PRO RETINA FOUNDATION

Pro Retina – Stiftung
zur Verhütung von Blindheit

17th PRO RETINA RESEARCH-COLLOQUIUM POTSDAM

Conference Report | March 31– April 1, 2023

Retinal Degeneration

New aspects in personalized and molecular medicine

An Interdisciplinary Dialogue

THIS MEETING IS SUPPORTED BY

PRO RETINA FOUNDATION

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CONFERENCE REPORT

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PRO RETINA DEUTSCHLAND E. V. & THE PRO RETINA-FOUNDATION FOR PREVENTION OF BLINDNESS

WHO WE ARE

The patient-organisation, "PRO RETINA Deutschland e. V.", was founded in 1977 as „Deutsche Retinitis Pigmentosa-Vereinigung" by patients and their relatives intended to organize help for themselves. The four objectives mentioned in the constitution are to actively support research, to give psychological and social advice for its members, to strengthen public awareness and to stand in for patient interests in politics, society and health care system.
Every member can join one of the 60 regional groups, which are spread throughout Germany. At present (2023), PRO RETINA Deutschland e. V. counts more than 6,500 members. The Board, the counsellors, the leaders of the regional groups and all active members are working on a non-profit basis, but they are supported by a fulltime working staff at our office which is located in Bonn (www.pro-retina.de).

WHAT WE DO IN RESEARCH

The jewel of all this work is the PRO RETINA-Foundation for Prevention of Blindness, which was founded in 1996.
From the early beginning we have created a stable network with researchers and ophthalmologists for joined information and advice. We support research projects with direct financial funding – since the "Foundation for Prevention of Blindness" was established in 1996, more than two million Euro have been donated. We actively initiate research projects and therapy tests and contribute to their implementation.

Every year, we award two research prices and organize and support national and international seminars and conferences on relevant topics. We are financing PhD grants in order to foster research activities and networking between researchers.

We are consulted by a Scientific and Medical Advisory Board and a Working Group on Clinical Issues. In this Working Group scientists of different medical and other relevant disciplines are taking part.

The main objective is to secure a long-term support for research activities, e. g. by granting financial means for the development of new research projects or by financing the initial phase of relevant projects.

It is envisaged to increase the capital of the foundation to a minimum of Euro 5,000,000, which are to result in a steady source of funding for the support of research, independent from changing income of donations.
We guarantee that the benefits of the Foundation will only be dedicated to the research of retinal diseases, with the wider objective to develop applicable therapies for the patients.

PROGRAMME

Friday, March 31, 2023

13:00 – 13:05 **Welcome remarks**
Franz Badura (Amberg, Germany)

13:05 – 14:30 **SESSION 1**
Selected poster presentations
Eight abstracts to be selected

14:30 – 15:10 **Keynote lecture**
Bart Leroy (Ghent, Belgium)
Why do clinical trials fail in ophthalomolgy?

15:10 – 16:00 Coffee break with scientific chit-chat

16:00 – 17:30 **SESSION 2 – IRD & RP**

16:00 – 16:30 Knut Stieger (Giessen, Germany)
Developing tools for genome editing based therapies in IRD

16:30 – 17:00 Christina Zeitz (Paris, France)
Approaches to discover novel gene defects in progressive inherited retinal disorders

17:00 – 17:30 Carlo Rivolta (Basel, Switzerland)
Genomics and bioinformatics to identify retinal disease genes

17:30 Dinner

19:00 – 19:30 **EVENING LECTURE**
Bernhard Weber (Regensburg, Germany)
Know the score – Genetic testing in times of change

19:30 – open Swingin' poster session

PROGRAMME

Saturday, April 01, 2023

09:00 – 10:40 **SESSION 3 – Artificial Intelligence in ophthalmology**

09:00 – 09:25	Gregor Reiter (Vienna, Austria) AI-based fluid monitoring in the real-world
09:25 – 09:50	Karsten Kortuem (Munich, Germany) How an AI empowered retina clinic will change patient care
09:50 – 10:15	Andreas Maunz (Roche – Basel, Switzerland) What makes AI interesting for clinical research and development?
10:15 – 10:40	Katharina Stingl (Tübingen, Germany) Chorioretinal atrophies after Luxturna gene therapy

10:40 – 11:15 Coffee break

11:15 – 12:55 **SESSION 4 – Hot topics in AMD research and clinical translation**

11:15 – 11:40	Przemyslaw (Mike) Sapieha, (Montreal, Canada) Neutrophil extracellular traps target – senescent vasculature for tissue remodeling in retinopathy
11:40 – 12:05	Andrew Lotery (Southhampton, UK) The potential to treat AMD with Complement Factor I
12:05 – 12:30	Natalia Pashkovskaia (Tübingen,Germany) Assembloid and microfluidic approaches for in vitro modeling of human retinal diseases
12:30 – 12:55	Olaf Strauss (Berlin, Germany) Complement and AMD: Finally a new role of the terminal complement complex?

12:55 – 13:00 Concluding remarks

13:00 Farewell Lunch and end of meeting

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Characterization of DNA repair mechanisms in undifferentiated neurons and the effect of TET3 expression

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Introduction: Inherited retinal dystrophies are a group of heterogeneous disorders, which vary in severity and progression. There are more than 270 genes that have been associated with these diseases, most of them being expressed in photoreceptors. The genome editing field, in which the CRISPR-Cas9 technology is used to introduce DNA double strand breaks (DSB) that are subsequently repaired by the cells own repair systems, has gathered much attention in recent years. The major pathways for DSB repair are non-homologous end-joining (NHEJ) and homology directed repair (HDR). The main obstacle for its application in the retina is the lack of adequate information of DNA repair efficacy in photoreceptors. Our goal is to identify and to study DNA repair and to improve genome editing efficacy in mature neurons and to determine the impact of the Tet3 protein, a master regulator of neuronal cell type specific gene expression.

Material and methods: Transfections were done in iNGN cells, which are inducible pluripotent stem cells that differentiate to mature neurons upon activation of the TET ON system by adding doxycycline to the culture media. Undifferentiated iNGN wildtype (WT) and iNGN-TET3KO, in which the TET3 protein is knocked out, were transfected with a custom-made BRET reporter plasmid for quantification of editing events, and the corresponding gRNA/Cas9 plasmid. In addition, we used a CRISPR-Cas version that is inducible with 4-hydroxy tamoxifen. As a control, we transfected HEK293T cells using both systems. Moreover, we quantified the DNA repair events using bioluminescence resonance energy transfer (BRET) assay.

Results: The frameshift rate was calculated from the BRET assay data obtained from the transfections with both Cas systems using 3 different guides (In8T3, In8T2 and In6T1) being 68.6%, 36.01%, and 29.99%, respectively. for iNGN TET3KO with Cas9. On the other hand, the rate for iNGN WT was 29.78%, 9.89%, and 12.14%. Furthermore, the inducible Cas showed similar outcomes with iNGN TET3KO (53.66%, 39.17% and 39.58%) and iNGN WT (19.05%, 5.17% and 8.35% , respectively). The results showed an increase in the frameshift rate in the undifferentiated iNGN TET3KO cells in comparison to the iNGN WT.

Conclusions: The BRET assay data indicates that the Knockout of TET3 protein leads to the improvement of DNA repair activity in the undifferentiated iNGN cells.

Effect of VCP inhibition on degenerating cones in secondary cone degeneration models

*Ana Cristina Almansa Garcia[1,2], Bowen Cao[1,2], Merve Sen[1,2], Angela Armento[1], Sylvia Bolz[1], Ellen Kilger[1], Blanca Arango-Gonzalez[1] *, and Marius Ueffing[1]**
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Materials and methods: *Rd10* mouse eyes were enucleated either at the beginning (PN9) or at the peak of rod degeneration (PN18) and cultured in serum-free conditions. With the aim to determine the best treatment time window, three different treatment paradigms were chosen from onset to progression of degeneration (PN9-18, PN18-30, PN18-40). Medium was changed every two days, and the VCP inhibitors ML240 20 µM or NMS-873 0,5 µM were added into the medium simultaneously. PR degeneration was assessed by TUNEL assay, immunostaining, ONL (outer nuclear layer) rows, and cone density quantification. Human retinal explants were obtained after informed consent from an eye enucleated from a patient with uveal melanoma and maintained in culture for four days *in vitro* (4DIV) following the same protocol and analysis.

Results: The *rd10* retinas treated with VCP inhibitors showed more remaining ONL nuclei rows and lower TUNEL-positive cells percentage than the control *rd10* retinas in all treated groups. VCPi neuroprotection in the total number of remaining PRs in *rd10* explants was higher when the treatment started at the beginning of retinal degeneration (PN9-18 group). We did not find significant differences in the cone density in the PN9-18 and PN18-30 groups; however, in the PN18-40 (when cone degeneration starts), we observed a drop in the number of cone PRs in the control retinas, while in the treated retinas, cone PR density was maintained at similar levels when compared to those at PN30. In human retinal explants, PR cell death as a conquence of axotomy, loss of circulation as well as culture conditions was reduced by VCPi (more ONL nuclei rows and fewer TUNEL-positive cells). There were no differences in the number of cone PRs after 4DIV, but in the ML240 group, cone PRs showed significantly longer outer segments. No signs of inflammation or cytotoxic effect were observed on the human retina after VCPi treatment.

Conclusions: Our results suggest that VCP inhibition can prevent, or at least delay, secondary cone degeneration in the *rd10* model, where the cones degenerate as a consequence of rod degeneration. Furthermore, preliminary data based on human retinal explants suggest, that cone PRs can profit from VCPi and that without apparent cytotoxicity when applied to the human retina.

This study was supported by the ProRetina Foundation, the Foundation Fighting Blindness FFB (Grant PPA-0717-0719-RAD), the Kerstan Foundation, and the Maloch Stiftung.

iPSC-RPE cells carrying the Complement Factor H (CFH) Y402H polymorphism show an impaired response to oxidative and ER-stress

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Purpose: Age-related macular degeneration (AMD) is a degenerative disease of the macula and leading cause of blindness in the elderly population. It is caused by an interplay of diverse risk factors (genetic predisposition, age and lifestyle habits). One of the main genetic risks is the Y402H polymorphism in the Complement Factor H gene (*CFH*/FH), an inhibitor of complement system activation. We could demonstrate that FH loss in RPE cells disturbs cell homeostasis and that those damaged RPE cells cause retinal degeneration (Armento et al., 2021). Here, we investigate the impact of additional stressors on iPSC-RPE cells carrying the *CFH* AMD risk polymorphism Y402H.

Methods: Following differentiation of iPSC cells into mature RPE cells, we investigated the impact of *CFH* 402H (high risk) vs *CFH* 402Y (low risk) in iPSC-RPE cells. Cells were treated with Hydroquinone (HQ), an immunotoxic stressor and major component of cigarette smoke, and Tunicamycin (TM), an inducer of endoplasmatic reticulum (ER) stress. The response to stress was assessed *in vitro* by cytotoxicity and caspase activity assay (mean ± SEM, $n > 4$). Alterations in signaling pathways, such as unfolded protein response (UPR), apoptosis and autophagy were investigated by Western Blot.

Results: RPE cells *CFH* 402H showed increased cytotoxicity compared to *CFH* 402Y when exposed to stress condition (HQ, 1.1 ± 0.1 vs 2.1 ± 0.3, $p < 0.001$; TM, 1.5 ± 0.2 vs 2.7 ± 0.3, $p < 0.001$). While HQ induced caspase activation in both *CFH* 402Y as well as *CFH* 402H cells (5.8 ± 0.8 vs 7.3 ± 0.9, $p < 0.001$), TM induced caspase activation only in the *CFH* 402H cells (1.2 ± 0.1 vs 2.2 ± 0.2, $p < 0.001$). RPE cells carrying *CFH* 402H showed increased levels of Bip (UPR marker), LC3 (autophagy marker) and reduced level of BCL2 (anti-apoptotic marker) compared to their homozygous *CFH* 402YY controls, when exposed to HQ and TM.

Conclusions: RPE cells carrying the *CFH* 402H risk variant are susceptible to stress insult and more prone to stress induced apoptosis. Our data support the hypothesis that complement associated risks influence the endophenotype of RPE cells specifically and point to a yet enigmatic role of complement factors within cells of the retina promoting AMD pathology.

CD44 signaling in Müller cells affects photoreceptor survival in retinitis pigmentosa

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Purpose: Retinitis pigmentosa (RP), a group of inherited retinal disorders, is characterized by progressive degeneration of photoreceptor cells and several other pathological changes. For example, Müller cells, the major type of glial cells of the retina, undergo reactive gliosis. Gliotic Müller cells display dysregulations of their supportive functions, including glutamate uptake as well as metabolism, and show an upregulation of CD44. CD44, a cell surface glycoprotein, is preferentially localized in the microvilli of Müller cells. However, the function of CD44 in RP is not known. Thus, our goal was to analyze the role of CD44 in healthy and diseased retinas. In parallel, we shed light on the links between CD44, Müller cell glutamate metabolism and photoreceptor survival.

Methods: To generate RP mice which lack CD44, *Pde6b*$^{STOP/STOP}$ mice were crossed with *Cd44*$^{-/-}$ mice. First, we characterized the retinas of *Cd44*$^{-/-}$ and *Pde6b*$^{STOP/STOP}$ *Cd44*$^{-/-}$mice by quantifying the thickness of the outer nuclear layer (ONL), the length of the inner and outer cone segments, and the number of microglia at different time points of disease progression. Next, in order to reveal cell type-specific changes in the proteome, retinal neurons and Müller cells were isolated from *Pde6b*$^{STOP/STOP}$ and *Pde6b*$^{STOP/STOP}$ *Cd44*$^{-/-}$ retinas by magnetic activated cell sorting, and analysed by label-free liquid chromatography-tandem mass spectrometry based proteomics.

Results: We identified CD44 to be persistent upregulated in Müller cells from *Pde6b*$^{STOP/STOP}$ mice via immunoblot, quantitative real-time PCR, and immunohistochemistry. Importantly, CD44 was also upregulated in other RP mouse models. To gain a more detailed understanding of CD44's role in healthy retinas, we analyzed *Cd44*$^{-/-}$ mice with no expression of CD44. These mice reveal a normal retinal structure in comparison with wildtype mice. On the other hand, *Pde6b*$^{STOP/STOP}$ *Cd44*$^{-/-}$ retinas showed a significantly thinner ONL and shorter cone inner and outer segments than *Pde6b*$^{STOP/STOP}$ mice. Consistent with the accelerated disease progression, we found a higher number of activated microglia in the *Pde6b*$^{STOP/STOP}$ *Cd44*$^{-/-}$ mice. Additionally, proteomic analysis revealed that the glutamate transporter 1 (EAAT2) is significantly downregulated in the Müller cell fraction of *Pde6b*$^{STOP/STOP}$ *Cd44*$^{-/-}$ mice compared to *Pde6b*$^{STOP/STOP}$mice.

Conclusions: In RP, CD44 is dramatically elevated and an upstream driver of EAAT2 in gliotic Müller cells. Consequently, loss of CD44 leads to a downregulation of EAAT2 and thus excessive glutamate. In conclusion, CD44 is a key regulator of glutamate homeostasis and its loss accelerates photoreceptor degeneration.

All-in-one AAV vectors for CRISPR/SpCas9 genome editing

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Purpose: Adeno-associated virus (AAV) vectors are important delivery vehicles for therapeutic genome editing tools but are severely limited by their packaging capacity of maximal ~4.8 kb. The size of the gene encoding *Streptococcus pyogenes* Cas9 (SpCas9) (~4.1 kb) combined with the single guide RNA (sgRNA) scaffold precludes packaging into a single AAV vector. To overcome this limitation a dual AAV system is used, although simultaneous delivery of multiple vectors may reduce efficacy and increase safety risks. Here, we describe an approach to generate an all-in-one single AAV vector co-expressing SpCas9 and an sgRNA targeting exon 3 of the bestrophin-1 (BEST1) gene.

Methods: To drive sgRNA expression, vector constructs were generated by restriction cloning to contain either a short CMV promoter (159 bp) or a full RSV promoter (226 bp) for SpCas9 expression in combination with a truncated (99 bp) or a full-length (241 bp) U6 promoter for sgRNA expression. The cleavage efficiency of sgRNA_BEST1$_{exon3}$ in the constructs was determined in vitro by (1) a fluorescence-based assay in HEK293T cells using a pCAG-EGxxFP vector for EGFP fluorescence reconstitution and (2) CRISPR-induced double-strand break (DSB) analysis following viral particle production and viral transduction of HEK293T cells.

Results: Quantification of reconstituted EGFP fluorescence revealed that the all-in-one AAV construct containing the full-length U6 promoter in combination with the truncated CMV promoter resulted in SpCas9 activity comparable to that of the dual vector application. In contrast, the presence of a truncated U6 promoter in combination with the full-length RSV or the truncated CMV promoters resulted in relatively inefficient SpCas9 activity. For the optimized all-in-one AAV-U6$_{full\text{-}length}$–CMV$_{159bp}$–SpCas9 construct, particle production and targeted cleavage were observed after viral transduction of HEK293T cells, although to a lesser extent than with the dual vector system.

