MASARYK UNIVERSITY

FACULTY OF MEDICINE

Utilizing Retinal Organoids to Understand the Development, Function, and Diseases of the Human Retina

Habilitation Thesis

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Abstract

Retinal organoids are miniature 3D models of the retina routinely generated from pluripotent stem cells. These structures mimic the complexity and functionality of the retina, containing all cell types found in the retina, such as photoreceptors, ganglion cells, and bipolar cells. Retinal organoids represents invaluable tools for studying retinal development, function, and diseases, providing a platform to investigate the underlying mechanisms of retinal disorders, test potential therapies, and develop personalized treatment strategies. This thesis explores their potential in understanding retinal development, function, and disease modelling, highlighting key contributions from my research group and collaborating researchers.

Declaration

I hereby declare that this thesis with title **Utilizing Retinal Organoids to Understand the Development, Function, and Diseases of the Human Retina** I submit for assessment is entirely my own work and has not been taken from the work of others save to the extent that such work has been cited and acknowledged within the text of my.

Brno May 31, 2024

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Tomáš Bárta

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I would like to express my deepest gratitude to my family—my wife and children—who have provided constant support throughout my research journey. Their patience and encouragement have been invaluable to me.

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Thank you!

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Glossary

2D	– Two-dimensional
3D	– Three-dimensional
ESC	– Human embryonic stem cell
Fst	– Follistatin
GCL	– Ganglion cell layer
INL	– Inner nuclear layer
ipRGC	– Intrinsically photosensitive retinal ganglion cell
iPSC	– Induced pluripotent stem cell
miRNA	– microRNA
ONL	– Outer nuclear layer
PSC	– Pluripotent stem cell
RGC	– Retinal ganglion cell
RP	– Retinitis pigmentosa
RPE	– Retinal pigment epithelium
SCN	– Suprachiasmatic nucleus
Shh	– Sonic hedgehog
TuD	– Tough decoy
USH1B	– Usher type 1B

1 Introduction

The world around us is a kaleidoscope of colours, shapes, and movements. These visuals, however fleeting or persistent, are captured and processed by our eyes, allowing us to interact with our environment and navigate through life. The ability to perceive visual stimuli is one of the most sophisticated and important senses that has evolved in the majority of animal species.

Throughout evolutionary history, the ability to detect light has granted species a crucial advantage, from escaping predators to locating food. The primitive light-sensitive patches found in ancient organisms gradually evolved, leading to the formation of complex ocular systems seen in high animals.

Situated deep within the eye, the retina acts as the primary stage where the vision unfolds. This thin layer, densely packed with photoreceptor cells, captures incoming photons of light and transmutes them into electrical signals. These signals are then processed, refined, and relayed to the brain, resulting to the visual experiences we perceive.

However, the retina is far more than just a passive screen. It is a dynamic structure, containing various cell types, each with a specialized role. From the rod and cone photoreceptors that detect light and distinct colours, to the ganglion and bipolar cells that process and transmit visual information, the retina represents a centre of cellular activity and information integration.

Retinal organoids have significantly advanced the field of ophthalmology. Due to the unavailability of human retinal tissues for experimental purposes, retinal organoids have emerged as invaluable tools. They faithfully recapitulate human retinal development, mimicking the structure and function of the human retina. Moreover, by utilizing patient-specific human pluripotent stem cells (PSCs), researchers can model retinal diseases, harnessing the inherent mutations carried by PSCs.

In this thesis, I explore the broad potential of retinal organoids to deepen our understanding of the human retina development, its functions, and disease modelling.

Additionally, here I summarize three publications, generated by my research group and collaborators, which have significantly contributed to the exploration of human retinal organoids and their application in research.

2 Theoretical Background

2.1 Human Retina

2.1.1 Development and structure of the retina

The genesis of the human retina starts as an outpouching from the forebrain known as the optic vesicle around the fourth week of embryonic development. As the adjacent ectoderm thickens to form the lens placode, the optic vesicle starts to invaginate, giving rise to the optic cup, with its inner layer forming the neural retina and its outer layer forming the retinal pigment epithelium (RPE). Once the optic cup is formed, the neuroblastic layer of the inner optic cup, destined to become the neural retina, undergoes differentiation and lamination (**Fig. 1**). This results in the formation of three main layers: Ganglion cell layer (GCL), Inner nuclear layer (INL), Outer nuclear layer (ONL). These layers are delineated by two plexiform layers, where synaptic connections occur. (**Fig. 4**)[1].

Rods and cones, the photoreceptors of the retina, arise from a common pool of progenitor cells presented in the forming neural retina. Cones begin to develop first, followed by the rods. By the 18th week of gestation, the differentiation of foveal cones begins, and by the 27th week, rods begin to appear around the fovea[2].

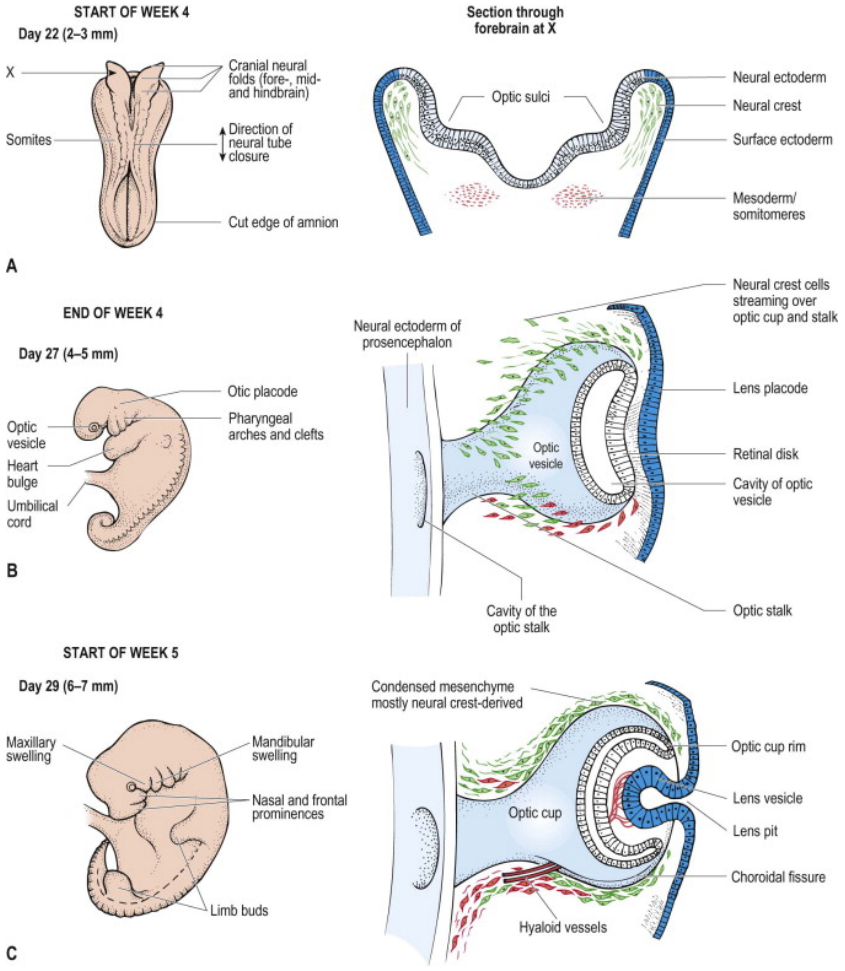

Figure 1: Illustrative overview of eye development. *Stages spanning from day 22 to the 8th week of embryogenesis (panels A-C). On the left side, the overall morphology of the embryo during this timeframe is depicted. The diagram employs a color-coded scheme to denote the germ layers, highlighting their origins and eventual roles in forming the ocular and surrounding periocular structures.*

