DISSECTING PROTEIN TRAFFICKING IN RETINAL NEURODEGENERATION BY SUPER-RESOLUTION IMAGING ON ANIMAL MODELS AND HUMAN IPSCS

Roser Gonzàlez Duarte
Facultat de Biologia UB
Pablo Loza Alvarez
ICFO - Institut de Ciències Fotòniques
Slaven Erceg
Centro de Investigación Príncipe Felipe Valencia

1. Abstract

Background/main objective: Inherited retinal dystrophies are highly genetically heterogeneous neurodegenerative disorders leading to blindness. Over two hundred genes alter the function of rods and cones, which are highly differentiated neurons. Besides, photoreceptors are extremely difficult to maintain in vitro and no available cell lines reproduce their complexity. In vivo protein trafficking studies in these highly polarised cells is still fragmentary, particularly on the connecting cilium (a mutational hot spot), through which all photoreception and phototransduction proteins reach the outer segment. The identification of protein constituents in ciliary trafficking by proteomic studies and the application of cryoelectron tomography has represented a qualitative leap. However, a gap remains at visualizing the dynamics of protein trafficking in mouse models of disease-causing mutations and their validation in the human context. We aim to fill this gap by focusing on two retinal dystrophy genes, *RD3* and *CERKL*, at the intersection of mutational analysis in gene-edited mice, transient transgenic mouse retinas, and the generation of retinal organoids derived from human iPSCs, using super-resolution and state of the art imaging techniques.

Methodology: We will combine mouse genetic techniques for gene editing, transient transgenesis in the retina by in vivo DNA electroporation, the generation of photoreceptors from iPSCs from patients, and innovative super-resolution imaging techniques: multicolour STED imaging and light sheet microscopy.

Expected results: To establish a fine resolution map of photoreceptor protein trafficking, highlight the key steps of RD3 and CERKL in ciliary pathways in living and fixed murine retinas, to understand the yet unknown genotype-phenotype correlations in murine tissue and human ihPSCs, and to quantify the alterations caused by light and oxidative stress conditions, to unveil new therapeutic approaches for treatment of retinal neurodegeneration.

2. Results

This project has provided relevant insights into the pathophysiology of Leber congenital amaurosis 12 (LCA12), a very severe form of inherited blindness caused by mutations

in *RD3*. Our results indicate that the Ca2+ sensor proteins GCAPs mediate the pathophysiology by sensing the abnormal drop in intracellular Ca2+ and interfering with the autophagy pathway. While GCAPs are not a good therapeutical target themselves, this study may uncover therapeutic targets in the autophagy pathway. We expect this conclusion to be extended to gene defects converging at causing closure of the cGMP channels and therefore causing low Ca2+ (light-equivalent disorders). The study on IMPDH1 has uncovered an unexpected complexity of this enzyme regulation in vivo, stressing the relevance of its allosteric regulation by product, mediated through the CBS protein domain. Studies will go on to determine the effect of mutations on enzyme activity and regulation in vivo, in collaboration with crystallographers. An important conclusion of this work is that *IMPDH1* mutations would result in constitutive enzymatic activity by disrupting GDP/GTP allosteric control. Therefore, therapeutic approaches for RP10 and adLCA should be based on the development of novel and more specific IMPDH1 inhibitors.

More than 15 years have passed by since the identification of *CERKL* and the aetiopathogenesis of genetic blindness due to mutations in this gene is far from being understood. To this end and within this project, we have generated different models, from gene-edited mice to transient transgenic mouse retinas, to characterize the function of CERKL in the retinal response to light and oxidative stress, which are very useful to dissect particular physiological functions of the gene. However useful these models may be, they do not fully mimic all the phenotypic traits shown by human patients. Therefore, the generation of retinal cup organoids derived from fibroblasts of a patient bearing the most common mutation in *CERKL* and a control sibling was one of the most important milestones of the project. It has been reached and we are currently studying these organoids through many technical approaches, including high resolution 3D-imaging and high throughput transcriptomics.

All the groups involved in this project have developed and consolidated advanced methodologies, such as the generation and neuronal differentiation of retinal organoids in vitro, as well as the generation of imaging algorithms to reconstruct biological relevant volumes (organs). Overall, the project has been instrumental to produce important results in basic science as well as in technological progress that can be used to approach other biomedical challenges.

In summary, the most relevant results of this project are:

1) Drawing a detailed map of the protein interactions, modification and trafficking of RD3 in cell culture, genetically modified mice and in transient transgenic retinas.

2) Contributing to elucidate the physiological role of *CERKL* in the protection and resilience of photoreceptor cells in response to oxidative stress, and the confirmation that CERKL is a highly dynamic protein. We have established several models, particularly gene-edited mice by CRISPR/Cas9.

3) High resolution imaging of the localization and trafficking of these proteins, opening new avenues to dissect the cellular function of these proteins

4) By generating retinal organoids in vitro, we have generated a human model and have shown that human patients bearing mutations in CERKL suffer from higher oxidative and luminic stress. These human retinal organoids are an instrumental resource to study the molecular basis of retinal neurodegeneration.

5) Furthering the applications of light sheet microscopy in the 3D imaging of neuronal organs.

