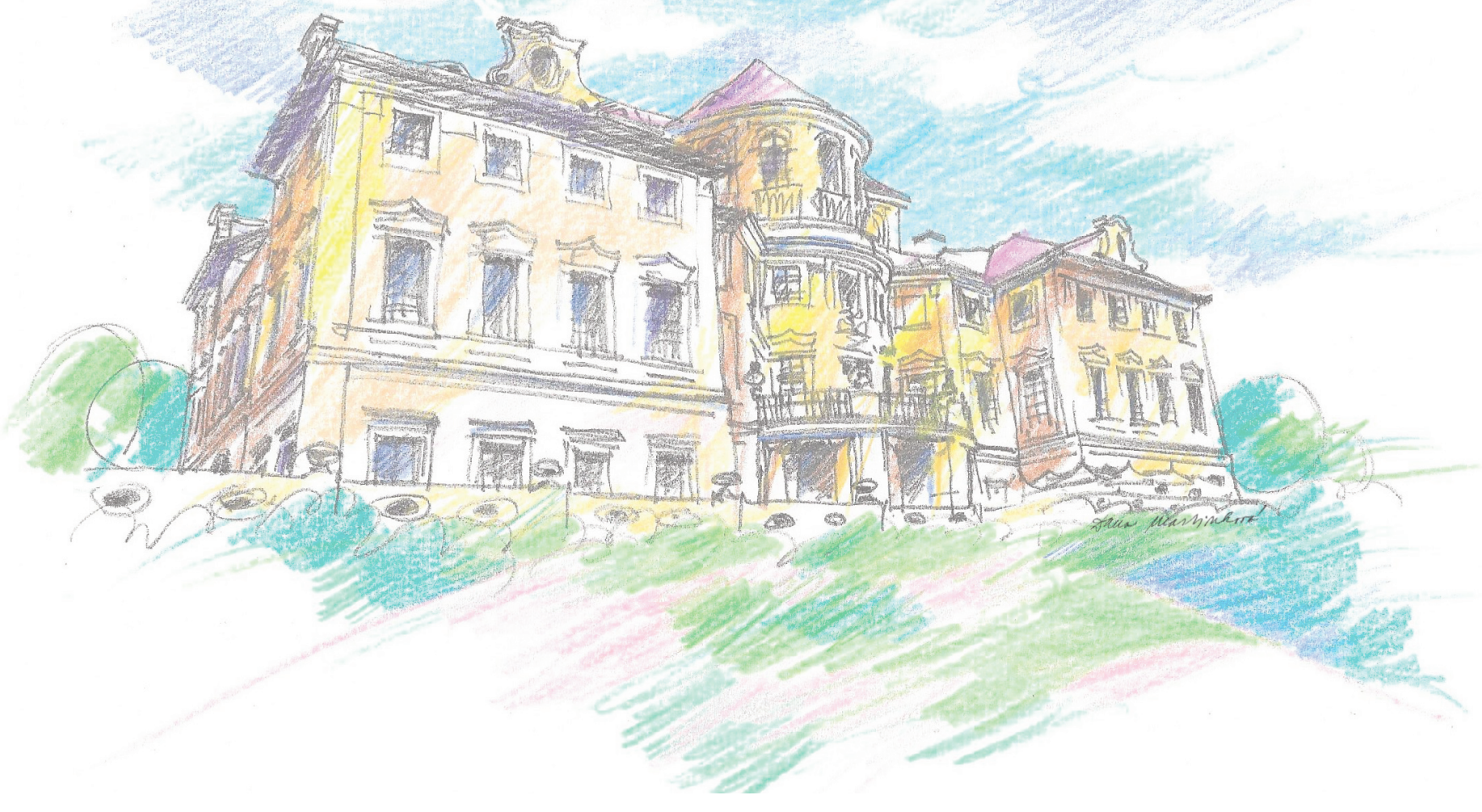
The PIGMOD Center, Institute of Animal Physiology and Genetics CAS

CELL THERAPY OF AMD GENE THERAPY OF USHER AND STGD

RESEARCH WORKSHOP

Molecular and Cellular Therapies for Usher Syndrome, Stargardt Disease and Age-Related Macular Degeneration

Conference Centre of CAS, Liblice castle,
4 – 6 December 2, 2022



Ladies and gentlemen, dear colleagues,

it is a great honor for us that we can welcome you all again in the unique hospitality of the Liblice Castle, which is also the Conference Center of the Czech Academy of Sciences.

Intensive and very successful cooperation throughout the year 2022 allowed us to focus the portfolio of our workshop program not only on "Age related macular degeneration" and "Usher Syndrome", but also to add another serious eye disease "Stargardt disease".

The organization of our second workshop was only possible thanks to significant support from the USHER 2020 Foundation and Roland Consult Company. As well as both grants from:
Technology Agency of the Czech Republic TO 01000107 and Czech-Norwiegen grant in the KAPPA Programme and Czech Agency of the Czech Republic 19-09628J.

Jan Motlík
Director of the PIGMOD Center

Miroslav Franc
Chairman of the Organizing Committee

Ústav živočišné fyziologie a genetiky AV ČR, v.v.i. | Rumburská 89 | 277 21 Liběchov
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The workshop is accompanied by presentations of companies in molecular and cell biology.

Speakers include

Marius Ader	Dresden, D	Jennifer Lentz	New Orleans, USA
Aziz El-Amraoui	Paris, F	Dunja Lukovic	Valencia, ES
Hannah Auch	Munich, D	Lyubomyr Lytvynchuk	Giessen, D
Tomáš Bárta	Brno, CZ	Stylianos Michalakis	Munich, D
Andrea Bähr	Munich, D	Mariya Moosajee	London, UK
Elvir Becirovic	Zurich, CH	Brigitte Müller	Giessen, D
Miquel Cambra	Munich, D	Andreas Parzefall	Munich, D
Lucie Casalta	Munich, D	Goran Petrovski	Oslo, NO
Lars Eide	Oslo,NO	Ruchi Sharma	Bethesda, USA
Slaven Erceg	Valencia, ES	Jessica Schäfer	Mainz, D
Georgina Faura Muñoz	Oslo, NO	Knut Stieger	Giessen, D
Andrea Fischer	Munich, D	Zbyněk Straňák	Prague, CZ
Boris Greber	Langenfeld, D	Hana Studenovská	Prague, CZ
Santosh Gupta	Oslo, NO	Václav Vencovský	Prague, CZ
Marian Hruska-Plochan	Zurich, CH	Kerstin Nagel-Wolfrum	Mainz, D
Nikolay Klymiuk	Munich, D	Uwe Wolfrum	Mainz, D
Igor Kozak	Abu Dhabi, UAE	Erwin van Wijk	Nijmegen, NL

PROGRAMME OF THE WORKSHOP

Sunday, December 4

Arrival and Registration

*18.00 - **Welcome Drink***

Monday, December 5

8.00 - 8.10

Opening – Jan Motlik, IAPG CAS Liběchov, PIGMOD Centre

Zdeněk Havlas, *Czech Academy of Sciences*

Session 1 - Cell Therapy of Age Related Macular Degeneration

Chaired by: Goran Petrovski

8.10 – 8.40

Keynote Lecture:

Ruchi Sharma, National Eye Institute Bethesda

"Development of autologous iPSC-derived retinal pigment epithelium therapy for the treatment of age-related macular degeneration patients."

Lectures:

8.40 – 9.10

Boris Greber, Research & Development iPSC/CT, Catalent Cell and Gene Therapy

"GMP-compliant iPS cell lines and differentiation workflows for cell therapy."

9.10 – 9.30

Igor Kozak, Moorfields Eye Hospitals UAE, Abu Dhabi, U.A.E.

"Current surgical options in the management of inherited retinal disorders."

9.30 – 9.50

Lyubomir Lytvynchuk, Department of Ophthalmology, Justus-Liebig-University Giessen, University Clinic Giessen and Marburg GmbH, Gießen, Germany

Zbyněk Straňák, Ophthalmological Clinic, Královské Vinohrady University Hospital Prague

"Libechov mining model in eye research: future perspectives in subretinal implantation of the cell carrier."

9.50 – 10.10

Hana Studenovská, Department of Biomaterials & Bioanalogous Polymer Systems Institute of Macromolecular Chemistry AS CR

"Steps in preparing a nanofibrous membrane for subretinal implantation."

