a progressive loss of retinal ganglion cells (RGC) (Richter et al., 1998) leading to a substantial disorganization of the ganglion cell layer (Berger et al., 1996). In the outer retina of \(Ndp^{yl}\) mice areas were observed with folds, with nuclei of the outer nuclear layer that are displaced into the outer plexiform layer, or with complete absence of photoreceptors (Berger et al., 1996; Ohlmann et al., 2005; Richter et al., 1998). In addition, ERG analysis of \(Ndp^{yl}\) mice detected significant alterations of rod a- and b-waves (Ruether et al., 1997). Not all of the structural changes of retinal neurons may be caused by lack of retinal capillaries and oxygen supply, as similar alterations in the outer nuclear layer were already detected in some \(Ndp^{yl}\) mice at P1-P4 (Richter et al., 1998), a time when retinal capillaries are also absent in wild-type mice. Degeneration of RGC was first observed at P14, and at P20 a 60% decrease in the number of RGC was detected when compared to wild-type littermates (Richter et al., 1998). Quite intriguingly, the loss of RGC in \(Ndp^{yl}\) mice occurs in the inner retina, which is in contact with numerous blood vessels, thus ruling out lack of oxygen as a likely cause of RGC degeneration. Also RGC damage because of a defect blood brain barrier and leaky capillaries in the inner retina is an unlikely scenario, as no RGC damage has been observed in Frizzled-4 mice that show similar structural changes in the blood vessels of the inner retina. In \(Ndp^{yl}\) mice with ectopic transgenic overexpression of Norrin, RGC degeneration is completely rescued (Ohlmann et al., 2005). Moreover, in Norrin-overexpressing transgenic mice, the number of RGC is substantially increased in both

![Fig. 5. Norrin prevents neovascularization following oxygen-induced retinopathy. Representative retinal whole mounts after perfusion with FITC-labeled dextran of J81-Crystallin-Norrin mice (B, D) and wild-type littermates (A, C) at P17 after induction of oxygen-induced retinopathy (magnification bars: 500 \(\mu\)m). The area of preretinal tufts (red in C and D) was quantified at P17. From (Ohlmann et al., 2010).](image-url)
To follow up on the hypothesis that Norrin may have a neuroprotective role for RGC that acts independently from its function in retinal angiogenesis, we applied excitotoxic N-methyl-D-aspartate (NMDA)-induced damage to the retina of mice (Seitz et al., 2010). In the mouse retina, both RGC and amacrine cells carry NMDA-receptors (Shen et al., 2006). In the optic nerves of eyes three weeks after intravitreal injection with a combination of Norrin and NMDA, approximately 80% more axons were observed as compared with NMDA-treated eyes (Fig. 6), an effect that involved a decrease in RGC apoptosis and was mediated via activation of Wnt/β-catenin signaling (Seitz et al., 2010). In addition, in eyes treated with combined Norrin/NMDA, an increased retinal expression of leukemia inhibitory factor (LIF), endothelin-2 (Edn2), and the neuroprotective factors fibroblast growth factor-2 (FGF2), brain-derived neurotrophic factor (BDNF), lens epithelium-derived growth factor (LEDGF) and ciliary neurotrophic factor (CNTF) was detected (Fig. 7). A similar activation of Wnt/β-catenin signaling and an increased expression of LIF and neuroprotective factors were observed in cultured Müller cells after treatment with Norrin, effects that again could be blocked by adding DKK-1 (Seitz et al., 2010).

An interesting aspect of these findings comes from studies in experimental photoreceptor degeneration, in which the secretion of neuroprotective factors such as FGF2 was shown to be induced via a crosstalk between Müller cells which express LIF, and photoreceptors which signal to Müller cells via Edn2 (Joly et al., 2008; Rattner and Nathans, 2005). In the inner retina, Norrin may similarly enhance the secretion of neuroprotective factors from Müller cells via an increased expression of Müller cell-derived LIF and of Edn2 (Seitz et al., 2010). At the moment it is not clear if Norrin-induced Edn2 derives from photoreceptors or from neurons in the inner retina, a scenario that might be distinctly possible, as an increase in Edn2 secretion has been found in the retina of mice with RGC damage in glaucoma (Howell et al., 2011). Edn2 may be a general stress signal of retinal neurons that signals to Müller cells to induce the secretion of neuroprotective factors such as FGF2, BDNF, and CNTF. The secretion may be substantially enhanced leading to protection of neuronal structure and function, if Edn2 signaling is amplified by LIF secretion and Norrin-induced activation of Wnt/β-catenin signaling in Müller cells (Fig. 8).

