PRO RETINA FOUNDATION

8th PRO RETINA
Research-Colloquium Potsdam

CONFERENCE REPORT

Retinal Degeneration

Accelerating Progress in Research and Translation

An Interdisciplinary Dialogue

March 22nd/ 23rd, 2013

Seminaris SeeHotel Potsdam

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supported by
BMBF-Project „HOPE 01GM1108A"

SPONSORED BY THE
Federal Ministry of Education and Research

PRO RETINA FOUNDATION
FOR PREVENTION OF BLINDNESS

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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 three objectives mentioned in the constitution are to actively support research, to give psychological and social advice for its members and to strengthen public information.
Every member can join one of the 60 regional groups, which are spread throughout Germany.
At present (2013), PRO RETINA Deutschland e.V. counts more than 5,600 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 Aachen (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 ("Wissenschaftlicher und Medizinischer Beirat", WMB) and a Working Group on Clinical Questions ("Arbeitskreis Klinische Fragen", AKF). 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.

PRELIMINARY PROGRAMME

Friday, March 22nd 2013

13:00–13:05 **Welcome remarks**
Franz Badura (PRO RETINA Foundation, research division)

13:05–13:50 **Keynote lecture**
Gerald Chader, Los Angeles: *RD research: Moving from scientific darkness to the enlightened era of clinical trials*

13:50–15:30 **Session 1** **Chairman: Olaf Strauß**

13:50–14:15 Antje Grosche, Leipzig: *The role of the Müller glia cell in health and disease*

14:15–14:40 Volker Busskamp, Boston: *Genetic reactivation of photoreceptors*

14:40–15:05 Antje Biesemeier, Tübingen: *Melanin and lipofsucin granules in the RPE and their impact on future therapies*

15:05–15:30 Souska Zandi, Genf: *The influence of macrophage polarization on choroidal neovascularization creates novel treatment options*

Coffee break with scientific chitchat

16:30–18:30 **Session 2** **Chairman: Klaus Rüther**

16:30–17:00 Ulrich Dirnagl, Berlin: *(Failure of?) Bench to bedside translation: Lessons learned in stroke research*

17:00–17:30 Michael Brand, Dresden: *Retina regeneration in zebrafish – how do they do it ?*

17:30–18:00 Peter Charbel-Issa, Bonn: *Gene therapy – from bench to bedside*

18:00–18:30 Bernhard H. F. Weber, Regensburg: *High-throughput analysis in DNA testing*

18:30 **Dinner**

19:30–open **Swingin' poster session**

PRELIMINARY PROGRAMME

Saturday, March 23rd 2013

08:45 – 10:45 **Session 3** **Chairman: Bernhard Weber**

- 08:45 – 09:15 Peter Westenskow, La Jolla / San Diego: *Emerging therapeutic approaches for AMD*
- 09:15 – 09:45 Christoph Wierling, Berlin: *Computational tools and mathematical modeling of cellular systems for personalized medicine*
- 09:45 – 10:15 Heiko Fuchs, Düsseldorf: *Regulating the regulators: microRNA in translational research*
- 10:15 – 10:45 Linda McCarthy, GlaxoSmithKline, London: *Genome-wide genetic approaches to drug target identification, and drug development support*

10:45 – 11:30 **Coffee break**

11:30 – 12:55 **Session 4**
Selected poster presentation and poster award 2013
Six poster presentations: Three PRO RETINA awardees

12:55 – 13:00 **Concluding remarks**

13:00 **Lunch and end of meeting**

PRO RETINA
POTSDAM-MEETING

SPEAKER ABSTRACTS

RD Research: Moving from scientific darkness to the enlightened era of clinical trials

Gerald J. Chader, Doheny Eye institute, USC School of Medicine, Los Angeles, CA, USA

Purpose: This is a time of great opportunity in retinal degeneration (RD) research with good progress in new areas of investigation, translation to clinical trials and even some available therapies.

Methods: Treatment depends on whether photoreceptor cells are alive or dead. When sufficient numbers remain viable, 1) Neurotrophic Therapy 2) Nutritional Therapy and 3) Gene Therapy may be applied for sight preservation. However, when most/all cells are dead, 4) Transplantation/ Regenerative Medicine 5) Optogenetics and 6) Prosthetic Therapy can be used to replace the cells or their function.

Results: 1) Neurotrophic Therapy uses neurotrophic agents to save photoreceptors and improve their function. Clinical trials are now in progress with CNTF on RP and dry AMD patients. Results imply prolonged photoreceptor cell survival. In the future, many such agents are available for testing both singly and in combination. 2) Nutritional Therapy, using antioxidants, has been successful in RD animal models. A small clinical trial has concluded on RP and AMD patients. As with neurotrophic agents, many potent antioxidants are known and could be tested singly or in combination in RD animal models and, subsequently, in clinical trials. 3) Gene Therapy is showing success in clinical trials. Sight restoration is apparent in RPE65 gene replacement therapy. In the future, many genetic types of RD can be studied. 4) Early Transplantation Therapy studies yielded marginal results but more recently, specialized cell use (as with early progenitor cells) has given more positive pre-clinical results. Similarly, stem cell transplantation has seen good success in RD animal models. In the future, use of adult stem cells, re-programming of adult cells (rods, Muller glia, cornea limbal cells, etc.) can all be applied to photoreceptor regeneration. 5) Optogenetics is relatively new but making great progress in sight restoration. Many optical photoswitches (such as channelrhodopsin) are available for testing in remaining retinal cells. 6) In Artificial Vision, implanted electronic prostheses stimulate remaining secondary neurons to produce light-directed signals, brain images and sight restoration. Several types of prostheses are being tested in the human with one now commercially available. In the future, improvements in prosthesis design are being made such as the use of wider arrays to give more peripheral vision, increased numbers of electrodes to give better visual acuity and novel advances such as using the retina's "neural code" to reach the level of normal vision.

Conclusion: This is an exciting time for RD research - for the basic researcher, the physician and the patient. Some treatments are already available, several are in clinical trial and basic science is giving us many more options for testing and ultimate sight preservation and restoration.

The role of the Müller glia cell in health and disease

A. Grosche[1], *T. Pannicke*[1], *A. Bringmann*[2], *A. Reichenbach*[1]

[1]*Paul Flechsig Institute of Brain Research, University of Leipzig, Germany*
[2]*Department of Ophthalmology and Eye Clinic, University of Leipzig, Germany*

Purpose: Müller cells, the principal retinal macroglia cells, fulfill housekeeping functions such as the maintenance of the extracellular ion- and volume homeostasis, but evidence is accumulating that they also modulate the neuronal information processing by the release of gliotransmitters such as glutamate or purines. We aimed to increase the knowledge about the relevance of intact Müller cell functions for the tissue integrity of the healthy and diseased retina.

Methods: To assess information about the physiological properties of normal and gliotic Müller cells, a broad spectrum of methods such as microfluorimetric measurements to identify mechanisms of Müller cell volume regulation, electrophysiological recordings and an enzyme-linked assay detecting glutamate release from single Müller cells, were applied and a model of retinal ischemia/reperfusion in mice was included.

Results: The efficient volume regulation of Müller cells was tightly linked to their pronounced Kir4.1 channel-mediated potassium conductance, but also depended on the activity of a glutamatergic-purinergic signaling cascade. These functions were considerably altered in Müller cells of the postischemic retina – they lost their capability to counteract osmotic imbalances and their Kir-currents were greatly reduced. We identified the G_{q11}-coupled ATP/ADP-sensitive $P2Y_1$ receptor as a key player in the context of these processes. Its activation was essential for the volume regulation in healthy Müller cells and its stimulation by exogenous ATP restored the capability of volume regulation in gliotic Müller cells. Interestingly, we could also demonstrate a $P2Y_1$ receptor-dependent ATP-induced glutamate release from Müller cells which possibly resembles a positive feedback-mechanism in their volume regulatory cascade. The $P2Y_1$ receptor was discussed to regulate the gliotic activation of glia cells, thus we included $P2Y_1^{-/-}$ mice in our study on an ischemia/reperfusion model. There were less pronounced gliotic changes in the Müller cell physiology in these mice. While the Kir4.1 channel-mediated inward currents were significantly down-regulated in the postischemic retina of wildtype animals, they remained at nearly normal levels in the $P2Y_1^{-/-}$ mice. Howsoever, we found an enhanced apoptotic activity and cell loss especially in the photoreceptor layer in $P2Y_1^{-/-}$ mice.

Conclusion: The $P2Y_1$ receptor is essential for correct Müller cell functions, but also modulates adaptive changes of Müller cells under pathological conditions, thereby affecting the survival of surrounding neurons.

Genetic reactivation of photoreceptors

Volker Busskamp, PhD

Genetics Department, Harvard Medical School, Boston, USA

Purpose: In retinal degenerative diseases such as *Retinitis pigmentosa* (RP) rod photoreceptors degenerate whereas light-insensitive, morphologically altered cone photoreceptors persist for a longer period. I will present that a single gene therapeutic intervention to cones in RP retinas restores light sensitivity.

Methods: Adeno-associated-virus (AAV)-mediated gene transfer was used to express microbial halorhodopsins (hyperpolarizing opto-genes) specifically in cones in two mouse models of RP; one representing a fast (f-RD) and one a slow form (s-RD) of retinal degeneration. Photoreceptor-specific promoters restricted the opto-gene expression to cones. Resensitized retinas were analyzed by molecular biological, imaging and electrophysiological techniques. Furthermore, this approach was successfully translated to *postmortem ex vivo* human retinas.

Results: The targeted expression of hyperpolarizing opto-genes to cones could substitute for the native phototransduction cascade and restored their light sensitivity. Resensitized photoreceptors activated all retinal cone pathways (the natural ON and OFF pathway), drove sophisticated retinal circuit functions including directional selectivity, activated cortical circuits and mediated visually guided behaviors. Halorhodopsins could also reactivate light-insensitive human photoreceptors in human *postmortem ex vivo* retinas.

Conclusions:
These results demonstrate that, despite the diverse genetic origin of RP, the targeted expression of a single gene to persisting cone photoreceptors can restore significant functionality to the visual system following degenerative changes.

References:
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Lipofuscin granules in the RPE and their impact on future therapies

Antje Biesemeier, Sylvie Julien, Ulrich Schraermeyer

Section for Experimental Vitreoretinal Surgery, Institute and Department of Ophthalmology, Schleichstr. 12/1, University Hospital Tübingen

Purpose: The Retinal Pigment Epithelium (RPE) serves important functions for retinal survival and metabolism. Pigments like lipofuscin and melanolipofuscin accumulate with age in the RPE. They are phototoxic and thought to be one major cause for the onset and progression of atrophic Age related Macular degeneration (AMD) and also Stargardt's disease. Until now, no therapy for such lipofuscin associated RPE and retinal degeneration is available.

Methods: Several treatment options are discussed to elucidate new therapy options for the restoration of RPE function and the prevention of geographic atrophy. Focus will be led on strategies that reduce or even eliminate toxic lipofuscin accumulation.

Results: The following four treatment strategies will be focused: 1) To replace aged lipofuscin laden RPE cells by transplantation of new cells without lipofuscin. 2) To prevent further accumulation of lipofuscin by modulating or inhibiting the visual cycle in order to reduce the production of bisretinoids. 3) To prevent further accumulation of lipofuscin by inhibiting (auto) phagocytosis. 4) To eliminate already existing lipofuscin.

Conclusion: All these strategies have the power to either eliminate or prevent the production of phototoxic lipofuscin. Concurrent restoration of the RPE functions will prevent or even slow down the degeneration of the RPE and retina with age. The strategies are therefore all promising, but more research has to be done to make them safe treatment options for the prevention of blindness.

Effect of rho kinase on macrophage differentiation in AMD

S. Zandi[1, 2], S. Nakao[2, 3], F. Hafezi[1], A. Hafezi-Moghadam[2].

[1]Ophthalmology, HUG, Geneva, Switzerland; [2]Center for Excellence in Functional and Molecular Imaging, Brigham and Women's Hospital, and Department of Radiology, Harvard Medical School, Boston, MA.; [3]Ophthalmology, Kyushu University, Fukuoka, Japan.

Purpose: Macrophages are involved in the pathology of age-related macular degeneration (AMD). However, the function of ocular infiltrating macrophages and their subtypes in AMD is not fully understood. Here we show their contribution to choroidal neovascularization (CNV) and the influence of the Rho/Rho kinase (ROCK) signaling pathway on macrophage recruitment and polarization.

Methods: Paraffin embedded sections of human CNV membranes were stained with antibodies (Abs) against human CD206 (MMR), CD80 and human ROCK1 or ROCK 2. To further characterize macrophage phenotypes *in vivo,* flow cytometry for ocular-infiltrating M1- and M2-like macrophages during the development of CNV was performed at different time points. A total of 12 eyes were examined per group. The cells were stained with PE anti-mouse CD11b, FITC anti-mouse CD206 (MMR) and PE-Cy5 anti-mouse CD80 Abs.
To investigate whether ROCK inhibitors modulate leukocyte infiltration in CNV, we stained for the macrophage markers, F4/80 and CD11b three days after laser injury. Leukocytes transmigration was investigated using the Acridin-Orange Leukocyte Transmigration Rate Assay.

Results: In CNV membranes from human patients both, CD206 and CD80 were expressed and co-localized with ROCK 1 and 2.
We isolated cells from mouse CNV membranes and found that the number of CD11b(+)CD80(+) M1-like macrophages significantly increased on day 1 after laser injury and remained high through day 7. In contrast, the number of CD11b(+)CD206(+) pro-angiogenic M2-like macrophages was only significantly increased on day 7 after laser injury. ROCK 2 inhibition substantially reduced the CD11b(+) CD206(+) M2 population.
The number of accumulated F4/80(+) and CD11b(+) macrophages 3 days after CNV induction was significantly reduced by dual, but not by ROCK2 inhibition alone. However ROCK 2 inhibition decreased leukocyte transmigration, whereas dual ROCK inhibition did not.

Conclusions: This work elucidates the role of macrophage differentiation in CNV and the to-date unknown role of Rho-kinase in macrophage recruitment and polarization. We introduce Rho-kinase as an attractive molecular target in the prevention and treatment of AMD.

(Failure of?) Bench to bedside translation: Lessons learned in stroke research

Ulrich Dirnagl

Center for Stroke Research Berlin, Charité Universitätsmedizin Berlin

Stroke is a top cause of morbidity and mortality worldwide. Although some fellow researchers may disagree, stroke related research is well funded by national agencies, foundations, and the industry. Thousands of stroke researchers have produced tens of thousands of research articles over the last decades, and have greatly advanced our knowledge about the pathophysiology of stroke. Yet, breakthroughs in the treatment of stroke patients have not kept pace with the investment in and the urgency of the problem. Lindsay Symon, the famous british neurosurgeon, has been quoted with the pun "The outlook for stroke therapy is excellent ... if you are a rat". He said that in 1986. Thrombolysis and the treatment of stroke patients on dedicated wards ('stroke units') have substantially improved the outcome of stroke patients, but pharmacological neuroprotection, or even neurorepair, remain elusive. It appears that stroke is but one example for a critical and widespread problem of modern biomedicine: the difficulty of taking findings from the bench to successful large randomized controlled clinical trials and ultimately to the benefit of patients. 'Bench to bedside translation' is the mantra of modern biomedical research, and the 'translational bottleneck' its nemesis. In my talk I will discuss the following hypotheses: 1) Stroke pathophysiology is well advanced, but due to its complexity, many relevant issues remain unexplored and unresolved. 2) There is quantitative evidence that translational stroke research has a quality problem (which can be generalized to translational biomedicine. 3) There is quantitative evidence that preclinical stroke research can be predictive for human pathophysiology and clinical trial outcome: Translation works. 4) Research on specific diseases (e.g. stroke) has tunnel vision with respect to the organ which is primarily involved (e.g. the brain in stroke). However, most diseases have their origin in many systems of the organism (e.g. the heart and vasculature, the immune system, etc. in stroke), and the disease has important consequences for many systems of the body, which may become more relevant for outcome than the primary lesion itself (e.g. pneumonia and sepsis in stroke). 5) Both, the advances as well as the setbacks of stroke research may inform us how to overcome the translational roadblock. These lessons may have important implications for other biomedical fields.

Retina regeneration in adult zebrafish – how do they do it ?

Michael Brand, Sarah Hochmann, Anke Weber, and Stefan Hans

DFG Research Center / Cluster of Excellence for Regenerative Therapies Dresden, CRTD, and Biotechnology Center (BIOTEC), TU Dresden, Germany. michael.brand@biotec.tu-dresden.de

Purpose: Photoreceptor cell loss during adulthood is a major cause for blindness in humans. Studies of retinal degeneration and regeneration processes in regenerating model organisms are a powerful source of insight for retinopathy studies. Unlike mammals, zebrafish can regenerate their central nervous system and retina. The wealth of experimental tools and the cone-dominated retina of zebrafish make it a good model for studies of human retinopathies.

Methods: We established a transgenic, conditional genetic lesion model in the Fgf pathway for analyzing photoreceptor degeneration and regeneration specifically in adult retinal tissue. We employed BrdU birthdating experiments, apoptosis assays and immunocytochemistry as well as transgenes driving lineage-specific GFP expression to study this model. In addition, we developed a new focussed-light lesion model to study regeneration from endogeneous retinal stem cells.

