Differential gene expression in Ndph knockout mice in retinal development

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Abstract

PURPOSE. Mutations in the NDP gene impair angiogenesis in the eyes of patients diagnosed with a type of blindness belonging to the group of exudative vitreoretinopathies. With this study, we aimed to investigate differential gene expression caused by the absence of Norrin (the NDP protein) in the developing mouse retina to elucidate early pathogenic events. METHODS. A comparative gene expression analysis was performed on p7 (postnatal day 7) retinae from a knockout mouse model for Norrie disease using Affymetrix microarrays. Subsequently, results were verified by quantitative real-time PCR analyses. Immunohistochemistry was performed for the vascular permeability marker Plasmalemma vesicle-associated protein (Plvap). RESULTS. Our study identified expression differences in Ndph(y/-) vs. wild type mice retinae at p7. Gene transcription of the neutral amino acid transporter Slc38a5, apolipoprotein D (ApoD) and angiotensin II receptor-like 1 (Agtrl1) was decreased in the knockout, whereas transcript levels of adrenomedullin (Adm) and of the plasmalemma vesicle associated protein (Plvap) were increased in comparison to the wild type. In addition, we found ectopic expression of Plvap in the developing retinal vasculature of Norrin knockout mice on the protein level. CONCLUSIONS. These data provide molecular evidence for a role of Norrin in the development of the retinal vasculature. Expression of two genes, Plvap and Slc38a5, is considerably altered in retinal development of Norrin knockout mice and may reflect or contribute to the pathogenesis of the disease. In particular, ectopic expression of Plvap is consistent with hallmark disease symptoms in mouse and man.
Title: Differential gene expression in *Ndph* knockout mice in retinal development

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Differential gene expression in \textit{Ndph} knockout mice in retinal development

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\textbf{Purpose.} Mutations in the \textit{NDP} gene impair angiogenesis in the eyes of patients diagnosed with a type of blindness belonging to the group of exudative vitreoretinopathies. With this study, we aimed to investigate differential gene expression caused by the absence of Norrin (the \textit{NDP} protein) in the developing mouse retina to elucidate early pathogenic events.

\textbf{Methods.} A comparative gene expression analysis was performed on p7 (postnatal day 7) retinae from a knockout mouse model for Norrie disease using Affymetrix microarrays. Subsequently, results were verified by quantitative real-time PCR analyses. Immunohistochemistry was performed for the vascular permeability marker Plasmalemma vesicle-associated protein (Plvap).

\textbf{Results.} Our study identified expression differences in \textit{Ndph}\textsuperscript{\textminus/} vs. wild type mice retinae at p7. Gene transcription of the neutral amino acid transporter \textit{Slc38a5}, apolipoprotein D (\textit{ApoD}) and angiotensin II receptor-like 1 (\textit{Agtrl1}) was decreased in the knockout, whereas transcript levels of adrenomedullin (\textit{Adm}) and of the plasmalemma vesicle associated protein (\textit{Plvap}) were increased in comparison to the wild type. In addition, we found ectopic expression of Plvap in the developing retinal vasculature of Norrin knockout mice on the protein level.

\textbf{Conclusions.} These data provide molecular evidence for a role of Norrin in the development of the retinal vasculature. Expression of two genes, \textit{Plvap} and \textit{Slc38a5}, is
considerably altered in retinal development of Norrin knockout mice and may reflect or contribute to the pathogenesis of the disease. In particular, ectopic expression of Plvap is consistent with hallmark disease symptoms in mouse and man.

**Key words: Vitreoretinopathy • Angiogenesis • Gene expression • Norrie Disease • Ndph • Plvap**

### INTRODUCTION

Norrie Disease (OMIM 310600; ND) is an X-linked, recessive neurological disorder that presents with congenital blindness, progressive deafness and mental retardation\(^1\), and is caused by mutations in the Norrie disease pseudoglioma (NDP) gene\(^2,3,4\). The high phenotypic variability even within one family carrying the same mutation\(^5,6,7\) suggests the involvement of modifier genes or other factors, which might explain why no clear genotype-phenotype correlation has been described so far. In addition, mutations in this gene have also been associated with a variety of other recessive and sporadic vitreoretinal diseases, including exudative vitreoretinopathy (OMIM 133780; EVR), retinopathy of prematurity (ROP, stages 4b and 5) and Coats’ disease (OMIM 300216)\(^8,9,10,11\). These allelic clinical entities show remarkable similarities in their ocular phenotype especially with regard to abnormalities in the retinal vasculature.

The human disease phenotype is strongly resembled by the Norrie disease pseudoglioma homolog (Ndph) knockout mouse\(^12,13\). Although the retinal vasculature in humans develops around mid-gestation, and only postnatally in mice, the timing of events is comparable. In mice, retinal blood vessels start to develop around birth at the optic disc and spread radially inside the nerve fiber layer across the retina, until they reach the periphery around postnatal day 9 (p9). In addition, vessels start to sprout into the deeper layers of the retina at p7 to form two additional networks in the plexiform layers, parallel to the superficial plexus. Contrary to
the development of the retinal vasculature, the hyaloid vessel system, a transient developmental vasculature nourishing the developing lens, regresses. This process starts after its peak extension around p5 and lasts until p15, around which time the mice open their eyes and eye development is more or less complete\textsuperscript{14}. In the \textit{Ndph} knockout mouse, the inner retinal vessel development is severely impaired. The outgrowth of the superficial retinal vessel plexus is delayed and remains sparse, deep retinal vessels do not develop, and the regression of the hyaloid vasculature is delayed and incomplete. Furthermore, disorganization of the retinal ganglion cell layer and a reduction of retinal ganglion cells have been described\textsuperscript{12,15,13}. However, proliferation of fibrovascular material in the vitreous cavity is not as massive as the pseudoglioma described in patients suffering from severe ND.