Figure adopted and modified from: https://entokey.com/embryology-and-early-development-of-the-eye-and-adnexa/[3]

The human retina contains several distinct cell types, each playing a crucial role in the process of vision. Photoreceptor cells, encompassing rods and cones, are critical in the absorption of light photons, initiating the conversion of light into electrochemical signals. Rod cells, abundant in the peripheral retina, are highly sensitive to low light levels (scotopic vision) and lack the capability to recognize chromatic

stimuli, thus primarily contributing to achromatic vision. Cone cells, predominantly located in the foveal region, are responsible for high acuity vision under photopic conditions and enable chromatic discrimination through differential sensitivity to distinct wavelengths, corresponding to red, green, and blue light (**Fig. 2**).

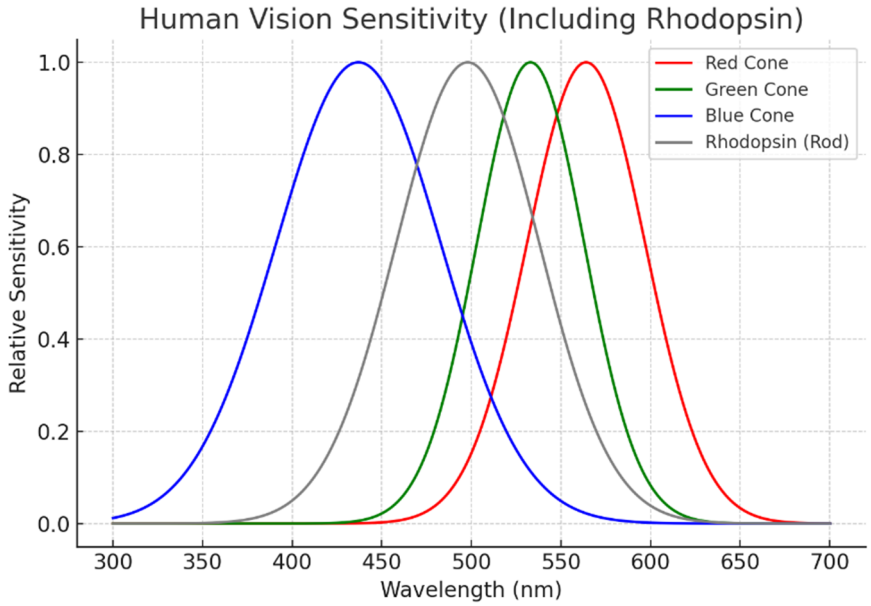

Figure 2: Graph showing relative sensitivity of individual human visual opsins.

Rods, cylindrical cells, possess an elongated structure with various specialized regions. Their outer segment, the site of phototransduction, comprises stacked membranous discs filled with rhodopsin, the photopigment responsible for light absorption. Adjacent to the outer segment lies the inner segment, containing cellular organelles important for metabolic processes. The cell body, situated further inward, accommodates the nucleus and other organelles essential for cellular maintenance. At the synaptic terminal, rods form connections with bipolar cells through ribbon synapses, facilitating the transmission of electrical signals (**Fig. 3**).

Cones, cone-shaped photoreceptors concentrated primarily in the fovea, exhibit similar structural components to rods, albeit with some distinctions. While cones possess outer and inner segments, cell bodies, and synaptic terminals akin to rods, their outer segments contain fewer membranous discs (**Fig. 3**). Additionally, cones display specificity in their photopigments, with distinct types sensitive to short-wavelength (S), medium-wavelength (M), or long-wavelength (L) light. This specialization allows cones to contribute to colour vision. Like rods, cones establish synaptic connections with bipolar cells through ribbon synapses at their synaptic terminals.

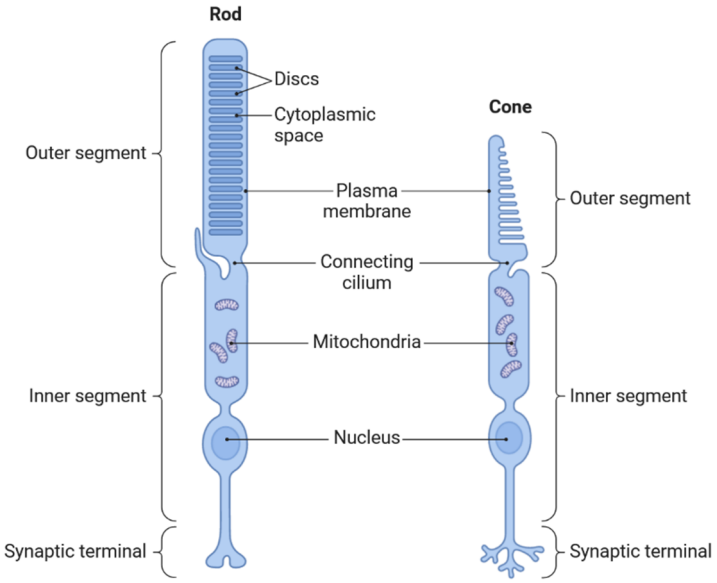

Figure 3: Schematic representation of morphology of human photoreceptors. *Figure generated by BioRender software.*

Bipolar cells represent the primary cells for signal transmission from photoreceptors to retinal ganglion cells (RGCs), effectively integrating synaptic input from multiple photoreceptors. This integration is crucial for the spatial and temporal modulation of the visual signal, facilitating to perceive visual contrast and detail.

RGCs, the output neurons of the retina, aggregate inputs from multiple bipolar cells. Their axons join to form the optic nerve, establishing the neural connection to

visual cortex of the brain. Notably, certain RGCs exhibit intrinsic photosensitivity, contributing to non-image-forming visual functions such as circadian rhythm synchronization and pupillary light reflex modulation.

Horizontal cells and amacrine cells contribute to lateral inhibition and signal refinement within the retinal circuitry. Horizontal cells modulate photoreceptor and bipolar cell interactions, enhancing contrast through lateral inhibition, while amacrine cells, interfacing with bipolar and RGCs, are instrumental in the modulation of signal transmission, particularly in the context of motion detection and adaptation to varying luminance levels.

Müller glial cells, traversing the full thickness of the retina, provide essential structural and metabolic support to neuronal components. These cells facilitate the homeostatic regulation of the retinal microenvironment, including neurotransmitter recycling, ion balance maintenance, and light guidance to photoreceptors, ensuring optimal conditions for phototransduction.

Retinal cells orchestrate highly specialized process of light signal conversion, enabling the perception of the visual stimuli. Each cell type, with its distinct structure and function, integrates into a network that underlies the capacity to perceive the visual environment with high precision and detail (**Fig. 4**).