10.10 – 10.30 - Coffee Break

10.30 – 10.50

Brigitte Müller, Justus-Liebig-University Giessen, Faculty of Medicine

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

10.50 – 11.10

Slaven Erceg, Príncipe Felipe Research Center Foundation Valencia

"Clinical grade of genetically corrected RPE cells for autologous cell therapy of hereditary retinal dystrophies."

11.10 – 11.30

Goran Petrovski, Oslo University Hospital, Department of Ophthalmology

"Retinal disease models in minipigs - application in translational and clinical studies."

11.30 – 11.50

Santosh Gupta, Oslo University Hospital, Department of Ophthalmology

"A method for enhancing melanogenesis in the retinal pigmented epithelium for research applications."

11.50 – 12.30

Lars Eide, Georgina Faura Muñoz, Oslo University Hospital, Department of Medical Biochemistry

"On the hunt for biomarkers to validate Retinal Pigment Epithelial cells for transplantation."

12.30 – 14.00 - Lunch

14.00 – 14.30

Marius Ader, Technische Universität Dresden, Center for Regenerative Therapies Dresden (CRTD)

"Human photoreceptor transplantation into mammalian models of retinal degeneration."

14.30 – 14.50

Tomáš Bárta, Masaryk University Brno, Department of Histology and Embryology

"Modelling ciliopathies using retinal organoid model."

14.50 – 15.10

Aziz El-Amraoui, Institute Pasteur Paris, Progressive Sensory Disorders, Pathophysiology and Therapy Laboratory

"Towards the development of gene therapies for hearing and vestibular deficits in mouse models of Usher syndrome."

15.10 – 15.30

Andrea Fischer, Ludwig-Maxmilians-Universität München,

"Update on multi-modality hearing screening in pigs."

15.30 – 15.50

Marian Hruska-Plochan, University of Zurich

"Human neural networks with sparse TDP-43 pathology reveal NPTX2 misregulation in ALS/FTLD."

15.50 – 16.20

Andreas Parzefall, Ludwig-Maxmilians-Universität München, Institut für Tierpathologie

"Tissue sampling strategies in porcine animal models in translational biomedical research."

16.20 – 17.00 - Coffee Break

20.00 – 22.00 - Evening programme Dinner and dance

Tuesday, December 6

Session 2 – Stargardt Disease

Chaired by: Knut Stieger, Stylianos Michalakis

8.00 – 8.30

Keynote Lecture:

Knut Stieger, Justus-Liebig-Universität Giessen, Eye Hospital

"Animal models of Stargardt disease."

Lectures:

8.30 – 9.00

Elvir Becirovic, Universitätsspital Zürich, Eye Hospital

"A gene therapy for Stargardt Disease using novel dual mRNA trans-splicing AAV vectors."

9.00 – 9.30

Stylianos Michalakis, Department of Ophthalmology, University Hospital LMU Munich

"Existing hurdles in retinal gene therapy and how to overcome them."

9.30 - 10.00 - Coffee Break

Session 3 - Gene Therapy of Usher Syndrom

Chaired by: Nikolai Klymiuk, Uwe Wolfrum

Lectures:

10.00 – 10.30

Erwin van Wijk, Radboud University Medical Centre Nijmegen, Department of Otorhinolaryngology

"Scrutinizing pathogenicity of the frequent USH2A p.Cys759Phe variant."

10.30 – 11.00

Mariya Moosajee, UCL Institute of Ophthalmology, Francis Crick Institute, Moorfields Eye Hospital and Great Ormond Street Hospital for Children, London

"Non-viral gene augmentation strategy for USH2A retinopathy."

11.00 – 11.30

Dunja Lukovic, Príncipe Felipe Research Center Foundation Valencia

"Dissecting Usher syndrome retinitis pigmentosa in iPSC-derived retinal models."

11.30 – 12.00

Uwe Wolfrum, Johannes-Gutenberg University Mainz, Institute of Molecular Physiology, Molecular Cell Biology

"Novel insight into cellular functions of USH2C VLGR1/ADGRV1 - in cell adhesion, cell migration, autophagy and Ca2+ homeostasis."

12.00 – 12.30

Kerstin Nagel-Wolfrum, **Uwe Wolfrum**, Johannes-Gutenberg University Mainz, Institute of Molecular Physiology & Developmental Biology and Neurobiology

"The Usher syndrome protein harmonin regulates canonical Wnt signaling."

12.30 – 14.00 - Lunch

14.00 – 14.30

Jennifer Lentz, Louisiana State University Health Sciences Center New Orleans, Neuroscience Center of Excellence

"USH1C: preclinical development of antisense therapies in mice and clinical natural history studies in patients."

14.30 – 15.00

Václav Vencovský, Faculty of Electrical Engineering, Czech Technical University Prague

"Distortion-product otoacoustic emissions as a tool for hearing assessment."

15.00 – 15.30

Nikolai Klymiuk team, Technical University of Munich, School of Medicine

Lucie Casalta, *"Prospectives of therapeutic gene editing in inherited retinal diseases."*

Miquel Cambra, *"Expression of USH genes in USH1C pig models."*

Hannah Auch, *(to be determined)*

Andrea Bähr, *"Peri-interventional management of Usher pigs during analysis of eye function."*

15.30 - Closing of the Workshop - Jan Motlik, *IAPG CAS Liběchov, PIGMOD Centre*

17:00 Dinner

LECTURES

Session 1 - Cell Therapy of Age Related Macular Degeneration

Keynote Lecture

Development of autologous iPSC-derived retinal pigment epithelium therapy for the treatment of age-related macular degeneration patients

Ruchi Sharma, Vladimir Khristov, Aaron Rising, Shekhar Jha, Arvydas Maminishkis, Juan Amaral, Irina Bunea, Kapil Bharti

National Eye Institute, Bethesda, Maryland

Age-related macular degeneration (AMD) is one the leading causes of vision loss in people above 60 years in the Western world. The disease is caused by progressive deterioration of the monolayer of retinal pigment epithelium (RPE) that supports photoreceptor cell function. Currently, there is no treatment available for the dry form of AMD, characterized by RPE cell death. We used induced pluripotent stem cells (iPSC) technology to develop an autologous cell replacement-based therapy for treating dry AMD patients. First, patients' blood cells were reprogrammed into iPSCs. Then, we differentiated iPSCs into RPE cells, using an efficient tri-phasic protocol developed in our lab. RPE cells were matured on a biodegradable polylactic co-glycolic acid (PLGA) scaffold for five weeks. Quality control assays were performed to confirm the purity, maturity, and functionality of the iPSC-RPE patch. Once the process and product were optimized and assessed for functionality, we moved to pre-clinical studies to test safety and efficacy of iPSC-RPE-patch in rats and pigs. Immune-compromised rats transplanted with a 0.5 mm iPSC-RPE patch didn't show any signs of tumor formation after nine months, confirming the safety profile of our iPSC-RPE patch. To evaluate the efficacy of the iPSC-RPE patch, we used a large animal model. We laser-injured the RPE monolayer in the visual streak of porcine eyes and after 48 hours we transplanted the patch. We performed different live retinal imaging techniques, such as optical coherence tomography (OCT), and confirmed the integration of the patch with the pig RPE layer. At the same time, multi-focal electroretinogram (ERG) showed that the retinal layers' electric response was much higher than the lasered area without the implant. This work led to the submission of an investigational new drug (IND) to the food and drug administration (FDA). FDA recently cleared a phase I/IIa IND application that allows the use of an autologous iPSC-RPE patch to treat AMD. This ongoing trial will test the safety, feasibility, and integration of the iPSC-RPE patch in 12 AMD patients.

GMP-compliant iPS Cell Lines and Differentiation Workflows for Cell Therapy

Boris Greber

Research & Development iPSC/CT, Catalent Cell and Gene Therapy

iPS cells are emerging as a powerful resource in regenerative medicine and immuno-oncology. The important issue of immune compatibility of these cells can be addressed using both autologous and allogeneic approaches, the latter being suited for targeting large numbers of patients in the population. We have generated a universal resource of GMP iPS cell lines from HLA-homozygous cord blood units. These master cell banks can provide the basis for off-the-shelf stocks and display a fully reprogrammed state characterized by unlimited self-renewal, broad differentiation potential, and high genomic integrity.

Furthermore, we have developed GMP-compatible protocols for the conversion of these cells into several cell types of medical relevance including retinal pigment epithelium (RPE), MSCs, and immune (NK) cells. This presentation highlights our progress with deriving RPE cells from iPSCs in a simple workflow at high efficiency.