Different hypotheses have been postulated about the etiology of these symptoms. It has been discussed that the extended presence of hyaloid vessels might impair the development of the retinal vasculature\textsuperscript{16,17}. However, blockage of the placental growth factor (PIGF) during early postnatal stages in the eye leads to delayed regression of the hyaloid vessel system, but not to changes in the retinal vasculature\textsuperscript{18}, indicating that persistence of the hyaloidea does not necessarily lead to defects in or lack of retinal vascularization. Another hypothesis was provided by a detailed analysis of the early development of the retinal vasculature in \textit{Ndph} knockout mice, which suggested two phases of disease progression \textit{in vivo}\textsuperscript{15}: In the early phase, the absence of functional Norrin (\textit{Ndph}\textendash protein) causes a defect in sprouting angiogenesis, which leads to a delayed outgrowth of the superficial vessels and prevents the formation of deep capillary networks in the retina. Then, in the later phase, the developmental lack of the deep vasculature leads to inner retinal hypoxia. This pathologic hypoxia may explain the observed clinical features of Norrie disease and might also be responsible for the similarities of the clinical phenotypes of the aforementioned diseases\textsuperscript{15}. In another study, the knockout phenotype could be rescued by ectopic overexpression of Norrin in the lens\textsuperscript{19}. Their authors suggested a direct effect of Norrin on vascular proliferation, since proliferation of
microvascular endothelial cells was increased after co-cultivation with the Norrin-expressing lenses\textsuperscript{19}.

Further, autosomal dominant and recessive forms of FEVR (familial exudative vitreoretinopathy), which are caused by mutations in Frizzled-4 (\textit{FZD4}) and LDL related protein 5 (\textit{LRP5}) in approximately 35\% of the patients\textsuperscript{20,21}, resemble the clinical pictures of the X-linked diseases\textsuperscript{12,13,22}. Prompted by these findings and the phenotypic similarities between \textit{Ndph} and \textit{Fzd4} knockout mice, Xu et al. could provide the first hint for a possible cellular function of Norrin. They showed that Norrin is a high affinity ligand of Frizzled-4 and activates the canonical Wnt/beta-catenin pathway in the presence of LRP5 in cell culture\textsuperscript{23}. This pathway eventually leads to transcriptional regulation of target genes under the control of TCF/Lef-binding sites. In summary, these data suggest that Norrin has an influence on transcriptional regulation of Wnt-target genes, is required for early angiogenic sprouting in the retina and the regression of the hyaloid vessel system, and is necessary for the formation of deep retinal capillary networks around postnatal day 7 (p7)\textsuperscript{15}.

The main purpose of the experiment reported here was to find differently expressed genes in retinae of p7 \textit{Ndph} knockout mice that are involved in the process of deep plexus formation and that could confirm Norrin’s hypothesized role on Wnt target genes \textit{in vivo}. Our findings support a role of Norrin in blood vessel development and provide evidence for a potential regulation of new target genes that were not considered so far. However, involvement of the Wnt/beta-catenin pathway could not be demonstrated by our experiments. One gene, the plasmalemma vesicle associated protein (\textit{Plvap}), has been characterized in more detail. This gene was described as a major structural component of fenestrated blood vessels\textsuperscript{24}, and here was found to be up-regulated in the retinal vasculature in the absence of Norrin. Thus, we discuss the question whether or not Norrin could also be involved in blood vessel integrity and the formation or maintenance of the blood-retina-/blood-brain-barrier.
MATERIALS AND METHODS

Animals

The *Ndph* knockout mouse line has been described before by Berger et al.\textsuperscript{12}. Briefly, exon 2 of the *Ndph* gene has partially been replaced by homologous recombination with a reverse oriented neomycin cassette, resulting in a loss of about half the coding sequence, including the signal peptide for protein secretion. The mutation is kept on a C57BL/6 background. Genotyping was performed by PCR analysis of tail DNA\textsuperscript{12}. The research was performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the Veterinary Service of the State of Zurich (Switzerland).

Tissue isolation and RNA preparation

Wild type and *Ndph*\textsuperscript{y/-} mice of different developmental stages (p5, p7, p10, p15, p21) were sacrificed by cervical dislocation. Five animals of each genotype were prepared and the retinas were frozen in liquid nitrogen. Total RNA was extracted using the RNeasy\textsuperscript{®}-Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions. DNase digestion was applied either directly on-column (p7 samples, RNase-free DNase Set, cat. no. 79254, Qiagen, Hilden, Germany), or separately after RNA extraction (RQ1 RNase-free DNase, p/n M610A, Promega, Madison, WI, USA). Both retinas of one animal were pooled into one tube of lysis buffer and homogenized with an Ultra-Turrax\textsuperscript{®} high-performance disperser (Ika-Werke, Staufen, Germany). RNA quality was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Delaware, USA) and a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). RNA Integrity number of all samples was greater or equal to 9.3.
Microarray experiment

Gene chip expression analysis has been carried out with RNA from p7 retinae (wt: n=5, ko: n=5), using the Affymetrix GeneChip® Mouse Genome 430 2.0 array (Affymetrix Inc., Santa Clara, CA, USA). On this chip, over 34,000 genes and ESTs are represented by ~45,000 probe sets. The Microarray experiment, from cDNA preparation to raw data processing, was performed at the Functional Genomics Center Zurich. Briefly, total RNA samples (2µg each) were reverse-transcribed without additional amplification (one-cycle protocol), purified, labeled and hybridized to the chip according to the manufacturer’s instructions. An Affymetrix GeneChip Scanner 3000 was used to measure the fluorescence intensity emitted by the labeled target.