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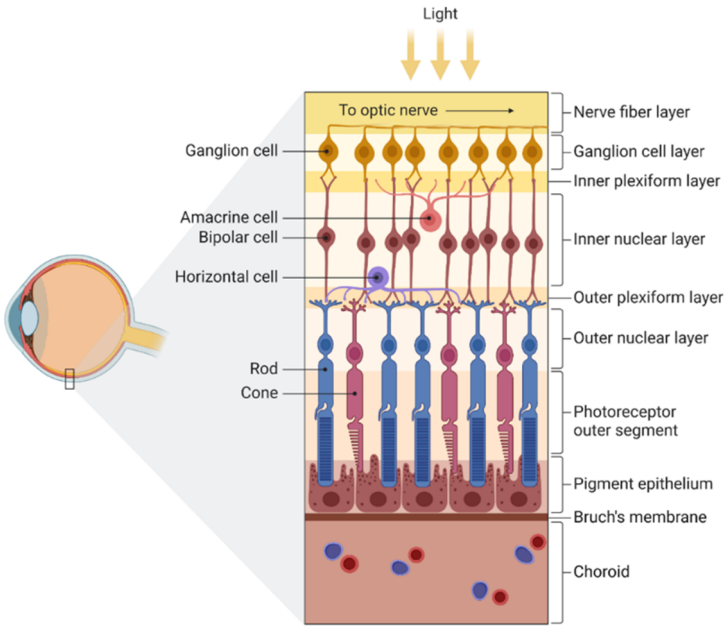

Figure 4: Schematic representation of the structure of the human retina.
Figure adopted from: Principles of Neural Science, Fourth Edition Eric R. Kandel, James Harris Schwartz, Thomas Jessell[4] and BioRender software

2.1.2 Development and function of the Retinal Pigment Epithelium (RPE)

The RPE is a specialized layer that originates from the outermost portion of the optic cup during embryonic development[5]. As the eye develops, the RPE differentiates into a single layer of pigmented cells. These cells, which are densely packed, perform several critical functions that are crucial for vision[6].

One of the primary responsibilities of the RPE is to provide support to the photoreceptors[6]. The RPE ensures that photoreceptor cells remain healthy and functional. It supplies necessary nutrients, removes waste, and protects the photoreceptors from potential damage caused by excess light[6].

Additionally, the RPE is actively involved in the process of phagocytosis[7]. As part of the natural life cycle of photoreceptors, the outermost tips of their segments, which are damaged or no longer functional, are periodically shed[8]. The RPE cells envelop and digest these shed tips, thereby ensuring that the photoreceptors can continue to function effectively without the accumulation of waste or damaged segments.

Furthermore, the RPE plays a significant role in the visual cycle. The visual cycle refers to a series of biochemical processes that regenerate visual pigments after they have been exposed to light[9]. The RPE cells help in the regeneration of these visual pigments, specifically the conversion of all-trans retinal to 11-cis retinal, a key step that allows the visual pigments to capture light[10].

2.2 Human Retinal Organoids

Retinal organoids are three-dimensional structures closely resemble the human retina. They represent a tool in ophthalmological research, allowing for more in-depth studies of retinal development, diseases, and potential treatments. These organoids are derived from PSCs, such as human embryonic stem cells (ESCs) or human induced pluripotent stem cells (iPSCs), which have the remarkable ability to differentiate into any cell type, including those found in the retina[11].

The significance of retinal organoids lies in their ability to model the developmental processes of the human retina, which was previously a challenging task due to limited access to human retinal tissues and the ethical concerns surrounding the use of such tissues. The application of retinal organoids in research allowed to study the complex processes of human retinal development.

Moreover, retinal organoids have bridged a critical gap in vision research by providing a more physiologically relevant and human-specific model. Prior to their application, most retinal research depended on animal models or two-dimensional human cell cultures, which often failed to fully replicate the human retinal environment. The three-dimensional nature of retinal organoids allows for a more accurate representation of the structure of the human retina and its function, making them critical tool for studying retinal diseases.

2.2.1 Differentiation and Structure of Retinal Organoids

A pivotal study by Nakano et al. (2012)[12] demonstrated the feasibility of creating self-organizing optic cups from human embryonic stem cells, a major milestone in the field. Following this, several protocols have been developed to optimize the differentiation process, aiming to produce organoids that closely resemble the human retina in structure and function[13].

The generation of retinal organoids is a complex process that begins with the differentiation of PSCs into retinal cells. This process mimics the natural development of the retina in the human embryo. Numerous methods exist for this differentiation, with three primary approaches being predominant: I) The most widely used technique involves the cultivation of 3D spheroids from PSCs. These spheroids are cultured under conditions that simulate the embryonic development of the retina[11,14] (**Fig. 5**). II) The second method entails the formation of embryonic bodies, which are subsequently anchored to a culture dish. Following this, retinal organoids (optic cups), are carefully separated and grown in non-adherent plastic environments [15,16]. III) In the third approach, PSCs are initially grown in a 2D format within differentiation media. Upon the initiation of differentiation, clusters of ocular precursor cells are selectively excised and then cultivated in non-adherent conditions[17]. Each of these methods reflects a unique strategy to replicate the process of retinal development, offering diverse pathways for research and application in regenerative medicine.

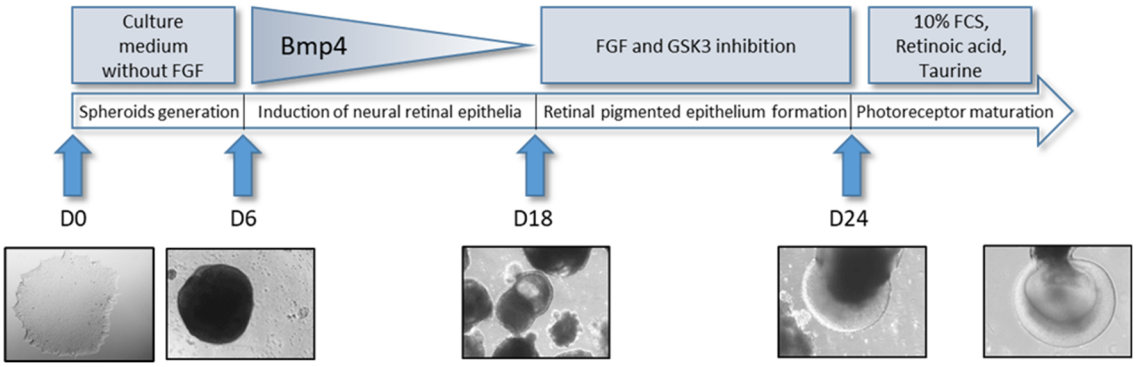

Figure 5: Schematic representation of the protocol for the generation of retinal organoids from 3D spheroids.

The resulting organoids consist of all cell types commonly found in human retina, including photoreceptors, retinal ganglion cells, bipolar cells, Müller glia, and retinal pigment epithelium. These cells are arranged in layers, replicating the organization of the retina. These stratified organoids are critical for studying retinal diseases allowing to observe interactions between different cell types and layers, as well as to investigate the progression of retinal disorders in a controlled environment (**Fig. 6, 7**).