Conclusions: Our study has identified an all-in-one AAV construct for targeted delivery of sgRNA/spCas9 in vitro, comparable in efficiency to the dual vector system. Preliminary results show that the all-in-one vector is sufficient to produce viral particles, although at a lower efficiency than the dual vector system, probably due to exceeding the AAV carrying capacity. Further investigations are needed to refine the all-in-one approach which may provide a more straightforward and less immunologically invasive alternative for the therapeutic application of AAV-guided CRISPR genome editing in vivo.

The translocator protein (TSPO) is a critical factor to support retinal and Müller cell homeostasis upon ischemia

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Purpose: TSPO is an integral membrane protein located in the outer mitochondrial membrane that is in the homeostatic retina predominantly expressed by Müller cells, microglia, blood vessels and pigment epithelial cells. Treatment of damaged retinae with agonistic TSPO-ligands show effects like dampened microglial reactivity and an improved preservation of retinal neurons. However, the mechanism by which TSPO agonists act remains largely unknown. Since TSPO appears to be involved in the import of cholesterol to the inner mitochondrial membrane – the place where steroidogenesis is initiated - we speculate that the beneficial actions of TSPO-agonists are mediated by Müller cells and work via an increased production of neurosteroids in the lesioned retina and thus lead to a better conserved Müller cell homeostasis and retinal function.

Methods: Using Müller cell-specific conditional TSPO knockdown, we first examined retinal function in the context of ischemia by electroretinography (ERG). Second, we probed changes in Müller cell physiology by analyzing the maintenance of mitochondrial membrane potential (JC-1 assay) in the ischemic retina. Furthermore, we investigated the response of Müller cells to hyperosmolar stress. Then, we performed single cell sequencing to test for potential changes in signaling pathways or compensatory effects. Finally, to further address our initial hypothesis, we measured neurosteroid levels in native and cultured retina by HPLC.

Results: In the post-ischemic retina, Müller cell-specific TSPO-KD leads to a greater decrease in the retinal light response measured by ERG compared to wild type. Consistent with this finding, we observed that Müller cell mitochondria have a lower membrane potential, whereas TSPO-KD has no effect on Müller cell volume regulation when exposed to hyperosmolar stress. Preliminary data from single cell sequencing of Müller cells revealed several transcriptomic changes suggesting a canonical influence of TSPO in response to retinal injury. Finally, we measured steroid hormone levels in native retina and observed changes in explants treated with finasteride or a TSPO ligand.

Conclusions: Our results show that TSPO in Müller cells is an important factor for the cells to fulfill their function in maintaining retinal homeostasis, but also to coordinate functional changes in response to tissue damage. Furthermore, loss of Müller cell TSPO appears to be detrimental to glial mitochondrial health, whereas the TSPO ligand enhances the production of potentially neuroprotective neurosteroids in retinal explants.

Evidence of potential early-stage ocular tertiary lymphoid tissue formation following high dose retinal AAV8 gene therapy

Kirsten Bucher, Daniela Süsskind, Martin Biel, Stylianos Michalakis, Bernd Wissinger, Ulrich Bartz-Schmidt, Tobias Peters, Dominik M. Fischer, Simon J Clark*, and the RD-CURE consortium*

Purpose: Retinal gene therapies with recombinant adeno-associated virus (rAAV) vectors are innovative approaches for the treatment of various retinal dystrophies. However, high vector doses can induce ocular immune activation and the accumulation of lymphoid cells in retina and choroid. Tertiary lymphoid tissues (TLT) are organized lymphoid aggregates with signs of proliferation forming in non-lymphoid organs during chronic inflammation. They can present transiently with their cellular organization varying from simple clusters of lymphocytes to structures reminiscent of secondary lymphoid organs. Data suggest that TLT serve as local sites of antigen presentation and lymphocyte activation. Here, we investigated potential TLT formation in eyes of non-human primates treated with high-dose retinal gene therapy with rAAV8.

Methods: TLT formation was investigated by immune-histological analysis of eye sections from a toxicology study of a clinical grade rAAV8 vector performed in Macaca fascicularis. Animals had been subretinally injected with either a lower dose of 1×10e11 vector genomes (vg), a higher dose of 1×10e12 vg or a sham injection. After three months the animals were sacrificed and their eyes processed for histology.

Results: Lymphoid aggregates were detectable in both retina and choroid in 5 out of 8 animals receiving the 1×10e12 vg dose, but none were observed in either the lower dose group nor the sham treated. All lymphoid aggregates contained large numbers of CD3+ T cells and CD20+ B cells. In all aggregates the cell membranes of CD3+ T cells formed close contacts with HLA-DR+ antigen presenting cells and both B cells and T cells showed evidence of proliferation as determined by co-staining with the proliferation marker ki67. The observed lymphoid aggregates are likely to represent early stage TLT as they did not stain positive for Lyve-1+ lymphatic vessels nor PNAd+ high endothelial venules, both markers of late-stage TLT formation.

Conclusions: High-dose retinal gene therapy with rAAV8 vectors can induce lymphoid aggregate formation featuring characteristics of early stage TLT. This implies a tolerable dosing limit of subretinal rAAV8 before the formation of early-stage TLT. However, the long-term effect of these structures, and whether they are beneficial or detrimental in the longer term, remains to be elucidated.

The characterization of a novel murine RP model carrying a deletion of isoleucine 255/256 in the rhodopsin gene

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Purpose: Rhodopsin gene (Rho) mutations are the most common cause of autosomal-dominant retinitis pigmentosa (adRP). The 3-bp deletion of one of the two isoleucine (Ile) monomers at codon 255 or 256 within the Rho gene (*Rho*^{Ile256del}) causes an early onset of retinal degeneration in patients. Unfortunately, the pathophysiology of *Rho*^{Ile256del}-associated adRP has long been elusive. Therefore, we generated *Rho*^{Ile256del} knock-in mice to study the progressive retinal degeneration and the mechanism of pathogenesis.

Methods: Changes in the outer retina of homo- and heterozygous *Rho*^{Ile256del} retinae (*Rho*$^{Ile256del/Ile256del}$ and *Rho*$^{Ile256del/+}$, respectively) and their WT counterpart (C57BL/6J mouse) over time were investigated using immunofluorescence and electron-microscopy (EM). In addition, the activation of apoptotic and non-apoptotic cell death markers was evaluated.

Results: *Rho*^{Ile256del} mice exhibit early and progressive photoreceptor degeneration, with increased TUNEL-positive cells and decreased nuclei rows in the ONL. The peak of degeneration was at PN18 in *Rho*$^{Ile256del/Ile256del}$ mice and at PN20 in *Rho*$^{Ile256del/+}$ mice, which exhibit a comparatively slower retinal degeneration. In both phenotypes, we found an accumulation of rhodopsin in the photoreceptor cell bodies and a shortening of the outer segments (OS) along with disorganized disc ultrastructure. In addition, a progressive and significant reduction in the number of cones was observed over time. The cyclic guanosine monophosphate (cGMP) signal intensity across the retina was elevated in the mutant photoreceptor segments. Both poly-ADP-ribose polymerases (PARP) and calpain activity were strongly increased in the *Rho*^{Ile256del} mutant retina. PARP activity peaked at P18 in *Rho*$^{Ile256del/Ile256del}$ and *Rho*$^{Ile256del/+}$ mice, being the peak of calpain activity delayed by two days in the *Rho*$^{Ile256del/+}$ animals but not in the *Rho*$^{Ile256del/Ile256del}$ mice.

Conclusions: We developed a *Rho*^{Ile256del} mouse model for studying rod-cone degeneration that mimics the RP phenotype in humans. More profound studies of this model, which shows mislocalization of rhodopsin, ultrastructural abnormalities, and activation of non-apoptotic cell death pathways, will lead to a better understanding of the pathology of the disease and the development of therapeutic strategies.

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Innovative enhanced-deletion genome editing-based splice correction for a frequent USH2A-associated deep-intronic mutation

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Usher syndrome is a rare autosomal recessive condition that causes hearing loss and retinal degeneration. Mutations in USH2A are responsible for ~ 50% of all Usher syndrome cases. The pathogenic deep-intronic variant USH2A:c.7595-2144A>G is a common mutation, accounting for ~ 4% of total USH2A alleles. This variant determines the activation of a cryptic splice site, leading to aberrant splicing by intron (pseudoexon) retention in the mature mRNA transcript, frame-shift and pretermination codon formation. CRISPR/Cas9 editing can correct aberrant splicing by targeting sequences involved in the faulty process, but the standard approach of generating a deletion containing the relevant deep intronic variant and pseudoexon sequences has drawbacks: using multiple gRNAs can activate P53-related pathways, increase the risk of off-target effects and chromosomal rearrangements, and require more components to deliver.

We therefore engineered Enhanced-Deletion Cas (EDCas) molecules to overcome these issues with standard CRISPR/Cas9 approaches. By using only one gRNA, EDCas molecules generate larger deletions at the targeted sites, inducing significant perturbation of sequences involved in the faulty splicing process. This in turn prevents their recognition by the splicing machinery, resulting in splicing correction.

We used EDSpCas9, derived from SpCas9, to rescue the splicing defect caused by *USH2A*:c.7595-2144A>Gin comparison to wild-type SpCas9. Using minigene assays in HEK293T cells higher splicing rescue rates (53.5 ± 11.8 % - 88.0 ± 1.9 %) were obtained when using EDSpCas9 coupled to 6 single gRNAs compared to SpCas9 (34.0 ± 25.3 % - 71.8 ± 30.0 %). Four lead gRNAs were validated in patient-derived *USH2A*:c.7595-2144A>G homozygous fibroblasts, showing high splicing rescue (85.7 ± 3.7 % - 92.4 ± 4.8 %), consistent with the minigene assay results. In contrast, wild-type SpCas9 coupled to the same gRNAs achieved lower splicing rescue (38.2 ± 5.4 % - 91.8 ± 3.2 %), with great variance from the minigene assay results. This suggests that while the efficacy of gRNA/EDSpCas9 strategies for splicing rescue can be evaluated using minigene assays, the results obtained for gRNA/SpCas9 may not predict their effectiveness in different cell lines.

Overall, the use of EDCas molecules to address splicing defects is showing promising preclinical results transferable to advanced disease models. Ongoing experiments are focused on assessing potential off-target effects, identifying repair mutational profiles at the genomic locus, and validating smaller EDCas molecules, fitting in AAV vectors.

Microglial and macroglial dynamics in a model of retinitis pigmentosa

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Purpose: In retinal neurodegenerative diseases, such as retinitis pigmentosa (RP), the characteristic photoreceptor cell death is preceded by changes of microglia and macroglia cells (astrocytes and Müller glia). Gene therapy, a promising therapeutic approach for RP, is based on the premise that glial cell remodeling does not impact vision rescue. However, the dynamics of glial cells after treatment at late disease stages are not well understood. Here, we tested the reversibility of specific RP glia phenotypes in a *Pde6b*-deficient RP gene therapy mouse model.

Methods: We analysed glia cell remodelling in *Pde6b*-deficient ($Pde6b^{STOP/STOP}$) mice during disease progression using different antibodies for microglia, Müller cells and astrocytes. In addition, we tested whether this remodelling is halted or reversed by genetic rescue.

Results: We found significant differences in the astrocyte network in our $Pde6b^{STOP/STOP}$ mutant mice compared to control. The astrocytes in 16- and 60-weeks-old mutant retinas showed a thickened appearance and displayed an irregular, "ragged" cell shape. Furthermore, we found an increase in microglia cells together with a decrease of the occupied area in the $Pde6b^{STOP/STOP}$ mutant animals. To assess the effects of genetic rescue in glial cells, $Pde6b^{STOP/STOP}$ mice were injected with tamoxifen at 12 or 16 weeks of age, when the ONL thickness had decreased by 67% and 82%, respectively. Treatment reversed Müller glial reactivity and microglia activation almost to WT levels.

Conclusions: We have characterized the reactive gliosis and microglia activation dynamics during and after photoreceptor degeneration in the $Pde6b^{STOP/STOP}$ mouse model of RP. We demonstrated an increased number of activated microglia, retraction of microglial processes, reactive gliosis of Müller cells, astrocyte remodeling and an upregulation of glial fibrillary acidic protein (GFAP) in response to photoreceptor degeneration. Importantly, these changes returned to normal following rod rescue at late disease stages, showing that therapeutic approaches restore the homeostasis between photoreceptors and glial cells.

Retinal microglia and their immunotoxic effects in AAV-based gene therapy

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Purpose: In recent years, gene therapy targeting inherited retinal diseases (IRDs) using adeno-associated virus (AAV) as vector have shown immense promise. After Luxturna®, an AAV-based treatment for a biallelic mutation in the RPE65 gene, gained approval by the FDA in 2017, multiple clinical trials have been started for treatment of other IRDs using various AAV-based approaches. However, despite the relatively low immunogenic potential of AAVs, and the immune privilege of the eye, numerous reports of inflammation after treatment show that AAV vectors are recognized as foreign invaders and elicit host-cell responses. Microglia, as the resident immune cells of the retina, are highly involved in these inflammatory processes. Our preliminary data together with data from other research groups have indicated that resident microglia are early responders to locally injected retinal AAVs, however, a comprehensive analysis in adult animal models is still missing. A deeper understanding of immune activation during retinal AAV gene therapy, as well as what factors determine the immunogenicity of the vector, is crucial to ensure the safety of these approaches.
Our hypothesis is that retinal immune cells such as microglia are activated very early in response to AAV and that their inflammatory responses negatively affect the success of ocular gene therapy. This hypothesis also considers the aspect that retinal microglia maintain a chronic pro-inflammatory state in the already degenerating retina and that innate immune memory effects might thus induce microglia to react more strongly against AAV in the future.

Methods: Two AAV capsids will be packed either with a non-coding stuffer sequence or the full-length coding sequence of murine Fam161a. Cx3cr1-GFP$^{+/+}$ reporter mice will be intravitreally injected with AAVs at three different titres and analysed at pre-determined time points post injection. In a comprehensive analysis combining in vivo retinal imaging that will allow to identify GFP+ microglia in these reporter mice alongside assessing retinal structure with histological and molecular approaches, we will quantify different key parameters of microglial reactivity and inflammation with regard to capsid, titre and transgenes. Based on these findings, an experimental AAV2 vector-based gene augmentation therapy in Fam161a-deficient mice under conditions of potent immunomodulation will be done.

Lipid metabolism in murine Stargardt disease models

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Stargardt disease is the most frequent inherited retinal dystrophy in both children and adults, with reported prevalence of 8-10/100'000. Autosomal recessive Stargardt disease (STGD1) is caused by pathogenic variants in the ATP-binding cassette (ABC) transporter 4 (*ABCA4*) gene. Impaired retinoid transport leads to accumulation of cytotoxic visual cycle byproducts in outer segments of photoreceptors and in the retinal pigment epithelium. Autosomal dominant Stargardt disease (STGD3) is caused by pathogenic variants in the ubiquitously expressed *ELOVL4* gene encoding the integral membrane fatty acid elongase ELOVL4, which elongates very long chain (VLC) saturated and polyunsaturated fatty acids (PUFAs). Previously, docosahexaenoic acid (DHA) supplementation was shown to delay retinal degeneration and to decrease subretinal bisretinoid accumulation in aging wild-type mice and a STGD3 mouse model (*Elovl4*$^{+/-}$).

Wild-type, *Abca4*$^{-/-}$ and *Elovl4*$^{+/-}$ were fed with either control chow, chow enriched with 5,000 IU vitamin A, chow enriched with 1% DHA, or enriched with both. Fluorescence lifetime imaging ophthalmoscopy (FLIO), optical coherence tomography (OCT), and electroretinography (ERG) at 2, 9, and 16 months was performed. After 18 months, we did histochemical staining and analyzed 47 single retinas by gas chromatography-coupled mass spectrometry (GC-MS) to assess 28 different fatty acids.

DHA supplementation improved photopic and scotopic responses in wild-type mice, but not STGD mouse models, as assessed by ERG. Vitamin A food supplementation lead to decreased photopic and scotopic responses in *Abca4*$^{-/-}$ mice, in line with previous observations and consistent with the increased lipofuscin deposits observed by FLIO. Interestingly, GC-MS showed increased eicosapentaenoic acid (EPA) concentrations in DHA-supplemented *Elovl4*$^{+/-}$ mice. This suggests that EPA, retro-converted from DHA, may be the preferred substrate for ELOVL4 to generate VLC PUFAs.

Establishment of an *ex-vivo* co-cultivation model to investigate retinal inflammation

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Background: Co-culturing retinal explants with retinal pigment epithelial (RPE) cells is essential to mimic diseases of the outer retina, such as age-related macular degeneration (AMD). In recent co-culture models, positive effects for the retinal explant, such as an increased survival rate of photoreceptors, have been observed alongside negative effects, such as a slight upregulation of inflammatory IL-1β levels. This study aimed to investigate the inflammatory effects induced by direct co-cultivation of functional primary porcine RPE monolayers and retinal explants, and to evaluate the potential of this model to simulate inflammatory retinal diseases.