### 3.3. The role of Norrin in the female reproductive system

Female Ndp⁻/⁻ mice are infertile, which is consistent with Norrin’s expression in uterus, placenta and ovary (Hsieh et al., 2005; Luhmann et al., 2005b). Investigations in pregnant Ndp⁻/⁻ mice showed placental abnormalities with an increased bleeding tendency starting at embryonic day (E) 7. In addition, a smaller decidual expansion and a rarefaction of decidual vasculature were discussed as reasons for infertility (Luhmann et al., 2005b). Infertility also has been reported for Fzd4⁻/⁻ mice, which show an insufficiency of their ovaries (Hsieh et al., 2005). Currently, it is not clear if the interaction of Norrin with Fzd4-4 is required for proper function of the female reproductive organs, or if other ligands and receptors are involved.

### 4. Different phenotypes of Norrin⁻, Fzd4⁻⁻, Tspan12⁻⁻ and Lrp5⁻⁻ deficient mice

While Norrin, Fzd4⁻⁻, Tspan12⁻⁻ and Lrp5⁻⁻ are all essential parts of a common angiogenic program that controls capillary growth in the developing retina, each of these molecules individually contribute to additional biological processes throughout the organism that very likely involve other ligands or receptors. Accordingly, the phenotypes of Norrin⁻, Fzd4⁻⁻, Tspan12⁻⁻ and Lrp5⁻⁻-deficient mice are largely comparable with regards to the absence of intraretinal capillaries and the persistence of hyaloid vessels, but are considerably different in other aspects. In contrast to Norrin, Fzd4⁻⁻ is widely expressed in multiple cell types and tissues of the body (Wang et al., 1996) including neurons in inner and outer retina (Wang et al., 2001). Still, Fzd4⁻⁻ mice neither develop obvious neuronal defects in the retina, nor suffer from a continuous loss of RGC as do Ndp⁻/⁻ mice (Junge et al., 2009; Ye et al., 2009). Functional changes were observed in Fzd4⁻⁻ mice by ERG, which could be rescued, however, when retinas were incubated in oxygen-containing cell culture medium indicating functional rather than structural retinal defects (Ye et al., 2009). It is tempting to speculate that Norrin is required for maintaining the structure and function of RGC during lifetime, and that this function is not mediated via Fzd4⁻⁻. In support of this hypothesis are in vitro data using a retinal neuronal cell line (RGC-5), which suggest a direct neuroprotective effect of Norrin that is only partially mediated via an activation of canonical Wnt/β-catenin signaling (Lin et al., 2009; Seitz et al., 2010). A different scenario may take place after acute RGC damage, e.g. after excitotoxic injury as applied by Seitz and coworkers, where the neuroprotective effects of Norrin are attenuated following blocking of the canonical Wnt/β-catenin signaling pathway (Seitz et al., 2010). Fzd4⁻⁻ is expressed in neurons of the inner retina and experimentally added high amounts of Norrin may well bind to the receptors to initiate a Wnt/β-catenin-mediated neuroprotective signaling cascade. Norrin is expressed in the retina at relative high amounts throughout lifetime and it is tempting to speculate that it is part of a protective signaling system to protect retinal neurons after injury.

Fzd4⁻⁻ is strongly expressed in Purkinje cells of the cerebellum and in mutant Fzd4⁻⁻ mice a dramatic apoptotic death of cerebellar granule and Purkinje cells occurs which leads to gait abnormalities and progressive cerebellar ataxia (Wang et al., 2001). Apoptosis of cerebellar neurons is likely induced by pronounced alterations of the blood brain barrier of cerebellar capillaries which are leaky and frequently rupture to form hemorrhages (Ye et al., 2009). In contrast, neither neuronal changes nor obvious alterations of the blood brain barrier were observed in the cerebellum of Norrin-deficient mice that only show a somewhat reduced vascular density in the cerebellum (Luhmann et al., 2008; Ye et al., 2009). In contrast to the retina, other ligands than Norrin appear to bind to Fzd4⁻⁻ in order to promote formation of the blood brain barrier in the cerebellum and Wnt7a and Wnt7b are likely candidates as they promote vascular development in the brain and formation of the blood brain barrier (Daneman et al., 2009; Stenman et al., 2008), and bind to Fzd4⁻⁻ (Lobov et al., 2005).

