Modeling complex blinding human disease AMD in an engineered tissue - to uncover disease mechanisms and develop personalized treatments.

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Abstract: Age-related macular degeneration (AMD) is the leading cause of irreversible visual impairment in older people. Susceptibility to AMD is dependent on a combination of genetic components and environmental factors with role attributed to retinal pigmented epithelium (RPE), immune cells and vasculature. Several genome-wide association studies (GWASs) have been applied to AMD, resulting in the identification of ~50 loci, that are significantly associated with increased risk. For most of these loci, the causal variants, their mode of action, and the affected target genes are unknown. This gap is partly due to the lack of an appropriate model to mimic AMD pathology. We propose to establish an engineered tissue model for the human RPE-vascular unit. This will allow us to substantially improve our understanding of AMD pathology and genetic predisposition by identifying causal risk SNPs within GWAS-determined AMD-risk loci, detect their target genes, and elucidate the biological processes that are affected by them. Moreover, modeling complex human diseases in an engineered tissue model will be an essential step toward developing and testing new therapies. The research strategy is based on the combination of new technologies; 3D printing of tissues, stem-cell and genome editing technologies and cutting-edge genomic techniques. This project holds great promise for AMD research: the identification of causal variants, target genes, and molecular pathways will improve our understanding of the molecular mechanisms involved in AMD; to significantly enhance our capability for early identification of individuals at high risk, intervention and disease prevention, all key visionary goals for personalized medicine.

Modeling complex blinding human disease AMD in an engineered tissue - to uncover disease mechanisms and develop personalized treatments.

Age-related macular degeneration (AMD) is the leading cause of irreversible visual impairment in older people, accounting for approximately 50% of legal blindness in western countries [1]. The hallmark of AMD is the progressive loss of central vision due to the gradual degeneration of the photoreceptors at the macula. The degeneration of the photoreceptors in AMD is attributed to the malfunction of several adjacent tissue types; the retinal pigmented epithelium (RPE), the RPE basement membrane termed the Bruch's membrane, and the underlying choroidal vascular capillaries.

AMD is classified into two main groups; a "dry" form (avascular) which seems to precede the rarer neovascular form termed "wet AMD". Dry AMD has no treatment and is characterized by the accumulation of lipids and proteins termed drusen between the RPE and the Bruch's membrane. The drusen depositions mechanically detach the RPE from the choroid vasculature and thus abrogate normal transport from the choroid, across the RPE and the Bruch's membrane to the retina. Combined with oxidative stress and inflammation, this results in RPE cell death (geographic atrophy), eventually leading to photoreceptor degeneration and vision loss. Neovascular AMD is characterized by the presence of vascular choroidal tissue inside the retina, which can leak fluid and bleed. This condition is deleterious to the photoreceptors and results in severe and rapid loss of the central vision [2, 3].

Susceptibility to AMD is dependent on a combination of genetic and environmental factors. The development of AMD pathology involves the interaction between several tissue types, with primary roles attributed to the retinal pigmented epithelium (RPE), immune cells (monocytes [4, 5]), and the vasculature changes [6]. To date, several genome-wide association studies (GWASs) have been applied for AMD. The recent and largest systematic study, conducted by the International AMD Genomics Consortium (IAMDGC), identified 52 single-nucleotide polymorphism (SNP) variants associated with AMD [7, 8]. Due to the haplotype structure of the human genome and the genetic association between SNPs within each haplotype block, variants identified by GWASs only serve as tag SNPs that define genomic loci associated with the disease. Any variant within these loci that is in high linkage disequilibrium with the tag is potentially a causal variant underlying the increased risk. The 52 AMD-associated loci contain 1,345 candidate risk SNPs for AMD [7]. While some of these SNPs alter protein-coding sequences and, therefore, most probably act by changing protein function, for most of these AMD loci, the genetic variants associated with elevated risk to AMD map to noncoding sequences in the genome. Despite these

important findings, the AMD-risk genes, the causal variants and their mode of action are currently unknown for most AMD-risk loci.

Our objective is to engineer a 3D tissue of the RPE-vascular unit aiming to improve our understanding of the molecular mechanisms by which risk variants detected by GWAS affect AMD pathogenesis, to understand the role of environmental factors and to develop a platform for testing novel therapies. This proposed study is a prerequisite for the translation of these discoveries into improved AMD treatment and prevention. In a recent study from the Ashery-Padan team, obtained through an interdisciplinary collaboration combining computational and experimental approaches, we obtained support for our hypothesis that a significant portion of the AMD-risk variants acts through modulation of cis-regulatory elements (CREs), which are regulated by tissue-specific transcriptional complexes required for the development and maintenance of the human RPE [9]. These insights were obtained by genomic and epigenetic analysis of transcription factors in 2D cultures of human RPE generated from stem cells (Figure 1, [9]). Nevertheless, for accurate disease modeling to extend our genetic studies to additional tissues involved in AMD, specifically the choroid vasculature, for developing cell replacement approaches and for testing new drugs, the prerequisite is to develop a real 3D tissue that combines both the human RPE and choroidal vasculature, thereby containing the wide variety of cell types that have been implicated in RPE physiology and with AMD pathology [10-12].

Notably, the laboratory of Dvir has utilized a personalized ECM-based hydrogel as a bio-ink for advanced 3D printing techniques [13-15]. The combination of the hydrogel and the patient's own cells was used to print thick, vascularized, and perfusable tissues that fully matched the immunological, biochemical, and anatomical properties of the patient. More recently, Dvir's group developed a process for fabricating a 3-layer retina-like structure, including the choroid layer, RPE layer, and photoreceptors (Figure 2; *Article in Submission*). As a proof of concept, the fabricated structure was composed of human endothelial cells, RPE and photoreceptor cell lines, and an ECM-based hydrogel. To generate a blood vessel with a 300 μm diameter and a capillary bed, endothelial cells were printed at room temperature in a sacrificial material that was surrounded by a printed ECM hydrogel (Fig. 2).