Methods: To confirm the establishment a functional RPE-monolayer, trans-epithelial electrical resistance (TEER) measurements and ZO-1 staining were performed. Retinal explants were then co-cultivated with functional RPE monolayers for 48 h. Cell death in the retinal explant was investigated with TUNEL-staining, ATP-measurements, and caspase-3/7-assays. Gene expression of several cell death markers *(p62, TNF-α, NfkB)* was also analysed. In parallel, the effects of the co-cultivation on the RPE cells were investigated by calcein/PI and ZO-1 staining, and MTS/caspase 3/7 assays. In addition, mRNA expression of the cell death markers *Bax, RIPK1,* and *RIPK3* was measured. The expression of inflammatory cytokines was further verified via qRT-PCR (*IL-1β, IL-6, IL-8*) as well as ELISA (IL-1β and IFN-γ).

Results: Direct co-cultivation of RPE monolayers resulted in inflammatory responses and cell death in the retinal explants and RPE cells. The increase in *IL-6* (+19-fold) and *IL-1β* (+5-fold) expression levels in the co-culture model resembled the inflammation observed in AMD. Cell death was evidenced by enhanced caspase 3/7 activity (+1.6 fold in retinal explants, +1.8 fold in RPE cells), increased number of TUNEL+ and PI+ cells, loss of cell viability (-40% in RPE cells), and reduced ATP levels (-45% in retinal explants). Furthermore, *Bax, RIPK1* and *RIPK3* expression was significantly higher in co-cultured RPE cells as well as *TNF-α* and *NfkB* expression levels in retinal explants. Co-cultivation also resulted in disruption of the RPE-monolayer, as demonstrated by the loss of tight junctions (ZO-1) and a reduction in TEER-levels.

Conclusions: Co-culturing with primary functional RPE-monolayers induced an inflammatory response resulting in monolayer loss and retinal cell death, as observed in enhanced caspase 3/7 levels, PI+ and TUNEL+ cells. Increased caspase activity, as observed in this model, contributes to the pathogenesis of AMD. Likewise, inflammation resembles a key element in AMD development, and enhanced IL-6, IL-8 and IL-1β levels are found in patients with neovascular AMD. In result, this direct co-cultivation model with a primary RPE monolayer can be used to study inflammatory retinal diseases, such as AMD, and to evaluate new therapeutic options.

Novel CRISPR/Cas editing strategies for unbiased rescuing of common splicing defects in *ABCA4* gene

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Stargardt disease is an inherited retinal disorder caused by mutations in *ABCA4*. The mutational spectrum is diverse and includes frequent pathogenic variants, affecting mRNA splicing. There is not yet an effective treatment for Stargardt disease, however, gene therapy using CRISPR/Cas technologies shows promise in permanently correcting genetic defects. The project aims to unbiasedly test novel CRISPR/Cas-based strategies to correct splicing defects due to common *ABCA4* variants, thereby restoring correct *ABCA4* mRNA processing and protein translation.

Individual minigene models harboring the common *ABCA4* c.5461-10T>C, c.5461-10T>G, c.5196+1013A>G, c.5196+1056A>G, and c.768G>T variants were cloned and validated in HEK293T. The occurrence of pathogenic splicing for these variants was confirmed by mRNA splicing analysis. To generate stable cell lines expressing the variants of interest, we then created two lentiviral "landing pad" vectors that allow the cloning of validated *ABCA4* fragments downstream of an antibiotic resistance marker and upstream of a fluorescence protein. The expression of the fluorescent protein will only occur if correct splicing takes place, streamlining the validation of CRISPR/Cas approaches by flow cytometry analysis. Next, a gRNA library composed of all possible single gRNAs encompassing sequences involved in faulty splicing will be generated. The established cell line expressing the splicing defects will be transduced with the gRNA library. Finally, novel Cas variants will be unbiasedly screened to assess their effectiveness in correcting splicing defects in combination with all designed gRNAs. Lead gRNA/Cas combinations will be further validated.

Our study found that mutant *ABCA4* minigenes had mRNA missplicing in HEK239T cells, consistent with prior research. The c.5461-10T>C and -10T>G mutations excluded exons 39 and 40, shortening the *ABCA4* transcript. The c.768G>A mutation elongated exon 6 by 36bp, while c.5196+1013A>G and c.5196+1056A>G mutations created pseudoexons within intron 36. The developed lentiviral “landing pads” vectors have the capability to multi-clone the validated *ABCA4* minigenes, which can be efficiently transduced into HEK293T cells to ease the screening of editing strategies.

This study provides a robust platform for consistently and objectively evaluating the efficacy of genome editing strategies in correcting splicing defects. By using clinically viable editing designs, the obtained results will have a high potential for translation into effective therapeutics to treat Stargardt disease.

Moderate PLVAP deficiency has no effect on the supply of RPE and photoreceptors by the choriocapillaris

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Purpose: In pathogenesis of geographic atrophy (GA) it is controversially discussed in which order retinal pigment epithelium (RPE) and choriocapillaris (CC) degenerate. We follow up on the hypothesis that primary impairment of CC causes secondary changes in RPE that will cause photoreceptor degeneration as seen in GA.

Methods: We used 4-weeks-old CAGG-Cre-ER/*Plvap*$^{fl/fl}$ mice and their *Plvap*$^{fl/fl}$ littermates of C57BL/6J background and induced a deficiency of plasmalemma vesicle-associated protein (PLVAP) via tamoxifen administration (5mg/ml 3x/d for 5 days). PLVAP forms diaphragms bridging fenestrated endothelium of the CC, thus its loss presumably results in CC-impairment. The animals were analyzed at an age of 8 or 12 weeks. PLVAP protein-synthesis was evaluated by Western blot analysis. The distribution of PLVAP-loss was observed by CC flat-mounts stained against PLVAP and PLVAP-positive area was quantified. The effect of PLVAP loss on CC fenestration was analyzed and quantified via transmission-electron-microscopy of 12-weeks-old mice. RPE-morphology was analyzed by CC/RPE flat-mounts stained against PLVAP and the tight junction protein Zonula occludens-1 of 12-weeks-old mice. Viability and health of RPE and sensory retina was evaluated by TdT-mediated dUTP-biotin nick end labeling (TUNEL) of sagittal-sections of 8- and 12-weeks-old mice and morphometric analysis of inner and outer nuclear retinal layers of 12-weeks-old mice.

Results: Western blot data verified that PLVAP protein-synthesis was significantly reduced to 42% in 8-weeks-old and to 30% in 12-weeks-old CAGG-Cre-ER/*Plvap*$^{fl/fl}$ mice in comparison to control littermates. CC flat-mounts revealed a not equally distributed PLVAP-loss, which was concentrated on 24% of the CC in 12-weeks-old mice, while 8-weeks-old mice showed no significant difference. PLVAP-deficient mice showed single capillaries without diaphragms and the calculated ratio of diaphragms/µm tended to be reduced to 80%, but the difference was not significant. RPE/CC flat-mounts showed a normal RPE morphology above PLVAP-deficient areas and demonstrated, that these areas are rather small and just as big as an overlying RPE cell. TUNEL assay and morphometric analysis of the retina showed no increased apoptosis or damage of RPE and sensory retina caused by PLVAP-deficiency.

Conclusions: 8 weeks after inducing PLVAP-deficiency, the PLVAP protein-level was reduced to 30% and this loss was concentrated on 24% of CC. This moderate extend of PLVAP-deficiency was not sufficient to cause damage of RPE and sensory retina as seen in GA.

Efficacy of a complement modulating gene addition therapy in the retinal ischemia/reperfusion model

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Purpose: Degenerative retinal diseases may have a variety of different causes, but come along with a similar inflammatory reaction, including activation of the complement cascade as part of the innate immune system. Inappropriate regulation of the complement system can cause further tissue damage. We aim to investigate if the expression of a complement inhibitor in the retina can decelerate degeneration in an ischemia mouse model by rebalancing overshooting complement activation. We consider Complement Factor H (CFH), the main inhibitory complement regulator, as a promising candidate to dampen excessive complement activation. Delivered into the eye by the injection of an adeno-associated viral (AAV) vector, a shortened, functionally optimized version of CFH (miniFH) was expressed directly in the immune privileged retina. We specifically addressed Müller cells for exogenous protein expression by using the Müller cell specific ShH10 capsid and limited the expression of the construct to Müller cell gliosis with the use of a GFAP promoter. This approach aims to limit retinal degeneration to a minimum, counteracting secondary complement-driven inflammation.

Methods: In this retinal ischemia/reperfusion (I/R) model, intraocular pressure is raised above systolic arterial blood pressure to occlude ocular blood flow for 60 minutes, followed by reperfusion. Eyes were intravitreally injected with AAV immediately after I/R surgery and analyzed 3 days and 14 days post ischemia (dpi). We used morphometric analysis in combination with cell death detection assays to evaluate cell survival and retinal integrity. Complement protein distribution, Müller cell gliosis and microglial activity were assessed by immunohistochemistry.

Results: By immunohistochemical staining, we were able to demonstrate EGFP reporter expression only in Müller cells. This was evidence of transgene expression specifically confined to our target cells. Although promising trends were observed, the analysis of retinal integrity and cell death by TUNEL staining did not show a significant protective effect on cell survival after ischemia. In particular, the thicknesses of the IPL, INL, and OPL seemed to be better preserved in miniFH-treated postischemic retinas at 14 dpi. Further histologic evaluation revealed that FH staining was predominantly localized to microglia in miniFH-treated ischemic eyes at 14 days. Astrocytes stained positive for C3 predominantly 14 days after ischemia. GFAP immunoreactivity in Müller cells of ischemic retinas was not altered by miniFH treatment.

Conclusions: We have successfully established an AAV-vectorized overexpression system for miniFH that allows effective and specific transduction of Müller cells. MiniFH gene addition resulted in trends toward improved cell survival in the acute and relatively severe model of I/R retinal

injury. Future studies should further validate the potential of this novel gene addition approach for its potential to slow down progression of multifactorial or inherited retinal diseases associated with complement overactivation.

Retinal degeneration, neuroprotection, and FGF2: Identifying the links

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Purpose: Retinal degenerative diseases, such as retinitis pigmentosa (RP), affect millions of people worldwide, and all experience partial or total blindness. So far, treatment options are limited, but gene therapy offers great hope for a cure. A previous study in an RP gene therapy mouse model *(Pde6b$^{Stop/Stop}$)* demonstrated that outer segments (OSs) of photoreceptors rescued at mid-disease stages do not recover their full pre-disease length. Thus identifying factors that support OS biogenesis is an important research priority. One candidate is fibroblast growth factor 2 (FGF2), which has been shown to enhance photoreceptor survival in preclinical studies. Therefore, this study aims to investigate whether FGF2 supports OS biogenesis and to analyze the mechanisms underlying photoreceptor protection by FGF2.

Methods: In the context of RP, FGF2 was studied on RNA and protein levels, and by immunohistochemistry staining of retinal sections at different disease stages. Further, *Fgf2$^{-/-}$* retinas and *Fgf2$^{-/-}$ Pde6b$^{Stop/Stop}$* double mutant retinas were characterized. Additionally, we performed absolute quantification to determine the absolute transcript copy number of each of the four FGF receptors.

Results: FGF2 is expressed in Müller glia cells in healthy retinas and predominantly binds to the tyrosine kinase receptor FGFR1. In *Pde6b$^{Stop/Stop}$* retinas, FGF2 becomes dramatically upregulated and is additionally expressed in rod photoreceptors. Interestingly, an FGF2 isoform shift was observed, from the high molecular weight (HMW) isoforms in WT to the low molecular weight (LMW) isoform in RP. To understand the physiological significance of FGF2 in the retina, important features of *Fgf2$^{-/-}$* retinas were characterized. Outer nuclear layer thickness, OS length, and cone density were not significantly different compared to the control. To unravel the role of FGF2 in the context of RP, we investigated *Fgf2$^{-/-}$Pde6b$^{Stop/Stop}$* double mutant mice. Our data showed that important morphometric features of those double mutant retinas were similar to those of *Pde6b$^{Stop/Stop}$* retinas. Further, we detected an upregulation of vascular endothelial growth factor b (VEGFb) in *Fgf2$^{-/-}$ Pde6b$^{Stop/Stop}$* double mutant retinas.

Conclusions: In this work, we studied the effect of FGF2 on photoreceptor survival in RP. We showed a massive upregulation of LMW-FGF2 in rod photoreceptors of *Pde6b$^{Stop/Stop}$* mice. Surprisingly, we discovered no detectable effects on retina morphology in *Fgf2$^{-/-}$ Pde6b$^{Stop/Stop}$* double mutant mice compared to *Pde6b$^{Stop/Stop}$* mice. We hypothesize that the loss of FGF2 was compensated by an upregulation of VEGFb. To test this, we will perform a CRISPR/Cas9-mediated knockdown of *Fgfr1*.

Identification of ciliary WNT inhibitors to improve RPE maturation and function

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Purpose: The retinal pigment epithelium (RPE) between the retina and choroid of the vertebrate eye is essential for visual function. iPSC-derived RPE tissue is currently being developed as a treatment for ocular diseases including ciliopathy-associated retinal degeneration. We previously found that the primary cilium regulates RPE development via modulation of WNT signaling which can be manipulated to improve iPSC-RPE maturation and function. Identifying novel compounds that expedite and improve this differentiation process is an important aspect of translational research since current inhibitors are unspecific, ineffective and little is known about their precise mode of action. Here, we developed a screening pipeline based on a fungal compound library to identify novel inhibitors of the WNT signaling pathway to specifically improve maturation of iPSC-derived RPE.

Methods: We investigated WNT inhibition and cytotoxicity via Luciferase- and MTT assays and purified promising compounds by mass spectrometry and high-performance liquid chromatography. Promising hits were further validated in different model systems by RT-qPCR and Western blot to analyze the expression of WNT compounds after treatment. To investigate the effects on RPE maturation, we used iPSC-derived RPE cells and treated them during the final step of differentiation using different concentrations and treatment periods.

Results: We identified a particularly promising candidate that displayed strong WNT inhibitory activity with minimal toxicity. Treatment resulted in downregulation of gene expression of all tested WNT targets in different model systems. Control iPSC-derived RPE cells showed enhanced maturation, even compared to treatment with the known WNT inhibitor Endo-IWR1. We observed improved RPE gene expression and polarization. Timing and concentration of application resulted in phenotypic changes.

Conclusions: In summary, we have established a screening pipeline that has enabled us to identify a new promising WNT inhibitory drug from a fungal library. Since we have screened it specifically for use in RPE, a potential application would be enhanced maturation of iPSC-RPE and treatment of ocular diseases such as retinal degeneration, age-related macular degeneration, and diabetic retinopathy. In future we will apply this compound to ciliopathy-patient-derived iPSC to determine whether we can rescue the disease phenotype.

Visual function is gradually restored during retina regeneration in adult zebrafish

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Purpose: In comparison to mammals, zebrafish are able to regenerate many organs and tissues, including the retina. Light lesions result in a loss of photoreceptors and the subsequent activation of Müller glia, the retinal stem cells. Müller glia-derived progenitors differentiate and eventually restore the anatomical tissue architecture within 4 weeks. However, little is known about how light lesions impair vision functionally, as well as how and to what extent visual function is restored during the course of regeneration, in particular in adult animals.

Methods: Here, we applied quantitative behavioral assays to assess visual function during homeostasis and regeneration in adult zebrafish. We developed a novel vision-dependent social preference test that allows the analysis of basic visual functions such as the detection of biological motion. Furthermore, we employed a quantitative optokinetic response assay with different degrees of difficulty, similar to vision tests in humans, that enables the assessment of more advanced visual functions. Finally, we compared the results of the functional analyses to morphological data obtained using classical histology as well as optical coherence tomography.

Results: With the aid of the newly developed behavioral assays, we could show that vision is massively impaired early after lesion. While basic visual functions were already restored within 7 days, the recovery of more advanced visual functions occurred gradually within 10 to 28 days. We found that vision under easier conditions with high contrast and low level of detail, as well as color vision, was restored around 7–10 days post lesion. Vision under more demanding conditions, with low contrast and high level of detail, was regained only later from 14 days post lesion onwards. Comparison of the obtained behavioral and morphological data revealed a close correlation between functional and morphological recovery and indicates that functional recovery is already observed before the completion of morphological restoration.

Conclusions: Taken together, we conclude that functional vision including contrast sensitivity, spatial resolution and the perception of colors is gradually but fully restored after light lesion in adult zebrafish.

Fluorescence Lifetime Imaging (FLIM) as a powerful tool to study Müller cell metabolism in health and disease

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Purpose: Müller cells perform a variety of functions in the retina, including maintaining a homeostatic environment. Here we analyzed the Müller cell metabolism in health and disease using transient retinal ischemia as an in vivo lesion model. We were also interested in the translocator protein (TSPO) – a mitochondrial membrane protein that has been implicated in a variety of cellular processes, including cholesterol transport and steroid synthesis. We hypothesized that the loss of TSPO in Müller cells also alters the metabolic state. Using Müller cell-specific TSPO Knockdown (KD) mice, we studied whether absence of TSPO altered the Müller cell metabolism and their metabolic response to tissue damage due to transient retinal ischemia.