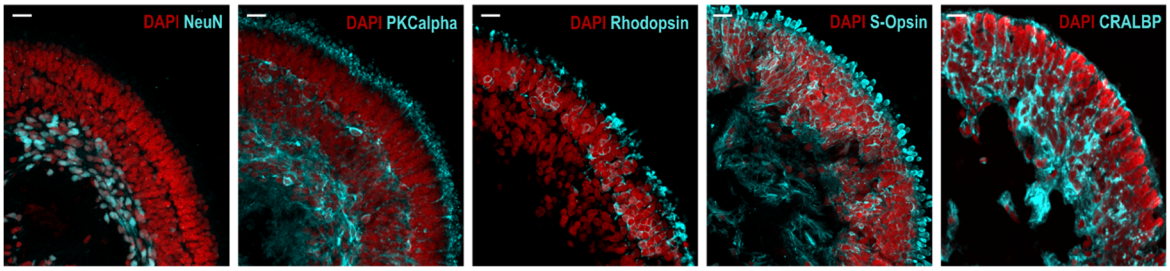

Figure 6: Expression of NeuN (Ganglion cells), PKCalpha (Bipolar cells), Rhodopsin (Rhods), S-Opsin (Blue Cones), and CRALBP (Müller glia) in retinal organoids *at day 180 of differentiation, as demonstrated using immunofluorescence staining. Scale bars = 20 µm. Figure adopted from Celiker et al., 2023*[18]

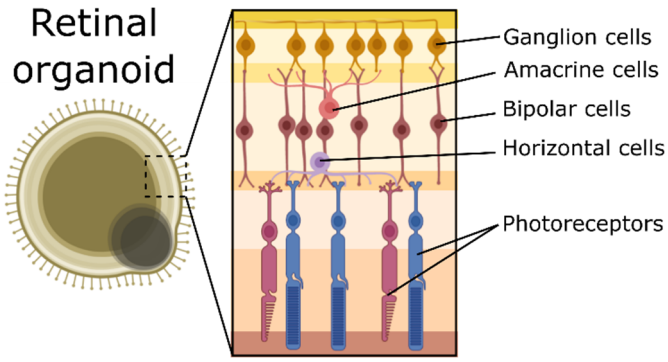

Figure 7: Schematic representation of the structure of retinal organoid. Figure generated using BioRender software

2.2.2 Retinal Organoids as a Tool to Study Development of the Human Retina

The development of the human retina is a highly orchestrated process involving a complex network of molecular regulators and developmental cues. Key transcription factors such as PAX6, SOX2, RAX and VSX2 play a pivotal role in the early stages of eye development and retinal cell fate determination. The elucidation of these developmental processes has been significantly advanced by the utilization of retinal organoid models.

PAX6, a highly conserved transcription factor, is crucial in the development of the retina[19]. It orchestrates the early differentiation steps and maintenance of various ocular cells. The role of PAX6 extends beyond the initial phases of eye formation; it is also involved in the maintenance of the adult eye, as its presence influences ocular cell function. It functions at the top of a genetic hierarchy, influencing the expression of genes like *MITF*, *SOX2*, and *CRYAA*, which are integral to eye and retinal development[20]. The role of PAX6 in retinal development is also crucial, as it influences retinal cell fate decisions and differentiation processes. It plays a role in the development of retinal ganglion cells, amacrine cells, and horizontal cells[21].

The findings from studies utilizing retinal organoids offer an explanation for the master control function of PAX6. This understanding is crucial for understanding the

homologous functions of PAX6 across the animal kingdom, where its misexpression can lead to the development of ectopic eye structures[22]. Furthermore, these insights into retinal self-organization and the role of PAX6 have profound implications for regenerative medicine, disease modelling, and drug development.

The spatial and temporal roles of *Pax6* in eye development were studied using mouse retinal organoids. For example, the authors Hung et al. [23] investigated the roles of *Pax6* isoforms, *Pax6a* and *Pax6b*, in mouse eye development and organoid differentiation. They found distinct spatial and temporal expression patterns for each isoform, with Pax6a correlating strongly with neuroretina gene *Sox2*, and *Pax6b* correlating with iris-component genes, such as *Foxc1*. During early development, Pax6b was expressed in the optic cup hinge and neighbouring mesenchymal cells, while Pax6a was absent in these regions. Both isoforms were expressed in the prospective iris and ciliary body by E14.5. As development progressed, Pax6 isoforms showed distinct expression patterns as lineage genes became more restricted. Using ESC-derived retinal organoids, the authors validated their findings and observed impaired spatial expression patterns of Foxc1 and Mitf in Pax6b-mutant organoids. This suggests the involvement of Pax6b-positive local mesodermal cells in iris development.

A study utilizing human retinal organoids investigated the potential of translational readthrough-inducing drugs (TRIDs) in treating congenital aniridia, a severe sight-loss condition often caused by heterozygous nonsense variants in the *PAX6* gene[24]. Utilizing iPSC-derived retinal organoids and 2D limbal epithelial stem cell (LESC) models from aniridia patients, the study demonstrated reduced PAX6 protein levels, mirroring the disease phenotype. Testing various TRIDs, including amlexanox and 2,6-diaminopurine (DAP), the authors found amlexanox most efficacious in increasing full-length PAX6 levels and rescuing the disease phenotype in both models.

The complexity of functions of PAX6 during eye development is enhanced by its interaction with a network of genes and signalling pathways, including TGFβ and Follistatin (Fst), forming a putative Turing network. This network is capable of initiating spontaneous pattern formation, a foundational process for the self-organization of the

retinal organoid. The Turing model, known for explaining how reaction-diffusion systems can lead to the emergence of complex patterns, suggests that the PAX6/Fst/TGFβ network can polarize the developing retina, establishing the primary axis for further development[25].

As the retina progresses in its development, transcription factors like CRX and NRL become instrumental in the differentiation of photoreceptors. CRX is particularly crucial for the development of cones and rods, and its mutations are associated with several retinopathies[26]. Pan et al. [27] investigated the pathogenic mechanism of CRX-associated autosomal dominant retinopathies, focusing on gene haploinsufficiency. Using human ESCs and retinal organoids, they created monoallelic CRX knockout models to study phenotypic differences. Their findings demonstrated delayed differentiation of the ONL, thinner ONL, and profound loss of photoreceptor outer segments, along with downregulated expression of genes for phototransduction and segment formation. Live-cell imaging revealed arrested translocation of monoallelic CRX^+ cells, suggesting a role for CRX in regulating postmitotic photoreceptor precursor translocation during human retinal development. These results confirm gene haploinsufficiency as the mechanism for CRX dominant pathogenicity and provide insights for the treatment of CRX-associated retinopathies.