Statistical analysis Raw data processing was performed using the Affymetrix GCOS 1.2 software (Affymetrix Inc., Santa Clara, CA, USA). After hybridization and scanning, probe cell intensities were calculated and summarized for the respective probe sets by means of the MAS5 algorithm25. To compare the expression values of the genes from chip to chip, global scaling was performed, which resulted in the normalization of the trimmed mean of each chip to a target intensity (TGT value) of 500 as detailed in the statistical algorithms description document of Affymetrix (2002). Quality control measures were considered before performing the statistical analysis. Differently expressed genes were calculated with the GeneSpring 7.2 software (Agilent Technologies, Santa Clara, CA, USA), filtered for presence (per Affymetrix presence/absence flags) in four of five samples in either one condition (wt or ko) and then subjected to a non-parametric test for differential expression (Mann-Whitney test without multiple testing correction (p<0.01), or with Benjamini-Hochberg multiple testing correction (p<0.1)).

Pathway analysis Pathway analysis was conducted with the online tool “Pathway express”, which is part of the “Onto-Tools”, hosted by the Wayne State University, Detroit, USA, using the default options26.
Gene ontology annotations

Gene ontology (GO) annotations are used to describe e.g. biological process or cellular component of a given gene product. Annotations for the top differently expressed genes (Table 3) were obtained from the Database for Annotation, Visualization and Integrated Discovery (DAVID) from the National Institute of Health.

Quantitative reverse transcription-PCR

Quantitative RT-PCR was performed not only to investigate differential expression during development, but also to verify the microarray results. For p7, the same samples as in the array experiment were used. Generation of cDNA was accomplished as follows: 1250ng total RNA were reverse transcribed in a 20µl reaction, using random hexamers pd(n)6 (Amersham Biosciences / GE Healthcare Europe, Otelfingen, Switzerland) and SuperScript III RT (Invitrogen, Carlsbad, CA, USA). For expression analysis of Slc38a5 and Mdm2, quantitative RT-PCR was conducted using 0.5µM of a forward/reverse primer pair (binding in the area that is recognized by the Affymetrix probes; Table 1) and SYBR®-Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA). TaqMan® probes and TaqMan® Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA) were used for quantification of Aass, Centd3, ApoD, Cldn5, Agtrl1, Adm and Plvap expression (Table 1). In each reaction, 10ng of transcribed total RNA were employed (except: Slc38a5 / 100ng).

Quantitative RT-PCR was carried out in five replicates in an Applied Biosystems PRISM® 7900HT system using the recommended standard cycling conditions: 45 cycles of denaturation (15s/95°C) and annealing/extension (1min/60°C). Gapdh and 18S (TaqMan®) or 28S (SYBR®-Green) rRNA were used as endogenous controls. Confidence intervals for wild type and knockout gene expression were calculated on basis of the mean ∆∆ ct-values at a significance level of 95% using Student’s t-test.
Table 1 Primers for SYBR®-Green assays and part no. of TaqMan® assays used in this study (Applied Biosystems, Foster City, CA, USA).

**Immunohistochemical staining and histology**

Cryosections were prepared from male *wt* and *Ndph*<sup>−/−</sup> mice aged p3, p5, p7, p10, p15 and p21. Eyes were dissected, fixed for 1h in PBS / 4% para-formaldehyde, washed in PBS and cryo-protected in Sucrose (30min in 10%, then o/n in 20%) at 4°C. They were embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek, Zoeterwoude, Netherlands), submersed in liquid nitrogen, cut into 8µm sections with a Leica Cryostat CM 3050S (Leica, Heerbrugg, Switzerland) and transferred onto microscope glass slides. After drying the sections for at least 3h, unspecific binding sites were blocked by incubation with 5% normal sheep serum (Sigma Aldrich, Steinheim, Germany) in PBST for 1h. Slides were incubated with primary antibodies in blocking solution (o/n, 4°C) in a wet chamber. Antiserum concentrations were 1µg/ml for anti-Plvap/MECA-32 (rat anti-mouse, Developmental Studies Hybridoma Bank) and 7µg/ml for anti-Collagen IV (polyclonal Col IV antibody, rabbit anti-mouse, no. 2150-1470, AbD Serotec/MorphoSys, Oxford, UK). Sections were washed and incubated with secondary antibodies in PBS (90min, RT). Cy3<sup>™</sup>-conjugated anti-rabbit IgG serum (Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK) was used at a concentration of 5µg/ml, Alexa Fluor® 488-labelled anti-rat IgG/M (no. A11006, Molecular Probes, Leiden,
Netherlands) was used at 2µg/ml. Slides were washed a last time and finally mounted in a DAPI-containing medium (Vectashield® Mounting Medium for Fluorescence with DAPI H-1200, Vector Laboratories, Burlingame, CA, USA). Images were taken with an Axioplan 2 microscope equipped with an AxioCam HRc digital camera (Carl Zeiss MicroImaging, Jena, Germany). Contrast in Plvap/AF 488 images was enhanced after acquisition with a photo-editing program.