Results: Using conditional dominant-negative transgene expression or focussed light lesion, we specifically ablated photoreceptor cells, while other cells are largely unaffected. After Fgf withdrawal or light lesion, photoreceptors gradually die and have disappeared within one week after onset of transgene expression. In time course experiments, we find early onset of cell death and regeneration with a peak of proliferation at 3 days post lesion. Importantly, the conditional transgene-induced degeneration is completely reversible in the adult zebrafish retina, and regeneration can be traced from dividing Müller glia progenitors into newly differentiated photoreceptors. Thus, a fast and profound regeneration response ultimately restores the layered retinal structure and vision by integrating new photoreceptor cells into the existing adult retina.

Conclusion: Adult zebrafish retina provides a powerful experimental framework for understanding the cellular and genetic requirements for retinal regeneration from endogenous Müller glia type stem cells. The new transgenic, conditional genetic lesion model for photoreceptors degeneration and regeneration for adult zebrafish retina allows us further to show that Fgf signaling is also a critical maintenance signal in the adult retina. This model will be used further to study the mechanisms that allow regeneration to occur in the adult vertebrate retina.

Hochmann, S., Kaslin, J., Hans, S., Weber, A., Machate, A., Geffarth, M., Funk, R. and Brand, M. (2012). Fgf signaling is required for photoreceptor maintenance in the adult zebrafish retina. PLoS One 7(1): e30365. doi:10.1371/journal.pone.0030365.

Gene therapy – from bench to bedside

Peter Charbel Issa

Department of Ophthalmology, University of Bonn, Bonn, Germany

The purpose of the presentation is to briefly review current gene therapy trials and to summarize challenges in translating gene therapy approaches from bench to bedside.
The genetic basis of inherited retinal dystrophies suggests that a gene therapy approach is logical either to replace or reduce the expression of defective genes. The first proof-of-concept clinical studies in patients with Leber's congenital amaurosis have suggested that retinal gene therapy is safe and potentially effective, at least for specific disease entities. However, potential difficulties of a gene therapy approach are numerous. They include the necessity to optimize cell tropism and transduction efficiency of viral vectors, or the challenge to package large genes such as *ABCA4* into viral vectors with limited space. Also, little is known on the impact of individual diseases on the efficiency of gene delivery and/or gene expression. From the clinical side, patient recruitment and selection may pose a challenge due to the rarity of certain diseases. Also, clinicians are needed with expertise or background knowledge in gene therapy, as well as in performing necessary surgical procedures and in defining meaningful endpoints for clinical gene therapy trials. The latter appears especially challenging because traditional outcome measures such as visual acuity testing appear inadequate for many instances. Last but not least, administrative issues dealing with gene therapy are novel to most getting involved.
Overall, the goal of translating gene therapy into clinical practice needs a multidisciplinary team, in which scientists and clinicians are required to understand each other's challenges.

High-throughput analysis in DNA testing

Bernhard H.F. Weber

Institute of Human Genetics, University of Regensburg, Germany

Purpose: In recent years, massive research efforts coupled with major advances in high-throughput technologies have greatly aided in the elucidation of the molecular causes of the Mendelian disorders. For many clinical entities, it has become evident that genetic heterogeneity is widespread rendering traditional genetic diagnostics approaches based on Sanger sequencing of single genes mostly insufficient. A prominent example of genetic heterogeneity offers the group of the retinal dystrophies with retinitis pigmentosa caused by mutations in probably more than 100 distinct genes. Therefore, DNA diagnostics with a reasonable efficiency in mutation detection requires parallel processing of many genes with a high level of accuracy.

Methods: Advanced technologies to analyze large-scale DNA sequences include deep sequencing approaches also known as next generation sequencing. Two approaches are available. One is a broad application addressing collectively all exons of the genome, the so-called exome. Alternatively, a targeted approach only analyzes the exons of selected genes. In principle, both approaches are feasible for DNA diagnostics but both have advantages and disadvantages which need to be discussed in light of economical and ethical considerations.

Results: As an integral part of customized healthcare, molecular diagnostics is essential to tailor medical care to an individual. In particular, it is needed to secure a clinical diagnosis, to establish carriers status, and to allow sub-classification of a given disease state. Clarification of an individual's genetic defect provides the basis for accurate evaluation of recurrence risk and paves the way for targeted treatment approaches. Available state-of-the-art technologies for high-throughput DNA analysis such as microarray-based resequencing and massively parallel sequencing are suited to address those needs of modern DNA testing. Such technologies have the capacity to yield sequence information of up to 600 Billion base pairs in a single analysis. Not surprisingly, the resulting depth of information and the required interpretation of the data pose enormous challenges to the analyst and the medical geneticist.

Conclusion: Considering the depth of data, a particularly demanding task necessitates the distinction of pathologically relevant mutations from a plethora of neutral sequence changes. Here, systematic approaches are needed to bring together available resources from bioinformatics, population-based information and functionally relevant data. The interpretation of such data will be complex as will be the task to communicate such a complexity to the patient.

Emerging therapeutic approaches for AMD

Peter D Westenskow, Toshihide Kurihara, Martin Friedlander

Purpose: We are interested in identifying genetic networks and novel molecular mechanisms that maintain the homeostasis of ocular endothelial cells and photoreceptors. The retinal pigment epithelium (RPE) provides essential and diverse supporting functions to both cell-types, and death or dysfunction of the RPEis characteristic of age-related macular degeneration (AMD) the leading cause of vision loss in industrial countries. As our knowledge of the pathogenesis of AMD improves, we can develop novel therapeutic agents to prevent retinal degeneration and, in the most severe cases, also treat the associated neovascularization.

Methods: To mimic the hypoxia induced in RPE cells as the choriocapillaris shrinks gradually in aging humans we performed inducible genetic gain and loss of function of the hypoxia inducible factors 1&2 (HIF1 and HIF2) and VEGF in adult transgenic mice. The mutants were analyzed using gene-profiling assays, histology, metabolomics, optical coherence tomography, and electroretinography. To treat rodent models of retinal degeneration, we generated RPE from induced pluripotent stem cells (iPS-RPE) and monitored grafted hosts using in vivo imaging and image-guided focal electroretinography. Perturbing the Ras signaling pathway using novel oligonucleotide-based therapies with anti-microRNA-132 limits endothelial cell sprouting and neovascularization in different disease models.

Results: The genetic loss of function of VEGF induces hypoxia in RPE cells, rapid atrophy of the choriocapillaris, and profound cone dysfunction in one week. Remarkably, the rod photoreceptors functional normally until 7-8 months post VEGF ablation before they begin to degenerate.Other phenotypes including metabolic irregularities, subretinal deposits, photoreceptor degeneration, and neovascularization are also observed. We generate human iPS-RPE that strongly resembles human primary RPE based on transcriptomic, proteomic, metabolomic, and functional assays. They also provide anatomical and functional photoreceptor rescue in rats with RPE-mediated degeneration (RCS rats).Ocular neovascularization can be strongly limited by preventing endothelial cell sprouting by injecting anti-miR-132.

Conclusions: We have demonstrated that the RPE is essential for maintenance of ocular vascular networks and photoreceptor homeostasis. The consequence of extreme hypoxia in rodents can be catastrophic and promote death of photoreceptors, especially in the cones. Cell transplantation strategies of healthy RPE cells that strongly resemble primary human RPE in atrophic retinas provides significant photoreceptor cell rescue. Finally, controlling the Ras pathway in endothelial cells with anti-miR-132 oligonucleotides effectively prevents pathological angiogenesis in the eye, a disorder commonly observed with multiple retinal degenerations.

Computational tools and mathematical modeling of cellular systems for personalized medicine

Christoph Wierling[1,], Hendrik Hache[1], Andriani Daskalaki[1], Thomas Kessler[1], Vikash Pandey[1], Alexander Kühn[2], Hans Lehrach[1]*

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The pathogenesis of many complex diseases such as cancer, diabetes or steatohepatitis is not yet well understood and the limited understanding of the underlying cellular interaction networks makes the identification of appropriate targets for new therapies difficult. This urges the need for appropriate computational models and advanced methods to understand the complex underlying networks. Much information is available on molecules and interactions of many cellular pathways. By processing literature information and pathway databases of different signaling pathways known to be relevant in many complex diseases such as cancer (like Wnt, Notch, BMP, Fas, EGF, Hedgehog, etc.), we have developed a large mathematical model of these pathways using the modeling and simulation software PyBioS (http://pybios.molgen.mpg.de). Although the development of large detailed mathematical models is difficult, the benefit one could gain using their predictive power is tremendous. However, the use of this data for the development of appropriate disease models is hampered by the lack of sufficient information about all the individual reaction kinetics along with their respective kinetic parameters in vivo. Hence, appropriate methods are needed to study these systems also in the absence of detailed information about most of the kinetic parameters. To overcome this bottleneck we have developed an approach, based on a Monte Carlo strategy, in which the kinetic parameters are sampled from appropriate probability distributions and used for multiple simulations in parallel. Results from different forms of the model (e.g., a model that resembles a certain mutation or the treatment by a drug) can be compared with the unperturbed control and used for the prediction of the effect of the perturbation. The modeling approaches are currently applied in the context of cancer, steatohepatitis and stem cell research. The established resources, tools, algorithms and models build a foundation for the application of systems biology strategies in medical and pharmaceutical research and, based on data from high-throughput genome, transcriptome, and proteome analysis, it enables the development of a personalized medicine.

Regulating the regulators: microRNA in translational research

Heiko Fuchs

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microRNAs (miRNAs) are short (20-24nt) single-stranded endogenously expressed RNAs that inhibit the translation of messenger RNAs predominantly by binding in the 3'-UTR of their target genes [1]. Due to their imperfect binding, each miRNA has the possibility to target and regulate multiple messenger RNAs. miRNAs play an important role in regulating developmental and physiological processes, lineage-specification as well as stem cell commitment [2,3]. A number of studies have shown that miRNA dysfunction is linked to severe diseases, including age-related macular degeneration (AMD), a degenerative disease of the retinal pigment epithelium (RPE) [4,5,6,7]. Angiogenesis, oxidative damage and inflammation have been reported as main factors that can induce RPE degeneration.
Here we want to present a number of selected miRNAs which have been previously reported to be involved in age-related macular degeneration. Furthermore, we want to present molecular tools which could be used to study miRNA function and how to control the expression of these miRNAs *in vitro*. Finally, we want to suggest how these miRNA tools could be used as miRNA therapeutics in translational research.

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POSTER ABSTRACTS

Prevalence of non-apoptotic cell death mechanisms in animal models for retinal degeneration

Arango-Gonzalez, Blanca[1]; Trifunović, Dragana[1]; Sahaboglu, Ayse[1]; Farinelli, Pietro[1,2]; Ekström, Per A.[2]; Ueffing, Marius[1]; Paquet-Durand, François[1]

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Purpose: An increasing body of evidence suggests that photoreceptor cell death in inherited retinal degeneration is governed by alternative cell death mechanisms. Since this issue is of major importance for the development of therapies for retinal degeneration, we set out to study markers for apoptotic and non-apoptotic cell death in different animal models of inherited retinal degeneration. These included human homologous models for autosomal dominant (*Rho*P23H and S334ter transgenic rats) and autosomal recessive Retinitis Pigmentosa (*rd1*, *rd2*, *rd10* mice), as well as for Achromatopsia (*cpfl1*mice).

Methods: We studied *in situ* a number of metabolic markers for apoptosis (BAX expression, cytochrome c leakage, activities of caspase-9 and -3), non-apoptotic cell death (activities of histone deacetylase [HDAC], poly-ADP-ribose-polymerase [PARP], calpain) and cGMP.

Results: We found that markers for non-apoptotic cell death (activation of calpain, HDAC and PARP as well as accumulation of PAR) were increased during photoreceptor degeneration in all analyzed animal models, along with cGMP. Withthe exceptionofthe*Rho* S334ter rat, markers for classical apoptotic pathwaysincluding BAX upregulation and translocation into mitochondria, cytochrome c leakage and activation of caspases-9 and -3 were not detectable.

Conclusions: Non-apoptotic cell death pathwaysappear as the common denominators of photoreceptor cell death along with mutations in different genes and in different rodent models for retinal degeneration. Its identification raises conceptual opportunities and targets towards development of mutation-independent treatments for inherited retinal degeneration.

Translocator protein 18 (TSPO) and microglia as a therapeutic target in retinal degeneration

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Background and Aim: Microglia plays an important role in maintaining retinal homeostasis. Pro-inflammatory reactions and chronic activation of microglia are common hallmarks of retinal degeneration and contribute to the progression of degenerative processes. Previously, we identified increased expression of the mitochondrial translocator protein 18 (TSPO) in activated microglial cells isolated fromretinoschisin-deficient and blue-light damaged retinas. In our study, we aimed to elucidate possible microglia-modulating effects of the specific TSPO ligand XBD173, which is used as an anxiolytic drug in neuropsychiatric disorders.

Results: *In vitro*, XBD173 shifted activated microglial cells towards a more ramified state, which is characteristic forneuroprotective M2 microglia. XBD173 significantly increased the phagocytic uptake of latex beads and apoptotic photoreceptor fragments in activated microglia. Microglial migration, a feature of pro-inflammatory microglia, was strongly reduced by XBD173 treatment.Accordingly, XBD173 treatment reduced the expression of the pro-inflammatory genes Ccl2, Il6 and iNOS in activated microglial cells in a dose-dependent manner. XBD173 treated activated microglia also showed reduced NO secretion and decreased neurotoxicity in a co-culture system with photoreceptor cells.

Conclusion: Based on our findings, TSPO expressed by activated microglial cells could be a therapeutic target to dampen retinal inflammation associated with degeneration. *Ex vivo* and *in vivo* experiments using the specific TSPO ligand XBD173 will be performed in the near future to support this concept.

MAGI2 links the periciliary Usher syndrome protein network to endocytosis

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The human Usher syndrome (USH) is the most common form of hereditary deaf-blindness. USH is a complex ciliopathy with at least 12 chromosomal loci assigned to 3 clinical subtypes, USH 1-3. We and others have previously demonstrated that all USH1 and USH2 proteins are organized into protein networks in the periciliary region.

To gain further insights into the molecular machinery of the periciliary adhesion complex we wanted to decipher its molecular composition. For this we adopted yeast-2-hybrid (Y2H) screens of a retinal cDNA library using the C-terminus of the USH1G protein SANS (scaffold protein containing ankyrin repeats and SAM domain) and of the USH2B protein VLGR1 (very-large G protein coupled receptor 1 = GPR98) as bait. In both screens, we identified the MAGUK protein MAGI2 (membrane-associated guanylate kinase inverted-2) as putative interaction partner to SANS and VLGR1. We subsequently affirmed their direct binding by independent, complementary interaction assays.

Previous studies on neurons have indicated that the scaffold protein MAGI2 participates in receptor endocytosis. Here we showed by selective knock down of MAGI2 and SANS that the MAGI2 mediated endocytosis is inhibited by binding of SANS to MAGI2. We consecutively provide evidence that the assembly of the MAGI2-SANS complex and thereby the monitored endocytosis is regulated by the phosphorylation of the complex partners by CKII kinase. In addition immunocytochemistry revealed partial co-localization of SANS, VLGR1 and MAGI2 in the periciliary region of photoreceptor cells. Furthermore the high resolution of immunoelectron microscopy demonstrated the association of the complex partners with endocytotic vesicles emphasizing their relation to endocytosis in this compartment.

In conclusion, we have identified MAGI2 as direct interacting partner of USH proteins providing the first evidence for a molecular link of the USH protein interactome to the endocytosis machinery in photoreceptor cells. Present data indicated a role of SANS-MAGI2-VLGR1 complex in endocytosis in the periciliary region of photoreceptor cells. These findings are in accordance with the recently emphasized endocytotic function of the ciliary pocket in primary cilia necessary for ciliogenesis and ciliary maintenance.

Supports: PRO RETINA Deutschland e.V.; DFG; FAUN-Stiftung; Forschung contra Blindheit; EU FP7 "SYSCILIA"

Characterization of Retinitis Pigmentosa (RP) mouse model, RD-10: A morphological and electrophysiological study

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The retina harbors the photoreceptors as well as a neuronal network that performs the first steps of information processing before the signals are relayed to the brain by the retinal output neurons, the retinal ganglion cells (RGCs). In different retinal diseases such as retinitis pigmentosa (RP), the photoreceptors degenerate over time but the retinal network, in particular the ganglion cells remain intact. This raises the possibility to stimulate electrically the retinal network or the RGCs with prosthesis such that they can relay information to the visual centers in the brain. Among many animal models of RP, the most extensively characterized animal is the *rd1* (Pde6b^{rd1}) mouse. The more recent identified Pde6b^{rd10} (*rd10*) mouse which carries a mis-sense mutation in the same gene, has a later onset and slower rate of photoreceptor degeneration than the *rd1* mouse. The slower degenerative time course makes *rd10* a more appropriate model of human RP, and presents a broader window of opportunity to test therapies for photoreceptor rescue.

In the present study we used anatomical and electrophysiological techniques to reveal modifications in the inner retina after photoreceptor loss. We investigated the effect of photoreceptors degeneration on anatomy and physiology of inner retina neurons in *rd10* mouse. Although there is a general assumption that the inner retinal cells do not suffer from photoreceptor death, we confirmed major changes both accompanying and after this process. Changes include sprouting of horizontal cells, lack of development of rod bipolar cells and progressive thinning and irregularity in INL (Fig.1).