NRL, on the other hand, is a key gene in rod photoreceptor development, and its absence can lead to an increased number of cone cells[28]. The authors Cuevas et al. [29] utilized retinal organoids derived from human ESCs to investigate the role of *NRL* in human retinal development. By engineering *NRL*-deficient human ESC lines using CRISPR/Cas9 gene editing, they observed that *NRL*-deficient organoids lacked NRL expression and failed to express markers associated with rod photoreceptors. Instead, they exhibited an abnormal number of S-opsin-positive cells, indicative of a default pathway leading to S-cone-like cell development. Another study confirmed the function of *NRL*. Using iPSCs-derived retinal organoid models the authors Kallman et al. [30] examined the developmental alterations in a human model of NRL loss. Consistent with its function in rod fate specification, they found that human retinal organoids lacking

NRL developed populations dominated by S-opsin-expressing photoreceptors. Additionally, they identified MEF2C as a potential regulator of cone development. Both studies provide the evidence in a human *in vitro* retinal organoid system that *NRL* is essential for defining rod identity and highlights the potential of gene-edited retinal organoids for studying photoreceptor specification in the context of retinal degenerative diseases.

Our contribution to this topic

- In our study[31] "***miR-183/96/182 cluster is an important morphogenetic factor targeting PAX6 expression in differentiating human retinal organoids***", we investigated the role of the miR-183/96/182 cluster in human retinal development, focusing on its regulation of *PAX6* expression in retinal organoids derived from human PSCs. We found that inhibition of this cluster leads to increased expansion of the neural retina at early differentiation stages. This suggests the miR-183/96/182 cluster plays a significant role in the morphogenesis of the neural retina, highlighting its importance in retinal tissue development and offering new insights into the mechanisms of retinal differentiation and morphogenesis.

2.2.3 Retinal Organoids as a Tool to Study Function of the Human Retina

Besides containing all cell types typically found in the retina, retinal organoids also possess the capacity to transduce light into an electrophysiological response.

Upon absorbing photons, a photoreceptor undergoes a biochemical cascade known as phototransduction. This process alters the cell membrane potential, leading to hyperpolarization and a change in neurotransmitter release at the synaptic terminals. Rods and cones release less neurotransmitter when they are activated by light, a signal that must be accurately interpreted downstream by bipolar cells.

Bipolar cells act as the intermediaries, conveying signals from the photoreceptors to the ganglion cells. They are distinguished into two main types based on their

response to light: ON and OFF bipolar cells. This distinction is crucial for understanding how the retina encodes and processes visual information.

ON bipolar cells respond to an increase in light intensity with depolarization. This counterintuitive response is due to the glutamate released by photoreceptors in the dark, which inhibits ON bipolar cells through a metabotropic glutamate receptor mechanism. When light reduces the release of glutamate by a photoreceptor, the inhibition of the ON bipolar cells is lifted, leading to their depolarization. As a result, ON bipolar cells are primed to signal the presence of light[32].

Conversely, OFF bipolar cells are hyperpolarized in response to an increase of light intensity. They are directly inhibited by glutamate through ionotropic glutamate receptors. In darkness, when photoreceptors release more glutamate, OFF bipolar cells are depolarized. Thus, they are tuned to signal decreases in light intensity, effectively encoding the absence of light or shadow edges in the visual field.

The signals from ON and OFF bipolar cells are then relayed to ganglion cells, which further process the visual information before transmitting it to the brain through the optic nerve. This layer of processing enriches the visual signal, enabling the brain to detect contrasts, motion, and other complex features of the visual environment[33].

Retinal organoids must react to light in a manner that replicates this complex cascade of events found in the human retina. Recent studies have demonstrated that retinal organoids derived from human PSCs exhibit these crucial functional characteristics, indicating their potential as models for studying retinal physiology and diseases. These organoids have been shown to contain photoreceptors that undergo phototransduction, changing their membrane potential in response to light and adjusting neurotransmitter release accordingly[18,34] (**Fig. 8**).

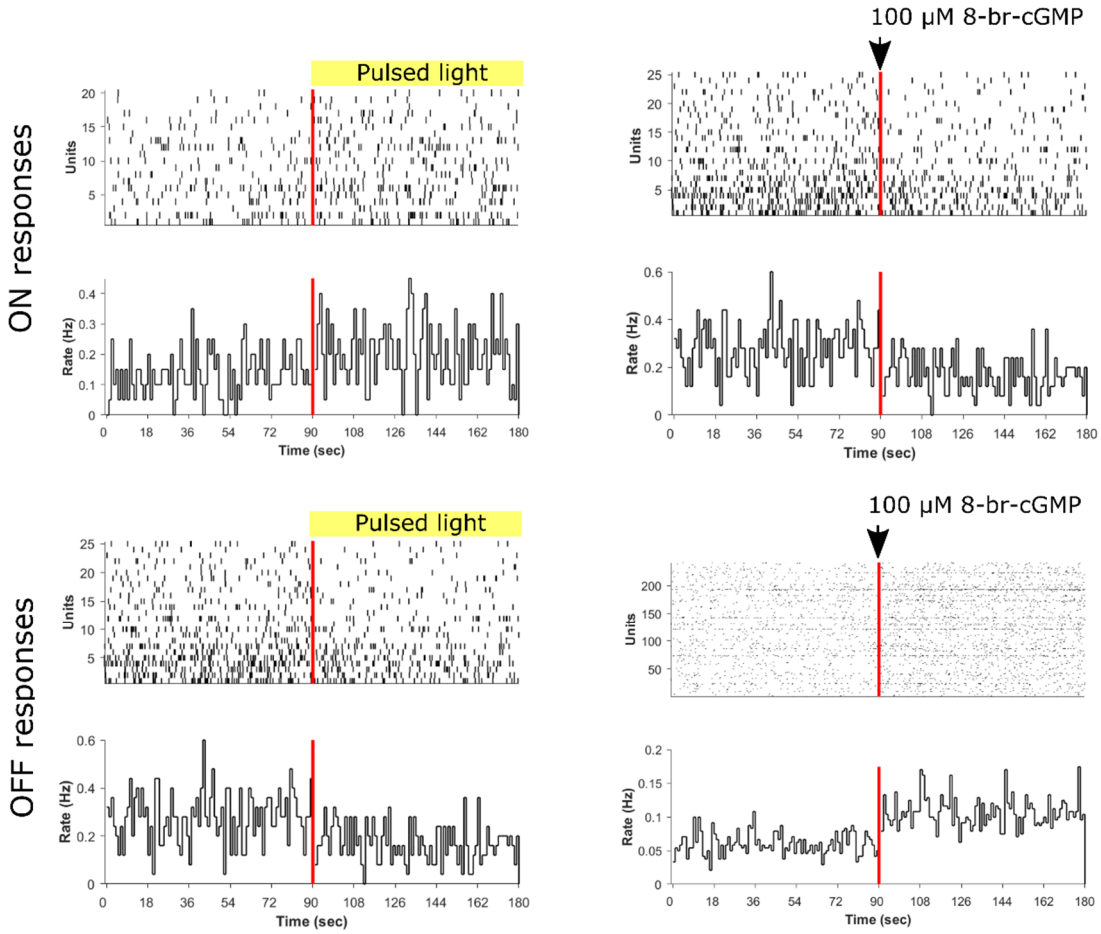

Figure 8: Electrophysiological properties of human retinal organoids*. In collaboration with Evelyne Sernagor laboratory (Newcatle University, Newcastle upon Tyne, UK) we measured electrophysiological properties of retinal organoids generated in my research group. Raster plots (top panels), firing rate histograms (bottom panels, bin size = 1 s) from RGCs which showed a 25% increase (ON responses) or decrease (OFF responses) in spiking activity in the presence of pulsed light or 100 µM 8-br-cGMP. In the raster plot, each small vertical bar indicates the time stamp of a spike, where each row represents a different RGC. The rate histogram illustrates the number of spikes per defined time window (here 1 s) divided by the total number of RGCs. The left half illustrates the activity before stimulus onset and, separated by the red line, the right half the activity when the organoids are exposed to pulsed light or 100 µM 8-br-cGMP. Figure adopted from Celiker et al., 2023*[18]