For retinal flatmounts, eyes were dissected and fixed for 15min in PBS/4% paraformaldehyde. Retinae were removed and post-fixed for additional 15min. After washing in PBS, unspecific binding was blocked by 10% normal sheep serum in PBST for 1h. Samples were then incubated o/n with the Collagen IV antibody. They were washed six times for 1h in PBST and incubated o/n with the secondary Cy3-labeled antibody. After repetition of the washing procedure, the retinae were finally flatmounted on microscope slides.

Sections (8µm) of paraffin-embedded eyes were hematoxylin-eosin stained as described elsewhere15 and observed under bright-light illumination.

**RESULTS**

In this study, we used the *Ndph* knockout mouse to investigate molecular pathways involved in early angiogenic sprouting in the retina and the formation of deep retinal capillary networks at around postnatal day 7 (p7)15. We hypothesized that Norrin is required for these processes, probably by transcriptional regulation of Wnt/beta-catenin target genes and/or other pathways.

**Retinal morphology at p7**

At p7, conventional microscopy of HE stained retinal sections did not reveal apparent differences in the retinal morphology of wild type and *Ndph* knockout mice (Fig.1A, B). In contrast, ColIV immunohistochemistry revealed that the retinal vasculature of the *Ndph*...
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knockout was strikingly altered, showing an intermediate phenotype to what we earlier observed at p5 and p10\textsuperscript{15}. The superficial vessel system appeared much sparser than in the wild type, its outgrowth was delayed, and missing vascular sprouts indicated a lacking development of deep retinal vessels (Fig. 1C-E). Since we argue that the vascular phenotype is the earliest and most obvious retinal defect caused by the absence of Ndph, we used this developmental stage to investigate the initial pathologic events on the molecular level, i.e. to identify genes involved in the initiation of the deep retinal capillary network by sprouting angiogenesis, which may be regulated by Norrin directly or indirectly.

**Microarray gene expression analysis**

To identify differently expressed genes in the retina of Ndph knockout mice, we took a global gene expression approach using microarrays. The gene chip experiment resulted in 26'817 expressed probe sets in p7 retinae. Differences in the overall expression levels between Ndphp\textsuperscript{-/-} and wild type retinae were rather moderate, both in absolute number and fold-change. In microarray studies, usually an arbitrary fold-change cut-off of 2.0 has been applied\textsuperscript{28}, still resulting in dozens or hundreds of differently expressed genes. Yet, expression changes of highly expressed genes may still be biologically relevant despite a fold-change lower than 2.0, while weakly expressed transcripts may show a fold-change higher than 2.0 due to their greater inherent measurement error\textsuperscript{28}. In this study, we reasoned that a cut-off of 1.6 might still be valid considering our use of five biological replicates (instead of the standard three) while giving us a decent list of differently expressed genes.

The list of expressed probe sets was subjected to a non-parametric Mann-Whitney test with multiple testing correction (Benjamini-Hochberg, \(p<0.1\)). This resulted in a list of 450 probe sets (with \(10\% = 45\) false positives), thirty-four of which showed a fold-change of at least 1.6. Each of the thirty-four represents a different gene (Table 2), including five currently unknown
cDNAs. Sixteen transcripts show an elevated expression in the knockout, while levels of eighteen genes are decreased.

Since only one of these 34 genes (Apcdd1) has been associated with the Wnt-signaling pathway so far, we attempted to find evidence for its involvement with a pathway analysis. Because the list of 34 genes was too small for this purpose, we subjected the list of expressed probe sets to a non-parametric Mann-Whitney test (p<0.01), but this time without multiple testing correction and fold-change cut-off. This resulted in a list of 872 probe sets (730 genes or ESTs) to be considered differently expressed between wild type and knockout. We then performed a pathway analysis based on these 730 genes by utilizing the online tool “Pathway express”\textsuperscript{26}. Also here, an involvement of Wnt-signaling was not striking. The five most prominent affected pathways were: the MAP-Kinase pathway, focal adhesion, calcium-signaling, tight junction, and only then, Wnt-signaling (Table 3). Due to the low statistical power of this analysis, these results must be treated with caution. While they might provide clues for future research activities, they have to be verified first by independent experiments.

In the study presented here, we decided to concentrate on the list of the 34 most differently expressed genes (Table 2).

As expected for a knockout, the Ndph gene appears in the list of lower expressed genes. However, its expression is only decreased by the factor of two. One possible explanation could be the general low retinal Ndph expression in the wild type, so the indicated fold-change could be the result of a measurement error. Second, the knockout is not a complete null-allele, but rather lacks most of exon 2, resulting in the loss of about half its coding region. Since the probes of the Affymetrix Mouse Genome 430 2.0 array predominantly target the 3’-region and a signal was detected in the knockout, it is possible that a transcript including this region is present in the retina of Ndph\textsuperscript{−/−} mice. In fact, a ko-allele derived, low expressed artificial transcript has been isolated from the brain of Ndph\textsuperscript{−/−} mice. Partial sequencing of this transcript revealed that no functional Norrin can be translated from this
RNA, as almost half of the open reading frame, including the signal peptide, is missing and no start codon is present in the relevant transcript area (data not shown\textsuperscript{29}).