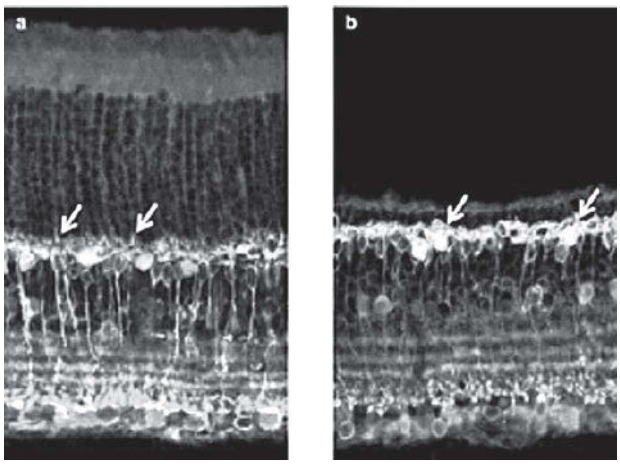

Fig. 1 a,b: The retinal staining representing rod bipolar cells (green), horizontal cells (red) and amacrine cells (blue). RD10 mouse retina shows a clear disappearance of photoreceptors and reduction in the rod bipolar cells complement dendrites at P32 (arrows in b), compared with their normal wild type counterparts (arrows in a).

Electrophysiological recordings by using multielectrode array (MEA), demonstrate neurophysiological properties of *rd10* retinas differ significantly from those of normal retina. This study investigated the resulting changes in the remaining network and ganglion cells. The presence of rhythmic burst in spontaneous activity of retinal ganglion cells (RGCs) and slow wave component (SWC ~5-7 Hz) are the basic difference observed in *rd10* mouse compared to normal mouse retina. A SWC with approximately 100 ms duration was also recorded along with spikes. We attempt to understand the mechanism of this SWC in degenerated retina by applying various synaptic blockers. Our results shows that SWC of *rd10* mouse is not the spike of ganglion cell but the excitatory post synaptic potential (EPSP) through the retinal synapses. Since, the bipolar cells properties are likely to alter due to photoreceptors degeneration: both excitatory inputs from photoreceptors and the feed-back inhibitory inputs from amacrine cells and horizontal cells to bipolar cells are likely to alter as well. Morphological studies also demonstrate that photoreceptor death is accompanied and followed by dendritic retraction in bipolar and horizontal cells, which eventually undergoes secondary degeneration.
The loss of photoreceptors during retinal degeneration is known to lead to an increase in basal activity in remnant neural networks. The rhythmic input to ganglion cells might hinder the successful stimulation of retinal ganglion cells via microelectrode prostheses. Thus, the SWC should be further studied and fully characterized to find a strategy to overcome its effects on retinal physiology.

Inflammation and AMD: Lipofuscin phototoxicity activates the NLRP3 inflammasome in RPE cells

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Purpose: Abnormal lipofuscin accumulation in the retinal pigment epithelium (RPE) as well as chronic local inflammation in the sub-RPE space has been implicated in progressive RPE dysfunction and degeneration in age-related macular degeneration (AMD). This study employs an in vitro model of lipid peroxidation-induced lipofuscinogenesis to investigate the effects of lipofuscin photoreactivity on RPE lysosomal membrane stability and activation of the NLRP3 inflammasome.

Methods: Lipofuscinogenesis was induced in human RPE-derived ARPE-19 cells and fetal humane RPE cells by incubation with isolated photoreceptor outer segments following modification with lipid peroxidation products. Lysosomal membrane permeabilization was induced by blue light irradiation (wavelength 455-460 nm; irradiance 0.8 mW/cm^2). Cytotoxicity was assessed by LDH release assay and DNA fragmentation assay. Following priming of cells with IL-1α, NLRP3 inflammasome activation was investigated by release of IL-1β. Specific inhibition of caspase 1 and cathepsin B was achieved by Z-YVAD-FMK and CA-074, respectively.

Results: Phagocytosis of HNE- or MDA-modified POS induced pronounced cellular lipofuscinogenesis. Subsequent blue light irradiation for 9 hours resulted in a reduction of cell viability to 17%. In contrast, control cells incubated with unmodified POS were unaffected by light treatment (viability 97%). Lipofuscin-associated cytotoxicity was mediated by apoptosis. Lysosomal membrane permeabilization occurred after blue light irradiation in a dose-dependent manner and was associated with activation of the NLRP3 inflammasome as evident from expression and release of mature IL-1β and IL-18. Inhibition of caspase activity using Z-VAD-FMK suppressed IL-1β release.

Conclusions: Lipofuscinogenesis induced by lipid peroxidation-related protein modifications renders RPE cells susceptible to light-induced lysosomal destabilization and pyroptotic cell death. Lipofuscin photoreactivity-mediated lysosomal membrane permeabilization is associated with activation of the NLRP3 inflammasome and release of proinflammatory IL-1β. Via this mechanism, lipofuscin accumulation in the RPE may contribute to chronic local inflammation in AMD.

Hyperosmotic induction of AQP5 and VEGF in retinal pigment epithelial cells

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Aim: During the diabetic state of hyperglycemia, the blood, ocular humors, and retinal extracellular fluid become hyperosmotic. High salt intake may result in raised blood pressure which is a risk factor of neovascular retinal diseases including diabetic retinopathy and age-related macular degeneration. Various factors and channels including VEGF and aquaporins (AQPs) may influence the development of retinal edema. We determined the effects of hyperosmolarity on the expression of AQP5 and VEGF in cultured human retinal pigment epithelial (RPE) cells.

Methods: Hyperosmolarity was induced by addition of 100 mM NaCl or sucrose to the culture medium. Alterations in the gene expression and secretion of VEGF were determined by real-time RT-PCR and ELISA, respectively. Phosphorylation of intracellular signaling molecules was determined by Western blotting.

Results: Hyperosmotic media increased, and hypoosmotic medium (60% osmolarity) decreased, the gene expression of AQP5 and VEGF in RPE cells. Hyperosmotic media also increased the secretion of VEGF from RPE cells. Triamcinolone acetonide (50 µM) prevented the hyperosmotic induction and secretion of VEGF. The stability of AQP5 and VEGF mRNAs was not different between normo- and hyperosmotic conditions, suggesting that hyperosmolarity induces an increase in the gene transcription. Hyperosmolarity induces phosphorylation of p38 MAPK and ERK1/2 in RPE cells. The hyperosmotic induction of AQP5 was decreased by inhibition of the activation of p38 MAPK and ERK1/2, respectively. Inhibitors of PI3K and JNK activation were without effects. Hyperosmotic media increase the gene expression of HIF-1α in RPE cells.

Conclusion: Hyperosmolarity induces increased expression of AQP5 and VEGF in RPE cells. High salt intake resulting in raised blood pressure and increased blood osmolarity may aggravate neovascular retinal diseases and retinal edema via stimulation of VEGF production in the RPE.

The sodium iodate model for transplantation of hESC-derived RPE

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Purpose: The Royal College of Surgeons (RCS) dystrophic rats are widely used for modeling human diseases characterized by RPE cell dysfunction or degeneration, and represent the standard pre-clinical model for RPE transplantation. However, there are disadvantages associated with this model, such as the early onset and fast progression of degeneration, and the ability of diverse cell sources to delay photoreceptor degeneration. Here we describe in detail the effect of sodium iodate (SI), a strong inducer of RPE cell death, on the mouse retina, and evaluate the utility of this model for transplantation experiments.

Methods: SI was injected systemically in 6-8 week old C57BL/6 mice. Functional characterization was done by ERG and by measuring visual acuity, 3, 7, 14, 21 and 28 days post-injection. Morphological characterization and RNA isolation were performed at the indicated time points. hESC-derived RPE cells were transplanted into the subretinal space 1 week post-injection. 3 weeks post-transplantation the retinae were isolated and analyzed.

Results: The effect of SI on RPE cells was very severe, leading to the complete loss of the RPE monolayer by day 7. The effect on photoreceptors was slower, with 10-33% reduction of ONL thickness by day 28. The ERG showed that 3 days post-injection the retinae were less sensitive and that by day 14 both a- and b-waves became unrecordable. Interestingly, light seemed to enhance the effect of SI, since ERG waves were better preserved when animals were kept in the dark. A reverse transcription followed by PCR using primers specific for genes involved in the phototransduction cascade and pigment regeneration showed a reduced expression of several genes by day 3. In SI-treated retinae protein mislocalization and dendrite sprouting of bipolar cells was observed. Interestingly, transplantation into the SI-model revealed that RPE donor cells were able to form a polarized monolayer on the free Bruch's membrane and to phagocyte shed OS.

Conclusions: Injection of SI is a reproducible protocol to cause RPE toxicity and, consequently, retinal degeneration. Additionally, it leads to altered gene expression and retinal remodeling. Our combined data suggests that the SI model is appropriate for analyzing the behavior of donor cells upon transplantation, but less suitable for functional studies.

Effects of bioflavonoids in human retinal pigment epithelial cells

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Objective: Bioflavonoid from vegetables and fruits are suggested to represent promising drugs for the treatment of cancer and retinal diseases. We compared the effects of various bioflavonoids (epigallocatechin-3-gallate [EGCG], myricetin and cyanidin) on physiological properties and viability of cultured human retinal pigment epithelial (RPE) cells.

Methods: The cell proliferation rate was determined by a bromodeoxyuridine immunoassay. Cell viability was studied with a trypan blue exclusion assay. Apoptosis and necrosis rates were revealed by DNA fragmentation ELISA.VEGF secretion was detected by ELISA. The phosphorylation of intracellular signalling proteins was explored by Western blotting.

Results: With the exception of EGCG, all flavonoids tested decreased dose-dependently the RPE cell proliferation and the secretion of VEGF. Myricetin induced a significant decrease in the cell viability at higher doses, via induction of caspase-3 independent cellular necrosis. The myricetin-induced RPE cell necrosis was mediated by calpain activation, oxidative stress, and activation of phospholipase $A_{2.}$ Cyanidin decreased the rate of RPE cell necrosis. Myricetin and cyanidin induced decreases in the phosphorylation levels of ERK1/2 and Akt protein.

Conclusion: The data show that EGCG has little effects on RPE cell proliferation, migration, and secretion of VEGF. The intake of myricetin as supplemental cancer therapy or in the treatment of retinal diseases should be accompanied by careful monitoring of the retinal function. Possible beneficial effects of cyanidin, which had little effects on cell viability, should be examined in further investigations.

High-resolution optical coherence tomography in mouse models of genetic and induced retinal degeneration

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The retina as a part of the nervous system has the unique advantage to be visualized through the transparent ocular media, which makes it one of the favoured regions to study neurodegenerative and -regenerative processes. Recent developments in the field of ophthalmic imaging such as optical coherence tomography (OCT) allow now non-invasive, three-dimensional investigation of retinal events over long time periods *in vivo*, even in small laboratory animals like mice. However, up to date a detailed analysis of how different degenerative processes of the retina are reflected in OCT images is still missing. Therefore, we perform experiments in which we use OCT to visualize the degeneration of photoreceptors and retinal pigment epithelium (RPE) cells of genetic and induced mouse models of retinal degeneration.

We use a self-developed high-resolution spectral domain OCT system for simultaneous dual-band imaging in the 0.8 µm- and 1.3 µm-wavelength range – the two most common spectral bands in biomedical OCT. This system is fiber-coupled to an ophthalmic scanning unit, which allows flexible imaging of the posterior and anterior eye segment with a high axial resolution of 3.2 µm and 4.3 µm in tissue. The field of view and the dispersion compensation can be adjusted to different eye lengths ranging from rats to zebrafish.

Until now, three different mouse models of retinal degeneration were investigated. These models divide into one genetic model with specific degeneration of the photoreceptors due to deficiency of rhodopsin ($Rho^{-/-}$), and two induced models in wild type mice. Induced retinal degeneration was achieved by systemic injection of sodium iodate ($NaIO_3$) leading to RPE degeneration and secondary loss of photoreceptors, and intense white light illumination causing light damage of the photoreceptors. The $NaIO_3$ model was carried out in C57BL/6 mice and the light damage was applied to Nrl-GFP mice. OCT imaging was performed daily or weekly, depending on the specific degeneration model, over a time period of up to 6 weeks. During that time, the degenerative processes caused significant changes in the retinal microstructure. Individual retinal layers that were affected by the specific degeneration could successfully be identified and monitored over the observation time period.

Therefore, longitudinal OCT studies deliver reliable information about the retinal microstructure and the time course of retinal degeneration processes *in vivo*. Hence, it will be a very useful tool for the investigation of retinal treatment response to novel therapeutic strategies in the future.

Cell replacement therapies for photoreceptor regeneration: Cultivation, modification and intraocular transplantation of photoreceptor progenitor cells

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Purpose: A recent clinical trial showing visual reinstatement via subretinal electronic prosthesis implies that even severely degenerate retinae may have the capacity for repair. While induced ploripotent stem cells (iPSc) may provide a renewable source of cells for patient-specific transplantation, being autologous, cells would display the same disease phenotype that is expressed in the patient. Characterisation, modification and intraocular transplantation of photoreceptor precursor cells (PPC) were here studied to provide preliminary proof of concept for development of ex-vivo gene therapy for retinal degeneration.

Methods: A system for long-term culture and expansion of rod PPC was established by comparison of varied culture conditions for early postnatal retinae, dissociated from Nrl.GFP mice, in which GFP is expressed specifically in rod photoreceptors. Cultured cells were transfected using florescent reporter minicircle DNA and AAV8 to assess expression of ex-vivo transfection of PPC. Magnetic assisted cell sorting was performed to enrich rod PPC in long-term culture via the cell surface antigen CD73. Long term cultured cells were lastly used for assessment of survival and integration in-vivo; transplanted into early postnatal C57/BL6 retinae.

Results: A reliable cultivation system was achieved by use of Neuronal growth media and incubation at 34°C. Rod PPC numbers significantly increased in vitro in the first 14 days and maintained high viability 31 days. Transfection of PPC was achieved by a florescent reporter minicircle DNA and by AAV8. Dissociated retinal cell cultures were enriched to over 85% Nrl.GFP positive rod PPC prior to transplantation, and integration of transplanted Nrl.GFP PPCs in the outer retina was observed within 14 days.

Conclusions: Long-term survival of rod PPC in-vitro will allow for a sufficient period of time for assessment of gene therapies before transplantation. This report presents, what is to our knowledge the first successful transplantation of long-term cultured PPC to the outer retina. The results discussed in this report afford a foundation for transplantation of genetically modifies and rod-enriched cells in murine models of retinal disease.

Functional analysis of HTRA1 variants associated with age-related macular degeneration (AMD)

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Introduction: AMD is a multifactorial disease of the central retina and a leading cause of irreversible vision loss in industrialized countries. On chromosome 10q26, over 20 common variants tagging a single risk haplotype have strongly been associated with the disease. The risk haplotype spans two gene loci, namely *ARMS2* (age-related maculopathy susceptibility 2) and *HTRA1* (HtrA serine peptidase 1). To date, it is still unclear which of the two genes in 10q26 is the target of the risk signals. In the current study, we are investigating the influence of risk-associated variants in and near *HTRA1* on transcription, translation, splicing, localisation and enzymatic activity.

Methods: To analyse the effect of risk variants on transcription of *HTRA1*, semiquantitative sequencing of *HTRA1* transcripts from human retinal/RPE samples heterozygous for the 10q26 risk haplotype was performed. In addition, qRT-PCR was performed with *HTRA1* cDNA from human retinal/RPE samples, lymphocytes and placentas, and reporter assays were performed with promoter constructs of non risk and risk associated *HTRA1* haplotypes up to 4.5 kb in size. RT-PCR with various primer combinations for *HTRA1* and cDNAs from human retinal/RPE samples of the 10q26 risk and wild type haplotypes was performed to identify unique splicing events for wt and risk variants. To assess an influence of the 10q26 haplotype on the HTRA1 protein COS-7 and HEK293 cells were transfected with risk and non risk HTRA1 variants. HTRA1 localization and translational efficiency were compared via immunocytochemistry and Western Blot. HTRA1 protease activity assays were performed with casein as substrate.

Result: *In vitro* and *in vivo* assays revealed no influence of the 10q26 haplotype on *HTRA1* transcriptional activity. There was also no effect of the 10q26 risk haplotype on splicing of HTRA1 mRNA. The synonymous risk-associated variants in exon 1 of *HTRA1* failed to show an influence on translational efficiency, protease activity or localization of the risk and non-risk derived HTRA1 protein.

Conclusion: So far, no significant influence of the 10q26 risk haplotype was observed on transcription, splicing, translation and localisation of HTRA1. Further studies are required to assess the functional effect of the AMD associated polymorphisms in 10q26.