Current surgical options in the management of inherited retinal disorders

Igor Kozak[1]

[1] *Moorfields Eye Hospitals UAE, Abu Dhabi, U.A.E.*

Inherited retinal dystrophies (IRD) are among the leading causes of visual disability. Few treatments have been approved by authorities for these diseases to halt progression of vision loss. However, most other treatments have been predominantly experimental. The approaches include using implantation of prosthetic devices, gene delivery or cell-based therapies. Prosthetic devices include both epi- and subretinal implants. For gene therapy, the agents can be delivered into the eye either via subretinal injection or intravitreal injection. In intravitreal injections, the agent is delivered into the vitreous cavity. While easier to perform and less risky, this method is more likely to cause immunological responses, because of a greater systemic spread of the agent. Subretinal injections deliver the agent in a more precise and localized manner and they are effective at targeting RPE and PR cells. However, they are more invasive than intravitreal injections, and the injection bleb must be carefully controlled in order to prevent the development of macular holes and retinal detachment. For cell-based therapies, regardless of origin, the therapeutic cells can be delivered via several different techniques. Subretinal delivery can be accomplished with dissociated cells in suspension either through the retina after pars plana vitrectomy (PPV) or through a transscleral approach without vitrectomy. Surgical complications are always a concern with application of these new therapeutic approaches.

In conclusion, several surgical techniques for drug and device delivery are available for both approved and experimental treatments of retinal dystrophies.

Subretinal Implantation of RPE on a Carrier in Minipigs - surgical technique

Stranak Z.[1], Lytvynchuk L.[2], Veith M.[1], Kozak I.[3], Taras A.[4], Motlik J.[4]

[1] *Ophthalmological Clinic, Královské Vinohrady University Hospital Prague*
[2] *Department of Ophthalmology, Justus-Liebig-University Giessen, University Clinic Giessen and Marburg GmbH, Gießen, Germany*
[3] *Moorfields Eye Hospitals UAE, Abu Dhabi, U.A.E*
[4] *IAPG CAS Liběchov, PIGMOD Centre*

Degenerative disorders of the retina (including age-related macular degeneration), which originate primarily at or within the retinal pigmented epithelial (RPE) layer, lead to a progressive disorganization of the retinal anatomy and the deterioration of visual function. The substitution of damaged RPE cells (RPEs) with in vitrocultured RPE cells using a subretinal cell carrier has shown potential for re-establishing the anatomical structure of the outer retinal layers and is, therefore, being further studied. Here, we present the principles of a surgical technique that allows for the effective subretinal transplantation of a cell carrier with cultivated RPEs into minipigs. The surgeries were performed under general anesthesia and included a standard lens-sparing three-port pars plana vitrectomy (PPV), subretinal application of a balanced salt solution (BSS), a 2.7 mm retinotomy, implantation of a nanofibrous cell carrier into the subretinal space through an additional 3.0 mm sclerotomy, fluid-air exchange (FAX), silicone oil tamponade, and closure of all the sclerotomies. This surgical approach was used in 55 surgeries (29 animals) over the past 8 years with a success rate of 92.7%. Anatomic verification of the surgical placement was carried out using in vivo fundus imaging (fundus photography and optical coherence tomography). The recommended surgical steps for the subretinal implantation of RPEs on a carrier in minipig eyes can be used in future preclinical studies using large-eye animal models.

Steps in preparing a nanofibrous membrane for subretinal implantation

Hana Studenovská, Jitka Nováčková, Luďka Machová, Vladimír Proks

Institute of Macromolecular Chemistry, Academy of Sciences of the CR, Heyrovský Sq. 2, 162 06 Prague 6, Czech Republic

In this work we focused on preparation of ultrathin, porous nanofibrous membranes for application in retinal tissue engineering. We compare two conventional culturing membranes for tissue culturing made either from polyethyleneterephtalate (PET, track-etched, thickness 10 µm, pore density 1×10^{8} cm^{-2}) or polyimide (PI, track-etched, thickness 7 µm, pore density $2{,}2\times10^{7}$ cm^{-2}) with cell carrier based on 400-nm-thick poly(L-lactide-DL-lactide) fibres (thickness 4 µm, porosity 80%). Nanofibrous membranes were prepared by electrospinning that easily allowed an embedding of a supporting frame. Such a frame enables not only handling without irreversible folding of carrier and keeping a side-orientation of the sample while seeded with cells, but also to regain membrane's flat shape when inserted into the subretinal space during surgery. ARPE-19 cells were seeded onto PET, PI and nanofibrous membranes. Viability of ARPE-19 cells was monitored in different stages of sample preparation. After cutting of samples from inserts by punch there was no significant difference in viability of ARPE-19 cells in all membranes. To keep sterility of samples, a femtosecond laser to cut the samples was applied. PET and PI membranes were impossible to cut by laser. In nanofibrous membranes viability of cells after laser cutting was similar to those after using a punch. Thus, a femtosecond laser proved as a powerful tool in preparation of samples for retinal tissue engineering in sterile conditions. Viability of ARPE-19 cells in nanofibrous membranes were tested also after loading to the surgical injector with a flat loading tube. Loss of cells during loading is as low as 15%. The presented ultrathin, highly porous, and surgically convenient cell carriers have key characteristics in order to improve the integration and the functionality of transplanted RPE cells.

Acknowledgement: The authors would like to thank the Technology Agency of the Czech Republic KAPPA Programme, Project Number TO01000107 for providing financial support.

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

Brigitte Müller[1], K. Stieger[1], T. Ardan, H. Studenovska, S. Erceg, J. Motlík, G. Petrovski, and all from the Czech-Norwegian KAPPA Grant Team

[1]*Ophthalmology, Faculty of Medicine, Justus-Liebig-University Giessen, Giessen, Germany.*

Purpose: Dysfunction of the retinal pigment epithelial cell layer (RPE) is primary in the pathogenesis of various eye diseases like age-related macular degeneration (AMD) or retinitis pigmentosa with MERTK-mutations. MERTK mutations reduce the ability of RPE cells to phagocytize the photoreceptor outer segments, which leads to accumulation of debris separating photoreceptors from RPE cells, resulting in their degeneration and loss of vision.

In this study, we continue with the implantation of human iPSCs derived RPE cells cultured on nanofibrous carriers into the subretinal space of the pig eye as a potential cell-based therapy for retinitis pigmentosa with MERTK mutation. **Methods**: Human iPSCs (hiPSCs) derived RPE cells were grown from dermal fibroblasts of healthy patients and patients with retinitis pigmentosa due to a MERKT mutation. Cells containing the MERKT mutation were corrected with the CRISPR/Cas technology and cultivated on ultrathin nanofibrous membranes prepared by electrospinning of poly(L-lactide-co-DL-lactide). Fourteen minipigs (8–9 months old) were used in the study supported by pre- and postoperative tacrolimus immunosuppressive therapy. In comparison to previous experiments, the immunosuppressive protocol was significantly changed, i.e., applied doses were increased as well as the number of applications (every 12 days).The observational period lasted 1, 2, and 6 weeks, and included in vivo optical coherence tomography (OCT) of the retina, as well as post-mortem immunohistochemistry using the following antibodies: HNAA and STEM121 (human cell markers); Bestrophin and CRALBP (hRPE cell markers); peanut agglutining (PNA) (cone photoreceptor marker); PKCα (rod bipolar marker); Vimentin, GFAP (macroglial markers); and Iba1 (microglial marker). **Results**: First macroscopical analysis of the treated eye bulbs after enucleation and preservation showed some effects of the subretinal implantation on the condition of the treated retina. One week after implantation the nanofibrous carrier (NC) was detectable in the fundus and the retina above it appeared more translucent than the surrounding retina. In several eyes retinal detachment occurred and/or retina looks more whitish than normally. Two weeks post implantation (PI) concentrical zones with different whitish appearance around the implanted NC were visible in some eyes. These zones get more whitish with increasing distance from the NC. As seen in one week pigs the NC was well detectable in the nasal part of the fundus and the retina above did not show signs of gliosis resulting in a translucent appearance. Even six weeks PI no signs of gliosis were visible in the fundus. The NC was readily detectable in the nasal part of the fundus and the retina above it appeared more translucent compared to surrounding retinal areas. **Discussion**: Macroscopically the hiPSC derived RPE cells cultured on nanofibrous carriers and implanted into immunosuppressed minipigs seems to be more successful concerning integration in the subretinal space and no obvious gliosis or inflammation. In a previous study we detected survival of implanted hiPSC derived RPE cells only one week after surgery whereas no implanted cells were detected during the following weeks most likely due to insufficient immunosuppression. Therefore, the significantly altered immunosuppressive protocol in this experiment was effective. Immunohistochemical analysis will add more evidence soon.