**Verification of differential expression by qRT-PCR**

To validate the microarray results, qRT-PCR was performed for three of the most upregulated and six of the most downregulated transcripts using the same p7 retina RNA samples that were used for the array experiment. Relative quantification values were determined with 18S or 28S rRNA and Gapdh (not shown) as endogenous controls. Expression values in general seemed to correlate with the microarray results (Fig. 2). Five genes were proven to be significantly differently expressed. Three genes showed a decreased expression in the knockout: The neutral amino acid transporter *Slc38a5* (24x less), apolipoprotein D (*ApoD*; 2.4x less) and angiotensin II receptor-like 1 (*Agtrll*; 2x less). Increased expression was found for the plasmalemma vesicle-associated protein (*Plvap*; 4.2x higher) and adrenomedullin (*Adm*; 2.7x higher).

Despite the low fold-change threshold of 1.6 for our microarray statistical analysis, we were able to confirm differential expression of more than half the genes examined.

**Expression during development**

We further investigated expression for the two most differently expressed verified genes, *Plvap* and *Slc38a5*, during postnatal retinal development. In addition to p7, qRT-PCR was conducted on retinal cDNA from p5, p10, p15 and p21 (Fig. 3).

*Plvap* expression in the knockout was significantly increased at every developmental stage in comparison to the wild type. The difference increased about one order of magnitude in later stages (p15/p21) when compared to the earlier stages (p5-p10) (Fig. 3A).

*Slc38a5* expression was significantly decreased at every developmental stage in the *Ndph* knockout (Fig. 3B). Fold-change was about ten times lower at p5 and p15, about twenty-five
times lower at p7 and p10 and about three times lower at p21. Since the Slc38a5 locus is located within 8.6 Mb from the Ndph locus, we investigated whether the observed difference was caused by a different genetic background, because the knockout Ndph allele is derived from the 129P2 mouse strain, which has then been backcrossed to the C57BL/6 background. We could not exclude a background effect by analysis of a genetic marker inside the Slc38a5 locus (rs13483703), since it indicated the 129P2 allele at this position. Thus, we studied the expression of Slc38a5 by additional qRT-PCR on p7 retinae of 129P2 wild type males and found similar levels as in the C57BL/6 wild type group (data not shown). Consequently, we excluded an influence of the genetic background on the expressivity of Slc38a5.

In summary, these developmental studies, which were performed on array-experiment independent RNA samples, added additional proof to the obtained microarray gene expression data.

**Immunohistological staining of Plvap**

As gene expression differences do not always reflect consequences at the protein level, immunohistochemistry was also performed for Plvap at all developmental stages that have been investigated on the transcript level (plus p3 in addition). Eye cryosections of Ndph<sup>yic</sup> and wild type mice were co-immunolabeled with antibodies directed against Plvap, a high specific marker for endothelial cells (MECA-32)<sup>30</sup>, and collagen type IV (ColIV) to visualize the vascular beds<sup>23</sup> (Figure 4). In all stages investigated (p3-p21), ColIV staining has been observed in the choroid and the superficial retinal vasculature of both genotypes. Vessels of the inner retina, located in the outer and inner plexiform layers, were detected in later stages in the wild type, but not the knockout retina, as has been described before<sup>13,15</sup>. Plvap was localized similarly to the choroidal vessel system in both wild type and knockout animals at all investigated developmental stages. However, the Plvap staining pattern of the retinal vasculature differed considerably. In the wild type, labeling was very faint at most.
Traces were observed in the superficial network at p3, but not in later stages, and the deep vessel system appeared to be slightly stained only at p10 (Figure 4, arrows). In contrast, Plvap staining in the knockout was detected as early as p3, but even more obvious and with increasing intensity at all later stages.

Taken together, these data show that Plvap expression in Ndph-/- retinae is increased not only on transcript, but also on protein level, which in particular seems to be confined to the retinal vasculature.

**DISCUSSION**

**Norrin and the role of Wnt-signaling**

When Norrin has been shown to bind to the Wnt-receptors Frizzled-4 and LRP-523, it was suggested that signaling occurs through the canonical Wnt/beta-catenin pathway. Surprisingly, our study suggested only one gene among the most differently transcribed genes besides Ndph to be associated with this pathway: Adenomatosis polyposis coli down-regulated 1 (Apcdd1). This does not necessarily mean that Norrin is not exerting its function over binding to Wnt-receptors – but it may imply that Norrin does not work primarily as a transcriptional regulator via the canonical Wnt/beta-catenin signaling. However, this conclusion relies heavily on the number of currently known Wnt target genes, and on the assumption that this signaling actually occurs in p7 retinae - so it is still possible that this pathway is more prominent at another developmental stage. Furthermore, since we were not able to detect expression differences of two major angiogenesis-related genes albeit they have been shown to be regulated by Wnt-signaling (Tie-2/Tek31 and Vegfa32), it is possible that Norrin has a direct role in controlling expression of Wnt-target genes in a spatially and temporally restricted manner which was not detectable by our experimental approach.
On the other hand, we were able to confirm differential expression of five genes that so far were not shown to be Wnt-targets.