Pericentrin, linked to a growing list of human disorders, identified at the basal-body complex in mammalian photoreceptor cells

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Pericentrin (Pcnt), a conserved protein of the pericentriolar material (PCM), serves as a multifunctional scaffold for numerous proteins and plays an important role in microtubule organization. To date, three Pcnt splice variants from orthologous genes in mice and humans are known. We generated a specific anti-Pcnt antiserum detecting all known Pcnt splice variants, and examined the cellular and subcellular distribution of Pcnt in ciliated tissues of the mouse, the olfactory epithelium and the retina. For the first time, we identified Pcnt and its centrosomal interaction partners at the basal body complex of mouse retinal photoreceptors. Photoreceptors are morphologically and functionally subdivided into the light sensitive outer segment and the metabolic active inner segment, which contains the typical energy producing and protein synthesizing components of an eukaryotic cell. The two compartments are linked via a modified, non-motile primary cilium, the connecting cilium. Here, Pcnt colocalizes with the whole of the protein machinery responsible for transport processes between the two photoreceptor compartments. Surprisingly, photoreceptors express a small Pcnt splice transcript, which is not present in receptor neurons of the olfactory epithelium.
Recent studies indicate that Pcnt mutations are associated with a range of diseases including primordial dwarfism (like Majewski/microcephalic osteodysplastic primordial dwarfism type II - MOPD II) and ciliopathies. Diseases associated with mutations in the *PCNT* gene display heterogeneous clinical manifestations, making it difficult to pinpoint the functional role of PCNT. Western blot studies, which reported the loss of PCNT in human, used for detection an antibody recognizing the N-terminal part of PCNT. However, the usage of such an antibody causes an experimental problem because it might not detect all PCNT splice variants, e.g. Pcnt S or any other variant lacking the N-terminal region of PCNT. Therefore we used in our analyses the self-made antibody MmPeriC1, which detects all splice variants. Our findings suggest so far for the mouse and even for humans a patchwork of different Pcnt splice variants in different tissues, which may explain why mutations in the human *PCNT* gene generate a multitude of different phenotypes.

Support: DFG (GI770/1-1), Schmauser-Stiftung, Universitätsbund Erlangen-Nürnberg e.V.

Comprehensive proteomics analysis reveals new substrates and regulators of HTRA1, a peptidase implicated in AMD

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Purpose: The aggressive (wet) form of age-related macular degeneration (AMD) is characterized by the abnormal formation of leaky blood vessels underneath the retina. This symptom can rapidly lead to blindness. Current treatment of wet AMD targets vascular endothelial growth factor (VEGF), in order to prevent further growth of blood vessels. Recent results suggest that the trypsin family serine protease HTRA1 is directly involved in the initiation of angiogenesis. This study set out to identify novel binding partners (substrates and/or regulators) of HTRA1. Such an improved understanding of the (patho)physiological role of this peptidase may aid development of new therapeutic strategies.

Methods: HTRA1 interacting proteins were co-immunoprecipitated from human placental lysates and from various human cell lines either endogenously expressing HTRA1 (SKOV3), or after transient transfection with a plasmid coding for HTRA1 (ARPE-19, HeLa). Stable isotope labeling with amino acids in cell culture (SILAC) and isotope-coded protein label (ICPL) enabled a high-throughput quantitative proteome profiling of the precipitates. Candidates exhibiting the strongest binding properties were selected for further analyses.

Results: The combination of greatly selective antibody-based isolation of protein complexes and quantitative mass spectrometry revealed high confidence HTRA1 -interacting partners. These include proteins involved in collagen biosynthesis or in TGF-β signaling pathway. Besides, several IGF-binding proteins and serine protease inhibitors (SERPINs) were identified. We also demonstrate the specific cleavage/degradation of selected putative substrates.

Conclusions: Our data suggest that HTRA1 directly contributes to the pathogenesis of AMD. The discovery of new substrates may help understand the role of HTRA1 in AMD. Furthermore, the identification of its natural inhibitors holds promise for the development of novel therapeutics.

Cone-like photoreceptor transplantation into the mouse retina

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Purpose: Vision impairment affects around 314 million people worldwide. In diurnal organisms, day vision depends on cone photoreceptors (PR) and several eye diseases including age-related macular degeneration,lead to cone PR degeneration. Several therapeutic approaches, such as geneand cell-therapy, are currently being developed mainly focusing on rod dystrophies, leaving cone-dystrophy therapies not well studied. Thus, we evaluated the feasibility of cone-likePR transplantation into wild type and diseased mouse retinas and the possibility of functional recovery.

Methods: Cone PRaccount for only 3% of the cells in the mouse retina. Hence,a more comprehensive source of cone PRswas developed. We crossed $Nrl^{-/-}$mice, that contain no rods but only cone(-like)photoreceptors, with an actin GFP reporter line (aGFP).The resulting linetg($Nrl^{-/-}$, aGFP) was used as a source for cone-like PRs. Cone-like cells were sorted using Magnetic Associated Cell Sorting (MACS) using CD73 as a cell surface marker. Enriched $CD73^{+}$ cells were transplanted into the subretinal space of adult wild-type (WT) retinas. Integration efficiency was analyzed 2 weeks after transplantation. Cone-likePRs were then transplanted into age matched cone dystrophy model (Cpfl1 mutant mice) and WT retinas.Integrationrate and functional recovery (ERG measurements) were analyzed 4 weeks after transplantation.

Results: The generated reporter line showed rosette-like structures typical of a rodless retina and expressed cone-specific markers. tg($Nrl^{-/-}$,aGFP) showed comparable ERG measurements to $Nrl^{-/-}$ mice. Cone-like PRsexpressed CD73, which was used as a cell surface marker. MACS-CD73sortedcone-like cells were able to integrate into WThosts, having a peak of integration at post-natal day 4 (P4). Integrated cone-like cells are able to acquire a mature photoreceptor morphology.P4 MACS-$CD73^{+}$ sorted cells were then transplanted into cpfl1 hosts, showing similar integration rates as in WT retinasand an increased a- and b-wave amplitudes under mesopic and photopic conditions.

Conclusions: Cone-like cells can integrate in different types of host retinas having a peak of integration at PN4. Cone-like cells express cone specific markers, acquire mature photoreceptor morphology and partially rescue daylight vision.Hence, cone-like cell transplantation-might represent a promising strategy for the restoration of vision in cone-dystrophies.

Subretinal microbeads – A stem cell based intraocular delivery system

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Microbeads are genetically engineered and microencapsulated human mesenchymal stem cells with the potential as slow release system for biologicals in the eye. We studied the impact of subretinal microbead implantation on retinal integrity and monitored viability of the xenogenic cells in the mouse eye by noninvasive retinal imaging (Spectralis™ HRA+OCT) and upon the end of the study by light and electron microscopy.

The implanted GFP-marked cells encapsulated in subretinal microbeads remained viable over a period of up to 4 months. Studies on the retinal integrity following implantation showed focal damage due to the surgical implantation, as well as GFAP upregulation and opsin mistargeting in the immediate surrounding of microbeads.

The accessibility for routine surgery and its immune privileged state make the eye an ideal target for release system implants for therapeutic substances, including neurotrophic and anti-angiogenic compounds or protein based biosimilars. Microencapsulated human stem cells, such as the microbead system, promise to overcome limitations inherent with single factor release systems, as they are able to produce physiologic combinations of bioactive compounds.

Towards elucidating the molecular pathology of X-linked juvenile retinoschisis: The retinoschisin-Na/K-ATPase interaction

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Background: Mutations in the *RS1* gene are known to cause X-linked juvenile retinoschisis (XLRS), a juvenile macular degeneration in males. The protein encoded by *RS1*, retinoschisin, is exclusively expressed in the retina, where it is secreted and binds to photoreceptors. Studies with *RS1h* knockout ($Rs1h^{-/Y}$) mice show that retinoschisin is essential for maintaining the structural integrity of the retina (Weber et al., 2002); however its function and thus its role in the aetiology of XLRS are poorly understood. Our recent studies demonstrated that the retinal Na/K-ATPase (a heterodimeric complex of ATP1A3 and ATP1B2) is a direct interaction partner of retinoschisin, essential to anchor retinoschisin to the outer surface of plasma membranes (Friedrich et al., 2011). Consequently, our goal is to delineate the molecular events underlying the retinoschisin - Na/K-ATPase interaction and to study its relevance on retinal integrity. In a first step, we aim to investigate the effects of retinoschisin binding on the retinal Na/K-ATPase.

Methods: Retinal cryosections from wildtype and $Rs1h^{-/Y}$ mice at postnatal days 10 to 18 (P10 to P18) were labeled with antibodies against retinoschisin, ATP1A3, and ATP1B2. Enzymatic assays were performed to document Na/K-ATPase catalyzed ATP cleavage in membranes of murine retinas (wildtype and $Rs1h^{-/Y}$), and of cultured human retinoblastoma cells (Weri-Rb).

Results: From P14, $Rs1h^{-/Y}$ mice exhibit a significant reduction in ATP1A3 and ATP1B2 expression in the photoreceptor inner segments and a mislocalization of ATP1A3 and ATP1B2 in the outer retina. However, retinal membranes of $Rs1h^{-/Y}$ and wildtype mice (P8 and P14) exhibited no differences in Na/K-ATPase mediated ATP cleavage. Additionally, the binding of recombinant retinoschisin had no influence on the Na/K-ATPase activity in murine $Rs1h^{-/Y}$ retinas and Weri-Rb, or on the sensitivity against Ouabain, a specific inhibitor of the Na/K-ATPase mediated ion transport.

Conclusion: The mislocalization of the retinal Na/K-ATPase in $Rs1h^{-/Y}$ mice indicates a direct stabilizing effect of retinoschisin on the Na/K-ATPase complex. Thus, insufficient Na/K-ATPase activity may contribute to the progressive photoreceptor cell death observed in $Rs1h^{-/Y}$ mice. However, no influence of retinoschisin binding on ATP cleavage and sensitivity against Ouabain was observed. Further studies assessing other functions of the Na/K-ATPase (ion transport, signal transmission and intercellular adhesion) are required to elucidate the effect of retinoschisin on the retinal Na/K-ATPase.

Epigenetic analysis of risk-associated variants in age-related macular degeneration in the human retina

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Genome wide association studies and meta-analysis of genome wide association studies have identified multiple disease-associated haplotypes influencing risk of developing age-related macular degeneration (AMD). However, a number of those findings are not readily in a context of a protein-coding gene locus and thus lack obvious causal variants. It appears possible though that such intergenic variants influence gene expression of cis- or trans-localized genes by interfering with transcription factor binding at regulatory regions.

The present study aims to identify potential regulatory regions in the human retina by a genome-wide analysis of epigenetic markers. Therefore, we performed chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq) of three healthy human retinas with antibodies against histone H3 lysine 4 monomehtylation (H3K4me1) and histone H3 lysine 27 acetylation (H3K27ac). Regions within 2kb up- or downstream of known transcription start sites were excluded from analysis. Remaining sites that showed a more than threefold enrichment for H3K4me1 or H3K27ac were considered to be potential regulatory *hot spots*. We show that within these regions, the most abundant motif (found in approx. 30% of all sites) corresponds to the cone-rod homeobox (CRX) binding site. Additionally, we found several transcription factor binding motifs highly similar to the consensus sites for regulatory factors including CTCF, Mef2a/c or NR2E3. We further show that several AMD risk variants lie within those regulatory *hot spots* and can therefore potentially alter transcription factor binding and hence, influence the function of the regulatory element. The impact of AMD risk associated variants on transcription levels of near-by genes is currently subject to further analysis.

Adaptive optics SLO and micro-stimulator for high-resolution retinal imaging and function testing

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Recent advances in wavefront sensing and control have made it possible toimage the human retina in real-time with enough resolution to seeindividual cells. This remarkable capability was primarily made possible by correcting the eye'simperfect optics with adaptive optics – a technique borrowed from astronomers who strove toincrease the resolution of ground-based telescopes. In particular, the adaptive optics scanning laser ophthalmoscope (AOSLO) is likely to be the next generation imaging technology, providingclinicians and vision scientists with unparalleled resolution to probe the basis of vision at the cellular level *in vivo*. By incorporating image-based eye tracking and fast light switching control, the AOSLO can also be used to deliver light to targeted cells on the retina non-invasively, thereby creating the unique possibility to relate retinal structure to visual function directly. We present an AOSLO based microstimulator that holds immediate promise for use in a clinical environment. In the clinic, the AOSLO will help characterize retinal disease progression earlier and monitor pharmaceutical intervention at the level of individual photoreceptors in living subjects. This strategy lessens the need of animal models or inefficient, protracted histological validation in the development phase of novel treatments for retinal diseases. In addition to clinical applications, cell-level access to living neuronal tissue opens the door to study the basics of visual function with psychophysical methods at a previously inaccessible microscopic scale.

Knockout of complement regulator CD59a leads to dysregulation of complement factors and increased macrophage accumulation in the RPE/choroid of mice.

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Several polymorphisms in complement genes are associated with an increased risk to develop age-related macular degeneration (AMD), one of the most common causes for blindness in the elderly. This suggests that dysregulation of the complement system may be an important pathogenic mechanism in AMD. To investigate the consequences of altered complement regulation in the RPE/choroid and retina with age, we examined *Cd59a* complement regulator knockout mice between 4-15 months.
Cd59a$^{-/-}$ mice on a C57BL/6J background and age-matched C57BL/6J wildtype (wt) controls were assessed for increased fundus autofluorescence by *in vivo* scanning laser ophthalmoscopy. Fundus images were evaluated by two independent, masked graders. H&E stained paraffin sections and immunohistochemistry on RPE/choroidal flat-mounts were compared with expression analysesby quantitative RT-PCR of *C3*, *Cfb, Cfd*, *Properdin*, *Ccl2* and *KC*in RPE/choroid and retina at 4 and 9 months (n=6 per group) in order to understand inflammatory processes on the histological, cellular and molecular level.
In vivo imaging and quantification of autofluorescent spots in the fundus of wt and *Cd59a*$^{-/-}$ miceshowed a significant increase of spots from 4 to 15 months in both groups ($p<0.0001$, two-way ANOVA, Holm-Sidak, n(wt)=47, n(Cd59a$^{-/-}$)=62). This process was significantly elevated in *Cd59a*$^{-/-}$mice ($p<0.0001$), at 9 and 15 months, despite a grossly normal retinal histology. Immunohistochemistry with anti-CD45 and anti-Iba1 showed corresponding subretinal macrophages as a source of the autofluorescent spots. Relative qRT-PCR for *C3* showed a significant increase in the RPE/Choroid of 9 months old *Cd59a*$^{-/-}$mice ($p=0.042$) while levels in retina and 4 month old animals were not significantly altered. qRT-PCR analysis of *Properdin*, Ccl2 and *KC* showed no significant changes between the genotypes at 9 months.
This data suggest that the normal age-related accumulation of subretinal macrophages is more pronounced in *Cd59a*$^{-/-}$ mice in age. This may reflect a cellular inflammatory response to the local dysregulation of the complement system in the RPE/choroid of *Cd59a* deficient mice and supports the importance of a well regulated complement system for the maintenance of homeostasis in the ageing eye.

Support: PH: Deutsche Forschungsgemeinschaft He 6175/1-1

Conflict of interest: None

Chimeric human opsins activate a non-native G-protein pathway

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Objectives: Opsins are light-sensitive G-protein coupled receptor proteins, essential for vision, circadian rhythmicity and eye development. Opsins modify cellular function by activating G-protein second messenger systems. Rod and cone opsins activate $G_{\alpha t}$, while melanopsin (OPN4), the opsin of photosensitive retinal ganglion cells, activates $G_{\alpha q/11}$. The $G_{\alpha q/11}$ pathway is ubiquitous in neurons, while the $G_{\alpha t}$ is exclusive to photoreceptors. If inner retinal neurons, such as bipolar cells, were modified to become light sensitive, these cells could act as substitute photoreceptors in patients who have lost their photoreceptors. Chimeric opsins were created as potential candidates for gene therapy targeting bipolar cells in degenerate retinae.

Methods: Eight chimeric opsins were cloned into the plasmid pMT4: four based on rhodopsin (RHO) and four based on long wavelength sensitive (LWS) opsin. Each chimera had a variable extent of its wild type intracellular surface replaced by the corresponding sequence from OPN4. All chimeras featured a C-terminal 1D4 epitope. Immunohistochemistry was performed using an anti-1D4 antibody applied to transfected HEK293T cells. Calcium kinetics were imaged using Rhod-2 fluorescent dye. Wild type and chimeric opsins were purified using a sepharose column and underwent UV-visible spectroscopy.

Results: Immunohistochemistry showed chimeric opsins to be correctly transported to the plasma membrane. Four of the eight chimeras induced increases in intracellular calcium in response to light: LWS with the third intracellular loop replaced with OPN4 sequence, RHO with the second and third, first to third and complete intracellular surface replaced with OPN4 sequence. Chimeric opsins produced calcium responses that were of a similar time course, but less intense, than those of wild type OPN4. Spectroscopy of wild type LWS opsin produced a λ_{max} of 558 nm. However, spectroscopy of chimeric opsins has hitherto been unsuccessful.

Conclusion: Chimeric opsins based on RHO and LWS are transported to the plasma membrane, where some of them function to produce light-induced increases in intracellular calcium. Such responses suggest that changing the intracellular surface of a $G_{\alpha t}$ signalling human opsin to that from a $G_{\alpha q/11}$ signalling human opsin, can change the signalling pathway. Such a novel opsin, could be utilised as gene therapy to patients who have lost photoreceptors.

Activated blood coagulation factor X (FXa) induces angiogenic growth factor expression in human retinal pigment epithelial cells

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Objective: Choroidal neovascular membranes are surrounded by a rim of fibrin which may stimulate vessel growth. To reveal whether activated blood coagulation factors may induce angiogenic responses of retinal pigment epithelial (RPE) cells, we determined the effects of the activated blood coagulation factor X (FXa) on the proliferation, chemotaxis, and expression of angiogenic growth factors in cultured human RPE cells.

Methods: Gene expression was determined by RT-PCR in human RPE cells. Alterations in gene expression and secretion of growth factors were determined by real-time PCR and ELISA, respectively. Proliferation and chemotaxis were investigated with a bromodeoxyuridine immunoassay and a Boyden chamber assay, respectively. Activation of signaling proteins was determined with Western blotting.