Methods: We used two-photon excitation (DIVE) at 760 nm to excite NAD(P)H. The fluorescence lifetimes of free and protein-bound NAD(P)H are distinct, allowing a ratio between the two fractions to be calculated. A high free to bound NAD(P)H ratio (FBR) indicates a high rate of glycolysis, whereas a low FBR is thought to be associated with relatively high levels of OXPHOS. Freshly isolated Müller cells from wild-type and Müller cell-specific TSPO KD retinas 7 days after ischemia induced by transient elevation of IOP for 60 minutes were assayed in standard physiological buffer and again after 10 minutes with or without stimulation with 2-deoxy-D-glucose, an inhibitor of glycolysis, or pyruvate, a substrate that promotes OXPHOS.

Results: Establishing FLIM-based NAD(P)H imaging in wild-type mouse Müller cells first, we found that the NAD(P)H FBR was stable in baseline measurements and decreased upon stimulation with either 2-deoxy-D-glucose or pyruvate, as metabolism was shifted toward OXPHOS by both stimulants. Furthermore, a shift to a higher FBR was found in Müller cells from TSPO KD mice, indicating a higher rate of glycolysis. This may be indicative of impaired mitochondrial functionality and a putative role of TSPO in mitochondrial health and metabolism. Ischemia was shown to dramatically decrease FBR of Müller cells from TSPO KD mice, suggesting a higher energy demand after lesion. In the wild-types, no difference in baseline measurements was observed between cells from ischemic and non-ischemic eyes, likely due to the already high rate of OXPHOS.

Conclusions: FLIM-based NAD(P)H imaging is a useful tool for the investigation of the metabolic state of Müller cells and has allowed for the determination of the influence of 2-deoxy-D-glucose and pyruvate, respectively, on the metabolic state of wild-type Müller cells. In addition, the influence of specific genetic (TSPO-KD) or pathological (ischemic) alterations could be successfully studied and it could be shown that the energy supply of gliotic Müller cells is ensured by an increase in glial OXPHOS depending on functional TSPO.

CCN2/CTGF affects vessel loss and neovascularization in the oxygen-induced retinopathy (OIR) model

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Purpose: Retinopathy of prematurity is characterized by the formation of retinal neovascularization. These immature blood vessels are hyperpermeable and leaky, leading to ischemia in the surrounding tissue, and finally cause a degeneration of retinal neurons. The matricellular protein Cellular Communication Network Factor 2/Connective Tissue Growth Factor (CCN2/CTGF) is highly expressed in the mouse retina and critically required for retinal angiogenesis. Here we addressed the question whether CCN2/CTGF is involved in the formation of pathologic angiogenic processes as seen in retinopathy of prematurity.

Materials and methods: We used the oxygen-induced retinopathy (OIR) model to investigate the influence of CCN2/CTGF on retinal neovascularization. Therefore, dam with pups were exposed to 75% oxygen from P7 to P12 and afterwards returned to room air up to final analysis. Two different mouse models were used in course of the experiments: TheβB1-CTGF1 mouse model showing a lens-specific CCN2/CTGF overexpression, and the conditional inducible CAGC-Cre-ER^{TM}CTGF$^{Coin/Coin}$ mouse model. In the CAGCCre-ER^{TM}CTGF$^{Coin/Coin}$ mouse model CCN2/CTGF deficiency was induced by the application of tamoxifen-containing eyedrops (5mg/ml; 3x per day for 5 days). Wildtype CD1 littermates as well as CTGF$^{Coin/Coin}$ littermates were used as the control groups. Retinal flat mounts were visualized under the fluorescent microscope and area of vessel loss, of neovascular tufts and the deep vascular plexus of both mouse models and their control littermates were quantified and plotted as percentage of the total retinal area.

Results: βB1-CTGF1 mice showed a significantly smaller avascular area and area of neovascular tufts, in comparison to wildtype CD1 littermates. Additionally, the pro-angiogenic properties of CCN2/CTGF support the growth of the deep vascular plexus, resulting in a larger area of these intraretinal capillaries in βB1-CTGF1 mice compared to age-matched wildtype littermates. Finally, we could show that the avascular area was significantly increased in CAGCCre-ER^{TM}CTGF$^{Coin/Coin}$ mice compared to control littermates.

Conclusions: The results imply a crucial role of the levels of CCN2/CTGF in pathologic angiogenic processes in the OIR-model as the overexpression of CCN2/CTGF attenuates vessel loss and the formation of neovascularization. CCN2/CTGF thereby supports the growth of the deep vascular plexus, whereas the lack of CCN2/CTGF inhibits the regrowth of retinal vessels into the avascular area.

Desialylation as a trigger of microglial responses in the aging and degenerating retina

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Introduction: Age-related retinal degenerations are the major cause for vision loss and blindness. Hallmark of these diseases are chronically activated microglia due to a pro-inflammatory environment. Main contributors to the progression of retinal degeneration are reactive oxygen species (ROS) and complement components (C1q, C3) produced and released by microglia. The complement system and microglial responses are controlled by various factors with sialylation being one major checkpoint. Among aging, the amount of sialylation significantly reduces in humans, but the relevance for complement- and microglia-associated diseases including age-related macular degeneration (AMD) is unclear.

Purpose: This study aims to investigate the effect of reduced sialylation on retinal microglia response in a mouse model of induced retinal degeneration. For this purpose, Gne+ x Cx3cr1$^{GFP/WT}$ mice will be used in this study. Gne stands for UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase and catalyzes two reactions of the sialic acid synthesis in the cytosol, making Gne the key enzyme of sialic acid biosynthesis. Gne$^{+/-}$ mice show a sialic acid, specifically polysialic acid, reduction of 25% in many different tissue types including the murine retina. Additionally Cx3cr1$^{GFP/WT}$ allow for noninvasive *in vivo* monitoring of microglia.

Methods: Gne$^{+/-}$ x Cx3cr1$^{GFP/WT}$ mice are exposed to bright white light with an intensity of 10.000 and 15.0000 lux. Non-invasive spectral domain optical coherence tomography (SD-OCT) and confocal scanning laser ophthalmoscopy (SLO) are used to analyze microglial response and retinal thickness after light exposure. The sialylation state and microglia morphology is analyzed by *in vivo* fundus imaging and retinal flat mounts. Microglia activation marker expression in whole retinal transcripts is determined by qRT-PCR.

Preliminary studies: It was shown previously that white light damage induces retinal degeneration and creates an environment, which reactivates microglia. Moreover, in the retina of 9-month-old Gne$^{+/-}$ mice, sialylation was reduced and microglia density was increased as well as gene transcription of pro-inflammatory factors.

Small molecules, big effects: Inhibition of neovascularisation by GpC-NPs

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Purpose: Neovascularisation involves the formation of new blood vessels, which can be triggered by pathological processes in the eye. Neovascularisation is a characteristic feature of diseases such as wet age-related macular degeneration (AMD) and diabetic retinopathy. leading to visual impairment. Currently, there is no curative treatment option available for neovascular AMD. CpG oligodeoxynucleotides (CpGs) are short unmethylated DNA strands containing multiple cytosine-guanosine motifs. CpGs have demonstrated anti-angiogenic potential in ophthalmic research, making them a potential future drug candidate against neovascular AMD. The aim of this project was to investigate the anti-angiogenic effect of CpGs bound to lipid DNA nanoparticles (CpG-NP) compared to unbound CpGs.

Methods: The lipid DNA nanoparticles (NPs) developed by our laboratory can be easily loaded with various substances, thereby increasing the efficacy of the coupled substances. To investigate the effect of the NPs on CpGs, firstly a tube formation assay with human umbilical vein endothelial cells (HUVECs) was performed, in which CpG-NPs were compared to pristine CpGs. Additionally, the efficacy of CpGs and CpG-NPs in a retinal and choroidal angiogenesis model was tested using porcine eyes. To induce sprouting, 10 ng/mL VEGFA was added to the organ cultures. After 7-10 days, the growth of vascular structures was visible, and treatment with CpGs or CpG-NPs was initiated. The influence of CpGs or CpG-NPs on the outgrowths, as well as the expression of angiogenic markers (VEGF, Ang1, Ang2, bFGF, CD31) was determined in the organ cultures. Furthermore, the distribution and adhesion of CpGs by intravitreal injection of fluorescently labelled particles into porcine eyes (ex vivo) was investigated. In initial in vivo experiments with C57BL/6-mice, the animals received intravitreally applied CpGs or CpG-NPs as treatment after laser-induced choroidal neovascularization, and the lesion-size was determined after 7 days.

Results: Treatment with CpG-NPs significantly reduced tube formation in human endothelial cells. Two-day treatment of retinal explants with CpGs or CpG-NPs resulted in regression of sprout formation, with CpG-NPs showing a stronger anti-angiogenic effect. Likewise, the expression of various angiogenesis markers was significantly reduced in organ cultures after treatment with CpGs or CpG-NPs. In addition, uptake and adhesion to the retina were significantly improved in the CpG-NPs group compared to free CpGs. The first in vivo experiments in a CNV mouse model indicated that CpGs or CpG-NPs can modulate the healing process. Both DNA motifs reduced the expression of the lesions, whereby the NP-bound CpGs showed a significantly better effect.

Conclusions: In this study, CpG-NPs had a better anti-angiogenic effect compared to free CpGs. Furthermore, the adherence of CpG-NPs to the retina ex vivo was significantly higher than the adherence of free CpGs. Therefore, CpG-NPs are a promising potential anti-angiogenic treatment

option for neovascular-retinal diseases. Further in vivo studies are needed to confirm these first results.

Investigating the role of nitric oxide signaling in retinal ganglion cell degeneration after optic nerve transection

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Purpose: Retinal ganglion cells (RGCs) are the sole output neurons of the retina and thus integrate and transmit all visual information to the brain. RGC degeneration is the root cause of the disease Glaucoma, the second leading cause of blindness worldwide. Glaucoma is linked to damage of the optic nerve, *i.e.* damage to RGC axons. The underlying cellular mechanisms causing RGC degeneration are largely unclear, yet, this has been connected to an excessive production of the endogenous signaling molecule nitric oxide (NO). NO is produced by the enzyme nitric oxide synthase (NOS) and activates soluble guanylate cyclase enzyme (sGC), which subsequently produces the second messenger, cyclic guanosine monophosphate (cGMP). cGMP in turn activates protein kinase G (PKG). Earlier data from our group found that PKG inhibitors can prevent RGC degeneration after optic nerve transection. The aim of the present study was to investigate the role of NO/sGC-signaling in RGC loss.

Methods: To mimic the optic nerve damage suspected of causing RGC degeneration, organotypic retinal explants from wild-type post-natal day 12 mice were cultivated for different time periods after optic nerve transection (0, 0.5, 6, 12, 24, and 48 hours (h)). Retinal cross-sections were then stained using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL; to identify dying cells), RNA-binding protein with multiple splicing (RBPMS) immunostaining (to detect RGCs), and NADPH diaphorase assay (to detect active NOS), followed by microscopic analysis.

Results: The microscopic analysis of axotomized retinae at 0 and 0.5 h after optic nerve transection already identified an elevated activity of NOS, while cell death as assessed by the TUNEL assay was still very low. Furthermore, a high number of RBPMS positive cells were detected at these time points. After 6 and 12 h the activity of NOS was still high. At the same times the numbers of TUNEL positive cells started to rise, while the numbers of RBPMS positive cells decreased significantly. After 24 h, NOS activity and RBPMS positive cells decreased, whereas TUNEL positive cells reached a peak. Finally, after 48 h NOS activity, as well as RBPMS and TUNEL positive cells were almost undetectable.

Conclusions: Our data suggest an involvement of NO-signaling in RGC degeneration after optic nerve transection. Indeed, we found that NOS was highly active right after RGC axotomy, *i. e.* at a time which precedes RGC death. Overall this study makes it seem likely that the entire NO/sGC/cGMP/PKG signaling pathway may be involved in RGC death after optic nerve damage.

The long-term cultivation of cells of the retinal pigment epithelium on nanofibre nets

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Objective: Dead or defective retinal pigment epithelium (RPE) must be replaced to maintain the function and viability of the photoreceptors. One possibility is to cultivate RPE cells on a flexible polymeric carrier material on which it can be brought into the subretinal space in an orderly fashion. In the context of such an approach, special nanofibre meshes were tested for their suitability for the cultivation of RPE cells, with the focus on long-term cultivation.

Methods: Nanofibre nets made of polycaprolactone (PCL) or a co-polymer of PCL and collagen were prepared by using the method of electrospinning. Isolated RPE cells from pig eyes were cultured and after achieving a stable culture applied as a suspension onto the nanofibre meshes. Simultaneous culturing of RPE cells on collagen-coated plastic bottoms of the culture vessels served as a control reference. The cells of the reference cultures and the net cultures were regularly photographed using a microscope and quantitatively and morphologically evaluated. To detect release of inflammatory markers in an ELISA, supernatants of the cell cultures were taken at regular intervals.

Results: RPE cells could be cultivated well on the nanofibre nets using an optimised protocol. Within the observation period of 6 to 9 months, the cell density of the cultures remained relatively constant. While the cell density of the reference cultures had a cell density of approx. 900 to 1300 cells/mm^2, the cell density on the PCL-nets as well as on the PCL collagen nets was approx. 800 to 1000 cells/mm^2. During the observation period, the number of cells practically did not change. The morphology of the cells on the meshes remained constant, and no loss of pigmentation could be detected. RPE cells released IL-6 and TNF-α during the first days of cultivation. Whereas TNF-α could not be detected any more during the following months, RPE cells still release IL-6 on a low level.

Conclusions: RPE cells could be grown on the nanofibre networks over the unusually long period of half a year and beyond without major loss of cell density. Further investigations now relate to the release of other cytokines, the correct polarisation of the RPE cells and their functional properties.

Impact of healthy rods on neighboring mutant rods in a partial rescued retinitis pigmentosa mouse model

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Purpose: Retinitis pigmentosa (RP) is a group of genetic disorders and the most common inherited retinal dystrophy that leads to the loss of vision. It is caused by mutations in rod-specific genes, eg, Phosphodiesterase 6b (*PDE6B*), leading to patchy photoreceptor degeneration. Non-cell autonomous effects triggering loss of neighboring cells have been investigated previously. In this study, we evaluate the effect of healthy rods on neighboring mutant rods.

Methods: PDE6B expression was partially rescued in an RP mouse model ($Pde6b^{STOP/STOP}$) by Pax6αCre. Pax6α is a transcription factor expressed in progenitor cells that give rise to neurons in the peripheral retina. Visualization of Cre recombination was achieved using $ROSA^{nT\text{-}nG}$ or $ROSA^{mT\text{-}mG}$ reporter mice. We used immunofluorescence, *in situ* hybridization, FAC-sorting, qRT-PCR and western blot to investigate the effects of partial rescue on mutant photoreceptors.

Results: $Pde6b^{STOP/STOP}$, *Pax6αCre* mice have retinas with large, roughly segregated zones of recombined (GFP-positive) cells in the periphery and non-recombined (tdTomato-positive) cells in the center of the retina. At 4 weeks of age, PDE6B immunolabelling was present in GFP-positive rods and absent in tdTomato-positive rods. At 8 weeks of age, PDE6B was present in tdTomato-positive rods bordering the GFP-positive rods. Over time, PDE6B expression stepwise extended to the center causing the formation of a gradient in outer nuclear layer thickness. Importantly, Cre protein and Cre RNA were absent in adult photoreceptors at any time point. Next, RNA and DNA of PDE6B and tdTomato double positive cells were then analyzed after FAC sorting. Presence of Pde6b RNA was shown by qRT-PCR, while PCR of *Pde6b* DNA revealed both mutant and recombined alleles.

Conclusions: Partially rescued RP retinas have a graded outer nuclear layer thickness between recombined and non-recombined zones due to stepwise PDE6B expression over time. The communication between healthy and mutant rods plays a critical role during disease progression.

Early physiological changes of glia cells in a pig model for diabetic retinopathy

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Purpose: The metabolic changes associated with diabetes, such as hyperglycemia and insulin resistance, lead to vascular pathologies like stroke and coronary heart disease. In addition, diabetic retinopathy (DR) is a common comorbidity, resulting in breakdown of the blood-retinal barrier, vascular leakage, hemorrhage, ischemia, and eventual blindness. In this study, we used the *INS^{C94Y}* pig model of neonatal diabetes to study DR, not only because of its similarity in eye size and cone density to humans, but also because it closely mimics the symptoms of late-stage disease. We focused on the role of glial cells, especially Müller cells, and their role in the dysregulation of the neurovascular unit.

Methods: To gain a deeper understanding of the molecular and metabolic changes in different retinal cell types, we isolated Müller cells, microglia, and retinal neurons from 8- and 24-months-old diabetic and wild-type animals, distinguishing between cone-rich central and peripheral retina, and used them to generate proteomic datasets. Furthermore, we investigated the ability of Müller cells to withstand osmotic stress in acute retinal slices. Finally, the JC-1 assay was used to test the mitochondrial membrane potential and, by proxy, the health of individual Müller cells.

Results: Already in cells from 8-months-old animals, we found changes in the proteomic landscape related to mitochondrial metabolism. The proportion of differentially regulated proteins increased even more in cells isolated from 24-months-old animals. Interestingly, neurons seemed to respond earlier to disease progression, while Müller cells showed a stronger response in older animals. As a result, we did not find a difference in Müller cell volume regulation at 8 months. However, at this age, the mitochondrial fitness of Müller cells was already dramatically reduced indicating early metabolic changes.