Clinical grade of genetically corrected RPE cells for autologous cell therapy of hereditary retinal dystrophies

Slaven Erceg

Príncipe Felipe Research Center Foundation Valencia

The generation of genetically corrected RPE cells from patient-derived human induced pluripotent stem cells (hiPSCs) may provide a source for autologous therapeutically-relevant therapy for this rare retinal dystrophy as well to create the human cellular disease models. Recently we generated a faithful RPE model from patient bearing mutation in *MERTK* gene and genetically corrected these cells using CRISPR/Cas9 system. For further application in humans is necessary to differentiate the hiPSC toward RPE in clinical grade conditions.
The RPE cells from the genetically corrected patient-derived hiPSC (1 and 2 alleles) as well as patient´s RP-hiPSC and control healthy hiPSC were characterized using immunocytological methods for the differentiation and polarization markers, electronic microscopy, RT-PCR, TEER measurement, ELISA test, Western Blot and phagocytic assay. All generated RPE cells exhibit typical features of RPE cells. We demonstrated the reestablishment of the expression of full-length MERTK protein as well as the reversion of lost phagocyte function of hiPSC-RPE *in vitro*, which represents the first example of its kind in this field.
The generated hiPSC-derived RPE in clinical grade conditions display the typical characteristics of mature RPE cells re-establishing the function of phagocytosis in genetically corrected patient-derived RPE cells *in vitro*. Functional *in vivo* studies in animal models such as RCS rats or larger animals will confirm whether this strategy can completely restore the vision and be employed as a potential treatment for patients.

Retinal disease models in minipigs - application in translational and clinical studies

Goran Petrovski[1,2]

[1] *Center for Eye Research and Innovative Diagnostics, Department of Ophthalmology, Oslo University Hospital and Institute for Clinical Medicine, Faculty of Medicine, University of Oslo, 0450 Oslo, Norway;* [2] *Department of Ophthalmology, University of Split School of Medicine and University Hospital Centre, Split, Croatia*

The eyes of the minipigs show remarkable similarity to those of humans in regards to their anatomy and physiology. Retinal disease models in small animal models have already been described in detail, but they seem to be lacking in minipigs, which development is crucial for understanding disease pathogenesis and treatment discoveries.

A model for retinal ischemia and glaucoma is hereby presented to study the acute effects of these disorders *in vivo*, giving the lead to translational and clinical studies. Similarly, a model for inducing retinal pigmented epithelium (RPE) atrophy using laser ablation, as well as choroidal neovascularization using laser photocoagulation, that resemble the dry- and wet-form of AMD, respectively, are also described here.

A method for enhancing melanogenesis in the retinal pigmented epithelium for research applications

Santosh Gupta[1], Goran Petrovski[1,2]

[1] *Center for Eye Research and Innovative Diagnostics, Department of Ophthalmology, Oslo University Hospital and Institute for Clinical Medicine, Faculty of Medicine, University of Oslo, 0450 Oslo, Norway;* [2] *Department of Ophthalmology, University of Split School of Medicine and University Hospital Centre, Split, Croatia*

The retinal pigmented epithelium (RPE) is a specialized single-cell layer found in the posterior segment of the eye between the choroid plexus and the neural retina, performing critical functions in the visual cycle. One of the characteristic features of the RPE is its pigmentation due to melanin, which is organized in specialized compartments known as melanosomes. RPE protects the eye from the photodamaging effect of the light entering it due to the melanin pigmentation. These melanosomes are synthesized during the early stage of fetal development and eventually stop after the birth with no synthesis turnover. There exists no efficient treatment for inducing melanogenesis in RPE *in vitro*, *ex vivo* and *in vivo* using conventional pharmacological strategies. In this study, we developed a small molecule based chemical modality of inducing pigmentation in non-pigmented RPEs (the human ARPE-19 cell line). Upon chemical treatment, pigmentation was induced by Day 3, which by Day 5 was prominently visible under bright field microscope. Viability studies showed around 90% live cells by Day 3. Presence of melanosome-like particles (MLPs) was further confirmed by ultrastructural analysis using transmission electron microscopy. MLPs in various stages of maturation could be observed by Day 3 and remained present till Day 14. Our study confirms that pigmentation in non-pigmented RPEs can be induced using a chemical approach. In order to unravel the mechanism of this *de novo* MLP formation and its characterization, further studies are warranted.

On the hunt for biomarkers to validate Retinal Pigment Epithelial cells for transplantation

[1]Georgina Faura Munoz, [1]Marte Eikenes, [2]Hana Studenovská, [1]Hans-Otto Böhm, [1]Katja Benedikte Prestø Elgstøen, [3]Goran Petrovski, [1]Lars Eide*

[1]Department of Medical Biochemistry, Institute of Clinical Medicine, University of Oslo, Norway; [2]Institute of Macromolecular Chemistry, Czech Republic; [3]Department of Ophtalmology, Oslo University Hospital, Norway

Age-related macular degeneration (AMD) is major cause of visual impairment in elderly world-wide. In AMD, the epithelial layer separating circulating blood and the neural retina (blood-retina barrier) fails to function properly. This dysfunction, which is speculated to be a combination of age-related oxidative stress, mitochondrial dysfunction and improper mitophagy, leads to weakened diffusion of nutrients into the retina as well as export of compounds in the opposite direction, and predispose photoreceptors to cell death.

Retinal pigment epithelial (RPE) cells make up this epithelial monolayer on the Bruch's membrane and one of the most promising therapies against AMD is RPE-covered biodegradable patches. However, the success of such therapy depends on the quality of the primary RPE cells that are obtained from donors. In this project, we aim to identify biomarkers that can be used to assess RPE quality, and aid design of *in vitro* culture conditions to optimize RPE for transplantation.

We postulate that age-related mitochondrial dysfunction caused by oxidative damage on mitochondrial DNA (mtDNA) is a major contributor to RPE impairment, and that assessment of mtDNA quality and correlating extracellular biomarkers can be used to diagnose RPE quality.

Primary RPE cells freshly isolated from various donors were analysed for DNA integrity (nuclear and mitochondrial DNA damage, mtDNA copy number), and cultivated into mature epithelium *in vitro* and subsequently analysed for epithelial parameters, such as transepithelial electric resistance (TEER), pigmentation, apical/basolateral metabolites from glucose, protein and fatty acid oxidation. The results imply that TEER associates poorly with DNA integrity. However, the integrity of mtDNA integrity seems to correlate with apical secretion of 3-hydroxybutyrate, a biomarker of fatty acid oxidation. We are additionally evaluating the effect of hydrogen peroxide on RPE DNA integrity and mitochondrial metabolism, to identify novel RPE biomarkers that are characteristic of oxidative stress. We also investigate the impact of biodegradable (coated and uncoated) polylactate scaffolds, which are being used in transplantation.

Modelling Ciliopathies Using Retinal Organoid Model

Marija Dubaic[1,2], Kamila Weissova[1,3], Marek Hampl[1,2], Lucie Peskova[3], Canan Celiker[3], Natalia A. Shylo[4], Eva Hrubá[3], Michaela Kavkova[5], Tomas Zikmund[5], Scott D. Weatherbee[4], Tomas Barta[1,3] and Marcela Buchtova[1,2]

1) Laboratory of Molecular Morphogenesis, Institute of Animal Physiology and Genetics, Czech Academy of Sciences, Brno, Czech Republic
2) Institute of Experimental Biology, Masaryk University, Brno, Czech Republic
3) Department of Histology and Embryology, Faculty of Medicine, Masaryk University, Brno, Czech Republic
4) Department of Genetics, Yale University, School of Medicine, New Haven, CT, USA
5) CEITEC - Central European Institute of Technology, Brno University of Technology, Brno, Czech Republic

Primary cilia play a significant role in many developmental patterning events, including establishment of left-right asymmetry and organogenesis. The primary cilium consists of microtubular axoneme covered by cellular membrane, which is anchored to the cell through the basal body at the ciliary base. Recently, Transmembrane protein 107 (TMEM107) has been found to be an important factor for cilia formation during embryonic development. TMEM107 protein was found to be expressed in the transition zone, which is located at the base of the cilium. This zone selectively transmits proteins and thus regulates protein composition, morphology and function of the cilium.
Mutations in *TMEM107* have been described in patients diagnosed with Orofaciodigital syndrome, Meckel-Gruber syndrome, and Joubert syndrome. All described cases exhibited alterations in the number and/or length of cilia, confirming the critical role of TMEM107 protein in cilia formation. Additionally, the patients with mutations in *TMEM107* demonstrated distinct craniofacial defects including retinal and eye defects.
Here we used retinal organoids derived from human pluripotent stem cells as a model to closely investigate the role of TMEM107 in retinal development. We used shRNA and CRISPR/Cas9 approach to perform TMEM107 loss-of-function studies. TMEM107 deficiency results to: I) impaired differentiation towards retinal structures, II) profound structural changes to retinal organoids, III) absence of primary cilia on retinal cell types, and IV) impaired SHH signalling. Our results indicate that TMEM107 is crucial for retinal development and SHH signalling. We envisage to use retinal organoids to further explore the molecular mechanisms underlying the role of TMEM107.