Results: The cells expressed mRNA for FX and FXa receptors. The gene expression of FX was increased by hypoxia and prostaglandin E_2. FXa induced chemotaxis of RPE cells via activation of the p38 MAPK signaling pathway. FXa induced expression of VEGF, HB-EGF, and bFGF, as well as secretion of VEGF, bFGF, and TGF-ß1 from RPE cells. The stimulatory effects of FXa on the expression of growth factors and secretion of VEGF were prevented by inhibition of the TGF-ß activin receptor-like kinase, but not by the thrombin inhibitor hirudin. FXa induced phosphorylation of ERK1/2, p38, and Akt proteins.

Conclusions: FXa induces chemotaxis of RPE cells, as well as expression and secretion of angiogenic growth factors from RPE cells, including VEGF. The effects of FXa on the expression and secretion of VEGF are mediated by autocrine/paracrine TGF-ß signaling. The results support the assumption that activation of the blood coagulation cascade may facilitate neovascularization in part by induction of angiogenic factors in RPE cells.

Electrically evoked responses of retinal ganglion cells in wild-type and *Rd10* mouse retinas.

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Purpose: Over the years, retinal implants have been developed to restore limited vision in patients blinded by outer retinal diseases like retinitis pigmentosa (RP) through electrical stimulation of the surviving neurons. The recently identified *rd10* mouse, which has a relatively delayed onset and slower progression of degeneration is an appropriate model to study RP. To support ongoing efforts to optimize prosthetic retinal stimulation, stimulation paradigms need to be established for this new mouse line. Here we investigate retinal ganglion cell (RGC) responses to different stimulation paradigms in adult wild-type (*wt)* and *rd10* mice.

Methods: RGC spiking responses were recorded *in vitro* from patches of *wt* and *rd10* retina **epiretinally**, using a planar multi-electrode array (MEA, 60 electrodes, 200µm interelectrode distance, 30µm diameter, MCS, GmbH). The stimulus was delivered via one of the 60 electrodes on the MEA while the other electrodes recorded electrically-evoked responses. Stimuli consisted of square-wave, monophasic voltage pulses in incremental blocks with randomized pulse durations. The same protocol was applied during **subretinal** stimulation in which a flex-MEA (36 electrodes, 300µm interelectrode distance, 30µm diameter, MCS, GmbH) was placed on the subretinal side to deliver electrical stimulation while the planar MEA recorded RGC responses.

Results: Under **epiretinal** stimulation, the *wt* and *rd10* retina demonstrated voltage and duration dependence. For both mouse strains, a dependence of the response on duration was typically seen only for a few transitional (threshold) voltages. Additionally, RGC's responsiveness decreased with increased interelectrode distance from the site of stimulation. No significant differences were observed in stimulus threshold between *wt* and *rd10* retina. Finally, we present preliminary epiretinally-recorded responses during simultaneous subretinal stimulation in *wt* retina using a 'sandwich' approach in which a flex-MEA is pressed onto the photoreceptor side of a retina already mounted ganglion cell side down onto a standard MEA.

Conclusions: Our preliminary findings present one of the first examinations of electrical stimulation in *rd10* retina. Based on these findings, we propose tentative stimulation parameters appropriate for activation of *rd10* retina in our continued development of more efficient stimulation protocols for the Tübingen retinal prosthesis.

Revised diagnostic strategy for inherited retinal dystrophies

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Purpose: The diagnosis of inherited retinal dystrophies (RD) is based on morphologic and functional criteria. The recent introduction of high-resolution spectral domain optical coherence tomography (SD-OCT), near-infrared fundus autofluorescence and wide-angle autofluorescence imaging provides new means to evaluate early stages of retinal disease. We evaluated our development of a revised diagnostic strategy for inherited RD in our specialized center for rare retinal dystrophies.

Methods: Retrospective evaluation of diagnostic criteria in 467 patients with inherited RD(examined between 9/2008 and 2/2013) who at their initial examination were examined with SD-OCT. Additional examinations included clinical examination, blue and green fundus autofluorescence (FAF), near-infrared fundus autofluorecence (NIA), wide-angle imaging, full-field electroretinography (ERG) or multifocal electroretinography (mfERG). Molecular genetic evaluation was performed

Results: The most important diagnostic step for the initial diagnosis of RD is the suspicion of this differential diagnosis. A delayed diagnosis was usually due to the erroneous diagnosis of other ocular disorders, the time period from the start of the diagnostic process to the diagnosis of RD was up to 8 years. The most important ophthalmologic examination is a combination of retinal imaging (FAF, NIA, SD-OCT). More than 90% of patients with RD can be detected with one of these methods, however, a combination is required as subtle alterations may present only in one of the techniques in early disease stages. Normal findings of retinal imaging are most frequently observed in cone dystrophies and stationary retinal dysfunction (CSNB, ACHM). Especially in these cases ERG and mfERG are diagnostically important. Ophthalmological findings are only rarely sufficient to identify associated genes, molecular genetic evaluation has a major role in the differential diagnosis of RD. Additional information can be obtained during long-term follow-up and especially in syndromes with the experience of experts in other specialities.

Conclusion: The clinical approach to accelerate the diagnosis of RD is based on 5 steps: 1. Think of it. 2. Retinal imaging. 3. Electrophysiology for selected cases 4. Molecular genetic evaluation. 5. Detailed differential diagnosis based on long-term follow-up and additional examinations in other specialities. In our experience retinal imaging has replaced electrophysiology for the initial diagnosis of RD, which provides a means for early diagnosis of RD.

Autosomal recessive incomplete achromatopsia caused by a homozygous nonsense mutation in PDE6H

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Purpose: Achromatopsia is an autosomal recessive retinal dystrophy characterized by color blindness, photophobia, nystagmus and severely reduced visual acuity. Four genes, *GNAT2*, *PDE6C*, *CNGA3* and *CNGB3*, have been implicated in ACHM, and all encode functional components of the cone phototransduction cascade.

Methods: Following a functional candidate gene approach that focused on screening further genes involved in the phototransduction cascade we analyzed a cohort of over 650 index cases with ACHM or other cone disorders by PCR and Sanger sequencing. Analysis was complemented by haplotype reconstruction and immunohistochemical stainings of murine retinal sections.

Results: We detected a homozygous single base change (c.35C>G) resulting in a nonsense mutation (p.Ser12*) in *PDE6H*. The c.35C>G mutation was present in three individuals from two independent families with a clinical diagnosis of incomplete ACHM. Patients from the two families share a common haplotype of 301 kb supporting that the c.35C>G (p.Ser12*) mutation is due to an ancestral mutational event. Moreover, we show by immunohistochemical colocalization studies in mouse retina that the encoded protein is expressed in all retinal cone photoreceptors.

Conclusion: These findings add the novel ACHM gene to the growing set of genes involved in cone disorders, and demonstrate the important role of this protein in cone phototransduction.

Support: Deutsche Forschungsgemeinschaft DFG (KFO134-Ko2176/1-2 and KFO134-Ru571/5-2), the Bundesministerium für Bildung und Forschung (HOPE: 01GM0850, 01GM1108A), the Foundation Fighting Blindness USA (BR-GE-0510-0489-RAD, C-GE-0811-0545-RAD01), the Research Foundation Flanders FWO (FWO11/KAN/013-31524611).

Rhodopsin interacts with the small GTPaseRac1, a signalling protein involved in photo-oxidative stress

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Purpose: The monomeric G-protein Rac1, a member of the family of Rho/Rac/Cdc42 GTPases, can be activated in a light-dependent manner in photoreceptor outer segments. Activation of Rac1 is involved in light-induced photoreceptor degeneration, since depletion of Rac1 in mouse rod photorecptors protects cells from phototoxic effects (1). The specific mechanism, however, remained elusive. Typically, Rho-GTPases influence the dynamics and re-organisation of the cytoskeleton and degenerative effects involving active Rac1 could be linked to an impairment of protein translocation. Another possible scenario is that active Rac1 stimulates guanylate cyclase activity in a PAK-dependent manner (2) increasing cytosolic cGMP-levels above normal resulting e.g. in cytotoxic intracellular Ca^{2+}-concentrations. In the present study we address the question, whether Rac1 is present in photoreceptor cells, at which cellular concentration and how it participates in signalling.

Methods: The presence of Rac1 in rod outer segments (ROS) was probed by immunohistochemical staining of bovine retinae and western blot analysis of isolated ROS. We investigated the interaction of rhodopsin and Rac1 using co-immunoprecipitation and surface plasmon resonance (SPR)-spectroscopy under dark and light conditions. Using the same approach we tested, whether Rac1 and Transducin (G_t) compete for binding to rhodopsin.

Results: Rac1 is present throughout the whole retina except in the outer and inner nuclear layer, but it is strongly expressed in photoreceptor cells. Interaction studies revealed that Rac1 associated with rhodopsin in a light-independent manner. Furthermore, Rac1 and G_t seem to compete for binding to rhodopsin. Preliminary kinetic analysis indicates that binding of Rac1 occurs with lower affinity and pace than the association of G_t.

Conclusions: In dark-adapted rod cells Rac1 cannot compete with G_t for binding to rhodopsin and signalling can proceed normally. Under intense illumination, when G_t is translocated to the inner segment, Rac1 can interact with rhodopsin by outdoing G_t. This process could trigger activation of Rac1 leading to photo-oxidative stress.

Haruta et al. (2009) PNAS 106, 9397-9402
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Ex vivo model to study murine retina regeneration

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Introduction: Loss of retinal tissue is not restored in adult mammals. Recent studies suggest that in the mammalian retina Müller glia (MG) have the potential to regenerate neurons. Nevertheless, the results are controversial and the amount of regenerated neurons was low. To increase the amount of regenerated neurons as well as to reveal the underlying mechanism we studied the MG in murine retinas ex vivo.

Method: Juvenile murine eyes after end of retinogenesis were enucleated and the neural retina was cultured as a whole organ with the ganglion cells upwards on membranes. To stimulate and monitor MG proliferation, mitogen EGF and BrdU were applied, respectively. Retinas were analyzed by immunostaining and 3D-microscopy.

Results: The temporal and spatial response of MG proliferation (Sox9+ BrdU+), de-differentiation (Sox9+ Chx10+) and its generated progeny were analyzed in a retina explant culture over 12 days ex vivo (DEV). PROLIFERATION: Sox9 is expressed in retinogenesis by all progenitors and maintained by MG. At DEV4, 48%$\pm$12 SEM (N=7) of the MG had entered the cell cycle and Sox9+BrdU+ cells peaked at DEV6 with 67%$\pm$5 SEM (N=10). DE-DIFFERENTIATION: Chx10 is expressed in retinal progenitors at the onset of neurogenesis. At DEV3 the first MG upregulate Chx10 and at DEV4 already 43%$\pm$8 SEM (N=7) expressed Chx10 (Sox9+Chx10+). PROGENY: Strikingly, using transgenic mice enabling a MG-lineage trace (hGFAP-cre::RYFP) we observed at DEV6 YFP+BrdU+Calb1+ expressing progeny. Also BrdU+Prox1+ (DEV6; 407$\pm$59 SEM (N=4) cells per central retina section) and BrdU+ Ptf1a+ (DEV3/4; 20$\pm$4 SEM (N=4) cells per central retina section) cells have been found. Intriguingly, the response of MG decreases significant over postnatal-age (P) (e.g. progeny at DEV6: P8 = 21$\pm$4 SEM (N=4) Calb1+BrdU+ cells versus P12 = 2$\pm$0.3 (N=4) Calb1+BrdU+ cells; $p<0.01$).

Conclusion: Our results show that MG cell cycle re-entry, de-differentiation and progeny generation follow a defined time-course. Further, our data suggest that these processes are age-dependently restricted. Expression of Ptf1a and Prox1, both essential for embryonic neuronal retinogenesis, in MG and derived progeny followed by Calbindin-expressing progeny suggest neuronal regeneration. Thus, the retina regeneration ex vivo approach is a powerful way to study MG-derived neuronal regeneration.

Support: CRTD seed and core grant (M.O.K.); ProRetina (K.L.)

Treatment of microglia with interferon-ß (IFN-ß) induces an anti-inflammatory phenotype

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Background and Aim: Microglia cells represent the resident macrophage population of the brain and retina. While a short period of controlled microglial activation is considered to be neuroprotective, chronic activation may lead to neuronal degeneration. This phenomenon is a hallmark of retinal degenerations in mice and humans.In a recent study, we identified interferon-α/-ß receptor signaling in microglial cells of retinoschisin-deficient mice, indicating that IFN-ß-dependent anti-inflammatory signaling may occur in retinal microglia. Our aim in this study was to elucidate IFN-ß target genes and functional characteristicsof *in vitro*cultured microglia cells.

Results: Stimulating ES cell-derived microglia (ESdM), a novel tool to analyze microglial function *in vitro*, and the cell line BV-2 with IFN-ß showed an upregulation of pro-and anti-inflammatory genes like Mx1, iNOS and Il6. The upregulated genes could be classifiedinto different functional categories such as metabolism (e.g. Apol9a), cell adhesion (e.g. Cd11b), migration (e.g. Cxcl10, Ccl2), cell differentiation (e.g. Slfn5) and immunoregulation (e.g. Il-6, iNOS, Mx1).On a functional level, we observed a ramified morphology in IFN-ß treated BV-2 and ESdM cells as shown by phalloidin staining. Moreover, ESdM and BV-2 cells showed increased phagocytosis of latex blue beadsand apoptotic photoreceptor fragments after stimulation with IFN-ß and lipopolysaccharide (LPS).

Conclusion: We conclude thatIFN-ß may induce an alternatively activated, potentially neuroprotective phenotype in microglial cells. Modulating microglia with the cytokine IFN-ß could be beneficial to protect from neuronal degeneration. *Ex vivo* and *in vivo* experiments will be necessary to support these preliminary observations.

In vitro and *in vivo* screen for Macrophage Migration inhibitory factor (MIF) and Dickkopf (Dkk) mediated neuroprotection

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Based on previous mass spectrometric analysis of the retinal Müller glial (RMG) cell secretome, we investigate the putative neuroprotective effects of the signalling molecules MIF and dkk in the degenerating retina.
It is known so far that MIF is involved in glucose metabolism and initiation of the innate immune response while Dkk influences gene expression towards an increased survival probability. Therefore both proteins are promising novel candidate factors for neuroprotection and were therefore used for our in vitro retinoprotection screen.
We use C3H mice that suffer from photoreceptor degeneration and collect complete retinas five days after birth. These retinas are then cultured for 14 days in the presence or absence of dkk and MIF. The application of MIF preserved an outer nuclear layer (ONL) of averagely 32 ± 8 µm, while Dkk led to an ONL of 32 ± 7 µm, compared to the ONL thickness of 9 ± 3 µm in the untreated control group. Given that the average ONL thickness in healthy C57/Bl6 mice is about 61± 4 µm, the results indicate that both substances slow the progress of degeneration for about 50%.
Additionally to the qualitative screen for neuroprotection, we use cultivated retinas also to determine downstream targets of the applied proteins. We focussed on transcription factors that may adapt retinal gene expression to counteract the degeneration process. Western blot analysis revealed that both substances induce the phosphorylation or expression of a variety of transcription factors. Additionally frozen sections of cultivated retinas were stained for these downstream targets to validate the western blot results. Which specific genes are particularly affected is under investigation.
Though the results are promising we cannot address questions about the proteins impact on functional vision using cultivated retinas. Consequently, we will take the next step and express the proteins in rd10 mouse retinas in vivo using Adeno-Associated-Viruses.

In vivo imaging of fluorescent probes linked to antibodies against human and rat vascular endothelial growth factor (VEGF)

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Purpose: To investigate fluorescent molecular probe linked to antibodies against VEGF for *in vivo* imaging of VEGF.

Methods: Bevacizumab (a humanized monoclonal antibody, Roche), antiRatVEGF (a polyclonal antibody against rat $VEGF_{164}$, R&D Systems) and B20-4.1.1 (a polyclonal antibody against human and rat VEGF, Genentech) were covalently attached to indocyanine green (ICG), a near-infrared dye, yielding soluble conjugates. Binding properties were assessed by an *in vitro* proliferation assay. Using confocal scanning laser ophthalmoscopy (cSLO), *in vivo* reflectance and fluorescence imaging was performed in Dark Agouti rats that had undergone argon laser photocoagulation to induce choroidal neovascularisations (CNV). Retinal uptake and fluorescence were recorded following intravenous and intravitreal injection of the dye conjugates for up to 100 days.

Results: *In vivo* imaging before dye application showed ill-defined retinal lesions at day 7. Immediately following intravenous and intravitreal injection a strong fluorescence was visible. Twenty-four hours following injection an accumulation of the antibody-conjugate at the site of the roundish laser lesions for all antibody-conjugates were observed. Furthermore, multiple fluorescent spots were visible up to 35 days following intravenous injection of Bevacizumab-ICG and for up to 70 days following intravitreal injection of Bevacizumab-ICG, B20-4.1.1-ICG and antiRatVEGF-ICG. No fluorescent spots occurred after intravenous injection of B20-4.1.1-ICG or antiRatVEGF-ICG. Over time, a continuous decrease of the fluorescence intensity was observed for all antibody-conjugates.

Conclusion: Pharmacokinetics of fluorescent-labeled bevacizumab, antiRatVEGF and B20-4.1.1 can be investigated *in vivo* following intravenous and intravitreal injection. Strong accumulations in the site of the laser lesion were observed for all antibody-conjugates. This novel molecular imaging approach of VEGF may be applicable in patients for earlier diagnosis and more refined individualized anti-VEGF therapies with the aim of optimizing functional outcomes.