In addition to defects in retina, inner ear and cerebellum Frzd4⁻⁻ deficient mice show defects in esophageal peristalsis and gastric sphincter function that lead to esophageal dysfunction. The defect is related to the fact that a variable length of the lower esophagus, ranging from the lower one-fourth to nearly the entire length of the esophagus, is devoid of the normal sheath of skeletal muscle (Wang et al., 2001). Other phenotypic changes of Fzd4⁻⁻ deficient animals are seen in the ovaries in which the corpora lutea do not develop normally, a defect that results in infertility (Hsieh et al., 2005). In addition, Fzd4⁻⁻ mice suffer from changes in coat color, presumably caused by a reduced migration or expansion of melanocytes during neural crest migration (Wang et al., 2001). Lrp5⁻⁻ mice do not express obvious phenotypic changes of retinal neurons (Xia et al., 2008), while in Tspan12⁻⁻ mice the thickness of the outer nuclear layer in the retina is consistently reduced in adult animals (Junge et al., 2009). So far, no other...
Fig. 6. Norrin protects against NMDA-mediated loss of retinal ganglion cells. A–D. Representative sagittal semi-thin sections through optic nerves (A–D) of eyes three weeks after intravitreal injection of 3 μl NMDA [10 mM] (A, B), or NMDA [10 mM] plus Norrin [5 ng/μl] (C, D) in the fellow eye. Three weeks after treatment with NMDA alone, a substantial loss of axons and the formation of an extensive glial scarring (asterisks) is observed in the optic nerve of the NMDA-treated eye (A, B). In contrast, in the optic nerve of the eye injected with NMDA in combination with Norrin (C, D), the loss of axons and the area of glial scarring is considerably smaller. Several axons exhibit a more intense staining of their myelin sheath indicating axonal degeneration (arrows). E, F. Transmission electron microscopy of remaining axons of both NMDA, and NMDA plus Norrin treated animals show structurally intact morphology with mitochondria (arrows), microtubules and neurofilaments (Fig. 1E). Several axons which stain as intense dark spots by light microscopy (arrows, Fig. 1B, D) are degenerated and consist of myelin whorls (arrow, Fig. 1F). G. The total number of optic nerve axons was quantified and plotted as the relative number of optic nerve axons (mean ± SEM). Magnification bars: A, C. 50 μm; B, D. 10 μm; E. 1 μm; F. 1.5 μm. From (Seitz et al., 2010).
Tspan12/C0 mice. Outside the eye, Lrp5/C0/C0 mice show bone defects associated with a reduced bone mass and osteopenia caused by a reduced osteoblast proliferation and bone matrix deposition (Kato et al., 2002). Moreover, delayed mammary gland development associated with reduced mammary ductal stem cell activity was reported (Lindvall et al., 2006) as were increased plasma cholesterol levels along with a markedly impaired glucose tolerance (Fujino et al., 2003).