**Plvap as an early indicator for vascular permeability**

Our results suggest an early involvement of Plvap (plasmalemma vesicle-associated protein) in the pathogenesis of NDP-related retinopathies. We found increased mRNA expression of *Plvap* throughout development (Fig. 3A) and also conclusive differences between immunostained cryosections of wild type and *Ndph<sup>1/4</sup>* mice (Figure 4). Plvap is endothelial cell specific, and has been described as integral membrane glycoprotein associated with the stomatal diaphragms of caveolae, transendothelial channels, vesiculo-vacuolar organelles and the diaphragms of endothelial fenestrae<sup>24,33</sup>. Enhanced prevalence of endothelial fenestrae might result in reduced vascular integrity. Both have been observed in retinæ of *Ndph<sup>1/4</sup>* mice as soon as p14<sup>13,15</sup>. Plvap expression further has been shown to be negatively correlated with the differentiation of the blood-brain-barrier<sup>30</sup>. Hence, elevated expression of Plvap may indicate enhanced vascular permeability and break-down of the blood-retina-barrier. It has previously been proposed that blood vessel leakiness is a result of hypoxia-driven VEGF (vascular endothelial growth factor A) upregulation<sup>15</sup> and thus is a secondary effect due to low oxygen supply. Interestingly, *Plvap* itself has been suggested to be a VEGF target<sup>34</sup>, rendering a correlation between vessel leakiness and Plvap more likely. Thus, elevated expression of *Plvap* in later developmental stages (p15 onwards; Fig. 3A, 4), when a malformed/underdeveloped vasculature causes a pathologic hypoxic condition in the retina, was not surprising. Development of the superficial vessel system, however, is driven by a physiological hypoxia through induction of a periphery-to-center gradient of VEGF<sup>14</sup>. Noteworthy, this hypoxia-driven VEGF expression does not, or only marginally, yield visible Plvap expression in the wild type situation that is detectable by immunohistochemistry. Therefore, we think that an increased Plvap expression in early developmental stages (p3-p10;
Differential gene expression in Ndph knockout mice in retinal development

Fig. 3A, 4) of Ndph<sup>−/−</sup> mice is not secondary due to low oxygen supply and subsequent VEGF upregulation, but rather due to a lack of Norrin that may have an important influence on vascular development. This is supported by the observation that Plvap upregulation is not solely dependent on hypoxia or VEGF, but is also triggered by other activation of the Erk1/2 MAPK pathway<sup>35</sup>. In addition, also fenestrae induction has been shown to be VEGF-independent, as it is regulated through cytoskeletal remodeling by actin depolymerization<sup>36</sup>. However, we could not detect differences in total- and phosphorylated ERK1/2 MAP-kinase levels on Western blots with retinal protein extracts from p7 Ndph<sup>−/−</sup> wild type and knockout animals so far (data not shown). Additionally, differences in Plvap expression may be due to a mechanism of endothelial cells to compensate the impaired blood vessel development, because evidence has been provided before that Plvap might have a role in angiogenesis<sup>37</sup>. Thus, it remains to be shown whether a possible mode of action for Norrin, besides transcriptional regulation via the Wnt/β-catenin pathway, might be a direct modulation of the microtubule cytoskeleton<sup>38</sup> or Plvap itself. While we are aware of these alternatives, we hypothesize that transient Plvap expression and fenestration occur during maturation of the retinal vasculature and that Norrin signaling is possibly required for its suppression. This might be indicated by the faint staining of the superficial and deep vasculature in the wild type, which was only observed during early development of the respective network (p3 & p10), and the retinal expression pattern in the Ndph<sup>−/−</sup> knockout. Since mRNA and protein levels in the knockout have been higher at each developmental stage examined, Norrin might act as a suppressor of Plvap expression, and thus may be involved in sprouting angiogenesis as well as in the formation and maintenance of the blood-retina- or blood-brain-barrier.

Differential gene expression reflects impaired blood vessel development

Besides Plvap, we were able to confirm differential expression of four more genes. All but one of them seem to emphasize Norrin’s hypothetical role in blood vessel development.
Agtr11 (angiotensin II receptor-like 1) is a venous marker that has been shown to be important for retinal angiogenesis. Therefore, its two fold reduction correlates well with the observed retinal phenotype of the Ndph knockout mouse. However, we cannot exclude that this decreased expression is merely an indicator for the already reduced overall vessel density or, considering the venous/capillary nature of the deep vessel system, a lack of development of this particular network. The same applies to the 2.4x reduction of ApoD (apolipoprotein D).

Nevertheless, ApoD could be implicated in the observed angiogenic defect of Ndph⁻⁻ mice, because it has been reported to stimulate proliferation and migration of vascular smooth muscle cells, probably through modulation of the cellular response to PDGF-BB. ApoD expression was suggested to be induced by PDGF-BB itself. Interestingly, Pdgfb and Pdgfrb expression have been shown to be decreased in Ndph⁻⁻ mice in early stages (p5/p10) before, which could indicate reduced angiogenic activity. Although Pdgfb has been shown to be regulated by VEGF, Vegfa levels were not different at p5 and even higher at p10, suggesting an alternative, VEGF-independent transcriptional regulation of Pdgfb, and thus ApoD, in Ndph knockout mice. The possibility of a functional relationship between Norrin and ApoD might be supported by the finding of co-expression of these two genes in disease-affected organs. APOD has been shown to be upregulated in human endometrium during implantation, a process that is disturbed in female homozygous Norrin knockout mice. Further, ApoD expression was also reported in the inner ear, another affected organ in Norrie disease patients and mice, where it has been suggested to be implicated in cochlear fluid homeostasis. Taken together, Norrin may influence angiogenesis either by direct transcriptional regulation of ApoD, or indirectly via Vegfa-independent regulation of Pdgf-β.