Expression of recombinant ARMS2 and binding to self-cell-surfaces via glycosaminoglycans

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Purpose: Age-related macular degeneration (AMD) is the most common cause of blindness in developed countries. The disease is characterized by irreversible vision loss and the degeneration of retinal pigment epithelial cells due to the accumulation of deposits (drusen) at the macula. ARMS2 is one of three genes on chromosome 10 that are strongly associated with AMD. Recent data indicated that ARMS2 is causally related to the deterioration of vision in individuals carrying the risk alleles. However, reports about cellular expression and localization of the ARMS2 protein are inconsistent and the physiological role of this protein is still unclear. Therefore, we express recombinant ARMS2 in human embryonic kidney cells (HEK) and investigated the localization of the protein. Furthermore, we analyze whether the recombinant ARMS2 binds to cell-surfaces.

Methods: ARMS2 cDNA was cloned into the pCA vector coding for an ARMS2 protein fused with green fluorescent protein (GFP). HEK cells were transfected with this plasmid and ARMS2 expression was followed via GFP fluorescence detection. In addition ARMS2 expressed and purified from *Pichia pastoris* was incubated with wild type Chinese hamster ovary cells (CHO) and a mutant CHO strain (pGS-A) deficient in expressing GAGs on the surface. Subsequantly binding of ARMS2 was analyzed by flow-cytometry using a specific ARMS2 antiserum.

Results: The ARMS2-GFP fusion protein expressed in HEK cells was located intracellularly to small clusters in the transfected cells. Extracellular incubation of purified ARMS2 with CHO cells revealed that recombinant ARMS2 bound exclusively to those CHO cells that expressed GAGs on their surface and did not bind to pGS-A cells that are deficient in GAG expression.

Conclusion: ARMS2 binds specificly to GAG expressing CHO cells, thus indicating that ARMS2 harbors a heparin binding region and recognizes and binds to self-cell-surfaces. Furthermore, ARMS2 binds to living or late apoptotic/necrotic ARPE cells. These findings indicate, that ARMS2 is secreted into the extracellular space and subsequently acts as a surface bound protein.

Supported by the PRO RETINA Foundation

Translational read-through of retinal disease causing nonsense mutations

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Purpose: Disease causing nonsense mutations are frequent in patients suffering from nonsyndromic and syndromic retinitis pigmentosa (RP), including the human Usher syndrome (USH) which is the most frequent cause of inherited combined deaf-blindness. The over read of nonsense mutations by translational read-through inducing drugs (TRIDs) has become a promising treatment strategy for degenerative diseases. We formerly have focused *USH1C* and have shown that different TRIDs induce read-through of a nonsense mutation in *USH1C* in cell culture, organ culture and *in vivo*. Nevertheless previous data demonstrated that the read-through efficacy of nonsense mutations differ significantly within their genetic context. Here we aim to extend our research efforts to evaluate read-through efficacy of TRIDs in a series of nonsense mutations causing syndromic and nonsyndromic RP. The ultimate goal is to develop an effective treatment to cure retinal degeneration caused by nonsense mutations.

Methods: We screened the Leiden Open Variation Database (LOVD) database for USH causing nonsense mutations. We generated constructs coding for retinal disease-related nonsense mutations in *USH1C*, *USH2A*, *RP2* as well as *NPHP4*. HEK293T cells were transiently transfected with these constructs and TRIDs were applied into the culture media. Read-through experiments were analyzed by immunofluorescence microscopy and Western blot analyses.

Results: Our exemplary data base search revealed that approximately 20% of all USH cases are caused by nonsense mutations leading to the expression of truncated protein fragments or no protein at all. We demonstrated TRIDs induced read-through for different nonsense mutations causing USH. In addition, we validated the read-through efficacy TRIDs for further retinal diseases causing nonsense mutation.

Conclusion: The read-though efficacy of TRIDs on diverse nonsense mutations causing syndromic and nonsyndromic RP emphasizes the potential of TRIDs for the treatment of frequent causes of hereditary retinal disorders.

Supports: German Ministry of Education and Research (E-Rare-2, the ERA-Net for Research on Rare Diseases) "EUR-USH", FAUN-Stiftung, Nuremberg, the Foundation Fighting Blindness (FFB), EU FP7 "SYSCILIA" and "Treatrush".

Genetic cell ablation to study retina regeneration in the mouse retina

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Introduction: The adult mammalian retina undergoes, in contrast to fish and birds, no spontaneous neuronal regeneration upon retinal injury. Nevertheless, mammalian retinal Müller glia respond to neuronal damage by various molecular and cellular changes and some studies suggest that regeneration can be stimulated. Several investigations on retinal regeneration in fish and mouse suggested that a defined threshold of injury is required to lead Müller glia into cell cycle re-entry. The main aim of this work is to establish genetically controlled cell ablation to investigate the age-dependent regenerative potential of the mouse retina.

Methods: We crossed the alphaPax6-Cre::RosaAI9 strain with Cre-inducible diphtheria toxin receptor (Rosa-iDTR) transgenic mice. We chose the well-described alphaPax6-Cre mouse strain, as its Cre-expression is mainly restricted to peripheral retina cells, which provides a major internal control area free of cell ablation. Retinae from juvenile mice at postnatal day 5 (P5) and P10 were explanted and exposed to diphtheria toxin A (DTA) for 24h. To monitor cell proliferation BrdU was added. The explants were cultured for 1 to 5 days *ex vivo* and subsequently analyzed by immunostaining and imaging.

Results: With the Cre-inducible fluorescent-reporter AI9 we monitored the alphaPax6-Cre lineage and found a more widespread brain expression pattern than previously reported. DTA induced cell ablation was observed after 2 days (N=3) and was even more increased after 4 days (N=3). Immunostaining for iDTR provided a powerful tool to monitor cell ablation. According to the alphaPax6-Cre lineage we observed a decrease of RAI9 and iDTR positive cells as well as total Dapi cells over time. We confirmed cell death also by Caspase3 antibody staining. We found a time and age-dependent Müller glia activation (Gfap expression). A proliferative response in the control areas of ablated retinae was detected by cumulative labeling with S-phase marker BrdU.

Conclusion: We were able establish a new method for acute and accurate retinal damage to study retinal regeneration. Our ablation model provides the advantages of: 1) very specific local ablation; 2) an internal non-ablated control area; 3) easy ablation monitoring. Within this new ablation model we were able to show, that the juvenile mammalian retina is able to regenerate upon retinal damage.

Support: CRTD seed and core grant (M.O.K.).

Therapy strategies for Usher syndrome type 1C in the retina

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Purpose: The Usher syndrome (USH) is the most common form of inherited deaf-blindness. Three clinical subtypes (USH1-USH3) are defined according to the severity of the hearing impairment, vestibular dysfunction and the age of onset of retinitis pigmentosa (RP). USH1 is the most severe subtype with congenital severe to profound hearing loss and onset of RP during puberty. Currently no treatment of the senso-neuronal degeneration in the eye exists. In our studies we focus on *USH1C* gene which encode for the scaffold protein harmonin.

Methods: We assess three different gene-based therapy options to treat the retinal phenotype of USH1C patients, namely gene addition, gene-repair by homologous recombination mediated by endonucleases and translational read-through therapy using PTC124 and designer aminoglycosides.

Results: Our first approach is gene addition of harmonin isoforms using recombinant adeno-associated virus. However, our transcriptome analysis in human retinas demonstrates that *USH1C* is transcribed in various harmonin isoforms. The ambiguity concerning the activities of different harmonin isoforms makes USH a difficult target for gene addition approaches. Consequently we extend our research to alternative treatment strategies. Our 2nd approach is gene-repair by homologous recombination mediated by endonucleases, namely zinc-finger nucleases (ZFN) and TAL effector nucleases. We identified ZFNs for *USH1C* and proofed their functionality on the genomic and protein level in cell culture. In our 3rd approach we aim to develop a translational read-through therapy using PTC124 and designer aminoglycosides. Latter compounds target in-frame nonsense mutations which account for ~20% of all USH cases. We demonstrated the improved retinal biocompatibility of the designer aminoglycosides and PTC124 compared to "classical" aminoglycosides. We verified the read-through efficacy of the translational read-through inducing drugs for an USH1 causing nonsense mutation in cell culture, retina cultures and *in vivo* in murine retina.

Conclusion: All analyzed gene-based therapy strategies lead to the restoration of USH protein expression. These adjustments may be sufficient to stop or at least slow down the progression of retinal degeneration, which would greatly improve the life quality of USH patients.

Support: German Ministry of Education and Research (E-Rare-2, the ERA-Net for Research on Rare Diseases) "EUR-USH", European Community FP7 "SYSCILIA", "Treatrush", FAUN-Stiftung, Nuremberg, the Foundation Fighting Blindness (FFB).

Temporal characteristics of photoreceptor degeneration in the retina: Implications for the causative cell death mechanisms

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Hereditary retinal degeneration relates to a group of human diseases in which the photoreceptors degenerate and die. We asked the question as to how long the process of photoreceptor cell death takes, from the beginning to the end. For this study, we used the *rd1* mouse model, which is characterized by phosphodiesterase-6 (PDE6) dysfunction and photoreceptor death triggered by high cGMP levels. We employed cellular data on the progression of cGMP accumulation, cell death, and survival, to create mathematical models that simulated the temporal development of the degeneration and the clearance of dead cells. The cellular data and the modelling suggested that for an individual cell, the degenerative process was rather slow taking approximately 80h to complete. The model predictions were tested using organotypic retinal explant cultures derived from wild-type animals and exposed to the selective PDE6 inhibitor zaprinast. Surprisingly, detectable cGMP accumulation occurred only 36h after the beginning of PDE6 inhibition, suggesting that initially cGMP levels were kept in check by compensatory feedback mechanisms. Cell death increased significantly only another 36h later. Together, the *in vivo* data, the mathematical modelling, and the *in vitro* data allowed the discrimination of three major stages in photoreceptor cell death: 1) the initiation phase taking up to 36h, 2) the execution phase lasting another 40h, and finally 3) the clearance phase lasting about 7h. Paradoxically then, *rd1* photoreceptor neurodegeneration, while very rapid at the tissue level, is governed by rather slow mechanisms. This has important ramifications for the potential underlying cell death pathways, since such a long time-frame of 80h is axiomatically incompatible with the execution of necrosis or apoptosis.

The role of Properdin in the development and progression of age-related macular degeneration

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Introduction: Age-related macular degeneration (AMD) is the leading cause for irreversible vision loss in the aging population in industrialized countries. The growing population of elderly emphasizes the need for effective treatment options and the development of preventive programs. Currently, monoclonal anti-VEGF antibody therapy slows down neovascular age-related macular degeneration, a late complication in about 10-15% of AMD patients. Importantly, there is no effective therapy option for atrophic AMD, the most common phenotype with slow bilateral progression of the disease. The various phenotypes of AMD result from contributions of genetic, environmental and aging factors. Complement factor H, a known risk-associated genetic factor in AMD, is a negative regulator of the alternative complement pathway while Properdin is the sole positive regulator.
The aim of the present study is to establish Properdin detection systems from human and murine samples and to investigate the functional role of Properdin in AMD.

Methods: BALB/c mice were immunized either with purified human Properdin or Properdin-specific sequences in virus-like particles to generate highly specific and affine monoclonal antibodies against Properdin. Different immunological methods ELISA, Western Blot analysis and intracellular FACS staining were applied to characterize specific hybridoma clones. Commercially available and in house monoclonal antibodies were used for sandwich immunoassays to detect Properdin from buffer and serum samples.

Results: Newly generated monoclonal anti-Properdin antibodies mAb149 and mAb1340 detect highly sensitive native Properdin in an indirect ELISA format. Validation of sandwich ELISA assays show a specific detection of human Properdin from serum with a limit of detection in the lower ng/mL range. Consequently, we compared Properdin concentrations in serum samples from AMD patients and a matched control group. Murine Properdin was not detected from serum samples with the methods used for human specimen.

Conclusions: These initial results encourage further investigation into the functional role of Properdin in the development and progression of age-related macular degeneration. Further attempts are necessary to generate anti-Properdin antibodies against human and murine Properdin, which then can be used in AMD animal models and humans in parallel.

Age-dependent pathological vascular changes upon neurotoxin-induced degeneration of the mouse retina

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Background and aims: The retina has served as a model region of the CNS for landmark studies on pathologic neovascularization and angiogenesis. Here, we sought to study the interactions of neurons, glia and vasculature in the neuroretina after neuronal damage in vivo.

Methods: 5 mM of kainic acid (KA) was injected intraocular in mice at different ages. BrdU or EdU was administered daily to label proliferating cells. Retinal wholemount or sections were analyzed using immunostaining and confocal microscopy.

Results: KA-induced neurodegeration led to significant loss of the inner nuclear layer and inner plexiform layer after 7 days of post-lesion. Detailed analysis of transverse retinal sections showed that mostly amacrines and retinal ganglion cells are lost, but not Müller glia, bipolar, horizontals and photoreceptors. Interestingly, in juvenile retina (after end of embryonic retinogenesis), but not adult, we observed various types of major pathological vascular changes across the retina. Isolectin B4-staining revealed pathological vascular clusters (51±9 SEM clusters per retina, N=4) including the vascular sprouting, hypervascularization and vessel regression. Control injections of PBS (up to daily injections at 4 subsequent days) did not induce vascular defects. Meanwhile, many BrdU-positive cells (elongated shape) were visualized in pathological vascular clusters indicating that the cells within the clusters are able to re-enter into the cell cycle. Further, although substantial vessel regression and changes occurred, a significant amount of the vasculature defects were restored within three weeks. The vascular pathologic phenotype were age-dependent both occurring in juvenile and in comparison significantly less in adult mice.

Conclusion: We observed the induction of various pathologic vascular changes, includes sprouting, cluster formation and remodeling upon neurotoxic damage of the juvenile retina. Thus, we conclude that neuronal loss might lead to vascular pathologies. Furthermore, our data suggest that endothelial cells and pericytes are able to re-enter the cell cycle due to vascular injury, cell loss or angiogenic stimuli. Therefore, it will be of great interest to find out the underlying mechanism that (1) link loss of neurons to vessel damage, and (2) regulate and restrict vascular regeneration in the retina.

Funding: CRTD seed and core grant (M.O.K.), DZNE core funding (M.O.K.) and a DAAD Fellowship (S.B.P.).

Extracellular recordings from ganglion cells in the human retina

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Aim: The morphology and function of retinal ganglion cell (RGC) types have been characterized in all common laboratory animals. However, almost nothing is known on a functional level about human RGCs. Further, novel treatment approaches for retinal pathologies are usually tested in non-primate or even non-mammalian models. We believe that it is of great importance to study the function of human RGCs and to develop treatment testing paradigms with human tissue. We thus aimed at characterizing ganglion cell types in human retina. Additionally, we evaluated the usability of *post-mortem* tissue for testing of novel treatments such as optogenetics.

Methods: Human retinal tissue was donated by patients who had to undergo enucleation in the local eye clinic. We recorded the RGC responses with a 60-electrode multi-electrode-array (MEA). Various light stimuli were projected onto the photoreceptors of retinal patches, such as flashes of different contrast, white-noise-flicker, bars moving in eight directions, drifting sine wave gratings, and natural movies. We then characterized the response of each recorded RGC with various parameters such as polarity, latency, and tuning to spatial and temporal frequencies. The same paradigm was applied to pig retina after various ischemia times to evaluate the survival of *post-mortem* tissue.

Results: In one year we obtained 11 donated retinas. In 5 retinas we could record light responses. The recorded cells showed various properties such as different polarity (ON, OFF, ON-OFF), and distinct spatial and temporal tuning. The experiments with pig retina showed that light responses are present after at least up to 25 min of ischemia (i.e. photoreceptors still functional) and that spontaneous activity can still be measured after at least 12h of ischemia (i.e. ganglion cells still alive).

Conclusions: Different functional types of ganglion cells can be recorded in donated human retinas from patients. In the future, acquired expertise, improved collaboration with the eye clinic, and the recent implementation of a high-density MEA system (11000 electrodes) will allow further quick and comprehensive characterization of human RGC types. In addition, due to the long survival seen in the ischemia experiments, we believe that *post-mortem* human eye donations (expected: 50/year) are a powerful tool for testing of novel treatments. We are currently establishing the evaluation of optogenetic approaches for the treatment of blindness in such *post-mortem* tissue.

Stimulation of Müller glia proliferation in the mouse retina

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The mammalian retinal Müller glia (MG) are well known to undergo gliosis in all major types of retinal diseases – which sometimes may even lead to scar formation due to proliferative gliosis. Some studies suggest that in the mouse retina MG derived neuronal regeneration can be stimulated, but only to a very limited extent. Here, we started to investigate, if conditional immortalization might stimulate MG derived proliferative gliosis and /or neuronal regeneration. We ultimately want to find out if retinal gliosis is a deficient regenerative response or an independent entity.