Conclusions: Here we confirmed the validity of the *INS^{C94Y}* pig model for the study of DR. Our results suggest that molecular and metabolic changes in Müller cells can be detected at an early stage of disease progression. Further studies are needed to elucidate their role in older animals. The combination of these findings may help to develop new strategies and therapies to overcome the blinding effects of DR.

Polygenic risk scores (PRS) for age-related macular degeneration (AMD) in routine DNA testing

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Purpose: Age-related macular degeneration (AMD) is the most common cause of visual impairment and blindness in industrialized countries. Due to its complex aetiology routine DNA testing is challenging. Based on a plethora of genome-wide association studies (GWAS), polygenic risk score (PRS) calculations have entered the field of complex diseases including AMD. Here, we report on an established PRS algorithm which can be incorporated in a genetic counselling setting to estimate more accurately genetic AMD risk.

Methods: A well-established PRS model was used to count individual risk alleles of 47 out of 52 known AMD risk variants as reported by Fritsche and colleagues (2016). The risk alleles were weighted with the respective effect size. A PRS-to-disease-risk prediction for individual patients considering their respective age and gender was performed by applying a recently published machine learning approach, known as Mondrian Cross-Conformal Prediction (MCCP). The MCCP output reports a predicted disease status according to PRS, age and gender with a corresponding credibility value. Validation was performed with the data set of the International AMD Genomics Consortium (IAMDGC).

Results: Cross-validation with random samples and different sampling sizes confirmed a local training cohort of 1,667 AMD patients and 1,148 healthy controls to generate reproducible prediction results. Overall, female gender was more likely predicted to be afflicted by AMD than male gender. Furthermore, prediction outcome was highly related to age. The older the person tested the more likely the person was predicted to have an AMD condition. However, when the studied individuals were divided into PRS quintiles, this trend was shown only for the three middle PRS quintiles (quintiles 2 to 4). For individuals with a particularly low (quintile 1) or high (quintile 5) risk, a reliable predictive power was obtained regardless of age and gender.

Conclusions: The combination of PRS and a machine learning approach for a PRS-to-disease-risk prediction represents a valid approach to introduce this information into genetic counselling for AMD risk. Our data show that robust conclusions can only be made for individuals at very high or very low genetic risk for AMD. The interpretation for individuals at medium risk or on the borderline of high/low risk remains arbitrary and depends strongly on the age of the patient analyzed.

Pharmacological targeting of microglia ameliorates vasculopathy associated with attenuated PDGF-B/PDGFRβ signaling in retina

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Background: Ocular vasculopathies including diabetic retinopathy (DR) and wet age-related macular degeneration (AMD) cause vision loss. While these complications are mainly associated with retinal microvasculature, neuroinflammation is manifested early on in the progression of disease. Microglia, tissue resident macrophages of the retina, regulate inflammation and maintain retinal tissue homeostasis. However, sustained physiological stress can trigger overt activation of microglia, which contributes to tissue damage. In this study, two mouse models that mimic the vascular and neural complications of DR were used to decipher the molecular and cellular components underlying the disease.

Methods: C57BL/6J mouse pups received a single s.c dose of 30µg of anti-PDGFRβ mAb (clone APB5) at P1 to block recruitment of pericytes to endothelial cells. We generated the *Pdgfb*$^{i\Delta EC}$ mice by crossing Ve-Cadherin CreER^{T2} transgenic mice with PDGFB floxed mice (Pdgfb$^{flox/flox}$). For the treatment, mice received i.p injections of minocycline (45mg/kg) once daily from P5 – P9 or P27. The retinal vascular and neural changes at P10 and P28 were determined immunohistochemically. RNAseq was used to determine DEGs and for pathway analyses whereas qRT-PCR was used to validate and quantify gene expression. The thickness of the retina and vessel perfusion and leakage were assessed by SD-OCT and fluorescein angiography, respectively.

Results: Blocking the PDGFB/PDGFRβ signaling pathway had variable impacts on the mouse retina. Inadequate pericyte cover and loss of PDGFB produced an altered vascular network at P10 and P28, reminiscent of DR. Further, we observed microglia activation and impairment of retinal structure. Key biomarkers of inflammation and microglia activation such as *Ccl2, Inos, Lgals3, Tspo* and angiogenic factors *Pgf* and *Icam1* were upregulated in the diseased retina at P10. RNAseq identified *Edn2, Glycam1* and *Fgf2* as key factors driving pathology in adult mice (P28). Further, reactive gliosis and leaky vasculature were observed in the adult mice. Treatment with minocycline attenuated the expression of cytokines and angiogenic factors, reduced vascular leakage and preserved retinal structure.

Conclusions: Minocycline partially attenuates retinal inflammation and vascular abnormalities in mice with an ocular phenotype reminiscent of diabetic retinopathy.

Conditional Müller cell-specific knockdown of TSPO highlights its neuroprotective role in a retinal ischemia/reperfusion model

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Purpose: Translocator protein 18 kDa (TSPO) is predominantly expressed by Müller cells, microglia, endothelial and pigment epithelial cells in the mature retina. TSPO is a mitochondrial membrane protein that is implicated in cholesterol transport and steroid metabolism. This could be a possible explanation for the described neuroprotective effects of TSPO-stimulating agents, which include a reduction in neuronal degeneration, anti-inflammatory effects and attenuated microglial reactivity. We hypothesize that part of this TSPO-dependent neuroprotection is mediated by Müller cells, which express the highest levels of TSPO in the healthy retina (Mages et al., 2019). In order to confirm this hypothesis, we have generated a conditional TSPO knockdown (KD) mouse line specific for Müller cells and analyzed the survival of neurons and the inflammatory tissue response to retinal ischemia in this model.

Methods: First, we established and validated the newly generated Glast-Cre^{ERT2}, TSPO$^{fl/fl}$ mouse line which allows Müller cell-specific deletion of TSPO upon tamoxifen injection. Next, we performed morphometric measurements of retinal layers and immunohistochemistry to study neuronal subpopulations to elucidate the consequences of TSPO loss in Müller cells in the context of ischemic retinal damage. As TSPO-KD may also affect the inflammatory tissue response, we examined Müller cell gliosis and retinal microglial morphology.

Results: Our recombination protocol results in a 50% reduction of TSPO specifically in Müller cells, but does not lead to a reduction of TSPO expression in microglia or endothelial cells. We observed a general thinning of the retinal layers after ischemia/reperfusion and, importantly, a more pronounced neurodegeneration in the post-ischemic retina of Müller cell-specific TSPO KD animals. Detailed analysis of neuronal subtypes revealed the strongest effects of glial TSPO KD on ganglion and horizontal cell survival. Furthermore, Müller cell-specific TSPO KD does not appear to alter microglial response, consistently there was no change observed in Müller cell GFAP expression.

Conclusions: Müller cells specific TSPO seems to be a critical factor for Müller cells to support the survival of neurons after retinal ischemia. This substantiates our initial hypothesis and, together with previous literature showing that microglia-specific TSPO ablation is beneficial in a laser-induced mouse model of neovascular AMD (Wolf et al., 2020), underscores that TSPO has very cell type-specific functions.

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Analysis of hiPSC derived RPE cells cultured on nanofibrous carriers and implanted into immunosuppressed minipigs

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Purpose: Dysfunction of the retinal pigment epithelial cell layer (RPE) is primary in the pathogenesis of various eye diseases like age-related macular degeneration (AMD) or retinitis pigmentosa (RP). Loss of RPE cells during the pathogenesis leads to areas without function for which repopulation with RPE (and photoreceptors) is necessary. In this study, human iPSCs derived RPE cells cultured on nanofibrous carriers (NC) were implanted into the subretinal space of healthy pig eyes as a potential cell-based therapy for AMD or RP.

Methods: Human iPSCs (hiPSCs) derived RPE cells were grown from dermal fibroblasts of healthy patients and cultivated on ultrathin nanofibrous membranes prepared by electrospinning of poly (L-lactide-co-DL-lactide). Fourteen minipigs (8–9 months old) were used in the study supported by pre- and postoperative tacrolimus immunosuppressive therapy. The observational period lasted 1, 2, and 6 weeks, and included in vivo optical coherence tomography (OCT) of the retina, as well as post-mortem immunohistochemistry using the following antibodies: HNAA and STEM121 (human cell markers); Bestrophin and CRALBP (hRPE cell markers); ZO-1 (tight junction marker); peanut agglutining (PNA) (cone photoreceptor marker) Recoverin (labels rods and cones); PKCα (rod bipolar marker); Vimentin, GFAP (macroglial markers); and Iba1 (microglial marker).

Results: At all time points we were able to detect the implanted cells with human cell marker STEM 121 and HNAA. In two out of three eyes from each time point they build a monolayer and have round big nuclei. In most pig eyes the implanted hiPSC derived RPE cells look well integrated and tolerated by the underlying retina. They were detectable with common RPE markers CRALBP and Bestrophin and express tight junctions positive for ZO-1 antibody. Six weeks post implantation, the inner and outer segments of cone photoreceptors facing the hiPSC RPE cells recovered well from the retinal detachment. No signs of gliosis or inflammation were visible in the fundus. Some microglia activation did occur.

Conclusions: In most pig eyes the implanted hiPSC derived RPE cells look well integrated and tolerated by the underlying retina. Hence, our immunosuppressive protocol with increased doses of Tracolimus and number of applications (every 12 days) compared to previous experiments, was successful.

Longitudinal characterization of chorioretinal atrophies after subretinal gene augmentation therapy with Voretigene Neparvovec in *RPE65*-mediated retinal degeneration

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Purpose: *RPE65*-biallelic mutation-associated inherited retinal degeneration (*RPE65*-IRD) is the only IRD with an approved gene therapy: Voretigene Neparvovec (VN). Successful phase 1 – 3 and follow-up studies showed improved rod functionality. However, recent studies indicated that in some patients new or accentuated chorioretinal atrophies develop following treatment. This study aims to characterize the development, localization, and topography of such atrophies over time.

Methods: The data of all *RPE65*-IRD patients treated with VN at a single center were analyzed. All patients were assessed with multimodal imaging, including 55° fundus autofluorescence (FAF) and 55° infrared reflectance imaging (IR) at baseline and at approx. 3-, 6-, 9- and 12-months. Functional testing included best-corrected visual acuity (BCVA) and full-field stimulus threshold testing (FST). The FAF and IR images were manually segmented for the occurrence of new atrophies by two independent graders. The resulting regions were measured with pixel accuracy and converted to mm^2. From these areas, average atrophy sizes and growth rates were deduced. In addition, the localization of atrophic lesions with respect to the macula and the subretinal injection site was documented.

Results: A total of 30 eyes of 19 patients with a mean age of 24.4 ± 9.6 years at baseline were analyzed. Mean BCVA was 1.15 ± 0.7 logMAR (n = 30 eyes) before surgery and 1.1 ± 0.8 logMAR 12 months after surgery (n = 24). Of all treated eyes, after one year 10/24 (42%) developed new chorioretinal atrophies, 10/10 eyes central atrophic areas, 8/10 eyes at the injection site, and 9/10 eyes peripheral areas. At the 12M, the median atrophy area visible in the IR images was 14.2 mm^2 (n = 10). For the eyes where IR and FAF were available, the median atrophy area was 14.5 mm^2 in the IR and 15.9 mm^2 in the FAF images respectively (n = 7). FST results improved in 8/10 eyes with new atrophic lesions after surgery.

Conclusions: Treatment with VN led to sustained improvements in overall rod functionality and no significant changes in visual acuity in the first year following treatment. In some patients progressing atrophic lesions developed. Whenever FAF is not possible, IR imaging is a suitable alternative to detect and quantify these changes. Here we show, that the atrophic lesions appear larger in FAF than in IR imaging.

Colony stimulating factor 1 (CSF1) receptor blockade as novel tool to limit microglia reactivity in the light-damage model of retinal degeneration

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Purpose: Reactive microglia are commonly present in retinal degenerative diseases. They can secrete neurotoxic substances and contribute to photoreceptor cell death. However, as a fundamental part of the regulatory immune system they also have supportive effects. In this project, we aimed at studying the effects of complete microglia depletion and repopulation in a mouse model of acute retinal degeneration using the CSF1R-antagonist PLX3397.

Methods: Cx3cr1$^{GFP/+}$ reporter mice that carried the light sensitive RPE65Leu450 variant received PLX3397 or control diet prior or immediately after white light exposure to induce retinal degeneration. The effects of PLX3397 treatment were analyzed after light exposure. The number and morphology of retinal microglia was analyzed by *in vivo* fundus imaging and retinal flat mounts. Microglia activation marker expression in whole retinal transcripts was determined by qRT-PCR. OCT was used to measure retinal thickness; TUNEL, and cone staining were used to quantify the extend of retinal degeneration and photoreceptor cell death.

Results: PLX3397 treatment effectively depleted microglia in healthy and light damaged retinas. Add to this mRNA expression of the constitutive microglia marker AIF-1 was strongly increased in light damaged retinas and this upregulation was absent in mice treated with PLX3397. Expression of microglia activation marker were also upregulated after light exposure, and PLX3397 treatment prevented this induction. OCT revealed a thinning of the outer nuclear layer in light exposed retinas, and this thinning was also detected in light exposed retinas under conditions of microglia depletion. However, PLX3397 treatment could not prevent photoreceptor cell death, and even increased the amount of TUNEL positive cells in the outer nuclear layer. Repopulated microglia showed an increased response to pro-inflammatory stimuli compared to naïve microglia.

Conclusions: Retinal microglia were efficiently depleted with PLX3397 under normal and light damage conditions. However, the absence of microglia did not change the extent of retinal degeneration. Moreover, the photoreceptor debris were not phagocytosed and accumulated in the retina. Repopulated microglia also showed no reduced immune response.

Ablation of the primary cilia results in RPE functionality defects

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Purpose: Primary cilia are microtubule-based signalling organelles, defects of which lead to ciliopathies, resulting in symptoms such as retinal degeneration. Numerous ocular cell-types display a primary cilium, one of which is the retinal pigment epithelium (RPE). The RPE is a monolayer of polarised cells located between the neural retina and the vascular choroid. It has various important functions, such as the phagocytosis of shed photoreceptor outer segments (POS) and being involved in the visual cycle. Therefore, the RPE is essential for photoreceptor integrity and function. Our lab previously identified the role of primary cilia during development of the RPE, in addition to influencing RPE phagocytosis. Here we investigated the influence of an ablated primary cilium on RPE functionality in a conditional mouse model.

Methods: Loss of IFT20 results in complete ablation of the primary cilium. Therefore, we used a conditional knockout mouse model in which primary cilia are exclusively ablated in the RPE using the Cre-LoxP system. We crossed the *Ift20* floxed mice with the Tyrp2-Cre driver. Loss of primary cilia in the RPE was validated via immunofluorescence staining and retina integrity via Transmission Electron Microscopy. RPE functionality was investigated via DC-ERG response and by performing retinal adhesion and phagocytosis assays. In addition, mRNA-Sequencing was performed to examine gene expression.

Results: Deletion of *Ift20* in the RPE led to primary cilia ablation without affecting retinal development. RPE from *Ift20^{null}; Tyrp2-Cre* mice displayed a decreased ability to phagocytose POS and a reduced direct-coupled ERG response in comparison to control. In addition, retinal adhesion between the RPE and POS was decreased. Further, mRNA-Sequencing revealed mis-regulation of the transcriptome of mutant mice at various time points, including RPE functionality-related genes.

Conclusions: Recent studies identified the role of primary cilia on RPE development and maturation. With the present data, we analysed the influence of cilia on RPE function and could show that ablation of primary cilia exclusively in the RPE *in vivo* leads to functionality defects. These findings are important to take into account when designing treatment options for cilia-related ocular disorders.

The toxicity of optogenetic tools for vision restoration

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Purpose: Optogenetics has been a rapidly growing field in recent years, moving away from its original use in augmenting neural network understandings, towards clinical vision restoration therapy. Channelrhodopsin (ChR) is among the most well-known of opsins, with its derivatives having already been pushed into clinical trials for vision restorative treatments (reviewed in: 1). With Channelrhodopsins being of microbial origin, an unintended transcriptomic dysregulation or an immunological provocation in the eye by means of ReaChR introduction could prove harmful. Hence, a broader understanding of ReaChR's effects, both at the transcriptomic and immunological levels is required. In this study, we investigated the toxicity effects of ReaChR for optogenetic gene therapy in vision restoration.

Methods: Herein, 12 retinal degenerate RD1 mice were utilized; 6 of which expressing ReaChR in ON-bipolar cells via Cre-Flox (Pde6brd1/rd1.Grm6iCre/wt.Rosa26loxReaChR/loxReaChR [2]) while the remaining 6 (littermates) lacking Cre, acted as a control (Pde6brd1/rd1. Grm6wt/wt.Rosa26loxReaChR/loxReaChR). The retinas of each were extracted and subject to RNA-sequencing (NovaSeq6000). Reads were aligned using STAR, differential expression analyses were performed using R and DESeq2, and GO analyses were carried out in GOseq.