In retinal organoids, cone photoreceptors, in particular, have displayed light-evoked electrical responses that mirror those observed in the adult primate fovea[15,34]. This includes the basic phototransduction mechanism and the ability to

adapt to varying levels of background luminance, a feature critical for high-acuity vision in humans. Such findings suggest that cone photoreceptors in retinal organoids can initiate the phototransduction cascade without the addition of external chromophores, relying instead on endogenous components synthesized during differentiation. Furthermore, the electrophysiological properties of these photoreceptors have been extensively compared with those of *ex vivo* primate retina[15]. The similarities in response kinetics, sensitivity, and adaptation to light between organoid-derived cones and natural foveal cones demonstrate the capability of organoids to accurately mimic human retinal function.

Beyond its primary function of processing visual stimuli, the retina plays a pivotal role in synchronizing our internal clock with the external environment. This critical function is largely attributed to a specialized type of retinal ganglion cell, known as intrinsically photosensitive retinal ganglion cells (ipRGCs). ipRGCs are unique in their ability to respond directly to light, independent of the photoreceptors traditionally involved in vision. These cells are most sensitive to blue light, with a peak sensitivity around 480 nm, which is prevalent in natural sunlight[35].

ipRGCs play a crucial role in regulating circadian rhythms, which are the physical, mental, and behavioural changes that follow a 24-hour cycle, responding primarily to light and darkness. They achieve this by directly projecting their axons to the suprachiasmatic nucleus (SCN) in the brain, the master clock that governs the circadian rhythms of human body. The SCN processes the light signals it receives from the ipRGCs to adjust the internal clock of the body, aligning sleep patterns, feeding behaviour, hormone production, and other important physiological processes with the day-night cycle.

The sensitivity of ipRGCs to blue light has significant implications for how artificial light exposure, especially during the evening, can disrupt our circadian rhythms. Exposure to blue-rich light sources like smartphones, tablets, and LED lighting at night can trick our brains into thinking it is still daylight, leading to delayed sleep onset and a host of sleep-related issues. This understanding highlights the importance

of managing light exposure to maintain healthy circadian rhythms and overall well-being.

Our knowledge about how light, particularly specific wavelengths, influences these retinal clocks is strikingly limited. This is a significant gap in our understanding, as most research in this area has been conducted on animal models, whose visual systems are fundamentally different from humans[36]. These models fall short in accurately depicting the features of human vision and its circadian regulation. Therefore, human retinal organoids could provide an accurate model to study the response of circadian system to light in humans.

Our contribution to this topic

- In our study[18] entitled: "***Light-responsive microRNA molecules in human retinal organoids are differentially regulated by distinct wavelengths of light***", we explored the complexity of light-responsive microRNA (miRNA) molecules in human retinal organoids. Our findings revealed that certain miRNAs are differentially regulated by various wavelengths of light and exhibit a rapid turnover, mirroring the dynamic response of the retina to fluctuating light conditions. Using our in-house-developed photostimulation device Cell LighteR, we identified 51 miRNAs that undergo significant expression changes in response to light, pinpointing their critical roles in visual processing and circadian rhythm modulation. Importantly, we demonstrated that these miRNAs are linked to the regulation of circadian rhythms, further emphasizing the impact of light on physiological processes. Additionally, electrophysiological recordings provided compelling evidence of responsiveness to light of human retinal organoids, strengthening our understanding of retinal adaptation and its underlying molecular mechanisms. This investigation provides an evidence

of miRNAs role in the adaptation of the human retina to light and opens new pathways for research into visual function of the human retina.

2.2.4 Applications in Disease Modelling and Drug Discovery

Retinal organoids have significantly advanced the field of disease modelling and drug discovery for retinal diseases. These organoids serve as a powerful tool for understanding the pathogenesis of complex retinal diseases including retinitis pigmentosa, Stargardt disease, and age-related macular degeneration[37]. They provide a more accurate and human-relevant model than previous tools, enabling the study of disease progression and the efficacy of potential treatments in a controlled, laboratory settings. They have become instrumental in modelling various retinal diseases, particularly those with genetic origins, as they can be generated from patient-specific iPSCs. This allows for the exploration of the role of genetic variants or mutations on retinal diseases.

Studies using retinal organoids derived from patients with retinitis pigmentosa (RP) have uncovered cellular pathologies, such as disrupted cilia morphology leading to photoreceptor degeneration[38]. Buskin et al. generated retinal organoids and RPE from RP patients with *PRFP31* mutations. Patient-derived RPE cells displayed significant decreases in the number of ciliated cells and cilia length. Photoreceptors appeared normal initially but exhibited a notable increase in apoptotic nuclei over time. Disrupted splicing of cilia genes correlated with abnormal cilia microtubule morphology and reduced cilia formation. *In situ* correction of specific mutations resulted in restored cilia morphology and increased cilia incidence in mature RPE and photoreceptors of derived retinal organoids.

In another study, the authors Leong et al. [39] investigated Usher syndrome-associated RP, particularly focusing on Usher type 1B (USH1B)-RP caused by *MYO7A* mutation. Utilizing retinal organoids derived from iPSCs of USH1B patients, they ob-

served increased differential gene expression over time without excessive photoreceptor cell death compared to controls. Dysregulated genes were initially associated with mitochondrial functions, followed by proteasomal ubiquitin-dependent protein catabolic processes and RNA splicing. Single-cell RNA sequencing revealed *MYO7A* expression in rod photoreceptor and Müller glial cells, correlating with upregulation of stress responses in NRL^+ rods and apoptotic signalling pathways in VIM^+ Müller cells. These findings suggest defensive mechanisms mitigating photoreceptor cell death in USH1B organoids, providing a human model relevant for developing therapeutic strategies against USH1B-RP.

Similarly, Huang et al. utilized retinal organoids to model X-linked juvenile retinoschisis, a condition characterized by progressive central vision loss, retinal splitting (retinoschisis), and detachment[40]. Retinal organoids derived from patient iPSCs replicated disease phenotypes, including retinal splitting and defects in ER-Golgi transportation, paxillin turnover, connecting cilium development, and outer segment formation. Correction of disease-causing mutations in patient iPSCs led to the restoration of normal phenotypes in retinal organoids, demonstrating the potential of gene correction strategies in ameliorating disease phenotypes *in vitro* and potentially *in vivo*[40].

Furthermore, retinal organoids have been instrumental in studying autosomal dominant mutations, demonstrating that knocking down mutant alleles can mitigate retinal dystrophy phenotypes. Chirco et al. derived retinal organoids from patients with autosomal dominant *LCA7*, showing photoreceptor dysfunction and decreased expression of specific markers[41]. By eliminating mutant protein expression, they restored normal phenotypes in differentiated photoreceptor cells. Similarly, Diakatou et al. targeted an allele-specific mutation in *NR2E3*, resulting in autosomal dominant retinitis pigmentosa[42]. Knocking down the mutant allele led to the restoration of normal photoreceptor development in retinal organoids.