We performed experiments with juvenile mouse retina (after retinogenesis is finished) in retina organ culture ex vivo. Upon mitogen stimulation EdU (S-phase marker) pulse-chase experiments revealed an increase in MG proliferation within the first two days, a peak of EdU+ cells at day 4 (14 +-2 SEM, N=4) and a decrease until day 6 (4 +-0.4 SEM, N=4) per 400µm of a central retina section.
Next, we used transgenic mice with drug-inducible expression of the proto-oncogene SV40 large T antigen (SV40LT). It is well reported that SV40LT binds several proteins including the tumor suppressor p53 and retinoblastoma thereby bypassing cell-cycle checkpoints. Induction of the SV40LT for 6 days ex vivo led to an overall increase in proliferation compared to control. The number of EdU+ cells was 8.5-fold increased (SV40LT: 23 +-6 SEM, N=4; control: 3 +-0.2 SEM, N=4; $p<0.05$) and the number of active cycling (Ki67+) cells was 6-fold increased (N=4; $p<0.05$) per 400µm central retina section. It was even possible to keep a Ki67+ cycling population until 10 days SV40LT (33.8 +-6.8 SEM N=3).

Our results so far suggest that induction of SV40LT not only overcomes the proliferative restriction of Müller glia but also maintains its progeny in the cell cycle over extended period of time. Surprisingly, major parts of the generated cell progeny formed gliotic cell clusters. In our current and future work we study the Müller glia and its derived progeny to determine the underlying mechanisms that enable neuronal regeneration and prevent gliotic scar formation.

Support: Funke-Foundation Fellowship (PS). CRTD and DZNE core grant (MOK).

Tolerance induction and activation mechanisms of Myeloid Derived Suppressor Cells during Experimental Autoimmune Uveitis (EAU)

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Purpose: To investigate the role of innate activation mechanisms of Myeloid-derived suppressor cells (MDSCs) in Experimental autoimmune uveitis (EAU). EAU is a murine model of human uveitis that is one of the leading causes of blindness worldwide. Whereas self-reactive Th1 and Th17 cells are substantially involved in the development of uveitis, regulatory cell subsets such as Foxp3+ regulatory T cells and Myeloid-derived suppressor cells (MDSCs) can downregulate immune responses and prevent autoimmune disease. MDSCs were shown to accumulate during different pathological conditions such as cancer or inflammation. Complete Freunds adjuvant (CFA) is an oily emulsion of mycobacterial components that is used as an adjuvant to induce EAU. Our previous work has shown that CFA, besides it well known pro-inflammatory effects, was able to activate regulatory T cells in an antigen-unspecific manner. Therefore, we questioned if the pre-administration of CFA before EAU induction could prevent or ameliorate the development of uveitis and if this is accompanied by the induction MDSC.

Methods: B10.RIII mice were pre-treated with CFA, seven days before induction of EAU by immunization with the retinal antigen interphotoreceptor-binding-protein (IRBP), emulsified in CFA. The induction of different MDSC subpopulations ($CD11b^{+}Ly6G^{+}Ly6C^{low}$ and $CD11b^{+}Ly6G^{-}Ly6C^{high}$) and EAU disease scores were examined by flow cytometry and funduscopy.

Results: Pre-treatment with CFA a few days before EAU induction protected the mice from EAU. Furthermore, the CFA pretreatment was accompanied by an expansion of MDSCs in peripheral lymphoid organs, especially in the spleen. The frequency of both MDSC subpopulations was increased by about 15% in comparison to the control group.

Conclusion: CFA, beside its well-known pro-inflammatory capacity, can activate regulatory cell subsets in an antigen independent manner and protect from EAU, when administered before disease induction. This is accompanied by an expansion of regulatory MDSCs.

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Mutations in the gene coding for the ATP-binding cassette A4 (ABCA4) trans-membrane transporter cause autosomal recessive Stargardt disease, cone-rod dystrophy or retinitis pigmentosaand they may, in addition, modify the phenotype of other genetic and/or multifactorial retinal diseases, including age-related macular degeneration (AMD).
Stargardt disease, the most common hereditary macular dystrophy affecting children,is usually diagnosed within the first two decades of life and leads to progressive irreversible loss of central vision and in almost all cases to blindness by age 50. No cure or treatment is available. Areas of hyperautofluorescence are frequently observed throughout the retina. Fundus autofluorescence, which arises primarily from the retinal pigment epithelium (RPE), has been used as a clinical marker of disease progression related to age and/or inherited macular degeneration. At the histological level, degeneration of photoreceptors and the underlying RPE occurs within and near the macula. The reason for the death of RPE cells, which are responsible for the maintenance of photoreceptors and phagocytosis of their aging outer-segments, is an excessive accumulation of lipofuscin containing various toxic by-products of the visual cycle. Briefly, lipofuscin represents cellular debris with harmful properties that is stored away in storage compartments where it accumulates over a lifetime in cells unable to divide and thereby regenerate, such as RPE cells. Numerous fluorophores have been detected in RPE lipofuscin such as the pyridiniumbisretinoid isomers A2E and isoA2E, oxidized derivatives of A2E, and conjugates such as alltrans-retinal dimer-phosphatidylethanolamine. The photo-toxicity of A2E to RPE cells is well-established and ultimately leads to the initiation of a cell death program.
Since lipofuscin is a hallmark of Stargardt disease, removal of lipofuscin from the RPE may be a potential strategy to ameliorate clinical symptoms resulting from *ABCR* genetic defects.
Several small molecules have been shown to decrease the new formation of the bisretinoidlipofuscin of RPE by inhibition of the visual cycle but so far, there was no drug known to remove existing lipofuscin efficiently from the RPE. Although it is questionable whether inhibition of the visual cycle is a good strategy to improve vision, these substances are currently under development as therapeutic options to treat Stargardt disease or dry AMD.
We recently discovered that a small molecule (Remofuscin), belonging to the tetrahydropyridoethers class of compoundswas able to remove lipofuscin *in vivo* from RPE cells in monkeys and *in vitro* from cultured primary human RPE cells. Moreover, Remofuscin did not interfere with the visual cycle and it did not show any toxicity in a long term monkey study after oral application for 1 year.
Therefore, Remofuscin appears to be an ideal candidate for developing a new treatment modality for Stargardt disease.

Cellular Reprogramming – An approach to drive cells towards retinal cell fate

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Introduction: Our goal is to reprogram mouse embryonic fibroblasts (MEF) into retinal progenitor cells. For that purpose we want to use eye field transcription factors (EFTF). They are known to be necessary and in some cases sufficient to induce ectopic eye development. We see the *ex vivo* generation of retinal progenitors as a promising step towards providing retinal cells to develop new strategies to study retinal disease pathomechanisms and treat retinal diseases.
Cell reprogramming using a defined set of transcription factors has been shown to be able to induce pluripotent stem cells from fibroblasts. Further studies investigated the possibility to reprogram fibroblasts into any desired cell type by finding the right factor combination and culture condition. This has been shown to be possible for some cell types as for example for neural stem cells or cardiomyocytes, but so far it was not achieved for retinal progenitor cells.

Method: As gene transfer system we use a replication-incompetent vector system based on the human immunodeficiency virus type 1 (HIV-1), which employs the modulated envelope protein of the vesicular stomatitis virus (VSV-G) for pseudotyping. Single EFTF were cloned into the transfer vector, which also encodes for an EGFP reporter gene. Generation of the viral particles and infection was done as previously reported by the Lindemann group. Mouse embryonic fibroblasts (MEF) were isolated from E13.5 mouse embryos, propagated in culture and virus infected. Three days past infection the medium was changed to media for embryonic retina culture conditions. MEF were cultured for 20 days and analyzed with fluorescent microscopy and reverse transcriptase-PCR.

Preliminary Results and Outlook: We designed a screening strategy to investigate cell reprogramming of MEF into retinal progeny by defined combinations of transcription factors. To facilitate the screening for reprogrammed retinal progenitors we generated MEF from transgenic reporter mice (Chx10-Cre::Rosa26-AI9 mice). Chx10 is a transcription factor expressed at the onset of neurogenesis in retinal progenitors. Induction of AI9, a red fluorescent protein,

will be the first read out to find successful reprogrammed cells. Further, we cloned the desired reprogramming factors into the p6Nst50 transfer vector and generated infectious retroviral particles. The infection efficiency of the particles transferring the transcription factors is similar to the control virus, which yields up to 85%. The eye field transcription factors in the infected cells are expressed, which we confirmed on mRNA and protein level.
To test, whether our exogenous genes are functional, we overexpressed Otx2 in mouse embryonic retinal progenitor cells in culture. Otx2 is known to be able to drive cells towards rod fate. And indeed, Otx2 overexpression led to an upregulation of recoverin positive cells, proving its functionality.
Since we now have established our reprogramming method, as a next step, we want to screen combinations of factors to identify effective ones to generate retinal progeny. To further characterize the resulting cells we defined several levels of analysis including a RT-PCR screen, gene array analysis and cell differentiation analysis to investigate the potential induction of progenitor cells.

Acknowledgement: This work was financially supported by a CRTD seed grant (D.L. & M.O.K.; 2011-13) and CRTD core funding (M.O.K.).

Optogenetic approaches to detect cyclic nucleotides in cultured cells and in the intact retina

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Purpose: The cyclic nucleotides, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), are important second messengers in many cell types of the retina. They are involved in a variety of signaling processes mostly by activating protein kinases or by binding to ion channels. cGMP is crucially involved in visual signal transduction in photoreceptors. So far, there is no common view of the contribution of cAMP and cGMP to retinal adaptation or to the modulation of signaling processes in other retinal cell types, although many attempts to investigate those processes have been made in the past. Therefore, cyclic nucleotides are an attractive topic in the field of retinal research.

Methods: Using stimulation of the retina with the nitric oxide donor SNAP followed by immunohistochemistry with an antibody against cGMP, we could demonstrate cGMP synthesis in a variety of cell types of the intact mouse retina. However, this method does not enable us to resolve the dynamics of this process. To study the dynamics of cGMP and cAMP production and degradation, we plan to express the genetically encoded FRET-sensors Epac (for cAMP) and Cygnet (for cGMP) in the mouse retina. The genetically encoded sensors will enable us to monitor the production of cyclic nucleotides in real time in isolated cells or in retinal slices using imaging techniques. To introduce the cDNA encoding the sensors into retinal cells, we use adeno-associated viruses (AAV) as gene shuttles. We examined the transduction efficiency and specificity of different AAV serotypes by immunocytochemistry in retinal cultures *in vitro* as well as upon injection of AAVs into the intact eye *in vivo*.

Results & Conclusion: We could express Epac and Cygnet functionally in HEK293 cells that served as model systems. Upon stimulation with appropriate ligands, Epac revealed an increase in the intracellular cAMP concentration. In cells transfected with Cygnet, we could monitor an increase in cGMP upon activation of membrane-bound guanylate cyclases.
We tested a total of 6 AAV serotypes carrying the gene for the green-fluorescent protein (GFP) for transduction efficiency of retinal cells both *in vitro* and *in vivo*. All serotypes successfully transduced retinal cells but did not show clear specificity for retinal cell types.
In our future experiments we will express Epac and Cygnet in retinal cells using the established AAV gene transfer technique. Since little is known about the regulation of cAMP and cGMP in cells of the inner retina, this is a promising field of retinal research. Our results should foster our understanding of retinal adaptation and signal modulation. We made first successful steps on our way to investigate those processes by means of optogenetic approaches.

Effects of optineurin deficiency on retinal neurons *in vitro* and *in vivo*

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Purpose: Mutations in Optineurin are correlated to the development of primary open angle glaucoma and amyotrophic lateral sclerosis. Optineurin is interacting with various proteins implicated in diverse functions including membrane trafficking, protein secretion, NF-κB signaling, cell division and bacterial and viral immune responses. To investigate the localization and function of Optineurin in retinal neuronswe studied Optn promotor activity in Optn-LacZ reporter mice, generated a stable cell line of retinal neurons (RGC-5) with Optineurin deficiency and an Optineurin knockout mouse.

Methods: The subcellular localization of Optineurin was studied in RGC-5 cells and primary RGCs isolated from mature mice. A stable RGC-5 cell line with constitutive Optineurin deficiency was generated using siRNA. The cells were studied by confocal microscopy and transmission electron microscopy. The amounts of ciliary neurotrophic factor (CNTF) and neurotrophin-3 (NT-3) were analyzed by ELISA. Apoptosis was determined using ELISA or FACS, and recombinant NT-3 was used for rescue experiments. Optn-LacZ mice were used to investigate Optn promotor activity, while the retinal phenotype of Optn knockout mice was analyzed by microscopy and TUNEL labeling.

Results: In primary retinal ganglion cells and RGC-5 cells, Optineurin localizes to the Golgi complex and to vesicular structures in the cytoplasm. Optineurin deficiency in RGC-5 causes fragmentation of the Golgi complex, an increase in apoptosis and decreased secretion of NT-3 and CNTF. Adding exogenous NT-3 to the culture medium to achieve amounts seen in control cultures completely prevents the increase in apoptotic cell death. β-Galactosidase staining in Optn-LacZ reporter mice indicates strong promoter activity in the outer nuclear layer (ONL) and the retinal pigment epithelium. Optn -/- knockout mice show a significantly reduced thickness of the ONL as well as a slightly higher number of apoptotic cells compared to wild-type mice, while no differences in the number of optic nerve axons was observed.

Conclusion: Optineurin is required for the structure of the Golgi complex and secretion of neurotrophins in cultured retinal neurons. In vivo, Optineurin appears to have an important function in photoreceptors.

Supported by DFG Research Unit FOR 1075

Multifocal ERG with OCT after local retina laser coagulation on C57Bl/6 mice

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Purpose: The aim was to test the feasibility of multifocal electroretinogram (mfERG) by laser coagulation model for mouse retinal degeneration. The laser coagulation is the simple approach to generate the local retina dystrophy. The mfERG recording was simultaneously performed with OCT (optical coherence tomography), angiography and SLO (scanning laser ophthalmoscope) imaging of mouse eyes for structural and functional analysis of the retina at the laser damage.

Methods: Six C57Bl/6 mice were treated with argon laser with two laser spots on one eye, 300µm spot size and energy of 30J/cm^2 and two different residence times of 20ms or 270ms. MfERG were recorded 6 months after treatment under photopic conditions using an array of 19 equal-sized hexagons. The central hexagons were placed on top of the optic nerve head guided by means of SLO imaging. After the ERG recording, the OCT mapping was performed with the same alignments. Finally fluorescence angiography was performed. The mfERG were evaluated by statistical analysis of P1 amplitudes as function of retinal locations. For statistical analysis, the amplitude at the optic nerve head served as standard.

Results: When comparing the structural changes and the functional alterations we found differences in laser-induced retinal damage between the residence time 20 ms and 270 ms. OCT screens in a high resolution were obtained at the laser damage spots. Fluorescence angiography demonstrated formation of new vessels in and around the laser damage areas accompanied with severe retinal degeneration at residence time 20ms whereas intact vessels and weak retinal damage were observed at residence time 270ms. The ERG responses at local damage areas and at the optic nerve head were decreased compared to those in the surrounding areas. The amplitudes of the ERG responses at the damage sites were significantly reduced ($p < 0.003$; $n = 3$) with severe damages of choroid/retina in animals treated with residence time 20 ms. No significant amplitude reductions were detected in mice with weak retinal damage treated with residence time 270 ms. It was possible to successively perform SLO fundus imaging, mfERG, OCT and angiography with the same animal.

Conclusion: The multifocal ERG technique can be used to detect retinal damage as local changes in ERG function in mice. The usage of the multifocal ERG technique in combination with OCT allows a correlation of defects in retinal function with structural alterations.

Protein networks related to the Usher syndrome participate in the ciliary transport in photoreceptor cells

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Human Usher syndrome (USH) is the most common hereditary form of combined deaf-blindness. USH type I (USH1), the most severe form, is characterized by profound congenital deafness, constant vestibular dysfunction and pre-pubertal onset of *retinitis pigmentosa (RP)*. Our previous studies provided several lines of evidence that USH proteins participate in the ciliary transport of photoreceptor cells. To gain further insights into the mechanisms of the ciliary transport and the pathomechanisms underlying USH, we focused on the USH1G protein SANS interactome and the molecular composition and organization of the transport machineries of photoreceptor cells.

For this we adopted yeast-2-hybrid (Y2H) screens of retinal cDNA libraries and validated putative interactions by complementary assays, namely GST-pull downs, co-IPs and co-transfection assays. The relation of SANS to microtubules was tested in microtubule destabilization and spin-down assays. To analyze the spatial distribution of the identified proteins and complexes in photoreceptor cells we accessed immunofluorescence, immunoelectron microscopy and proximity ligation assays (PLA).

We identified and validated the interaction of SANS with multiple components of the dynein-dynactin motor-complex. Microtubule depolymerisation altered SANS localization in cultured and retinal cells and spin down assays demonstrated direct binding of SANS to microtubules. *In situ* co-localization analysis and PLA revealed complexes of dynactin, canonical cytoplasmic dynein components and SANS along the microtubule transport tracks in inner segments and the periciliary region of photoreceptor cells.

The present data strengthen our hypothesis that USH protein networks participate in cargo transport to its ciliary destination. We demonstrated that SANS is part of the cytoplasmic dynein motor complex, a molecular machine shipping outer segment cargo along microtubules through the inner segment to the periciliary region of photoreceptor cells. Defects in this molecular transport machinery may lead to the photoreceptor dysfunctions introducing the degenerative processes underlying USH.

Supports: DFG, EU-FP7 SYSCILIA, FAUN-Stiftung, Forschung contra Blindheit, PRO RETINA Deutschland.

Daily regulation of genes involved in photoreceptor survival

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Acknowledgements: The study was supported by grants from the Naturwissenschaftlich-MedizinischenForschungszentrum (NMFZ) of the University of Mainz.