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Towards the development of gene therapies for hearing and vestibular deficits in mouse models of Usher syndrome

Aziz El-Amraoui, PhD

Progressive Sensory Disorders, Pathophysiology and Therapy Unit

Institut Pasteur, Université Paris Cité, Institut de l'Audition, 63 rue de Charenton, F75012, Paris, France.

The Usher target organ, the inner ear, is responsible for both hearing and balance. These functions are dependent on the correct functioning of mechanosensitive hair cells, which convert sound- and motion-induced stimuli into electrical signals conveyed to the brain. Hearing impairment, with and without balance deficits, is the most frequent sensory deficit in humans of all age groups, from children (1/500) to the elderly (1 out of 3 over 70 years old). Over the last two decades, the study of inherited deafness forms and related animal models have been instrumental in deciphering the molecular, cellular, and physiological mechanisms of sense deterioration. Hearing loss is currently palliated by rehabilitation methods: conventional hearing aids and, for more severe forms, cochlear implants. Efforts are continuing to improve these devices, to help users to understand speech in noisy environments and to appreciate music. However, neither approach can mediate a full recovery of hearing sensitivity and/or restoration of the native inner ear sensory epithelia. New therapeutic approaches based on gene transfer and gene editing tools are being developed in animal models.

Recent work on two tetraspan-like proteins, member of the clarin family, in humans, mouse and zebrafish highlights the key role of clarin-1 and clarin-2 in the inner ear. Constitutive or conditional inactivation of either, or both, clarin genes lead to sensory deficits that reflect different clinical conditions observed in humans, with hearing loss being congenital and profound or post-natal and progressive, and sometimes also associated with severe balance deficits. As gene therapies continue to evolve with promising success for the treatment of rare diseases, we used these distinct clarin-deficient models to monitor and document the beneficial outcomes of viral-mediated gene replacement according to the type of sensory deficits. Our findings pinpoint the importance of the therapeutic window, and a significant correlation between the extent of sensory recovery and the type and age at onset of targeted deficit.

Update on multimodalitiy hearing screening in pigs

Andrea Fischer

LMU Munich

Background: There are multiple methods available for objective assessment of hearing. Many of those are being developed and fine-tuned for use in newborn hearing screening in humans. These are designed to provide objective and reliable results for hearing threshold and, in addition, an estimate of the audiogram. Diagnostic tests are usually performed during a short period of rest or sleep in newborns, but can also be done in the sedated or anesthetized patient. Hearing screening in a research setting in experimental animal models faces specific challenges because multiple procedures are frequently scheduled besides audiometry in the same patient, and all of these under general anesthesia, thus prolonging anesthetic duration and experiment time. In experimental Usher research, vision and auditory systems are studied in detail in the same animal with a bunch of time-consuming sophisticated methods. With regard to audiometry, the auditory brainstem response (ABR) is considered the gold standard in objective hearing assessment. It provides a hearing threshold for a broad-band click stimulus. Furthermore, an audiogramm can be obtained if thresholds are repeatedly assessed with the use of tone burst stimuli for each frequency under investigation (usually in the range of 1 – 4 kHz). Other methods are in use for hearing screening, which are less time consuming and independent of reviewer interpretation, but they rely on different concepts: One of these are otoacoustic emissions (OAE) which are recorded by insertion of a combined loudspeaker and microphone probe in the ear canal. Otoacoustic emissions record the outer hair cell response to a paired acoustic stimulus in a specific frequency range. The other one are auditory steady state responses (ASSR) which record the neural response of the brain to frequency- and amplitude modulated stimuli in a specific frequency range. The ASSR can be recorded with the same electrode setting as the ABR and detection of the signal follows an algorithm and is independent from reviewer interpretation. Choice of methods for hearing assessment has an important impact on anesthetic duration and length of the experiment in the individual animal. However, reliability and consistency of the data obtained need also to be considered when planning future experiments. We performed extensive hearing screening with multiple modalities in pigs with Usher syndrome, wildype and heterozygous pigs in a controlled and a less controlled environment with equipment from Path Medical, Germering. Specific attempts were undertaken to minimize any acoustic noise in the examination room and also from the animal e. g. excessive breathing or snoring or from the anesthetic equipment. Our results showed that comprehensive hearing screening of both ears incooperating multiple modalities for threshold assessment (ABR click threshold, ABR tone bursts, DPOAE, ASSR) can be done within 45 minutes. Use of bilateral stimulation was a great advantage and significantly shortened examination times. There was agreement between all methods in the overall scoring of hearing when performing the experiments in a noise-controlled environment. Thereby we obtained click, OAE and ASSR thresholds as low as 30 dB nHL for wildtype and heterozygous animals, while responses were absent in the homozygous Usher animals. Comparing ease and reliability of the recordings ASSR proved significant advantages when compared to the other methods and provided rapid assessment of hearing thresholds up to 8 kHz, independent from reviewer interpretation and less interference with acoustic noise. ASSR could be used in future experiments together with ABR click threshold for time-efficient objective hearing tests over a wide frequency range. OAE proved suitable as a rapid initial screening test for intact hearing in a noise controlled environment.

Correspondence: Andrea Fischer, Prof., Dr. med. vet., Dr. habil., FEAN, Dipl. ACVIM (Neurology), EBVS® European Specialist in Veterinary Neurology, Centre for Clinical Veterinary Medicine, Clinic of Small Animal Medicine, Veterinärstr. 13, D-80539 Munich, andreafischer@lmu.de; +49-89-21806236

Human neural networks with sparse TDP-43 pathology reveal NPTX2 misregulation in ALS/FTLD

Marian Hruska-Plochan[1], Katharina M. Betz [1,2,3*], Silvia Ronchi[4*], Vera I. Wiersma[1], Zuzanna Maniecka[1], Eva-Maria Hock[1], Florent Laferriere[1], Sonu Sahadevan[1], Vanessa Hoop[2], Igor Delvendahl[2], Martina Panatta[1], Alexander van der Bourg[5], Dasa Bohaciakova[7], Karl Frontzek[6], Adriano Aguzzi[6], Tammaryn Lashley[8,9], Mark D. Robinson[2,3], Theofanis Karayannis[5], Martin Mueller[2], Andreas Hierlemann[4], Magdalini Polymenidou[1#]

[1]*Department of Quantitative Biomedicine, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland*
[2]*Department of Molecular Life Sciences, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland*
[3]*SIB Swiss Institute of Bioinformatics, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland*
[4]*Department of Biosystems Science and Engineering, ETH Zürich, Mattenstrasse 26, 4058 Basel, Switzerland*
[5]*Brain Research Institute, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland*
[6]*Institute of Neuropathology, University of Zurich, Schmelzbergstrasse 12, 8091 Zurich, Switzerland*
[7]*Department of Histology and Embryology, Faculty of Medicine, Masaryk University Brno, Kamenice 3, 62500, Brno, Czech Republic*
[8]*Queen Square Brain Bank for Neurological diseases, Department of Movement Disorders, UCL Institute of Neurology, London, WC1N 1PJ, UK*
[9]*Department of Neurodegenerative Disease, UCL Institute of Neurology, Queen Square, London, WC1N 3BG, UK*

*These authors contributed equally to this work; #Author for correspondence: magdalini.polymenidou@uzh.ch

Human cellular models of neurodegeneration require reproducibility and longevity, which is necessary for simulating these age-dependent diseases. Such systems are particularly needed for TDP-43 proteinopathies, which involve human-specific mechanisms that cannot be directly studied in animal models. To explore the emergence and consequences of TDP-43 pathologies, we generated iPSC-derived, colony morphology neural stem cells (iCoMoNSCs) via manual selection of neural precursors. Single-cell transcriptomics (scRNA-seq) and comparison to independent NSCs, showed that iCoMoNSCs are uniquely homogenous and self-renewing. Differentiated iCoMoNSCs formed a self-organized multicellular system consisting of synaptically connected and electrophysiologically active neurons, which matured into long-lived functional networks. Neuronal and glial maturation in iCoMoNSC-derived cultures was similar to that of cortical organoids. Overexpression of wild-type TDP-43 in a minority of iCoMoNSC-derived neurons led to progressive fragmentation and aggregation, resulting in loss of function and neurotoxicity. scRNA-seq revealed a novel set of misregulated RNA targets coinciding in both TDP-43 overexpressing neurons and patient brains exhibiting loss of nuclear TDP-43. The strongest misregulated target encoded for the synaptic protein NPTX2, which was consistently misaccumulated in ALS and FTLD patient neurons with TDP-43 pathology. Our work directly links TDP-43 misregulation and NPTX2 accumulation, thereby highlighting a new pathway of neurotoxicity.