Results: A median read depth of 29.99 [IQR: 28.27-33.85] million reads per sample was achieved. One sample had to be disregarded from the analysis for high percentage of low quality reads. Interestingly, despite the relatively high read depth, only a limited number of genes were identified as differentially expressed (DE) even at low threshold. Nevertheless, read counts of the tope DE genes strongly correlated with the read counts observed for ReaChR supporting the idea of a possible causal relationship. Gene Ontology pathway analyses indicated that genes related to cell-substrate adhesion (GO term 0010812) were over-represented amongst the DE genes. Conversely, averaged read counts of the genes summarized under that GO term ReaChR gene counts ($R = 0.57$ $p < 0.005$).

Conclusions: These findings suggest that ReaChR may induce transcriptomic discrepancies, mostly related to cell adhesion. Disruptions in cellular adhesion have long been implicated as carrying a considerable role in retinal remodelling (3). Whether the observed changes represent deleterious remodelling or even reflect ReaChR-induced protection against remodelling is yet unknown. In ongoing studies we now aim to clarify these results using immunohistochemical techniques and furthermore focus on transcriptomic changes inside ReaChR expressing cells.

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Characterisation of *Egr1* activation in the Rho$^{P23H/+}$ mouse model of Retinitis Pigmentosa

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Retinitis Pigmentosa is a large heterogeneous group of inherited retinal diseases linked to different genetic mutations in rod-specific genes, thus triggering a cascade of cell death affecting rod photoreceptors first, and later also cone photoreceptors, leading eventually to complete blindness.

Egr1 (Early Growth Response 1) is an immediate-early gene whose induction is rapidly triggered by a variety of endogenous and exogenous factors, and its response is heavily regulated by the MAPK pathways. In the healthy retina, light seems to trigger *Egr1* expression in cells of the inner nuclear layer and ganglion cell layer. Upon degeneration in the rd10 mouse, *Egr1* shifts its expression from the inner to the outer retina becoming the most highly upregulated transcript in both rods and cones, suggesting a possible key regulatory function of *Egr1* in degenerative events.

In previous *ex vivo, in vitro* and *in silico* studies, *Egr1* transcription and translation were shown to be sensitive to retinal damage, indicating that the immediate-early response might be a common phenomenon in retinal degeneration. To our knowledge so far, one study correlated *Egr1* to degenerative events in retinal explants of rd1 mice *ex vivo,* but *in vivo* data is still needed to reliably resolve the complexity of *Egr1* activation in the ONL.

The aim of this project is to describe the role of *Egr1* in the degenerating retina and define its implications on photoreceptors survival *in vivo*. To reach this goal, we will modulate its expression levels specifically in rods or cones either via transgenic methods, such as RNA interference and gene overexpression, or via CRISPR-mediated knockout and activation. These methods will be tested in the Rho$^{P23H/+}$ mouse model of adRP because its degenerative phenotype develops only slowly, which will give us a wide time window for delivery and assessment of the treatments. In this model, we detected the same shift of EGR1 expression to the outer retina, with the inner retina ceasing to express EGR1, suggesting that activation of the gene in dying photoreceptors is not model-specific but may be common to retinal degenerative events. Therefore, understanding EGR1 functions and mechanisms of action is of great interest and may yield valuable insights on retinal degenerative processes.

Gene therapy for best vitelliform macular dystrophy based on haplotype-specific CRISPR/Cas editing

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Purpose: Best vitelliform macular dystrophy (BVMD) is a hereditary maculopathy caused by dominant-negative mutations in the Bestrophin-1 (BEST1) gene. So far, there is no treatment available. Here, we report a suitable gene therapy approach by haplotype specific CRISPR/Cas editing.

Methods: Genetic variants across the BEST1 locus were established based on the 1000 Genomes Project (data release Feb. 2009). Single-nucleotide-based haplotypes were established, and frequencies were estimated by the Hapview software. Single guide RNAs (sgRNA) targeting single nucleotide polymorphisms (SNPs) of the mutation-associated but not the mutation-free haplotypes were designed. Efficacy and allele specificity were determined in vitro (1) by a fluorescence-based assay in HEK293T cells using the pCAG-EGxxFP/pX330-sgRNA/Cas9 plasmid system to targeted reconstitution of EGFP fluorescence and (2) by analyzing CRISPR-induced double strand breaks in RPE cells differentiated from patient-derived human induced pluripotent stem cells (hiPSCs-RPEs).

Results: Analysis of SNP heterozygosity across the BEST1 locus revealed 69 common SNPs with minor allele frequencies > 0.05. Eleven major haplotypes with a frequency > 0.01 in the general population of European descent were established. We designed 26 sgRNAs for targeting the six most common haplotypes. Quantification of reconstituted EGFP fluorescence revealed six sgRNAs with high efficacy and sufficient allele specificity. For a further 14 sgRNAs allele specificity could be increased by shortening the sgRNA sequence length from 20 to 17 and/or by incorporating an additional base mismatch into the sgRNA sequence. High editing efficacy, ranging from 29 to 68 %, was confirmed at the on-target allele in hiPSC-RPEs while the off-target allele remained unaffected for all sgRNAs tested.

Conclusions: The current study identified a set of twenty allele-specific sgRNAs to specifically target haplotypes carrying a mutated BEST gene. This approach covers a significant fraction of BD patients independent of an individual mutation. The exclusion of genome-wide off-targets in edited hiPSC-RPEs is in progress.

Virtual-reality based gaze training for improved visual performance in Retinitis Pigmentosa patients

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Motivation: People living with tunnel vision caused by Retinitis pigmentosa (RP) oftentimes face challenges navigating and avoiding obstacles due to a severely decreased Field of View. Previous studies have shown that gaze training – guided tasks to encourage specific gaze behavior – helps to improve visual performance in patients with limited Field of View. We propose a virtual-reality based, at-home gaze training – to the best of our knowledge the first of its kind – with the goal to increase effective eye movements and navigation performance of RP patients in a real-world obstacle parkour.

Methods: Eight RP patients (7 female, 1 male; aged 20– 60, average 47.1 ± 13.2) took part in our study. The study consists of two phases: In the 'training phase', participants carried out the developed virtual-reality gaze training for a total of 10 hours over the course of four weeks. In the 'control phase', no training occurred. Before and after each phase, participants were asked to walk through randomized real-world obstacle parkours (20 trials per session). Performance differences before and after each phase were measured and evaluated using linear-mixed-models. Eye tracking data was acquired both during gaze training and during the real-world parkour trials.

Results: On average, the time required to move through the parkour was decreased by 17.0 % from 37.2 s before training to 30.9 s after the training phase ($p < 0.001$), the number of collisions was decreased by 50.0 % from 0.513 collisions per trial to 0.256 collisions per trial ($p < 0.001$) and the dynamic visual field size – the visual area that participants scanned over a fixed amount of time – was increased by 3.69 % ($p = 0.002$) after training. In comparison, the time required to move through the parkour was decreased by 5.9 % ($p < 0.001$) after the control phase, collisions were decreased by 10.4 % ($p = 0.56$) and dynamic visual field size was increased by 0.82 % ($p = 0.43$).

Conclusions: The performance increase over the training phase significantly surpasses the natural learning effects found in the control phase, suggesting that Virtual-Reality based gaze training can improve navigation performance and effective gaze movements of patients with RP.

Activation of $GABA_A$ receptors but not $GABA_B$ receptors abolishes the pathological oscillations and improves stimulation efficiency in retinitis pigmentosa mouse model *rd10*

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Purpose: In retinitis pigmentosa mouse model *rd10*, an intrinsic oscillatory activity is observed that compromises the efficiency of electrical stimulation by retinal prostheses [1]. In this study, we confirm that targeting $GABA_A$ receptors but not $GABA_B$ receptors can abolish these oscillations.

Methods: *In vitro* recordings of local field potentials (LFPs) and spiking activity of retinal ganglion cells (RGCs) from the retina of 5 – 6 months old wt and *rd10* mice were obtained using multi electrode arrays. The effect of pharmacological drugs on the oscillatory activity was determined.

Results: In *rd10* retina, the oscillatory activity is seen at a frequency of 3 – 6Hz in the LFPs and RGC firing. The inhibitory neurotransmitter GABA and $GABA_A$ receptor modulators like benzodiazepines abolish these oscillations and at the same time increase the stimulation efficiency significantly.
THIP, a $GABA_A$ agonist that targets a specific isoform of the receptor also abolishes oscillations. On the other hand, targeting $GABA_B$ receptors with $GABA_B$ agonist baclofen or $GABA_B$ antagonist CGP54626 did not have any effect on the oscillations.

Conclusions: While $GABA_B$ receptor ligands are without any effect, $GABA_A$ receptor agonists like benzodiazepines and THIP can be used to abolish oscillations and thereby improve stimulation efficiency. Our study shows the possibility of using clinically approved drugs to achieve better stimulation efficiency by a retinal implant.

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The role of somatic chromosomal abundance in risk and prognosis of age-related macular degeneration

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Purpose: Age-related macular degeneration (AMD) is the leading cause of visual impairment in western societies, affecting the central part of the retina, the macula. Currently, AMD reported cases exceed 200 million individuals worldwide, while AMD cases are expected to increase to 280 million by the end of the decade. Even though several risk factors, like aging, inherited genetics, and smoking among others have been identified little is known about somatic changes in the DNA that occur after fertilization. Post-zygotic mutations in nucleated blood cells lead to mosaic phenotypes with large genomic differences that have been implicated in age-related diseases like cancer and Alzheimer's disease. Recent studies identified that mosaic loss of the Y chromosome is an important factor in increasing the risk to develop late-stage AMD, independent of age and other environmental factors.

Methods: Building on these reports, we computed the chromosomal abundance of all available individuals from the International AMD Genomics Consortium (IAMDGC) and the UK Biobank to assess the role of chromosomal abundance in the risk of developing AMD. Utilizing the MoChA (Mosaic Chromosomal Alterations) pipeline, which uses the intensities of genotyping arrays to estimate chromosomal abundance for sex chromosomes. Following the computation of chromosomal abundance for sex chromosomes, we assessed the associations of chromosomal abundance of X and Y chromosomes for females and males, respectively.

Results: We found that the chromosomal abundance of X and Y is associated with late-stage AMD ($P = 6.425698 \times 10^{-14}$) and overall AMD ($P = 4.06989379 \times 10^{-14}$), while the chromosomal abundance of X is associated with late-stage AMD ($P = 2.1 \times 10^{-14}$) and overall AMD ($P = 2.9 \times 10^{-15}$), independent of age, AMD risk score, and the first ten principal components of ancestry. Finally, we computed a novel and accurate AMD phenotype for UK Biobank participants from the general practice data and successfully replicated our findings in this cohort.

Conclusions: In conclusion, we present significant associations of the chromosomal abundance of sex chromosomes with overall AMD risk and its sub-types independent of confounding factors.

Innate immune response in atrophic AMD: Inflammasome and microglia activation by lipofuscin-mediated photooxidative damage

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Introduction: Age-related macular degeneration (AMD) is the leading cause of blindness in all industrialized countries. The disease is characterized by accumulation of insoluble material at the level of the retinal pigment epithelium (RPE) in the form of both intracellular lipofuscin and extracellular drusen. Atrophic AMD is characterized by well-demarcated atrophic lesions resulting from loss of RPE cells followed by degeneration of photoreceptor cells, which results in vision impairment. Chronic local inflammation including inflammasome and microglia activation plays a central role in AMD pathogenesis.

Purpose: This study aims to investigate the role of NLRP3 inflammasome activation in a mouse model mimicking aspects of AMD, i.e. light-induced retinal degeneration (LIRD) in *Abca4/Rdh8* double knockout and *Abca4/Rdh8/Nlrp3* triple knockout mice that accumulate excessive lipofuscin in the RPE.

Methods: We plan to induce LIRD by white light exposure of the mice following pupil dilation. Subsequently, markers of retinal inflammasome activation will be analysed, such as mRNA and protein expression of IL-1β, IL-18, NLRP3, ASC and caspase-1. Cell death will be assessed by TUNEL staining, and retinal microglia activation and subretinal migration by Iba1 staining. We expect increased light damage and inflammasome activation in *Abca4/Rdh8* double knockout mice compared to wildtype and *Abca4/Rdh8/Nlrp3* triple knockout controls.

Results: In previous in vitro experiments, white light exposure of lipofuscin-loaded murin and human RPE cells results in pronounced NLRP3 inflammasome activation with release of IL-1β and IL-18 as well as secondary cell death by pyroptosis. Activated complement component C5a increases RPE cell susceptibility to inflammasome activation via C5a receptor. Apically secreted cytokines induce microglia activation and migration. Selective NLRP3 inhibitors are capable to suppress these processes in vitro and thus show promise as therapeutic strategy in atrophic AMD.

In vitro modelling of Sorsby fundus dystrophy using patient-derived iPSC-RPE cells

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Introduction: Sorsby fundus dystrophy (SFD) is a rare autosomal dominant macular dystrophy which may lead to bilateral loss of central vision in the fourth to fifth decade of life. While it is known that SFD is caused by mutations in the *TIMP3* gene encoding the tissue inhibitor of metalloproteinases 3 (TIMP3) protein, the molecular pathomechanisms of SFD still remain elusive. To gain a better understanding of SFD pathobiology, we generated an *in vitro* model of patient derived retinal pigment epithelium (RPE) cells following the induced pluripotent stem cell (iPSC) technology.

Methods: Peripheral blood mononuclear cells (PBMCs) were obtained from three individuals who were diagnosed clinically and genetically with SFD. PBMCs were reprogrammed to iPSCs and differentiated into RPE cells. SFD-patient derived RPE cells as well as three control cell lines not showing pathogenic variants in *TIMP3* were characterized by immunocytochemistry, Western blot analyses and quantitative reverse transcriptase (qRT) PCR.

Results: We generated SFD-patient derived RPE cell lines harboring the following pathologic variants in *TIMP3*: NM_000362.5(TIMP3):c.92C>G, p.(P31R), NM_000362.5(TIMP3):c.484 G>A, p.(E162K), and NM_000362.5(TIMP3):c.545A>G, p.(Y182C). After six, nine, 12 and 15 weeks in culture *TIMP3* mRNA expression, intracellular as well as extracellular matrix deposited TIMP3 was analyzed. No differences between SFD lines and controls were seen in *TIMP3* mRNA expression, but an increase in TIMP3 protein was evident in patient-derived cell lines. While normal TIMP3 and variants p.(E162K) and p.(Y182C) presented as two molecular weight species migrating at around 20 and 25 kDa under denaturing and reducing conditions in Western blot analyses. Mutant TIMP3 protein from the p.(P31R) line showed an additional band at a higher molecular weight. Upon enzymatic deglycosylation all TIMP3 variants migrated as a single protein species at around 20 kDa, showing that all proteins are modified by posttranslational glycosylation and indicating that the p.(P31R) variant may lead to the generation of an additional glycosylation site. Analysis of oxidative stress response gene expression revealed slightly but statistically significantly higher mRNA expression of *SOD2* and *NOQ1* in SFD patient derived RPE cells under basal culture conditions.

Conclusions: Our iPSC-RPE cell lines from three SFD patients will be valuable to shed further light on the molecular pathology of this disease. Given the different genetic variants represented in the cell lines including the newly described TIMP3 variant p.(P31R), it will help to uncover both, pathological features shared between SFD patients, as well as genotype-phenotype correlations.

Characterization of a mouse model of complex outer retinal pathology by injection of HBEGF and TNF

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Purpose: Retinal degenerative diseases are characterized by photoreceptor cell death leading to vision loss. Currently, there is no treatment to re-establish sight as photoreceptors do not regenerate. Transplantation of human iPSC-derived photoreceptors is a potential therapeutic approach to replace cells in the retina and restore vision (Gasparini et al., 2022). Several retinal degenerative diseases, like age-related macular degeneration (AMD), present a complex pathology, however, no preclinical animal models have been established that recapitulate this complex physiopathology. Such models are required to better understand the microenvironment of the degenerated retina and to develop novel treatment strategies. Recently, the Karl lab demonstrated that combined application of HBEGF and TNF-alpha (H+T) induces complex retinal pathology in human iPSC-derived retinal organoids, as severe glial pathology and photoreceptor degeneration via cell extrusion was observed (Völkner et al., 2022). Using this strategy, we aim to generate a complex retinal pathology in vivo model by combined application of H+T into the adult mouse eye.

Methods: The eyes of 16 and 52 weeks-old wild-type (C57BL/6j) mice received intravitreal injections of HBEGF and TNF for 2, 3, or 4 consecutive days. Cryostat sections of experimental eyes were analysed 4 or 30 days after treatment by TUNEL assay and immunohistochemistry using primary antibodies against: GFAP (astrocytes, reactive Müller glia), SOX2 (Müller glia), and L/M opsin (cone outer segments).

Results: Major retinal remodelling with foldings/tubulations, reactive gliosis in Müller glia cells, and cell death in the outer nuclear layer (ONL) were present 4 days after 2, 3 and 4 consecutive days of H+T injections and remained for at least 30 days. Photoreceptor outer segments presented early signs of pathology changes after 4 consecutive days of injections.