Nearly three times elevated transcript levels in the knockout were observed for adrenomedullin (Adm) by quantitative RT-PCR. It has been described to be a hypoxia induced vasodilating peptide that might have a vascular protective function. Adm probably exerts its protective role through a reduction of oxidative stress and has further been shown to
inhibit vascular remodeling\textsuperscript{51}. It is tempting to speculate that elevated expression of \textit{Adm} itself could lead to a developmental defect due to its anti-angiogenic properties, and that improper transcriptional regulation might be the cause for it. Hence, also differential expression of \textit{Adm} correlates well with the \textit{Ndph}\textsuperscript{\textsuperscript{\textregistered}} phenotype.

Surprisingly, and in contrast to the other genes discussed here, the gene most differently expressed has not directly been linked to angiogenesis. \textit{Slc38a5} (solute carrier family 38, member 5), which showed a twenty-five times decreased expression at p7 in \textit{Ndph}\textsuperscript{\textsuperscript{\textregistered}} mice (Fig. 3B), has been reported to be a main glutamine transporter in retinal Müller cells (system N2)\textsuperscript{52}. It therefore may play an important role for the neuronal signal transduction through regulation of the glutamate/glutamine household. Its decreased expression in \textit{Ndph}\textsuperscript{\textsuperscript{\textregistered}} mice may indicate an involvement of Müller glia cells as a target of Norrin signaling. This may be reflected by the reduced synaptic activity that has been observed by ERG measurements in later stages\textsuperscript{53} and is consistent with our observation that \textit{Slc38a5} expression is decreased at every developmental stage investigated, including the rather mature p21 (Fig. 3B). Besides their importance for neural function, Müller cells also play a role as guidance structures for the developing deep retinal vasculature\textsuperscript{14}. As such, one could speculate that communication between Müller cells and blood vessels might be disturbed in Norrin knockout mice. Interestingly, it has been reported earlier that blood vessels of the ganglion cell layer are frequently not surrounded by glia in p14 \textit{Ndph}\textsuperscript{\textsuperscript{\textregistered}} mice\textsuperscript{13}. However, another possible explanation for reduced \textit{Slc38a5} expression could be a secondary, neuroprotective response of Müller cells to oxidative stress. While this seems to be a likely mechanism at later, hypoxic stages in development (p15/p21), we think a ten-fold decreased expression of \textit{Slc38a5} at p5 is unlikely to be exclusively the result of hypoxia-induced neuroprotection (Fig. 3B). Besides this, no evidence for hypoxia at p5 has been provided so far: Western blots for the glial stress marker GFAP and the hypoxia-inducible factor 1-alpha (Hif1\textalpha) were shown to be similar up to p15 and p10, respectively, and no significant expression differences could be found for
VEGF\textsuperscript{15} at this stage. But \textit{Slc38a5} is not only expressed in the retina: it is also abundant in astrocytic end feet in the brain, the highest levels being found in neocortex, hippocampus, striatum and the cerebellum\textsuperscript{54}.

Thus, differential expression of this molecule might be a hint for a possible pathogenic mechanism also in the brain that could be associated with the mental retardation phenotype of ND. Noteworthy in this context, a microdeletion including \textit{SLC38A5} and the neighboring \textit{FTSJ1} has been found in three brothers with moderate to severe mental retardation\textsuperscript{55}.

\textbf{Conclusion}

Consistent with data obtained from other developmental stages\textsuperscript{15}, our examination of p7 \textit{Ndph} knockout mice showed insufficient retinal capillarization and lack of deep vessel formation. For the first time, vascular permeability has not been observed only in later, nearly adult stages, but was noted throughout development, as indicated by elevated mRNA and protein expression data for \textit{Plpav}.

Because of the early developmental stage investigated here and the lack of neovascularization in later stages, we tend to consider a role of VEGF itself in the early pathogenesis of \textit{NDP}-related diseases rather unlikely and ask whether Norrin instead might modulate the cellular response to VEGF. Norrin’s hypothetical role as a transcriptional regulator of Wnt/\(\beta\)-catenin target genes is not obvious from the array experiment. However, our recent and previous studies suggest that Norrin is important for retinal angiogenesis. Here, we could identify several genes probably implicated in blood vessel development that may be transcriptionally regulated by Norrin, directly or indirectly. These genes include \textit{Agtrl1}, \textit{ApoD}, and \textit{Adm}, while \textit{Slc38a5} might represent an interesting candidate with regard to future research of aberrant neuronal development and the massive reactive gliosis that may be the basis for the often described pseudogiomas in ND patients\textsuperscript{56}. Finally, due its proposed role in angiogenesis and
blood vessel integrity\textsuperscript{24,37}, our findings suggest an important contribution of $Plvap$ to the pathogenesis of Norrin-associated diseases.