5. Histopathology and clinical signs of Norrie disease

Mutations in NDP are causative for Norrie disease, which was first described by Gordon Norrie as hereditary bilateral Atrophia oculi congenita (Norrie, 1927) characterized by congenital amaurosis or blindness in early childhood. About 40 years later, Mette Warburg reported in detail on Danish patients with Norrie disease suffering from bilateral hereditary congenital amaurosis, and described X-linked inheritance as well as mental retardation and sensorineural deafness as extraocular manifestations in about one-third of cases (Warburg, 1961, 1963, 1966). Even though Norrie disease is a very rare disorder, its exact incidence is unknown, several histopathological and clinical reports about structural changes in eye, inner ear and brain are available. In the anterior segment of enucleated eyes, various structural defects were described, such as a missing corneal endothelium, corneal opacities, iris atrophy, neovascularization of the iris, anterior and posterior synechia, goniodygenesis, and cataract (Nadol et al., 1990; Phillips et al., 1986; Rehm et al., 1997; Warburg, 1966). In addition, an epithelial to mesenchymal transition (EMT) of the RPE, and an atrophic or hyperplastic choroid have been reported (Nadol et al., 1990; Rehm et al., 1997; Warburg, 1966). In the posterior eye, the structural changes strongly depend on the duration of Norrie disease. In amaurotic enucleated eyes, severe pathological changes were observed in the vitreous body, sensory retina and retinal pigment epithelium (RPE), such as a persistent hyperplastic primary vitreous (PHPV), fibrous masses, retinal membranes, retinal gliosis, or complete lack of the retina (Nadol et al., 1990; Phillips et al., 1986; Rehm et al., 1997; Warburg, 1966). In the posterior eye, the structural changes strongly depend on the duration of Norrie disease. In amaurotic enucleated eyes, severe pathological changes were observed in the vitreous body, sensory retina and retinal pigment epithelium (RPE), such as a persistent hyperplastic primary vitreous (PHPV), fibrous masses, retinal membranes, retinal gliosis, or complete lack of the retina (Nadol et al., 1990; Phillips et al., 1986; Rehm et al., 1997; Warburg, 1966). In the posterior eye, the structural changes strongly depend on the duration of Norrie disease. In amaurotic enucleated eyes, severe pathological changes were observed in the vitreous body, sensory retina and retinal pigment epithelium (RPE), such as a persistent hyperplastic primary vitreous (PHPV), fibrous masses, retinal membranes, retinal gliosis, or complete lack of the retina (Nadol et al., 1990; Phillips et al., 1986; Rehm et al., 1997; Warburg, 1966). In the posterior eye, the structural changes strongly depend on the duration of Norrie disease. In amaurotic enucleated eyes, severe pathological changes were observed in the vitreous body, sensory retina and retinal pigment epithelium (RPE), such as a persistent hyperplastic primary vitreous (PHPV), fibrous masses, retinal membranes, retinal gliosis, or complete lack of the retina (Nadol et al., 1990; Phillips et al., 1986; Rehm et al., 1997; Warburg, 1966). In the posterior eye, the structural changes strongly depend on the duration of Norrie disease. In amaurotic enucleated eyes, severe pathological changes were observed in the vitreous body, sensory retina and retinal pigment epithelium (RPE), such as a persistent hyperplastic primary vitreous (PHPV), fibrous masses, retinal membranes, retinal gliosis, or complete lack of the retina (Nadol et al., 1990; Phillips et al., 1986; Rehm et al., 1997; Warburg, 1966). In the posterior eye, the structural changes strongly depend on the duration of Norrie disease. In amaurotic enucleated eyes, severe pathological changes were observed in the vitreous body, sensory retina and retinal pigment epithelium (RPE), such as a persistent hyperplastic primary vitreous (PHPV), fibrous masses, retinal membranes, retinal gliosis, or complete lack of the retina (Nadol et al., 1990; Phillips et al., 1986; Rehm et al., 1997; Warburg, 1966). In the posterior eye, the structural changes strongly depend on the duration of Norrie disease. In amaurotic enucleated eyes, severe pathological changes were observed in the vitreous body, sensory retina and retinal pigment epithelium (RPE), such as a persistent hyperplastic primary vitreous (PHPV), fibrous masses, retinal membranes, retinal gliosis, or complete lack of the retina (Nadol et al., 1990; Phillips et al., 1986; Rehm et al., 1997; Warburg, 1966).
It should be noted, however, that all of these changes in the anterior or posterior eye were observed in very advanced Norrie disease and may well be secondary changes rather than part of the primary pathogenetic mechanism. In support of this assumption, the histopathological changes in eyes of neonates that suffered from Norrie disease for a short period of time and the associated clinical findings are less severe. Accordingly, an anterior-posterior stalk that attaches to the retina, unbranched vessels in a dysplastic retina, and avascular retinal areas with underlying alterations of the RPE were observed (Drenser et al., 2007). No structural changes were detected in the eyes of a fetus at 11 weeks (Parsons et al., 1992), which is consistent with the fact that the vascularization of the retina forms substantially later in fetal development (Provis, 2001). Interestingly, the few histopathological studies that are available from enucleated eyes of infants with Norrie’s disease show marked similarities with the phenotype of Ndp<sup>−/−</sup> mice including evidence for RGC damage. In the eye of a 9-month-old infant evidence for a persistent hyaloid vascular system was seen and a very marked reduction in the number of optic nerve axons (Phillips et al., 1986). In tissue from a six-year-old boy, which contained central as well as peripheral parts of the retina, a severe reduction in the number of RGC was visible. Except for a loss of photoreceptor outer segments in some areas, outer nuclear and photoreceptor layers displayed only minor morphological changes, and only a few retinal blood vessels, mostly capillaries, were visible (Schoeder et al., 1997). In summary, the histopathological data that are available from human samples are consistent with an impaired retinal angiogenesis, persistent hyaloid vasculature, and degeneration of RGC. Quite intriguingly, the major structural changes in human eyes with early Norrie disease markedly resemble the major phenotypic changes in the eyes of Ndp<sup>−/−</sup> mice. Mutations in NDP may also cause X-linked familial exudative vitreoretinopathy (FEVR), a vitreoretinal dystrophy characterized by premature arrest of vascularization of the peripheral retina (Chen et al., 1993). FEVR may also be inherited as an autosomal-dominant trait caused by mutations in Frizzled-4 (Robitaille et al., 2002) or Lrp5 (Toomes et al., 2004). Comparable to observations in Frizzled-4-deficient mice, the available histopathological data obtained from human patients with FEVR indicate an absence of intraretinal capillaries, but no obvious changes in RGC (Benson, 1995).