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Tissue sampling strategies in porcine animal models in translational biomedical research

Prof. Dr. Andreas Parzefall

Institute of Veterinary Pathology, Center of Clinical Veterinary Medicine,

Ludwig-Maximilians-Universität München, Munich, Germany

In translational medical research, porcine models have steadily become more popular. The anatomical similarity to humans and the larger body size of pigs, as compared to rodents, is particularly beneficial in studies of sensory functions, such as sight, hearing or equilibrium.
Considering the high value of individual animals, particularly of genetically modified pig models, and the often-limited number of available animals of these models, comprehensive tissue sampling strategies adapted to the peculiarities of porcine anatomy were established in order to warrant the standardized generation of representative, high-quality samples from different organs and tissues, optimally processed for a broad range of different scheduled downstream analysis types (including *e.g.*, including cryohistology, paraffin- and plastic-histology; immunohistochemistry, *in- situ* hybridization, electron microscopy, quantitative stereology, as well as molecular analyses of DNA, RNA, proteins, metabolites, and electrolytes).
The determination of the sampling locations and of the numbers of tissue samples to be generated, as well as their orientation, size, processing and trimming directions, are relevant factors determining the usability of the specimen for molecular, qualitative, and quantitative morphological analyses.
Adequate tissue sampling is therefore essential for the reproducibility of results and their comparability between different studies and investigators.
The presentation summarizes and illustrates the underlying sampling principles for several organs and tissues in different study types and provides examples for tissue sampling regimes that enabled the successful identification of morphological pendants to molecular analysis results in multi-OMICS studies of different porcine animal models.

Session 2 – Stargardt Disease

Keynote Lecture

Animal models of Stargardt disease

Knut Stieger

Justus-Liebig-Universität Giessen, Eye Hospital, Germany

Stargardt disease is the most frequently occurring inherited juvenile maculopathy. Mutations in the ABCA4 gene are associated with Stargardt disease type 1. Other genes associated with rare forms of Stargardt disease are CNGB3, ELOVL4, PROM1 and PRPH2. Since phenotypic manifestations are associated with special human anatomic features of the retina such as the cone enriched macula, animal models of disease in most cases only partially mimic pathologic features in human patients. Animal models of Stargardt disease have been developed and/or characterized in zebrafish, mice, rats, dogs, and pigs. This lecture aims at summarizing the current state of the art with regard to these animal models and their use (i) in deciphering the pathology of the disease, (ii) in the development of potential biomarkers, and (iii) in therapeutic applications.

A gene therapy for Stargardt Disease using novel dual mRNA trans-splicing AAV vectors

Elvir Becirovic

Universitätsspital Zürich, Eye Hospital, Switzerland

Adeno-associated viral vectors (AAVs) are currently the gold standard for gene therapy applications. However, due to limited genome packaging capacity of AAVs treatment of diseases caused by mutations in large genes require so-called dual AAV vectors. One example for a retinal disease caused by defects in genes exceeding the AAV packaging capacity is Morbus Stargardt, which is typically associated with mutations in the ABCA4 gene. Currently applied dual AAV vectors focus on reconstitution of split genes at the protein or genome level, but have some drawbacks in terms of their efficiency, flexibility, or safety. We have developed a technology that can reconstitute split fragments at the transcript level (reconstitution via mRNA trans-splicing, REVeRT) and circumvent these drawbacks. In this study, we supplemented ABCA4 using dual REVeRT AAV vectors in a mouse model of Stargardt disease and examined ABCA4 protein expression as well as retinal degeneration and function.

Abca4$^{-/-}$Rdh8$^{-/-}$ mice (n = 4) were injected intravitreally at postnatal day 21 (P21) with dual titer-matched AAV2.GL vectors, each expressing one half of the human ABCA4 gene under the control of the rod-specific rhodopsin promoter. The contralateral eye was sham-injected and served as an internal control for all measurements. Fundus cSLO, OCT, and ERG measurements were performed on treated mice ten weeks after injection. In one mouse, typical lipofuscin deposits were detected in nontreated eye, but were completely absent in the treated eye. ABCA4 protein expression was examined post mortem by Western blotting (n = 2) and immunolabeling (n = 2). Treated eyes from three of four mice showed robust expression of ABCA4. All eyes with detected ABCA4 expression showed delayed retinal degeneration and an improvement of scotopic and photopic light responses.

In conclusion, in this work we provide the first evidence of efficient gene therapy for Stargardt disease after intravitreal injection using new dual AAV vectors with high therapeutic potential.

Existing hurdles in retinal gene therapy and how to overcome them

Stylianos Michalakis

Department of Ophthalmology, University Hospital LMU Munich, Germany

Inherited retinal disorders are severe eye diseases that lead to visual impairment and even blindness. They are caused by defects in genes encoding key proteins of the visual process, most of which are specifically expressed in photoreceptors or pigment epithelial cells of the retina. With the advent of efficient gene therapy vectors based on non-pathogenic recombinant adeno-associated viruses (AAV), it is now possible to develop potentially curative therapeutic approaches for these previously incurable eye diseases.

In my talk, I will provide an introduction to AAV biology, discuss current therapeutic approaches to gene therapy for ocular diseases, and highlight key unmet needs. I will then present new technologies currently being developed to overcome key remaining challenges, such as the use of alternative, less invasive delivery routes and the ability to transfer large genes.

Session 3 - Gene Therapy of Usher Syndrom

Scrutinizing pathogenicity of the frequent USH2A p. Cys759Phe variant

Erwin van Wijk

Radboud University Nijmegen Medical Center, The Netherlands

The *USH2A* variant c.2276G>T (p.(Cys759Phe)) has been described as a frequent cause of autosomal recessive retinitis pigmentosa (arRP). However, the pathogenicity of the c.2276G>T variant was questioned after the identification of two asymptomatic individuals homozygous for this variant. Consequently, cases homozygous for the c.2276G>T did not receive a conclusive genetic diagnosis and as a result, they were not considered to be eligible for receiving future *Ultevursen*-mediated exon 13 skipping therapy, in case this would reach the market. We therefore assessed pathogenicity of the *USH2A* c.2276G>T variant using extensive genetic and functional analyses. Whole genome sequencing and optical genome mapping were performed for three arRP cases homozygous for *USH2A* c.2276G>T to exclude alternative genetic causes. A minigene splice assay was designed to investigate the effect of c.2276G>T on pre-mRNA splicing, in presence or absence of the nearby c.2256T>C variant. Moreover, an *ush2a*$^{p.(Cys771Phe)}$ zebrafish knock-in model mimicking human p.(Cys759Phe) was generated and characterized using functional and immunohistochemical analyses.