We are still working on the project, using the technical resources and tools as well as the knowledge gathered to go more deeply into the neurodegeneration of the retina, the visual organ of the central nervous system, and the most affected tissue in retinal neurodegenerative disorders. We believe that our results will open new avenues for the design of novel therapeutic strategies for these incapacitating pathologies.

3. Relevance of the results and expected impact

Retinal and macular neurodegeneration are the major genetic cause of vision impairment, with an immense social and economic impact. Inherited retinal diseases, with a prevalence of 1:3,000 worldwide, are characterized by high genetic and clinical heterogeneity, which poses a considerable challenge for diagnosis, genetic counselling, and disease management. Degeneration of photoreceptors due to genetic mutations

lead to untreatable progressive blindness. Nonetheless, precision medicine through the development of new specific therapies offers hope for effective treatments. Patients and their families are increasingly concerned about the availability and efficacy of genetic diagnosis and future therapeutic treatments to halt the progression or even cure retinal diseases. For instance, *Luxturna*, the first commercial gene therapy for any IRD, has been recently approved to treat patients bearing bi-allelic mutations in *RPE65*, but several gene and cell therapies for other IRDs are now in the pipeline. Therefore, a better understanding of the physiological function of causative genes is a pre-requisite for precision medicine in these devastating diseases.

In this context, all the genes and proteins we have characterized in this project are causative of inherited retinal neurodegeneration. Mutations in *RD3* cause childhood blindness (Leber congenital amaurosis) and mutations in *IMPDH1* and *CERKL* cause retinitis pigmentosa, characterized by progressive neurodegeneration of rods first, and later cones. Even though the association of these genes to retinal pathology is indisputable, their physiological role and the effect of the pathogenic mutations in photoreceptor function and homeostasis is far from understood. This project combined advanced methodologies in the generation of retinal models (from the generation of gene-edited mice and transient retina transgenesis in mouse, to human retinal organoids differentiated from iPSCs derived from patients) as well as in high-resolution imaging (three-dimensional reconstructed images and light sheet microscopy), which has allowed us to progress in the characterization of genes involved in protein trafficking and light/oxidative stress response. Only through the accurate dissection of the functional roles of the causative genes (such as *RD3, IMPDH1* and *CERKL*) and the disruptive effect of their pathogenic mutations, efficient therapeutic approaches can be designed in the future, which will certainly impact in the design of more effective, precision medicine therapies.

4. Literature (only international peer-reviewed publications)

GROUP 1

de Castro-Miró M, Tonda R, Escudero-Ferruz P, Andrés R, Mayor-Lorenzo A, Castro J, Ciccioli M, Hidalgo DA, Rodríguez-Ezcurra JJ, Farrando J, Pérez-Santonja JJ, Cormand B, Marfany G, Gonzàlez-Duarte R. Novel Candidate Genes and a Wide Spectrum of

Structural and Point Mutations Responsible for Inherited Retinal Dystrophies Revealed by Exome Sequencing. PLoS One 11:e0168966 (2016)

López-Begines, Plana-Bonamaisó and Méndez. Molecular determinants of Guanylate Cyclase Activating Protein subcellular distribution in photoreceptor cells of the retina. Scientific Reports vol. 8:2903 (2018)

de Castro-Miró, M.; Tonda R.; Marfany G, Casaroli-Marano RP, Gonzàlez-Duarte R. Novel mutations in the choroideremia gene and multi-Mendelian phenotypes in Spanish families. British Journal Ophthalmology 102:1378-86 (2018)

Trigueros S, Domènech EB, Toulis V, Marfany G. In Vitro Gene Delivery in Retinal Pigment Epithelium Cells by Plasmid DNA-Wrapped Gold Nanoparticles. Genes 10, pii: E289 (2019).

Mirra S, Marfany G. Mitochondrial gymnastics in retinal cells: a resilience mechanism against oxidative stress and neurodegeneration. Adv Exp Med Biol. (in press)

"CERKL, a retinitis pigmentosa gene, is relevant for retinal cell resilience to light and oxidative stress" (MS in preparation)

"Characterization of a new murine model for CERKL" (MS in preparation)

GROUP 2

Omar E. Olarte, Jordi Andilla, Emilio J. Gualda, and Pablo Loza-Alvarez, "Light-sheet microscopy: a tutorial," Adv. Opt. Photon. 10, 111-179 (2018)

D. Artigas, D. Merino, C. Polzer, P. Loza-Alvarez, "Sub-diffraction discrimination with polarization-resolved two-photon excited fluorescence microscopy," Optica 4, 911-918 (2017)

Merino D, Mallabiabarrena A, Andilla J, Artigas D, Zimmermann T, Loza-Alvarez P. STED imaging performance estimation by means of Fourier transform analysis. Biomed Opt Express. 8:2472-2482 (2017)

"Optimised light-sheet imaging of human retina organoids", in preparation.

GROUP 3

Aranzazu Bolinches-Amorós, Marian León, Gemma Marfany, Roser Gonzalez, Slaven Erceg, Dunja Lukovic. Generation of iPSC lines from retinitis pigmentosa patient with mutation in CERKL and healthy sibling. Stem Cell Research (in press).

"Identification of retinal cell types in iPSC-derived 3D retinal organoids by LSFM" (MS in preparation)