Photoreceptor cells face the challenge of surviving under marked changes in ambient light intensity. In order to achieve a better understanding of this process, this study aimed at identifying genesunder daily regulation which can have an impact on photoreceptor survival.
The genes that meet this demand are
(1) *Kcnv2*, a channel subunit important for visual function and cone survival
(2) *Grk1* (*G protein-coupled receptor kinase 1*),a kinase essential for visual processing and intact scotopic vision
(3)*Pgc-1α*(*peroxisome proliferator-activated receptor γ, coactivator 1alpha*), a transcriptional coactivatordecreasing the light damage susceptibility of the retina and
(4) *Esrrb*(*Estrogen-related receptor beta*) a nuclear orphan receptor known to protect rod photoreceptors from dystrophy.
According to their regulation the genes identified could be clustered in two groups: Those promoted to cycle as a result of light/dark transitions and those which are predominantly driven by a clock. The present results support a concept in which homeostasis of photoreceptor cells over the 24-h period is ensured by light- and clock-dependent transcriptional regulation of genes with impact on photoreceptor survival.

Human retina tissue culture – Towards an optogenetic treatment of blindness

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Aim: There are no existing treatments for blindness caused by retinal degeneration. Animal studies suggest that optogenetic approaches (i.e., expression of light-sensitive proteins in retinal neurons) hold a lot of promise to restore light sensitivity to the retina and therefore vision to the affected individual. Here, we established tissue culture conditions with the goal to evaluate optogenetic approaches in the human retina in-vitro. This is an important step towards the translation of this promising new treatment strategy for blindness.

Methods: We cultured retina from three different species: mouse, pig and human. Human retina was donated by patients that had to have an enucleation. We evaluated the degree to which culture conditions preserved the state of the retina by recording light-evoked and spontaneous ganglion cell activity on multi-electrode arrays (MEA).

Results: Mouse and pig retina that was brought into culture immediately after enucleation, stayed light responsive for at least 48h (mouse) and 72h (pig), and showed spontaneous ganglion cell activity for at least 120h (pig). Human retina showed spontaneous ganglion cell activity after at least 48h in culture.

Conclusions: MEA date from mouse, pig and human retina suggest that our culture condition maintain the retina in healthy physiological state for at least 48 hours. This time period should be sufficient for lentivirus-mediated plasmid expression after transfection, such that optogenetic approaches can be assessed in this *in-vitro* system.

Testing of a new gene therapy construct for X-linked juvenile Retinoschisis

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Background: X-linked juvenile Retinoschisis (XLRS) is an inherited form of macular degeneration in males leading to severe vision loss. It is caused by mutations in the *RS1* gene suggested to cause a functional loss of the encoded protein, retinoschisin. Retinoschisin is exclusively expressed in the retina and essential to maintain the structural integrity of the retinal layers. First gene therapeutic trials on *RS1* knockout (*Rs1h*$^{-/Y}$) mice were promising: In our institute, subretinal delivery of an AAV-based *RS1* vector had a long-term rescue-effect for the retina (Min et al., 2005; Jansen et al., 2008). The next challenge will be to express Retinoschisin in a physiological context. The regulatory elements controlling human *RS1* expression, an upstream CpG island and two opposing CRX-bound regions, were identified in our institute (Krauss et al., 2011) and now integrated into a novel gene therapy construct for XLRS. In this study, we characterized strength and developmental regulation of *RS1* expression from this construct in cell culture and *Rs1h*$^{-/Y}$ mice.

Methods: We generated a gene therapy construct (carrying AAV2 inverted terminal repeats) for XLRS using the endogenous RS1 regulatory elements to drive *RS1* expression. This construct was first tested in retinal cell lines (661W, Y-79, Weri-Rb), and HeLa cells. Subsequently, the construct was electroporated into explanted retinas of newborn *Rs1h*$^{-/Y}$ mice. Finally, *in vivo* electroporation into retinas of newborn *Rs1h*$^{-/Y}$ and wild-type mice was performed. Retinoschisin expression was assessed *via* qRT-PCR, Western Blot and immunohistochemistry.

Results: Retinoschisin was not expressed from of our gene therapy construct in the retinal cell lines (consistently with a lack of endogenous RS1 expression in these cell lines). Weak Retinoschisin expression was observed in HeLa cells cotransfected with the retina specific transcription factor CRX. Explanted retinas of *Rs1h*$^{-/Y}$ mice did not express Retinoschisin from the gene therapy construct, in contrast to a weak endogenous Retinoschisin expression in wildtype retinas. Similarly, exogenous Retinoschisin expression was not observed in *in vivo* electroporated retinas at postnatal days 0 to 8. Later stages are still to be analysed.

Conclusions: The novel gene therapy construct has the potential to drive Retinoschisin expression activated by CRX. However, Retinoschisin expression from this construct differs from endogenous Retinoschisin expression in the murine retina. This could be explained by different regulatory mechanisms involved in human and murine *RS1* gene regulation, or it could indicate that *RS1* expression from the gene therapy construct is not in a perfect physiological context.

Role of ApoE and its isoforms in AMD

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Purpose: Age-related macular degeneration (AMD) is a frequent, complement associated disease in elderly people. It is characterized by an irreversible loss of vision which is caused by the degeneration of retinal pigment epithelial (RPE) cells. Characteristic for AMD is the appearance of extracellular cell debris, called drusen which induce a state of chronic inflammation. The drusen are composed of many different proteins and lipids, including lipoproteins like apolipoprotein E (ApoE), amyloid-beta peptides (Aβ) and proteins of the complement system. Polymorphisms and mutations in the genes coding for complement proteins where identified as risk for AMD, which demonstrates the pivotal role of complement in AMD. Recently ApoE, a lipid transport protein, has be genetically linked to AMD. The human ApoE exists in three isoforms which differ in two amino acids, Arg and Cys, at the positions 112 and 158. A pooled studies analysis found the E4 haplotype to be associated with a decreased risk of late AMD, whereas E2/E2 homozygous individuals carried a significantly increased risk of late AMD. However, the role of the ApoE variations, ApoE2, ApoE3 and ApoE4 which are major compartments of drusen, is still unclear. To study the function of ApoE and its isoforms in AMD binding of ApoE2, ApoE3 and ApoE4 to its ligand Aβ was determined as well as the ability of Aβ to induce innate immune reactions.

Methods: A complement activation assay based on an enzyme-linked immunosorbent assay (ELISA) was used to investigate Aβ in activation of the classical or alternative complement pathway. Binding of ApoE variants to Aβ was performed by using an ELISA.

Results: Aβ incubated in NHS induces both complement pathways, the alternative and the classical pathway. All three isoforms of ApoE bind Aβ, whereby ApoE3 shows the strongest binding to Aβ.

Conclusion: The ability of Aβ to activate the complement system and to recruit ApoE raises the question how the ApoE isoforms are involved in these inflammatory process.

Endozepines influence retinogenesis

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Introduction: Endozepines are a family of peptides derived through proteolytic cleavage from the diazepam binding inhibitor (DBI). In the retina, DBI has been shown to be expressed by retinal progenitor cells, both at early and late stages of retinogenesis. Further, endozepines have been shown to regulate proliferation of a variety of cell types, including neural stem and transit-amplifying cells of the postnatal mouse subventricular zone. These observations suggest that endozepines are involved in the regulation of retinal development.

Methods: The effects of octadecaneuropeptide (ODN), a natural DBI processing product, on retinal progenitor cell fate and proliferation were investigated using embryonic mouse retina explant culture, as well as immunostaining and flow-cytometry.

Results: At embryonic day (E) 16, ODN treatment decreases numbers of cells expressing HuCD, Calretinin and Calbindin by 43% ± 7 (SD, n=3) after 6 days ex vivo, suggesting a change in amacrine cells. We found that numbers of gabaergic (GABA- and Nurr1-positive) and glycinergic (Ebf-positive) amacrine subtypes are reduced by ODN. However, other retinal cell fates are not affected. Interestingly, ODN treatment does not affect retinal progenitor proliferation, as numbers of mitotic cells (phosphohistone-3-positive) and cell cycle length remained unchanged. Further, ODN differentially affects early and late retinal retinogenesis, as numbers of amacrine cells are not reduced at E9.

Conclusion and Outlook: Our results suggest that endozepines regulate amacrine cell fate, but do not affect progenitor proliferation in embryonic mouse retinal explant culture. However, the mechanisms underlying these effects are currently unclear.
To study the role of endozepines in retinal development in vivo, we now aim to overexpress DBI in retinal progenitor cells using electroporation in neonatal mice.

Funding: CRTD core grant and DZNE core grant to M.O.K..

Towards identifying the gene associated with North Carolina Macular Dystrophy in 6q14-q16.2

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Objective: North Carolina Macular Dystrophy (NCMD) is an autosomal dominant macular dystrophy showing variable expressivity but complete penetrance that rarely progresses. The clinical course can be classified into three different stages: the range from drusen-like lesions in the central macular at the level of the retinal pigment epithelium (grade 1) to larger lesions with confluent drusen in the retinal pigment epithelium (grade 2) to macular staphyloma and hypertrophic fibrous tissue and pigmentation on the edge of the lesion (grade 3). Following genetic linkage analysis, the gene for NCMD was mapped within chromosome 6 in q14-q16.2 and was named Macular Dystrophy, Retinal, 1 (MCDR1). Further refinement localized the gene to an interval of 1.8 Mb of DNA between D6S1716 to D6S1671. Sanger sequencing of all *RefSeq* protein-coding genes within the 6q14–q16.2 region of interest has not revealed NCMD-associated mutations. The overall aim of our work is identifying the genetic defect in NCMD.

Methods: The entire candidate interval was analyzed in two affected patients (mother and daughter) and the unaffected father by *next generation sequencing*. To reveal NCMD-associated candidate variants bioinformatic evaluation and sophisticated filtering of the raw data were performed and verified by Sanger sequencing. Further, potential disease causing transcripts were amplified in several human tissues and retinal cell culture by RT-PCR. Full length sequence of the transcript and its gene structure were identified by RACE-analysis.

Results: *Next generation sequencing* and verification by Sanger sequencing on genomic level revealed 13 unique candidate variants. They range from single base pair substitutions to deletions and insertions of up to twenty base pairs. However, mapping these sequence variants to the genomic sequences on chromosome 6, no variant was localized to a protein coding gene sequence. Although, we could show that five variants localized to intronic regions of a yet unknown spliced gene were highly expressed in human retina and brain. No expression was detected in other human tissue and retinal cell culture analyzed so far.

Conclusion: These findings suggest that untranslated gene regions, intronic sequences or non coding elements at the 6q14-q16.2 locus are likely candidates for NCMD-causing effects. This may provide insight into the role of the newly discovered gene in the pathogenesis of macular dystrophy in particular and autosomal dominant retinal diseases in general.

In depth analysis of Usher syndrome protein networks provide insights into network functions and links to other ciliopathies

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The genetically heterogeneous human Usher syndrome (USH) is the most frequent cause of combined hereditary deaf-blindness and can be divided into three clinical subtypes, USH1-3, We and others have recently demonstrate that all USH1 and 2 proteins are organized into protein networks by USH scaffold proteins. This may explain why defects in proteins from different families cause similar phenotypes.

Preliminary data on the relation of USH proteins to ciliary function prompted us to access in depth analyses of the interactome related to USH by yeast-2-hybrid screens of retinal cDNA libraries and tandem affinity purification (SF-TAPs) in HEK293 cells. Putative direct interaction and complex partner proteins are validated by different complementary methods (e.g. co-expression and membrane targeting assays, GST pull-down) and protein interactions are demonstrated *in situ* in retinal photoreceptor cells by proximity ligation assays (PLA). Furthermore, we investigate the spatial distribution by light and electron microscopy.

Our studies indicate that Usher protein networks participate in diverse but overlapping cellular functions. Recent analysis of non-rodent photoreceptor cells including human and amphibian photoreceptors demonstrate a prominent USH protein network in calycal processes which mechanically stabilizes the outer segments. However, our results also indicate that other USH protein complexes are involved in endocytosis and ciliary cargo transport to the photoreceptor outer segments.

Our data include additional molecular links of USH proteins to molecules related to other ciliopathies, including isolated retinal dystrophies and syndromes like the Bardet-Biedl syndrome. In ongoing studies we are elucidating the functional relevance of the protein networks in ciliary transport and ciliogenesis by experimental approaches, such as cargo FRAP and RNAi knock down experiments. Our preliminary data provide evidence that USH is part of retinal ciliopathy group with molecularly related functions and common targets for future therapeutic interventions.

Supports: DFG; EU FP7 "SYSCILIA" and "TREATRUSH"; FAUN-Stiftung; Forschung contra Blindheit; ProRetina Deutschland.

Cone-rod dystrophy related mutations in in rod and cone guanylate cyclase impair the Ca^{2+} dependent regulation of phototransduction

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Purpose: Progressive visual impairment leading to blindness is often associated with inherited retinal dystrophies like Retinitis Pigmentosa, Leber´s congenital amaurosis (LCA) and cone-rod dystrophies (CORD). These disorders correlate very often with mutations in genes coding for proteins of the visual transduction system in rod and cone photoreceptor cells. One key enzyme in photoreceptor cells is the membrane bound guanylate cyclase ROS-GC1 encoded by the gene GUCY2D, which activity is regulated by guanylate cyclase-activating proteins (GCAPs) in a calcium-dependent manner. The catalytic active form of ROS-GC1 is a dimer and several CORD-related mutations in the GUCY2D gene have been localized to the dimerization domain of ROS-GC1. In order to understand the cellular consequences of critical mutations in the dimerization domain we investigated biochemical properties of point mutant proteins harbouring the following amino acid substitutions Q847L⟶K848Q, Q847L and K848Q.

Methods: The gene product ROS-GC1 was heterologously expressed in HEK cells and guanylate cyclase activity was measured in the absence and presence of the Ca^{2+}-sensitive regulators GCAP1 and GCAP2. Protein folding of the dimerization domain was investigated by CD spectroscopy of wildtype and mutant protein samples.

Results: Wildtype and mutants of heterologously expressed ROS-GC1 were correctly translocated to the plasma membrane. However, the mutations affected the catalytic properties of ROS-GC1 in different manners: 1) all mutants had a higher basal guanylate cyclase activity, but lower x-fold activation; 2) incubation with wildtype GCAP1 and GCAP2 revealed for all ROS-GC1 mutants a shift in Ca^{2+}-sensitivity; 3) activation of the mutant K848Q by GCAPs was severely impaired. Further, CD spectra of the dimerization domain showed that wildtype and mutants clearly had an α–helical content, but exhibited clear differences in thermal stability.

Conclusion: Our results indicate a significant imbalance of the cyclic GMP-calcium homeostasis in ROS-GC1 mutants. Minor changes in the correct dimer conformational arrangement lead to a severe impairment of the cyclase catalytic properties and even induce significant changes of Ca^{2+}-sensitive regulation of cyclase activity triggering probably Ca^{2+}-dependent apoptotic processes.

Supported by a grant from the DFG (KO948/10-1)

Norrin mediates its protective properties against OIR via induction of IGF-1

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Purpose: To analyze *in vitro* and *in vivo* if norrin, a growth factor that activates the canonical Wnt/beta-catenin pathway, mediates its angiogenic properties via induction of IGF-1.

Methods: Human dermal microvascular endothelial cells (HDMEC) and rat Müller cells were incubated with Norrin and/or Dickkopf (DKK)-1, an inhibitor of the canonical Wnt pathway. The expression of IGF-1 was analyzed by quantitative real-time RT-PCR and dot blot analysis. In *in vivo* experiments, transgenic mice with an ocular overexpression of Norrin (betaB1-Norrin) and wildtype FVB-N were investigated for retinal IGF-1 mRNA expression during retinal development and following an oxygen-induced retinopathy (OIR) that was induced by incubation of the mice with 75% oxygen from postnatal day (P) 7 to P12. At P13, 1 day after return to room air, IGF-1 expression in wild type and transgenic animals was additionally examined by western and dot blot analyses. Moreover, oxygen-treated mice at P12 were intravitreally injected with inhibitory anti-IGF-1 antibodies and vasoobliterated areas were quantified at P14.

Results: After treatment with 20 ng/ml Norrin, a significant induction of IGF-1 mRNA was detected in both HDMEC and Müller cells when compared to untreated controls. With higher concentrations of Norrin, IGF-1 expression was less prominent, indicating dose dependency of Norrin treatment. After an additional treatment of the cells with DKK-1, the Norrin-mediated effects on IGF-1 expression were significantly blocked.
During retinal development between P7 and P13, only negligible changes in IGF-1 mRNA expression were detected in retinae from FVB-N mice. With the onset of oxygen exposure in the OIR model, IGF-1 mRNA levels in wild type and betaB1-Norrin mice were significantly reduced compared to mRNA levels at P7. However, after oxygen exposure at P13, IGF-1 mRNA and protein levels increased substantially in transgenic mice when compared with wild type littermates. Finally, in oxygen-treated transgenic mice with an injection of anti-IGF-1 antibodies into the vitreous body at P12, a significant increase in vasoobliterated retinal areas was observed at P14.

Conclusion: Norrin induces the expression of IGF-1 via activation of the classical Wnt/beta-catenin pathway *in vitro* and *in vivo*. The protective effects of Norrin against OIR are mediated, at least partially, via induction of IGF-1.

Supported by DFG Forschergruppe 1075 and PRO RETINA-Foundation.

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THIS MEETING IS FUNDED BY

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PRO RETINA-RESEARCH FOUNDATION FOR PREVENTION OF BLINDNESS

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THIS MEETING IS SUPPORTED BY

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BMBF-PROJECT „HOPE 01GM1108A"

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