Non-viral gene augmentation strategy for *USH2A* retinopathy

Mariya Moosajee[1,2,3]

[1]Development, Ageing and Disease, UCL Institute of Ophthalmology, London, UK
[2]Ocular Genomics and Therapeutics, The Francis Crick Institute, London, UK
[3]Department of Genetics, Moorfields Eye Hospital NHS Foundation Trust, London, UK

USH2A mutations are a common cause of autosomal recessive retinitis pigmentosa (RP) and Usher syndrome type II, for which there are currently no approved treatments. Gene augmentation is a promising therapeutic strategy for treating retinal diseases, however conventional adeno-associated virus (AAV) vectors cannot accommodate cDNAs exceeding 4.7kb, such as the 15.6kb-long *USH2A* coding sequence. Our aim is to use a non-viral gene delivery strategy using episomal DNA plasmid vectors containing a scaffold/matrix attachment region (S/MAR) and the human *USH2A* cDNA to generate USH2A protein (usherin) expression in human cellular and zebrafish models.
USH2A-S/MAR vectors were generated by inserting the *USH2A* coding sequence into the pS/MAR backbone in five cloning steps. HEK293 cells and *USH2A*$^{-/-}$ patient-derived dermal fibroblasts were transfected using the Neon transfection system. Wild-type and *ush2a*^{u507} zebrafish were microinjected with USH2A-S/MAR vector at the single-cell stage of development. Expression of GFP and usherin was assessed in both cell lines and zebrafish using qRT-PCR, immunostaining and Western blot analysis. Localisation of adgrv1, a periciliary membrane *complex* protein, was examined in zebrafish photoreceptors.
USH2A-S/MAR vectors were generated, containing a GFP reporter gene and CAG (pS/MAR-CAG-USH2A) or CMV (pS/MAR-CMV-USH2A) promoters, reaching a size of 23kb. The vectors produced persistent transgene expression in HEK293 and patient fibroblasts up to 240 hours post transfection, and in zebrafish with up to 12 months of GFP expression detected in zebrafish retinal photoreceptors. Expression of adgrv1 was restored in pS/MAR-injected *ush2a*^{u507} photoreceptors.
USH2A-S/MAR vectors generated expression of full-length functional usherin *in vivo* and *in vitro*, showing promise as a novel non-viral retinal gene therapy and warranting further translational development into higher order animal models.

Biography:

Professor Mariya Moosajee is a clinician scientist, she is a Consultant Ophthalmologist in Genetic Eye Disease at Moorfields Eye Hospital, London, and Professor of Molecular Ophthalmology at UCL Institute of Ophthalmology, and Group Leader of Ocular Genomics and Therapeutics at the Francis Crick Institute in London. She graduated with First Class Honours in Biochemistry and Molecular Genetics in 2000, Medicine (MBBS) in 2003, and was awarded her PhD in Molecular Ophthalmology in 2009 all from Imperial College London. She has been awarded over 30 international and national prizes for her research and has over 100 peer-reviewed scientific publications. She has been named in top 100 most influential people in ophthalmology in The Ophthalmologist Power List for the past 3 years.
Her clinical focus is providing a genomic ophthalmology service for children and adults affected with pan-ocular genetic eye disease. Her clinical research involves deep phenotyping and natural history studies on molecularly confirmed rare disease patient cohorts to understand disease progression and define outcome metrics for clinical trials. In the laboratory, she is advancing our understanding of the molecular basis of ocular maldevelopment and inherited retinal dystrophies, using zebrafish disease models and human induced pluripotent stem cell derived retinal organoids. This permits the identification of potential therapeutic targets for development of treatment strategies, including small molecule drugs and non-viral gene therapy. Professor Moosajee is the President of the UK Eye Genetics Group, Research Lead for the Education Committee and elected member of the Academic Committee of the Royal College of Ophthalmologists, and President of Women in Vision UK.

Dissecting Usher syndrome retinitis pigmentosa in iPSC-derived retinal models

Dunja Lukovic

Príncipe Felipe Research Center Foundation Valencia, Spain

Mutations in USH2A are the most common cause of autosomal recessive non-syndromic retinitis pigmentosa (RP) and Usher syndrome (USH). Vision loss in these disorders has no effective treatment up to date. The iPSC derived retinal organoids are increasingly getting attention as retinal developmental and disease models which allow mechanistic studies and testing therapeutics. It is so far unknown to what extent the expression of USH2A is retained in retinal organoids. We generated iPSCs from patients affected by mutations in USH2A and studied its expression pattern and photoreceptor features in iPSC-derived retinal organoids.

Novel insight into cellular functions of USH2C VLGR1/ADGRV1 - in cell adhesion, cell migration, autophagy and Ca^{2+} homeostasis

Krzysko, J., Güler, B. E., Linnert, J., Kusuluri, D. K., Knapp, B., Wolfrum, U.

Institute of Molecular Physiology, Molecular Cell Biology, Johannes Gutenberg University Mainz, Germany

Mutations in *ADGRV1* cause Usher syndrome (USH2C), a form of hereditary deaf-blindness, and have been additionally linked to childhood absence epilepsy. The *ADGRV1* gene encode for the very large G protein-coupled receptor 1 (VLGR1/ADGRV1), which is the largest member of the adhesion G protein-coupled receptor (ADGR) family. In the absence of tangible knowledge of the molecular function and signaling of VLGR1, the pathomechanisms underlying these diseases are still unknown. Applying affinity proteomics, we have identified numerous novel potential binding partners and ligands of VLGR1 indicative for diverse of functions of VLGR1 (Knapp et al. 2019, 2022). Based on our interactome data, we will focus here on aspects of three different functional cell modules, namely cell adhesion, autophagy, and Ca^{2+} homeostasis.

We investigated the role in adhesion complexes, particularly in focal adhesions, where VLGR1 acts as a metabotropic mechnosensor. Its deficiency leads to a reduced size and molecular turnover in focal adhesions affecting cell motility and spreading.

We also gathered evidence for a function of VLGR1 at internal membranes such as in the formation of autophagosomes. Absence or defects of VLGR1 leads to the upregulation of the key autophagy molecules and an increase in activity of the autophagy processes in the retinae of *Vlgr1* mutant mice and USH2C patient-derived cells indicating that VLGR1 is essential for controlling autophagy at internal membranes.

Moreover, VLGR1 is associated with the mitochondria-associated membranes (MAMs) of the ER. These ER-mitochondria contact sites are highly dynamic and relevant for autophagy but also for the Ca^{2+} homeostasis of the cell and dysregulations associate with diseases. We confirm the interaction of VLGR1 with core proteins of the MAM complex and the presence of VLGR1 in the MAM compartment. We show that the MAM composition and structure is altered in the brain and retina of *Vlgr1*-deficient mice. Furthermore, we provide evidence for an impaired Ca^{2+} transient between ER and mitochondria, a key function of MAMs, after siRNA-mediated knockdown of *VLGR1* in human cells, in USH2C patient-derived fibroblasts and in primary astrocytes of *Vlgr1* mutant mice.

Our data also shed new light on the role of VLGR1 in pathomechanisms leading to VLGR1-associated diseases, namely human Usher syndrome and childhood absence epilepsy.

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The Usher syndrome protein harmonin regulates canonical Wnt signaling

Jessica Schäfer[1], Nicole Wenck[1], Katharina Janik[1], Susanne Kohl[3], Kerstin Nagel-Wolfrum[1,2], Uwe Wolfrum[1*]

[1]Institute of Molecular Physiology, [2]Institute of Developmental Biology and Neurobiology, Johannes Gutenberg University Mainz, Germany; [3] Institute for Ophthalmic Research, Centre for Ophthalmology, University of Tubingen, Germany

Human Usher syndrome (USH) is the most common form of hereditary combined deaf-blindness. USH is a complex genetic disorder, and the pathomechanisms underlying the disease are far from understood, especially in the eye. The *USH1C* gene encodes the scaffold protein harmonin. Although so far only disease-related phenotypes are known in the eye and inner ear, *USH1C*/harmonin is almost ubiquitously expressed in our body.

Here, we show that harmonin binds to β-catenin, which is not only integral part of cell adhesion complexes but also a key regulator of the canonical Wnt (cWnt) signaling pathway. We also demonstrate that harmonin also interacts with the stabilized acetylated β-catenin, especially in nuclei. In HEK293T cells, overexpression of *USH1C*/harmonin a1 significantly reduced cWnt signaling, but a *USH1C* mutant form did not. Concordantly, we observed an increase in cWnt signaling in dermal fibroblasts from USH1C patients compared with healthy donor cells. RNAseq analysis revealed that both the expression of genes related to the cWnt/β-catenin pathway and cWnt-target genes were substantially altered in USH1C patient-derived fibroblasts. Finally, we show that the altered cWnt signaling was reverted in USH1C patient cells by the application of Ataluren, a small molecule suitable to induce translational read-through of nonsense mutations. Our results demonstrate a cWnt signaling phenotype in USH establishing USH1C/harmonin as a negative regulator of the cWnt/β-catenin pathway. The link found here between USH and the cWnt pathway opens the door for potential new targets for USH therapy.