**ACKNOWLEDGMENTS**

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We would like to thank Marzanna Künzli from the Functional Genomics Center Zurich for conducting the microarray experiment from reverse transcription to raw data processing.
Figure 1 Retinae of p7 Ndph$^{-/}$ mice in comparison to wild type. (A,B) Hematoxylin-eosin (HE) stained central sections of paraffin embedded retinae. No obvious difference in retinal organization could be detected. (C,D) Immunofluorescence images from retinal flatmounts. Images compiled from a Z-Stack of pictures acquired with a Zeiss ApoTome™. To visualize the retinal vasculature, an antibody against Collagen-IV was used. In the wild type p7 retina, deeper networks start to develop from veins and capillaries, but not arteries, as has been shown by other authors$^{14}$ (arrowheads). In contrast, outgrowth of the superficial capillary network in the knockout is delayed, appears to be much sparser, and no developing deeper network could be detected. (E,F) Higher magnification of ApoTome™ images from c,d. Superficial vasculature is shown in red, deep vasculature in green. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; A: artery; V: vein
### Table 2

Relative expression of genes that are differently expressed in comparison to the *Ndph* wild type with a fold-change $>1.6$. Affymetrix MAS5 probe set signals were calculated with the GeneSpring 7.2 software (Agilent Technologies), filtered for presence (per Affymetrix presence/absence flags) in four of five samples in either one condition (wt or ko) and then subjected to a non-parametric test for differential expression (Mann-Whitney test with Benjamin-Hochberg multiple testing correction, $p<0.1$). This resulted in 26,817 probe sets to be considered expressed, of which 450 were identified to be differentially expressed (with an error rate of 10%). 34 genes showed a fold-change of at least 1.6. GO terms listed may not include all annotations for each gene, but rather only parent or main annotations to facilitate a first impression of possible gene function.

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<th>Gene Ontology (GO)</th>
<th>Cellular Component (CC)</th>
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Unknown genes
- **A130079K24Rik**
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- **A130078K24Rik**
- **E330034G19Rik**
- **AW492805**

**Notes:**
- Biological Process (BP) and Cellular Component (CC) are listed as Gene Ontology (GO) terms.
- Affymetrix IDs are provided for each gene.
Verification of array results

Figure 2 Transcriptional analysis of selected genes in retinae of Ndph\textsuperscript{-/-} knockout (n=5) and wild type (n=5) mice. The average expression value of the wild type group was set to 1. Error bars indicate the confidence interval at a significance level of 95% (p<0.05). Quantitative RT-PCR results from p7 retinae for genes that showed increased or decreased expression in the array experiment are depicted in (A) or (B), respectively. (A) Expression of Adrenomedullin (Adm; 2.7x) and the gene for the Plasmalemma vesicle associated protein (Plvap; 4.2x) is significantly increased at p7. (B) The neutral amino acid transporter Slc38a5 (25x), Apolipoprotein D (ApoD; 2.4x) and Angiotensin II receptor-like 1 (Agtrl1; 2x) show significantly decreased expression at p7. Significant difference in Claudin 5 (Cldn5) expression is borderline. 18S / 28S rRNA were used as endogenous controls.

Relative expression in development

Figure 3 Transcriptional analysis of the two most differently expressed verified genes in retinae of Ndph\textsuperscript{-/-} knockout (n=5) and wild type (n=5) mice. The average expression value of the wild type group was set to 1. Error bars indicate the confidence interval at a significance level of 95% (p<0.05). (A) Plvap expression is significantly increased at every developmental stage from p5 to p21. Difference between wild type and knockout increases about one order of magnitude in later stages (p15/p21) when compared to the earlier stages (p5-p10). Insert shows expression data for p5-p10 in a different scale. (B) Slc38a5 expression is significantly decreased at every developmental stage. Variability of wild type expression is higher in later stages (p10-p21) than in early stages (p5, p7). Following endogenous controls were used: (A): 18S rRNA (B): Gapd.
**Figure 4** Immunofluorescence images of retinal cryosections of *Ndph*−/− mice in comparison to wild type. For each one of the indicated developmental stages (p3, p5, p7, p10, p15 and p21), co-immunolabeling with antibodies directed against Plvap (AF488; green) and Collagen-IV (Cy3; red) has been performed. Collagen-IV localizes to the extracellular matrix of endothelial cells and serves as a marker for blood vessels. In all stages investigated, staining has been observed in the choroid (CV, below the RPE) and the superficial retinal vasculature (RV, inside the RGC/nerve fibre layer). From p10 onwards, blood vessels of the secondary, deep retinal vasculature were detected in the OPL in the wild type, but not the knockout retina. In addition, at p15 and p21, the tertiary, intermediate vessel system in the IPL can be seen in the wild type. Plvap localizes to the choroid vessel system (CV) in both wild type and knockout at all developmental stages. Interestingly, Plvap expression has also been detected in the retinal vasculature (RV) of the knockout animals beginning at p3 and more intense over time. In contrast, the retinal vasculature of the wild type is not, or only very weakly, labeled by the Plvap antibody. In the superficial vessel network, staining in the wild type could only be detected at p3 (arrow), but not in later stages. At p10, slight staining of the deep vessel system was observed (arrows). RV: superficial / deep retinal vasculature; CV: Choroid; RGC: retinal ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium.
### Top 5 involved pathways

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Genes mapped to pathways: 75
cDNAs: 868

Table 3: Results of a pathway analysis performed with the online tool “Pathway express”, which is part of the “Onto-Tools”, hosted by the Wayne State University, Detroit, USA. Only 10% of the input genes could be mapped to a pathway based on the KEGG Pathway Database.
References