In addition to ocular changes, one-third of patients with Norrie disease suffer from mental retardation and sensorineural deafness (Warburg, 1966), and individual cases with additional structural changes, such as microcephaly, hypogonadism, growth retardation, immunodeficiency, epileptic seizures and peripheral venous insufficiency, have been reported (de la Chapelle et al., 1985; Donnai et al., 1988; Gal et al., 1986; Rehm et al., 1997). The structural changes in the inner ear of patients with Norrie disease included loss of inner and outer hair cells in the organ of Corti, as well as degenerative changes in the stria vascularis and spiral ganglion, whereas the vestibular organ was unaffected (Nadol et al., 1990). Histopathological abnormalities in cerebellum or cerebrum appear not to be a consistent finding in humans (Nadol et al., 1990; Phillips et al., 1986; Warburg, 1966).

6. Mutations in NDP

To date, more than 95 mutations in NDP have been reported that are mainly associated with Norrie disease; the molecular characteristics of the mutations have been reviewed elsewhere (Nikopoulos et al., 2010). While most of the mutations are causative for Norrie disease, some have been found to be associated with X-linked familial exudative vitreoretinopathy (FEVR), retinopathy of prematurity (ROP), persistent hyperplastic primary vitreous (PHPV) and M. coats (Nikopoulos et al., 2010).

Functional analyses of several mutations indicated an alteration in the Norrin-mediated activity of the Wnt/β-catenin signaling pathway. Accordingly, most mutations lead to a decrease in Wnt/β-catenin signaling, whereas only one mutation was found that appeared to enhance Norrin’s activity (Qin et al., 2008; Xu et al., 2004). Since a decreased secretion of mutated Norrin was excluded, the data strongly suggest that mutations in NDP cause a decreased capability of Norrin to bind to Frizzled-4 receptors, leading to reduced activation of the canonical Wnt/β-catenin signaling pathway.

7. Future directions

The available data indicate a promising therapeutic potential for Norrin. Accordingly, Norrin could be used to treat patients suffering from Norrie disease, a scenario which will require continuous release of Norrin in the eyes of patients, and is only expected to work in very young patients without pronounced secondary changes in the affected eyes. The retinal expression of Norrin could be induced via viral-mediated gene transfer in the retina of patients (Alexander and Hauswirth, 2008), or by applying devices that continuously release Norrin in the eye. Comparable devices have been tested with success for the release of other growth factors in the eye (Sieving et al., 2006; Talcott et al., 2011; Zhang et al., 2011). Along these lines, it would be worthwhile to test if Norrin or components of its signaling pathway could be used to develop treatment strategies for patients with retinopathy of prematurity or other ocular diseases that involve ischemia-induced neo-vascularization. Finally, the signaling network that is involved in the neuroprotective properties needs to be analyzed in more detail, as it appears to offer approaches to protect RGC from apoptotic cell death, such as seen in glaucoma. In addition, it would be worthwhile to explore, if Norrin exerts neuroprotective effects also on other neurons within and outside of the eye.

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