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Phenotypic characterization of USH1C pig retinae in perfusion-fixed specimens

Joshua Klein[1], Anna Gager[2], Hannah Auch[3], Andreas Parzefall[2], Nikolay Klymiuk[3], Uwe Wolfrum[1]

[1]Institute of Molecular Physiology, Johannes Gutenberg University Mainz; [2]Institute of Animal Pathology, LMU Munich; [3]Institute of Molecular Animal Breeding and Biotechnology, LMU/TU Munich

Purpose: The human Usher syndrome (USH) is the most common form of inherited deaf-blindness, accompanied by vestibular dysfunction. There is no therapy for the ophthalmic component of USH to date. We have successfully generated a humanized pig model for USH1C, which recaptures the human USH1 disease (Grotz et al. 2022). Our preliminary phenotypic characterization of the USH1C pig revealed striking morphologic phenotypes in retinal photoreceptor cells. Here, we aim to further describe the retinal phenotype of USH1C pigs by using improved preparation and fixation techniques for electron-optical studies. In our TEM analyses, we concentrated on examining the compartments in which we recently demonstrated localization of USH1C/harmonin in the human retina (Nagel-Wolfrum et al. 2022), specifically the rod OS, calyceal processes synapses, and adhesion junctions between photoreceptor and Müller glia cells

Methods: To improve the preservation of fragile structures, such as calyceal processes or membranous discs stacks in the outer segment (OS) of photoreceptor cells and the cell-cell contacts between retinal cells we introduced perfusion fixation establishing a protocol for "local" cranial perfusion fixation. Perfusion fixed eyes were further processed for transmission electron microscopy (TEM) analysis.

Results: In comparison to previous immersion fixation, the preservation of the overall retina structure was improved after perfusion fixation. Photoreceptor cells, and especially their OS, were preserved for detailed inspections. Fragile structures such as the basal processes of retinal pigment epithelium (RPE) cells, calyceal processes along the OS of photoreceptor cells and the microvilli of the Müller glia cells were preserved. Additionally, the cell-cell contacts between photoreceptor and Müller glia cells were well preserved. A comparison of WT and USH1C retinae further supports previous results of phenotypic alterations due to the lack of *USH1C*/harmonin. The OS disc structure of USH1C pigs revealed various deformations.

Conclusion: A perfusion fixation prior to the enucleation of the pig eye strongly improved the preservation of the tissue for high resolution TEM analysis. This allowed us to investigate morphological differences in detail, especially fragile structures of the USH1C pig model.

Supports: FAUN Foundation (Nuremberg), USHER2020, DFG SPP SPP2127 - Gene and cell based therapies to counteract neuroretinal degeneration.

USH1C: preclinical development of antisense therapies in mice and clinical natural history studies in patients

Bhagwat V. Alapure[1], Inga Kristaponyte[1], Grant Rauterkus[2], Dongjoon Kim[1], Micah Klumpp[3], Jonathan Crabtree[4], Anup Mahurkar[4], Ronna Hertzano[4,5], Dominik Fischer[6], Moises A. Arriaga[3,7], Maria Reinoso[8], Wadih M. Zein[9], Robert K. Koenekoop[10], Jennifer J. Lentz[1,7]

[1] *Neuroscience Center of Excellence, LSU Health Sciences Center School of Medicine, New Orleans, LA, USA*
[2] *Tulane University School of Medicine, New Orleans, LA, USA*
[3] *Hearing and Balance Center, Our Lady of the Lake Regional Medical Center, Baton Rouge, LA, USA*
[4] *Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, MD, USA*
[5] *Department of Otorhinolaryngology Head and Neck Surgery, University of Maryland School of Medicine, Baltimore, MD, USA*
[6] *Department of Ophthalmology, University of Oxford, Oxford, UK*
[7] *Department of Otorhinolaryngology and Biocommunications, LSU Health Sciences Center School of Medicine, New Orleans, LA, USA*
[8] *Department of Ophthalmology, LSU Health Sciences Center School of Medicine, New Orleans, LA, USA*
[9] *Ophthalmic Genetics and Visual Function Branch, National Eye Institute, Bethesda, MD, USA*
[10] *Department of Pediatric Surgery, Montreal Children's Hospital, McGill University Health Center, Montreal, CA*

Usher syndrome (USH) is a rare genetic disorder characterized by the multi-sensory loss of hearing, balance, and vision. Approximately 10% of Type 1 USH (USH1) is caused by mutations in the *USH1C* gene (USH1C), but nearly all cases among the Acadian populations in Canada and Louisiana are caused by the c.216G>A founder mutation (216A) in the *USH1C* gene. To study mechanisms and develop therapies, we engineered a mouse model to contain the 216A mutation and developed a treatment targeting this mutation using antisense oligonucleotides (ASO). USH1C mice treated with 216A-targeted ASOs show significant short-term rescue of hearing, balance, and vision. In parallel, we are conducting several natural history studies (NHSs) with USH1C patients at all stages of disease progression to improve our understanding of when the hearing, balance, and vision losses begin and how quickly they progress, and to identify potential clinical trial participants and robust outcome measures that can be used to guide future clinical trials. Demographic, eye and ear histories, genetic, patient-reported surveys, and longitudinal hearing, balance, and vision clinical data are being collected from pediatric, young-adult, and adult USH1C patients. Natural history and outcome measures data for USH1C patients are important to guide clinical trials. The approach to develop these studies in USH1 patients highlights challenges specific to this population.

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Distortion-product otoacoustic emissions as a tool for hearing assessment

Václav Vencovský[1], Jakub Fuksa[2,3]

[1] *Department of Radioelectronics, Czech Technical University in Prague, Technická 2, 166 27, Praha 6, Czech Republic*
[2] *Department of Otorhinolaryngology, 3rd Faculty of Medicine, Charles University, Faculty Hospital Královské Vinohrady, Srobarova 50, 100 34, Praha 10, Czech Republic*
[3] *Department of Auditory Neuroscience, Institute of Experimental Medicine, The Czech Academy of Sciences, Vídeňská 1083, 142 20 Praha 4, Czech Republic*

Distortion-product otoacoustic emissions (DPOAEs) are weak acoustical signals evoked with two pure tones. The place of DPOAE generation is the inner ear, which in therian mammals contains a snail shaped organ called the cochlea. The cochlea transforms mechanical vibrations into electrical signal, which is then fed into the brain. Cochlea is a very complex organ and many details about its functions have yet to be resolved. The mechanoelectrical transduction occurs in the organ of Corti, which contains, despite other types of cells, two types of hair cells: (1) Inner hair cells (IHCs) arranged in a single row and heavily innervated with afferent nerve fibers, and (2) outer hair cells (OHCs) arranged in three rows, mostly innervated with efferent fibers and with voltage dependent somatic motility. It is assumed that OHCs play an important role in large sensitivity and frequency selectivity of the hearing system.

The basilar membrane (BM) response is in the healthy cochlea strongly nonlinear, which leads to generation of distortion products (DPs). These DP wavelets form DPOAE. If the hearing is impaired then the DPOAE amplitude declines, which can be employed for hearing assessment. In all USH1C homozygote pigs we measured, we could not find measurable DPOAEs, which means that the technique can clearly determine sever hearing loss. However, we believe that if properly used, DPOAEs can be useful also for determination of small hearing impairments. First, we decided to verify whether the fact known from human DPOAEs that the final DPOAE signal is composed of two sources, which may destructively interfere and, therefore, decrease the DPOAE amplitude, is significant also in pigs. We measured DP-grams (DPOAE as a function of frequency) with small frequency step. We could conclude that the fine structure in the amplitude of DP-gram is in pigs probably relatively small, at least much smaller than it is typically known from human data. Therefore, it is not required to conduct experiments with very small frequency step, which decreases the time required for the measurement.

OAE probe is inserted in some distance from the ear drum and, therefore, standing waves in the ear canal emerge and complicate calibration. This issue is more prominent at high frequencies, which is the case of domestic pigs in which we measure DPOAEs (up to 16 kHz). Techniques were developed for the human ear canals to compensate this standing wave issue; however, the pig ear canal is narrower and the techniques may not work properly. We, therefore, conducted experiments with variable level of the L1 tone, to verify whether the calibration issue in some cases did not alter significantly true L1 and L2 levels presented into the ear. Another outcome of these experiments was that we could estimate the “optimal” L1 and L2 combinations, which yield the largest DPOAE amplitude for the given L2 level. In the pigs, we noticed that optimal L1 levels are at moderate L2 levels (45-55) dB sound pressure level (SPL) by about 15 dB larger. This is larger level difference than was shown for humans at moderate L2 levels. Therefore, we would advise to measure DP-grams in pigs with L1 levels by about 10 or 15 dB larger than the L2 levels. In conclusion, if properly used, the DPOAE should be adequate technique to probe possible differences in performance of the auditory periphery between wild type and USH1C heterozygote pigs.

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