In case of Stargardt disease, where mutations in the *ABCA4* gene affect cone cells, retinal organoids provide a superior model over traditional animal models by

allowing a focus on the affected cone cells[43]. This specificity facilitates a deeper understanding of the disease pathophysiology. Organoids have also been pivotal in studying and correcting genetic defects, such as splicing errors in the *ABCA4* gene, revealing their value beyond disease modelling to therapeutic applications[44].

Additionally, retinal organoids from retinoblastoma patients have revealed mechanisms of *RB1* mutations, demonstrating the fidelity of organoids to model native disease states[45]. Children with germline mutations in *RB1* are at a high risk of developing retinoblastoma and other cancers later in life. While genetically engineered mouse models share some similarities with human retinoblastoma, they differ in cellular differentiation. Noirrie et al.[45], generated iPSCs from 15 participants with germline *RB1* mutations and differentiated them into retinal organoids. After 45 days in culture, the retinal organoids were dissociated and injected into the vitreous of immunocompromised mice to support tumor growth. The resulting retinoblastomas had molecular, cellular, and genomic features indistinguishable from human retinoblastomas. They also conducted parallel experiments with targeted *RB1* gene inactivation using CRISPR-Cas9.

Genome editing in organoids reduces variability between individuals, allowing for precise evaluation of disease mutations and the efficacy of genome editing technologies like CRISPR-Cas9. These technologies have successfully corrected mutations in diseases such as retinitis pigmentosa and X-linked juvenile retinoschisis, offering hope for future therapeutic applications[40,46].

Research into ciliopathies like Leber congenital amaurosis and Joubert syndrome, caused by mutations in the *CEP290* gene, have been validated using retinal organoid cultures[47,48]. The study[48] has confirmed the role of CEP290 in retinal cell and ciliary transport and biogenesis.

In conclusion, retinal organoids have emerged as indispensable tools in disease modelling and drug discovery for retinal diseases. They offer a more accurate and human-relevant model than previous methods, enabling the study of disease pro-

gression and treatment efficacy in a controlled laboratory setting. Using patient-specific iPSCs, they can provide insights into the cellular and molecular dynamics of various retinal conditions, including those with genetic origins.

Our contribution to this topic

- In our study[49] "***Role of ciliopathy protein TMEM107 in eye development: insights from a mouse model and retinal organoid***", we explored the role of TMEM107 in eye development, focusing on its impact on the Sonic Hedgehog (Shh) pathway. We found that TMEM107 deficiency leads to aberrant Shh signalling in retinal cells. This was evident from the up-regulation of GLI1 expression, indicating an abnormal activation of the Shh pathway in the absence of TMEM107. Our findings suggest that TMEM107 plays a critical role in the regulation of the Shh pathway during eye development, influencing the formation of primary cilia and ultimately affecting eye morphology and development.

2.2.5 Limitations of retinal organoid technology

Retinal organoids possess considerable potential for advancing screening methods to assess the effects of therapeutic agents and toxicity, and for conducting detailed assays. Using additional techniques from other 3D organoid systems in drug screening can open new avenues for retinal organoid-based approaches and could significantly enhance the bridge between laboratory and clinical applications in drug development. However, challenges related to human PSC-derived retinal organoids, including their use in studying retinal development, disease modelling, and as a platform for drug screening, must be acknowledged.

Efforts to standardize organoid characterization and development stages are ongoing, yet reproducibility remains a major issue due to variability in the originating human PSCs. The process of deriving organoids from human PSCs is critical in determining their composition, leading to diverse outcomes in their composition and ma-

turity[15]. Efforts are being made to address these challenges by improving culture conditions, employing bioreactors, and utilizing microfluidic devices to mimic blood vessels and rectify the absence of the RPE layer[15,50]. However, the inherent heterogeneity among organoids, influenced by the epigenetic memory of the somatic cells from which they are derived, presents a significant barrier[34,51]. This variability affects the efficiency of reprogramming somatic cells into iPSCs and their subsequent differentiation into retinal cells. The differentiation efficiency varies significantly among cell types, with some cell types forming organoids more readily than others. This heterogeneity, alongside the chromatin state and expression of pluripotent markers, plays a crucial role in the reprogramming process and the ability of cells to differentiate into retinal lineages[52]. These considerations are vital when using retinal organoid for disease modelling and drug toxicity assessments.

The variable maturity of photoreceptors within organoids and their lack of integration with the RPE layer limit their functionality in light sensitivity[53]. Current strategies involve co-culturing organoids with RPE cells to improve connectivity and function. To model progressive retinal diseases, methods such as inducing cellular aging or applying stressors have been explored. Nonetheless, the variability in development time and stages of retinal organoids from human PSCs, alongside the need for comprehensive proteomic analysis, neuronal activity assessments, and metabolomic profiles, remains a challenge[54]. These factors are crucial for evaluating the quality of retinal organoids and their utility in disease modelling and drug development.

3 Commentary to published work

3.1 Commentary to published work - Annex 1

miR-183/96/182 cluster is an important morphogenetic factor targeting PAX6 expression in differentiating human retinal organoids

Original article published in Stem Cells, 2020, Q1, IF (2020): 6.277

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Different noncoding RNAs, including miRNAs, are crucial for development, acting tissue-specifically and regulating various biological functions. MiRNAs are pivotal in the timing of developmental events by modulating molecular networks. The miR-183/96/182 cluster, comprising miR-183, miR-182, and miR-96, is a significant, conserved group of miRNAs in bilaterians, expressing predominantly in pluripotent stem cells and sensory organs, suggesting its vital role in differentiating these cells into neural and sensory tissues.

Previous studies have suggested the involvement of miRNAs in various developmental processes; however, the specific mechanisms by which the miR-183/96/182 cluster influences retinal development remained poorly understood prior to our investigation. Our study demonstrates that the miR-183/96/182 cluster plays a crucial role in the differentiation of human PSCs into retinal organoids by targeting and regulating the expression of *PAX6*, a key gene involved in eye development. By employing miRNA tough decoy (TuD) approach for miRNA inhibition, we observed that inhibition of the miR-183/96/182 cluster resulted in significant morphological changes and an increased expansion of the neuroepithelium in the developing retinal organoids. This was associated by upregulation of neural-specific and

retinal-specific genes, highlighting the role of the miRNA cluster in retinal tissue morphogenesis.

Our findings further establish *PAX6* as a direct target of the miR-183/96/182 cluster. We utilized a *PAX6* 3'UTR reporter assay and demonstrated that inhibition of the miRNA cluster leads to the upregulation of *PAX6* expression. This interaction between the miR-183/96/182 cluster and *PAX6* suggests a fine-tuned regulatory mechanism essential for the proper development of the retina.

Our investigation utilized a variety of analytical techniques to validate our findings. Scanning electron microscopy provided detailed images of the retinal organoids, revealing structural changes induced by the inhibition of the miRNA cluster. Flow cytometry was used to assess the expression of stem cell markers, while Western blot analysis and RT-qPCR allowed for the quantification of protein and mRNA levels, respectively.