Conclusions: H+T injections rapidly induce a complex retinal pathology phenotype in adult and aged mice. While the mechanism of photoreceptor degeneration has yet to be studied in more detail, this model ultimately might help to further understand retinal degenerative diseases in patients. Further, model application might advance cell replacement therapy development by studying photoreceptor cell transplant survival and integration upon transplantation under complex pathological conditions in vivo.

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Development of novel AAV variants for improved transduction of retinal microglia

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Purpose: Recombinant adeno-associated virus (AAV) vectors have been widely used in gene therapy applications for central nervous system (CNS) diseases with favorable preclinical and clinical results. Indeed, these vectors can successfully transduce post-mitotic cells, e.g, neurons and astrocytes. Given the emerging central role of microglia, the major resident immune cells in the CNS, in the pathobiology of neurodegenerative disorders, these cells are increasingly becoming the focus of the development of new treatment approaches. However, microglia are generally refractory to transduction by viral and non-viral vectors. Currently, available AAV variants do not achieve high transduction rates and sufficient transgene expression levels in microglia, especially *in vivo*.

Methods: Here, we introduce a novel primary microglia culture protocol from mouse retina, which allows for good reproducibility, high cell numbers and long *in vitro* viability. We used this model as a screening platform to evaluate CMV-mediated GFP transgene expression of different engineered AAV capsids. To evaluate cell type specificity, we also tested the vectors on primary retinal macroglia cells. The engineered variants were based on AAV6, the AAV serotype with the highest reported efficiency in microglia transduction *in vivo*, or on AAV1, the most closely related serotype. The variants carried single-point mutations of surface-exposed tyrosine, lysine, threonine, serine residues, and/or arginine residues, or the peptide insertions of AAV2.GL and AAV2.NN, two previously described 12-mer peptide insertions that enhance retinal cell transduction when introduced into the AAV2 capsid.

Results: Our results indicate that the two novel variants AAV6.GL and AAV6.NN show an increased transduction efficiency of primary mouse retinal microglia (2 to 3,5-fold) compared to AAV6 wild type or the recently published triple mutant AAV6 TM6, while reduced transduction is observed in primary retinal macroglia. Notably, AAV1 mutant capsids showed even more promising transduction efficiencies in targeting these primary cells (up to a 4,5-fold increase over AAV6 WT).

Conclusions: Our study establishes a new *in vitro* platform for assessing microglial transduction and identified novel AAV1- and AAV6-based capsid variants with improved microglial transduction properties. Future studies will focus on combining the novel capsids with microglia-specific promoters before testing selected variants *in vivo*.

RPE-differentiation status affects complement components and cellular function: Implications for studying degenerative retinopathies

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Purpose: Retinal pigment epithelium (RPE)-cells require a specific polar and tightly connected architecture to perform their functions. When the differentiation status of RPE-cells is altered, it can result in functional impairment and the development of degenerative retinopathies, such as age-related macular degeneration (AMD) and proliferative vitreoretinopathy. In this study, we aim to elucidate the pathogenesis of RPE-associated diseases by investigating the relationship between RPE-differentiation and RPE-specific complement components.

Methods: Morphology, RPE-cell markers and complement components of short- and long-term cultures of induced pluripotent stem cell (iPSC)-derived RPE- and ARPE-19-cells, grown in various media, were characterized. AMD-risk genotyping, quantitative gene expression analysis, immunocytochemistry and multiplex-immunoassays were conducted. Results were compared to RNA-sequencing data from previosuly published datasets. A biserial correlation was used to explore the relationship between complement gene expression and RPE-differentiation. Complement activation was determined with Western blot analysis.

Results: The iPSC- RPE- and ARPE-19-cells differed in their AMD-risk genotype, with the iPSC-derived RPE-cells displaying a homozygous single nucleotide polymorphism in the *CFI*-gene, linked to an increased AMD-susceptibility. Notably, the morphology of RPE-cells cultured for varying durations was significantly different. In APRE-19- and iPSC-RPE-cells cultured for short periods, the polar RPE-specific structure and expression of functional markers were significantly reduced. In addition, we observed reduced expression of complement genes and increased expression of proliferation genes in short-term cultured RPE-cells, which was confirmed by previously published RNA-sequencing data. Correlation analysis showed a highly significant relationship between *C3*-expression and RPE-differentiation in both APRE-19- and iPSC-RPE-cells. At protein level, we detected distinct intracellular arrangements of RPE-markers, cellular transition markers, and complement components in short- and long-term cultured cells. Finally, we observed fragments of complement activation in short-term cultured cells without the influence of cell-independent complement components.

Conclusions: The differences in RPE-cell morphology and functional status observed in this cell culture model may resemble the early changes observed in the pathologic RPE. Our results suggest that RPE-differentiation and cell-based complement patterns are correlated independently of blood supply. Furthermore, our results highlight the importance of using differentiated RPE-cells to ensure that experimental results reflect RPE-biology, as the use of undifferentiated RPE-cells in the past may have led to results that do not accurately reflect physiological RPE-function.

Development and quantification of photoreceptor outer segment formation

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Purpose: Photoreceptor cell transplantation aims to replace or support degenerated photoreceptors of defective eyes by introducing healthy donor cells. To achieve functionality after transplantation the formation of proper photoreceptor outer segments (OSs) is important. These membranous structures transfer incident light into a biochemical signal which is the crucial first step in the process of vision. While qualitative evidence for OS formation by human donor photoreceptors after transplantation was shown, reliable methodology for quantitative analysis of OS formation has not been established yet. This project aims to develop quantification methods for OS formation and quality.

Methods: OS formation was examined during development of C57Bl/6JRj wild type mice to obtain qualitative and quantitative measurements. Eyecups of pups between postnatal ages P4 to P24 were analyzed via light and electron microscopy including OS-specific immunohistochemistry. Images with OSs from different developmental stages were immuno-stained for short wavelength (S-) opsin and used to train a robust classifier based on supervised machine learning. Characteristic features were extracted to classify the maturation stage of photoreceptor OSs. Electron microscopic images were used to establish an innovative ultrastructural quantification measurement for the alignment of OS membrane stacks. Images were analyzed using a custom written MATLAB code that extracts the orientations of membranes and their coherency from the image gradient of sliding image patches.

Results: Immunohistochemical analyses revealed that the expression patterns of OS-specific proteins changed most between P4 and P12. Major changes in the ultrastructure of the OSs occurred simultaneously. The trained classifier was able to analyze image data from wildtype mice immunostained for S-opsin according to their maturation stage. Additionally, a method for quantifying the orientation of membrane stacks within OSs on EM images has been developed.

Conclusions: A set of immunohistochemical markers which can be used for characterizing the structure and potential function of OSs has been identified. A classifier based on supervised machine learning has been developed to quantify OS formation. Furthermore, the quality of OSs can be quantified on an ultrastructural level. These analysis tools provide the basis to evaluate in depth photoreceptor OS formation and quality in development, degeneration and after photoreceptor transplantation.

Effects of the ARMS2 A69S polymorphism on extracellular matrix of retinal pigment epithelium cells in AMD

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Introduction: Age-related macular degeneration (AMD) is a complex and progressive disease of the macula that mainly affects the elderly population. Aging causes thickening and ultrastructural changes in the extracellular matrix (ECM) composition of Bruch's membrane (BrM) leading to changes in retinal pigment epithelium (RPE) cells. Recent studies strongly suggest that the major risk for AMD is linked to genetic variants in the *ARMS2* gene, particularly the ARMS2-69S variant. Previously, we found that ARMS2 functionally interacts with ECM proteins, which are also involved in the development of other maculopathies. The present study investigates, how ARMS2-A69S influences the production, structural organization and composition of ECM components in RPE cells.

Methods: The impact of ARMS2 variants was analysed in hTERT-RPE1 cells by overexpression of ARMS2-69A (low risk), ARMS2-69S (high risk) and an empty plasmid control. The changes in ECM deposition were monitored via immunostaining of ECM generated by RPE cells overexpressing ARMS2 constructs followed by decellularization of transwell membranes after four weeks of culture (n = 3 for fibronectin, laminin, elastin, and fibulin-3 staining). Moreover, the levels of ECM components (fibronectin, laminin, elastin, fibulins) and ECM regulatory factors (MMPs and TIMPs) were assessed via Western Blot and qPCR (mean ± SEM, n = 6).

Results: RPE cells bearing the ARMS2-69S variant displayed alterations in ECM composition, accumulation and disorganization of ECM proteins, compared to the ARMS2 low risk variant. In detail, the network of fibronectin fibers appeared homogenous in low-risk ARMS2 RPE cells, and fragmented in the high-risk RPE cells. The laminin, elastin and Fibulin-3 staining displayed extracellular aggregates in the ECM deposited by ARMS2-69S RPE cells. Overexpression of ARMS2-69S led to a significant downregulation of Fibulin-3 gene (6.3 ± 0.9, $p = 0.005$). At protein level, we observed a decrease in secreted MMP2 (4.3 ± 0.9, $p = 0.005$) and TIMP2 (2.3 ± 0.9, $p = 0.005$) in the supernatant of RPE cells overexpressing ARMS2-69S.

Conclusions: Our data show that RPE cells produce altered ECM when expressing the ARMS2-A69S variant. Our results confirm a role of ARMS2 in ECM regulation and suggest a link between the *ARMS2-A69S* polymorphism in RPE cells and ECM changes found in AMD patients.

Development of a therapeutic genome editing approach for the treatment of *EYS*-associated Retinitis pigmentosa

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Purpose: One leading cause for autosomal recessive RP (arRP) are mutations in the *Eyes shut homolog (EYS)* gene that account for 5 to 10 % of arRP cases.
Since RP is a genetic disorder, genome editing by gene replacement may be a favorable treatment strategy. Gene replacement is based on inducing a double strand break (DSB) to the target genomic locus followed by insertion of a corrected cDNA copy of the mutated gene via the cells DSB repair mechanisms.
EYS is the largest gene expressed in the human retina and the whole transcript with flanking untranslated regions is around 10.5 kb. This makes gene replacement of *EYS* challenging since it cannot be delivered by single AAV approaches.
The aim of this project is to develop a replacement strategy that will not replace the whole gene but only the 3' part of the gene plus a poly-adenylation (pA) signal. This shall inactivate native 3' exons and lead to expression of a correct transcript.

Methods: Four guide RNAs (gRNAs), each targeting one exon of exons 27 to 30 of *EYS*, were characterized for their function and efficiency in a plasmid-based bioluminescence resonance energy transfer (BRET) assay and with genomic DNA of induced pluripotent stem cells (iPSCs) by TIDE (Tracking of indels by decomposition) analysis.
A repair template corresponding to the gRNA targeting exon 27 was cloned to be evaluated for integration efficiency by multiplex PCR and NGS.

Results: All four gRNAs showed promising efficiency in the plasmid-based BRET assay. On the genomic level only gRNAs targeting exons 27 and 28 showed editing events beyond the background level in TIDE analysis.
The repair template was amplified out of *EYS* cDNA and cloned into a vector containing a pA signal. The native gRNA target sequence in exon 27 was replaced by a codon optimized microhomologous sequence and a SapI recognition site was added to the 5' end of the template for linearization.

Conclusions: In this study, first steps towards a therapy of *EYS*-associated Retinitis pigmentosa via partial gene replacement were made. We identified gRNAs that efficiently target 3' exons of *EYS* and created a repair template for integration via microhomology mediated end joining (MMEJ) to replace mutated upstream exons.

Differential monolayer formation of hiPSC-derived RPE subpopulations after subretinal transplantation into RPE-depleted mice

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Purpose: Retinal pigment epithelium (RPE) degeneration leads to photoreceptor dysfunction and loss causing visual impairment and blindness. Cell replacement using human RPE generated from pluripotent stem cells in vitro represents a potential therapeutic strategy, however cell cultures can be heterogenous. We therefore identified cell surface markers to detect, isolate and transplant RPE subpopulations and examined how the groups differ with respect to cell survival, monolayer formation and maturation after suspension transplantation into RPE-depleted mice.

Methods: RPE markers were identified by flow cytometry screening correlating side scatter intensity as primary read-out for pigmentation with expression of 371 cell surface markers (MACS® Marker Screen, v2). A subsequently validated combination of two markers was used for MACSQuant® Tyto® based cell sorting. Suspensions containing 50,000 cells from unsorted, target-enriched or target-reduced fractions were transplanted subretinally into RPE-depleted C57BL/6JRj mice, who had intravenously received 30 mg/kg $NaIO_3$ one week before. 80 µg triamcinolone acetonide were injected intravitreally for local immune suppression. Three weeks later, eyes were analysed by immunohistochemical staining, electron microscopy and bulk RNA sequencing.

Results: All cell fractions expressed RPE-specific markers, showed survival for three weeks and were able to generate an RPE-specific phenotype in vivo with a basal labyrinth, basal nuclei, melanosomes and apical microvilli. While all groups formed correctly polarized monolayers as required for proper RPE function, the extent of retinal coverage and monolayer formation varied greatly between groups. Target-enriched cells covered the largest retinal area (35 %) and exhibited the highest fraction of monolayer (72 % of graft area). Unsorted and target-reduced cells showed similar retinal coverage (30 % and 24 %), yet a significantly lower proportion of polarized monolayers (32 % and 38 %).

Conclusions: Here, we show that increased homogeneity of hiPSC-derived RPE cell compositions using surface markers based on flow sorting greatly affects the capacity for monolayer formation after transplantation into RPE-depleted mouse retinas. Remarkably, target-enriched human RPE formed monolayers covering up to 72 % of the graft and 25 % of the entire retinal surface, an important prerequisite for proper RPE functions like barrier formation and directed nutrient/ion transport. Our results thus suggest that cell transplantation approaches for the treatment of retinal degenerative diseases require careful selection of appropriate donor cell populations.

Towards deciphering pathomechanisms of complex neuropathologies via comparison of induced human retina organoid pathology models

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Purpose: Age-related macular degeneration (AMD) and some inherited retinal diseases involve pathologic changes in photoreceptors, Müller glia and other cells in the inner retina. Our lab has shown that a complex phenotypic pathology model can be induced in human retinal organoids (HROs) upon combined treatment with HBEGF and TNF (HT) (Völkner et al. 2022). This model shows several features potentially relevant for AMD and some other diseases, particularly, a novel pathomechanism of photoreceptor degeneration via cell extrusion, retinal remodeling, gliosis, and scarring. To validate the relevance of these mechanisms for patients, we aim to identify the key regulatory programs in HT model and propose here multiple approaches to achieve it.

Methods: We propose to establish experimentally inducible pathology models with differential phenotypes to facilitate the identification of key regulatory genes of pathologic processes via comparative transcriptomic, metabolomic and histologic studies. Therefore, we generated HROs from human induced pluripotent stem cells and applied different factors to 200-day-old HROs to induce pathologies. Secondly, we are performing single-cell RNA sequencing analysis of our model to determine gene and signaling pathway candidates. Additionally, we propose to study cell metabolism which might regulate cell extrusion.

Results: We present our strategy to decipher pathomechanisms of pathologic processes in HROs, and some preliminary data. We tested treatment of HROs with selected known inducers of retinal degenerations in animal models: sodium iodate and ATP. Qualitative analysis in related HRO experiments upon both factors show photoreceptor cell loss, cell death and reactive gliosis as observed by immunohistochemistry. Additional treatments, such as a regulator of cell extrusion (YODA1), and inhibitor and promoter of oxidative phosphorylation (CCCP and DCA), also led to pathologic changes.

Conclusions: We observed that treatment of HROs with selected pathologic challenges is sufficient to induce differential pathologic phenotypes. This provides a starting point for our proposed strategy to decipher the underlying pathomechanisms. Comparative transcriptomic and metabolomic analysis of these models and of HT-induced pathology model will facilitate dissection of common and differential regulators of pathologic processes potentially relevant for diseases in patients.

Preclinical human model for retinal cell replacement therapy: Photoreceptor transplants integrate pathology-dependently into human retina organoids

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Purpose: Cell replacement is a promising future therapeutic approach to restore loss of neurons and visual function in retinal diseases. In vivo studies showed that mouse and human cell transplants can integrate into rodent and non-human primate retina, but cell integration and visual restoration are not yet efficient. It remains unclear if this depends on the animal model, pathology stage and pathologic phenotype and if transplants even could integrate in humans. To facilitate translation, we aimed to develop a preclinical human cell therapy research system, to test whether human transplants might integrate into human retina models and to evaluate to which extent a pathology alters cell integration.

Methods: To achieve this, we generated 200-days old human retinal organoids from pluripotent stem cells. Human photoreceptor cells were isolated from organoids with a photoreceptor-specific CRX-mCHERRY reporter and transplanted onto human organoid hosts without reporter. Pathologic organoid hosts with intermediate and late stages of photoreceptor degeneration and glial scarring were experimentally induced by HBEGF-TNF treatment. Experiments were analyzed by live-imaging, viral labeling, and histology.

Results: We observed that six weeks after transplantation, subretinal rod and cone photoreceptor transplants spontaneously incorporated as large clusters into healthy hosts. We did not find evidences for material transfer using a dual reporter approach. Histological and ultrastructural analysis indicated structural integration into the host retina. Transplant incorporation frequency was comparably high in healthy and pathologic organoid hosts (mean ± standard deviation: 74 ± 21 %, n = 72, N = 3). However, at late pathology stages with glial scarring, transplants incorporated with a differential phenotype: Single photoreceptor cells were scattered across the host retina instead of one large cell cluster.

Conclusions: We established a preclinical human model system for cell replacement therapy and observed for the first time integration of human photoreceptor cells into a human retina model. Our results indicate that the pathology stage of the retina might alter cell therapy outcome. This human model might assist mechanistic studies and preclinical optimization of cell replacement therapy for various cell types and diseases of the retina.

Long term porcine retinal explants as an alternative to *in vivo* experimentation

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Purpose: The porcine retina represents an optimal model system for inherited retinal dystrophies due to the close anatomical similarities to the human retina, including a cone enriched *area centralis*. As large animal models require significant resources and are therefore often difficult to perform, the organotypic retinal culture might represent a relevant model system to study gene therapeutic approaches prior to *in vivo* application. However, to this date it appeared to be difficult to reproduce good quality explants that can be kept in culture for sufficient time to enable viral vector mediated genome editing experimentation. Here we established a protocol for a long term culture with good morphological preservation.

Methods: Four retinal explants each were obtained from the central part of the retina of locally sourced pig eyes and transferred onto a semipermeable polycarbonate membrane insert with the photoreceptors facing down. They were kept in culture for up to 28 days with Neurobasal-A medium containing 100 or 450 mg/dl glucose at 37° C and 5 % CO_2. For supplementing the medium we tried combinations of fetal calf serum, B-27 with or without insulin and N-2. For a better comparability and quantification we developed a tissue quality score based on immunofluorescence with robust markers such as protein kinase C alpha (PKCα), peanut agglutinin (PNA) and 4',6-diamidino-2-phenylindol (DAPI).

Results: We were able to keep good quality explants in culture up to 28 days with only little degradation as seen after immunohistochemistry at different harvesting timepoints. The best results were attained using Neurobasal-A medium containing 100 mg/dl glucose supplemented with 2x B-27 containing insulin, 1x N-2, 1x L-glutamine and 1x antibiotic-antimycotic, and a medium change every 48 hours. Eyes treated with heat for decontamination purposes showed less preservation compared to those obtained without heat treatment. For an easy preparation process, it is also necessary to minimize transport time and keep the eyes in ice cold buffer the whole time.

Conclusions: Using a standardized protocol, porcine retinal explants represent an easy to handle intermediate model between *in vitro* and *in vivo* experimentation. Using pig eyes from a local butcher renders this model system easily reproducible and contributes to the implementation of the 3R principle by Russell and Burch. This model system is currently tested with gene therapy vectors for efficient gene transfer.

Usher syndrome 1C harmonin suppresses canonical Wnt-signaling in cellular models, providing novel insights into the pathophysiology and opportunities to evaluate therapies

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Purpose: Usher syndrome (USH) is the most common cause of hereditary combined deaf-blindness in human. To date, no effective treatment for preventing or slowing-down blindness exists. Reasons for this are the lack of faithful animal models for the ocular phenotype and unknown pathomechanisms underlying retinal degeneration. Here, we focus on *USH1C* encoding the scaffold protein harmonin. In retinae, harmonin is associated with β-catenin, a cell adhesion component but also a key effector in canonical Wnt-signaling (cWnt). We aim to decipher the role of harmonin in cWnt-signaling and generate distinct patient-derived cellular models to further unravel the molecular mechanisms underlying USH and to test treatment options.

Methods: We applied interaction assays such as co-immunoprecipitations and *in-situ* PLAs; cWnt was stimulated by Wnt-medium or β-catenin overexpression and quantified by luciferase assays, Western blotting, and immuncyto-/histochemistry (IC) in HEK293T cells and USH1C$^{R31*/R80Pfs*69}$ patient-derived fibroblasts. Differential expressed gene (DEG) analysis was determined in USH1C patient-derived fibroblasts compared to cells of healthy individuals by RNA-seq. USH1C^{R31*} pig retinae were processed for IC. Ataluren was applied as a translational read-through inducing drug. Furthermore, induced pluripotent stem cells (iPSCs) were generated and differentiated into photoreceptor precursor cells and 3D retinal organoids.

Results: Harmonin interacted and translocated with β-catenin into nuclei. Harmonin overexpression significantly reduced cWnt-signaling activity, but the USH1C^{R31*} mutated harmonin did not. Concordantly, we observed an increase in cWnt-activity in USH1C fibroblasts. RNA-seq analysis revealed various differential expressed Wnt-pathway and target genes in USH1C patient-derived fibroblasts. Data were confirmed in USH1C pig retinae. The cWnt-signaling phenotype was reverted in HEK293T and USH1C patient-derived cells by Ataluren, a small molecule suitable to induce translational read-through of nonsense mutations. In order to prospectively confirm this molecular phenotype in a preclinical disease model, we produced iPSCs from USH1C patient-derived fibroblasts and differentiated them into photoreceptor precursor cells as well as 3D retinal organoids.

Conclusions: Overall, our results reveal that patient-derived cellular models provide a great opportunity for deciphering USH-related molecular phenotypes and examining treatment options. Here, we prove for the first time that harmonin is a potent suppressor of cWnt-signaling,

thereby leading to Wnt-signaling dysregulation in USH1C patient-derived fibroblasts and pig retinae. The novel role of *USH1C*/harmonin in the cWnt-pathway opens new paths in research to unravel pathomechanisms, identify new therapeutic targets, and evaluate treatment options, as demonstrated by the restoration of the cWnt-phenotype by Ataluren treatment in human cell models.

Effect of anti-VEGF medication on microglia cells in animal model of laser-induced choroidal neovascularization

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Purpose: Age related macular degeneration (AMD) is the leading cause of visual loss in the elderly western civilization. There are 2 main types of late AMD, geographic atrophy and neovascular AMD. Abnormal vascular proliferation occurs due to excess release of vascular endothelial growth factor (VEGF). Consequently, neovascular AMD is treated by intravitreal injections of anti-VEGF drugs, such as aflibercept. Unfortunately, some neovascularisations are resistant towards this treatment. The cause of non-responding is currently under research. Microglia as the resident immune cells play a crucial role in the development of AMD. However, the effect of anti-VEGF injections on microglia is not yet known. This project aims to understand the effects of anti-VEGF medication on microglial cells.

Methods: For this project, we used the experimental model of laser-induced choroidal neovascularization (CNV) in mice. 55 mice were randomly divided into 3 groups. All mice received laser treatment. The first group of animals was not further treated after laser treatment, whereas groups 2 and 3 received intravitreal injections of aflibercept or PBS, respectively, immediately after laser treatment. The development of the laser spots was monitored by *in vivo* imaging (optical coherence tomography, fluorescence angiography, scanning laser ophthalmoscopy) as well as by immunohistochemical staining of choroidal flat mounts. Immunohistochemical staining of retinal cryosections was performed for VEGF receptor-1, VEGF receptor-2, CD14 and TMEM119, and co-localisation for the microglia stained for Iba-1 was checked.

Results: *In vivo* imaging revealed a decreased size of the laser spots and a significantly decreased size of the leakage in the anti-VEGF group compared to the PBS injection and control groups. Immunohistochemical staining of the flat mounts showed a decreased migration of retinal microglia into the laser spots after anti-VEGF injection. In addition, we hypothesize that due to intravitreal anti-VEGF-injections the expression of VEGF-2 receptor, VEGF-1-receptor and CD14 is inhibited. Furthermore, we expect a lower level of TMEM119 in microglial cells in the laser spots.

Conclusions: Intravitreal injection of aflibercept leads to a reduced size of the CNV in our experimental model. Simultaneously, a reduced migration of microglial cells into the laser spots was found. Whether the reduced size of the CNV is a consequence of a reduced microglial migration or *vice versa*, remains to be elucidated.

Whole genome sequencing in inherited retinal dystrophies

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Purpose: Inherited retinal dystrophies (IRDs) are characterized by high phenotypic variability, making DNA testing indispensable to confirm diagnosis. Despite major advances in high-throughput technologies and bioinformatic tools in recent years, approximately 30 – 40 % of IRD cases still remain genetically unexplained. To close this gap, Whole Genome Sequencing (WGS) represents a promising approach in routine diagnostics by offering the advantage of capturing variants throughout the human genome, importantly by addressing non-coding and structural variants (SVs). Here, we have analyzed index IRD patient samples with no pathogenic findings after routine gene panel and/or whole exome sequencing.

Methods: As part of the multicenter study "Bavarian Genomes Network for Rare Disorders", the Institute of Human Genetics Regensburg analyzed 35 IRD families comprising 87 persons by WGS. Using the Exome Variant Analysis Database (EvaDB) (Available from: https://brand-fabian.github.io/evadb-docs/), WGS data were analyzed based on phenotype, mode of inheritance, variant type, allele frequency, computational predictions, and SV analysis.

Results: WGS analysis led to a genetic diagnosis in 20 % of families investigated (7/35). In the WGS analysis, SVs stood out as particularly interesting. Overall, 6 % of families (2/35) could be resolved based on SVs. In another 9 % of cases (3/35), the identified SVs are excellent candidates for disease causation. One such candidate SV represents a large deletion spanning two genes, namely *KLF11* and *CYS1*. Based on further functional information, *CYS1* emerged as a promising new IRD candidate gene.

Conclusions: Taken together, this study demonstrates the utility of WGS in DNA diagnostics and further underscores the advantages of WGS for detection of SVs. Although bioinformatic analysis is time-consuming, this method offers great potential for identifying previously unknown disease-causing genes and thus providing certainty for patients with so far unknown genetic causes.

Influence of PARP1 on CRISPR/Cas9 induced Double Strand Break repair

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Introduction: Modern therapeutic genome editing strategies using CRISPR/Cas9 rely on the induction of a DNA double strand break (DSB) at a specific genomic locus, which is subsequently repaired employing one of three major DNA repair pathways. Especially non-homologous end joining (NHEJ) leads to unwanted indel formation at the DSB site causing mutagenic repair. Although micro-homology mediated end joining (MMEJ) causes larger deletions due to end resection, it can together, with homology directed recombination (HDR) in presence of a wild-type repair template precisely correct a genomic mutation. Recent studies are focusing on the downregulation/overexpression of certain DNA repair pathway proteins to decrease NHEJ mediated mutagenic repair and increase MMEJ or HR respectively. Poly-ADP-Ribose-Polymerase 1 (PARP1) is one of DNA repair pathway proteins, which is involved in all three repair pathways, making it the perfect target to investigate the influence of Knock-out or overexpression on DNA repair.

Methods: CRISPR/Cas9 was used to downregulate PARP1 expression as well as a plasmid overexpressing PARP1. Previously described luminescent and BRET based reporter assays were used in this study to determine the repair pathway choice and to quantify the influence of PARP1 downregulation/upregulation on NHEJ/MMEJ/HDR repair activity at the DSB site in our model cell line HEK293T.

Results: We generated five different CRISPR/Cas9 based knock-out plasmids and tested their ability to induce a frameshift in the target sequence using a BRET reporter system. Further, PARP1 downregulation or overexpression was verified using a PARP1 ELISA and western blot, respectively. PARP1 knockouts resulted in an increase of NHEJ and MMEJ activity but HDR activity was not affected. In contrast, an overexpression of PARP1 lead to a decrease of NHEJ and HDR activity, while MMEJ activity was stable.

Discussion: Our study provides useful information on the effects of PARP1 interventions to optimize genome editing approaches based on a Cas9 meditated DSB reducing unwanted, mutagenic NHEJ and promoting MMEJ in combination with a wild type repair template.

Photoreceptors rely on glucose for ATP production

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Energy metabolism is critical for cell survival and function. The current working model of the metabolic ecosystem in the retina requires tight interactions between the retinal pigment epithelium, photoreceptors and Müller glia cells. Defects in the metabolic system especially in the ATP-demanding photoreceptors can lead to retinal diseases including age-related macular degeneration (AMD).

Here, we developed a model to analyze aspects of the metabolic ecosystem in a mostly intact tissue environment. Using this system, we aim to analyze energy metabolism of rods and cones at the cellular level and under physiological and pathophysiological conditions. Further, the influence of night (dark) and day (light) is of interest as it has been shown that ATP consumption not only varies between cones and rods but also depends on the light conditions. To address these questions and study energy metabolism of photoreceptors with their supporting cells present we use two-photon laser scanning microscopy of genetically encoded metabolic sensors in acute eye preparations.

The viability and functionality of the cells after tissue preparation was investigated by measuring changes of intracellular calcium levels in rods in response to activation with the two-photon laser beam. Using different imaging protocols, calcium traces of the fluorescence signal of GCaMP6s suggested that rods are viable and functional in our adapted preparation method and respond in this set up as if they would be stimulated by light.

Further support for cell viability was achieved by fluorescence lifetime imaging of a genetically encoded ATP-sensor. Additionally, we observed that ATP levels in photoreceptor cells change faster when glycolysis is blocked compared to neurons of the inner retina.

Overall, our results indicate that the cells in our preparations are viable and functional. We are able to show that there are differences in ATP levels between neurons of the inner retina and photoreceptors by blocking major sources of ATP production. Therefore, our model can be used to measure central metabolites in various conditions to gain a better understanding of the metabolic ecosystem of the eye in health and disease.

The role of Kv7 in KCNV2-associated retinopathy

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Purpose: KCNV2-associated retinopathy is a rare, autosomal recessive cone dystrophy. KCNV2 encodes for the voltage gated potassium channel subunit Kv8.2. By essential heteromerization with potassium channels from other families, Kv8.2 gives rise to a conductance termed IKx that counterbalances the dark current in both rods and cones. While it is today understood that members of the Kv2 family channels are heteromerization partners of Kv8.2 in rods and cones, there is a long-standing debate on a possible role also of Kv7 in I_{Kx}. A potential involvement of Kv7 in I_{Kx} and thus a role in the pathophysiology KCNV2-associated retinopathy, however, would be of relevance in a therapeutic perspective, given that there a lot of modulators available for these channels. We hypothesize that, Kv7 are expressed in photoreceptors and can interact with Kv8.2 to form I_{Kx}.

Methods: We have established a 30 minutes 4% PFA-fixation protocol for the preparation of mouse retinal cryosections. Retinal cryosections of wild-type and Kv7-knockout mice were used to check for Kv7 expression and Kv7-Kv8.2 co-localization in photoreceptors. Additionally, electrophysiological techniques were used to study the possibility of Kv7-Kv8 interaction in mammalian expression systems and the biophysical properties of the Kv7-Kv8 heteromers. Specifically, we have performed whole-cell patch clamp recordings, to measure the K+ currents of CHO cells expressing Kv7 and Kv8. Molecular biology techniques were used to create dominant negative (DN) pore mutants and Kv7-Kv8 concatemeric structures.

Results: Immunohistochemical stainings on mouse retinal cryosections, show expression of Kv7.4 in cone photoreceptors, but not in rods. This signal was present in wild-type and absent in Kv7.4-knockout tissue. Electrophysiological data, indicate a modulation of Kv7 currents when co-expressed with the Kv8 subunits, confirming that interaction of these subunits is possible. Additionally, the lack of current in CHO cells co-transfected with Kv8 and DN Kv7, supports the hypothesis that the above-mentioned subunits form heterotetramers with a single conducting pore.

Conclusions: Our results are consistent with the hypothesis of an interaction between Kv8.2 and Kv7.4 in the retina. On one hand we have shown the presence of Kv7 in photoreceptors and on the other hand, we confirmed that an interaction between Kv7 and Kv8 subunits is possible. Further experiments will be needed to show their interaction in native tissue.

Characterization of Müller glia and retinal ganglion cells in human retina and retinal organoids

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Background: The study of human retinal diseases is often hampered by the inaccessibility of retinal tissue from patients. Retinal organoids can serve as a potential model to study retinal pathophysiology in vitro. Müller glia and retinal ganglion cells (RGCs) are key cell types affected in various retinal diseases. Our study aims to characterize the development of Müller glia and RGCs in human-derived retinal organoids compared to human adult and fetal retinas to improve the retinal organoid model for future analysis of the human eye.

Methods: Our study is based on retinal organoids generated from human induced pluripotent stem cells. We used cryosections from these organoids to perform immunofluorescence staining with antibodies that detect known marker genes of Müller glia and RGCs. We compared their expression patterns at different developmental stages of our organoids. In addition, analogous stainings of adult and fetal human retinas (fetal human retinas figures from Luo, Z. et al. Stem Cells International 2019) were used for comparison.

Results: We found that some retinal markers that have been established for the adult human retina also show increasing signal intensity in our retinal organoid model from early to late developmental stages. For example, the signal intensity of the Müller cell markers RDH10 and CRALBP gradually increased as the organoids matured. Vimentin also showed specific signals in retinal progenitor cells and Müller glia during early and late maturation. However, AQP4 and SLMAP, additional Müller glia maker genes, did not show specific signals on Müller glia in early and mature stages. After comparing our findings with those of the human adult and human fetal retina from the literature, we confirmed their time-dependent expression in Müller glia during the development of the human retina in vivo.

Conclusions: We have established tools to follow Müller cell development in human retinal organoids. A better understanding of the latter, together with that of RGCs over time, will help to define how future manipulations to improve our organoid model affect the generation and differentiation of these two cell types.

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