In summary, this , this work enhances our understanding of the regulatory networks involved in stem cell differentiation and highlights the critical role of the miR-183/96/182 cluster in the morphogenesis of the neural retina, offering new insights into the molecular mechanisms of retinal development.

3.2 Commentary to published work - Annex 2

Light-responsive microRNA molecules in human retinal organoids are differentially regulated by distinct wavelengths of light.

Original article published in iScience, 2023, Q1, IF (2021): 6.107

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In the human retina, the response to light plays a pivotal role in both vision and circadian rhythm regulation. Here we provided significant insights into the miRNAs that modulate based on photostimulation in the human retina, using retinal organoids as a model system. Here we identified retina-specific miRNA families in these organoids. With the deployment of the Cell LighteR photostimulation system, we discovered that retinal organoids showed a remarkable ability to alter miRNA transcription levels when exposed to different wavelengths of light. Notably, the response time of this transcriptional change in the retina was found to be much faster than in other tissues or cell types.

Interesting discovery was the differential miRNA responses to various light wavelengths. For instance, while the exposure to red and green light prominently elevated the expression of the miR-183/182/96 cluster, blue light exposure seemed to be a stimulant for the miR-204 family. These responses offer valuable insights into how diverse light stimuli might have unique influences on the miRNA-mediated regulatory mechanisms within the human retina.

When comparing these findings with previous research, particularly those involving mice, we observed distinct differences in the number of identified light-responsive miRNAs. One plausible explanation for this discrepancy could be the more advanced sequencing technique employed in this research. This technique led to the

identification of a much broader and more comprehensive list of light-responsive miRNAs in humans compared to previous studies.

Here we also focused on the miR-182/183/96 cluster. This cluster plays an indispensable role in the retina and is involved in various stages of retinal development. Given its enhanced responsiveness to light, we speculate that this cluster could significantly influence the formation of the postnatal retina.

Furthermore, the connection of specific miRNAs to circadian rhythms was evident. For instance, nine miRNAs have a direct association with the regulation of circadian rhythms. The miR-182/183/96 cluster, for instance, have a direct influence on pivotal circadian genes. Similarly, the miR-194/192 cluster targets genes that are integral to circadian rhythms, like *PER1*, *PER2*, and *PER3*.

Taken together, here we studied the role of miRNAs in modulating the response of the human retina to light. These light-regulated miRNAs potentially have profound implications for the formation and function of the retina and for the larger framework of circadian timing. The findings here lay the groundwork for a deeper exploration of the complex interactions between light, miRNA regulation, visual function, and circadian rhythms in humans.

3.3 Commentary to published work - Annex 3

Role of Ciliopathy Protein TMEM107 in Eye Development: Insights from Mouse Model and Retinal Organoid

Original article published in Life Science Alliance, 2023, Q1, IF (2021): 5.781

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The primary cilium is a cellular structure found on most cell types, responsible for various sensory and signal processing functions. It plays a significant role in processes like neurogenesis and kidney formation, and is crucial in eye development. Disruptions in its biogenesis or function can lead to defects called ciliopathies, which manifest as visual and other developmental abnormalities. A specific protein, TMEM107, located at the base of the cilium, is critical in maintaining ciliary functions.

Mutations in *TMEM107* have been linked to a number of syndromes that display altered ciliary morphology and function. Both human patients and mouse models with *TMEM107* mutations show developmental defects, and while its involvement in craniofacial defects is known, its role in eye development is still not understood.

In this work, we used various experimental models such as mice, retinal organoids, and retinal cell cultures to study the function of TMEM107. We found that TMEM107 is highly expressed in the neural retina of the developing eye. When TMEM107 is absent, distinct eye abnormalities such as anophthalmia (absence of the eye) and microphthalmia (abnormally small eye) occur. This deficiency also alters expression of important genes involved in eye development. TMEM107 is critical for ciliogenesis and Shh signalling. Without it, primary cilia are disrupted, and Shh signalling becomes aberrant, which leads to the development of cysts.

Tmem107-deficient mice displayed eye malformations resembling those observed in humans. Patients with mutations in the *TMEM107* gene have been diagnosed with various ciliopathies, all of which exhibit eye-related defects such as

anophthalmia, microphthalmia, and other ocular anomalies. The severity of these symptoms often relates to the type of mutations the patients have.

Interestingly, human embryos with *TMEM107* mutations that result in the complete absence of the protein might lead to more severe manifestations than those that produce a truncated version of the protein. This is because a truncated protein might still retain partial functionality. Furthermore, certain defects found in *Tmem107*$^{-/-}$ mice like optic nerve hypoplasia are also observed in humans with specific syndromes.

In the absence of Tmem107, there are profound differences in the expression of SOX2, a transcription factor crucial for eye maturation. Additionally, another transcription factor, SOX1, which is essential for eye development, is absent in the lens area of these mutants. Previous findings also pointed to a reduced ciliogenesis in Tmem107-deficient animals, although the length of the primary cilia in this research was reduced, contradicting earlier studies. This suggests that the role of TMEM107 in ciliogenesis may be tissue-specific, especially vital in areas of the eye with high TMEM107 expression.

One significant observation was the formation of cysts in *TMEM107*$^{-/-}$ organoids. These cysts often contain lipids and accumulated fluid, a phenomenon seen in many ciliopathy patients. The SHH pathway, critical in ocular development, is found to be aberrantly activated in the absence of TMEM107. For instance, upregulated SHH levels in TMEM107-deficient animals lead to downregulated PAX6 in the optic cup regions, contributing to the various eye defects observed.

Moreover, recent studies highlight the relationship between primary cilia and early eye development. Without ciliary proteins, aberrant or non-existent primary cilia can lead to abnormal eye patterns and morphologies, often due to incorrect Shh signaling. In *TMEM107*$^{-/-}$ cells, the Shh pathway is affected, suggesting that the impact of Shh on eye development might be linked to the repression role of Gli transcription factors.

Finally, primary cilia play an essential role in regulating stemness. TMEM107 and another stem cell marker, Sox2, have been found to express similarly in neural retina, crucial for the development of the eye. Both are predominantly present in pluripotent cells and are reduced in differentiating cells.

4 Conclusions

The utilization of retinal organoids has emerged as a transformative approach in advancing our understanding of the complex processes underlying the development, function, and diseases of the human retina. Experiments using the retinal organoid technology uncovered deep insights into the cellular and molecular dynamics underlying retinal biology. These findings shed light on the complexities of retinal development and function and offer promising potential for the development of novel therapeutic strategies for retinal diseases. Continued exploration and refinement of retinal organoid technologies have the potential to study and treat a wide variety of retinal disorders, ultimately improving outcomes for patients worldwide.

This thesis summarizes our work and work from other research groups that use retinal organoid models to study the human retina. However, the essence of the work still lies in harnessing the power of retinal organoid models to understand the retinal development, function, and diseases. These models offer a unique opportunity to recapitulate human retinal biology *in vitro*, providing a platform for investigating disease mechanisms, testing potential therapeutics, and exploring personalized treatment approaches. Through collaborative efforts and ongoing refinement of organoid technologies, we will enhance our understanding and management of various retinal disorders.

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