Using the combination of RPE and vasculature generated from iPSC together with autologous ECM will allow us, in course of this study, to bio-print the personalized RPE-vascular unit. This will be important for extending the maturation and life span of the cells on the dish and thus to better mimic processes that occur over a long period of time most relevant to the aging human eye

[16]. Importantly, such a multi-tissue device that is generated from a specific genotype of an iPSC will be pivotal for screening and testing disease-associated reagents as well as for personalized drug screening purposes. These challenges will be accomplished by implementing the following three specific aims and research strategies:

Aim -1 Engineer a human 3D-RPE-vascular unit from human iPSCs

Here, we will study RPE-vascular interactions to improve the differentiation properties of RPE and vascular tissue involved in AMD. This aim is based on our hypothesis that modeling complex human diseases, such as AMD, will require implementing new technologies to mimic the endogenous interactions between RPE, Bruch's membrane, and choroid vasculature to generate an ex-vivo model to RPE-vascular retinal blood barrier.

Research plan: ***Tissue fabrication and assessment of morphology:*** iPSCs will be differentiated to endothelial cells as described by Dvir's lab [13]. -A blood vessel network will be printed in an ECM-based hydrogel as described [14]. Following, human, iPSC-derived RPE cells (prepared according to [17]), will be deposited dropwise on top of the printed choroid and allowed to self-organize and mature until the pigmentation and hexagonal morphology is evident. Further validation of tissue differentiation will be accomplished by immunolabeling against the tight junction protein (ZO-1), and expression of key RPE transcription factors, such as OTX1/2, Mitf,BEST1, and RPE65 and acquisition of cell polarity will be confirmed by detection of beta-catenin (basolateral) and Ezrin (apical process) distribution (Fig. 1). We will further examine the differentiation based on RNA-seq analysis (Fig. 1). The formation of Bruch's membrane will be analyzed antibody labeling to specific collagens and by co-staining the construct for CD31, and MITF. Furthermore, the interface of the choroid and the RPE layers will be imaged by transmission electron microscopy (TEM).

Assessment of tissue function: To assess the function of the engineered RPE layer, the tissue will be cultured with fluorescent latex beads (d= 1 μm), and the ability of the engineered RPE layer to perform phagocytosis will be demonstrated. Moreover, several key RPE functions, such as the transport of nutrients and waste products across it, the maintenance of the blood-retinal barrier, and the regulation of cell growth and differentiation, are regulated by calcium ions. Therefore, we will next assess calcium signaling after activation by adenosine triphosphate (ATP) and monitor the trans-epithelial electrical resistance (TEER) during 4-weeks of cultivation.

Significance: The human-based 3D-RPE-vascular unit will allow monitoring of late stages of complex organ maturation and physiology and thus provide a better model to study human disease

mechanisms. The 3D-RPE-vascular unit will provide a mean to study general principles of neurovascular unit formation and maintenance as well as grow sufficient human tissue for genomic and epigenomic analyses (Aim 2).

Aim -2 Determine the cis-regulatory regions in RPE and vasculature involved in AMD

The RPE-vascular 3D-Chip will generate relevant human tissue from key lineages involved in AMD. Using ChIP-seq technologies, we will generate the cistrome (the collection of all CREs bound by a transcriptional regulator) and transcriptional target genes of the key RPE transcription factors in human RPE and TFs of the choroid vasculature (Ashery-Padan) and correlate with AMD-risk SNPs [9].

Research plan: Using this approach on human RPE from embryonic stem cells, we have revealed the co-occupancy of several key RPE TFs, allowing us to infer the formation of a tissue-specific transcriptional complex with a major role in shaping the RPE transcriptional program. Importantly, we found that the LHX2–OTX2 cistrome in the RPE is significantly and specifically enriched for AMD-risk SNPs [9]. In this task, we will conduct similar analyses and validations for two additional key TFs for RPE (MiTF) and vasculature, to determine their interacting partners, cistromes, and target genes, and detect AMD-risk SNPs that act through modulation of the binding affinities of these TFs to specific CREs that are active in the RPE. **Significance:** Improving our understanding of the genetic basis of AMD is of special importance because genetic risk lies at the initiation of the causal chain of disease pathogenesis. Hence, successful accomplishment of our goals holds promise for pointing to novel preventive interventions and rational development of drugs for AMD that act by targeting specific biological processes which drive pathogenesis at its early stages.

Aim-3 Model AMD pathology in an engineered tissue.

Functional analyses of the identified candidate AMD-risk SNPs and regulatory regions associated with TFs and gene regulation in the RPE:

Research plan: Quantitatively evaluate the effects of noncoding variants/mutations on the interactions between TFs and CREs Allele-specific measurements of TF binding will be performed by mapping the ChIP-seq reads to each allele of heterozygous variants. We will further examine how each allele contributes to the chromatin's accessibility using ATAC-seq; this provides a measure of chromatin openness, which correlates with the activity level of the transcriptional regulatory elements. We examined the ATAC-seq dataset obtained by Wang et al. in human macular RPE [18], as we identified two heterozygous donors for a specific AMD-risk SNP (rs3809579). Notably, for both samples, the number of ATAC-seq reads that originated from the chromosome with the non-risk allele (the C allele) was significantly higher than the number of reads originated from the chromosome with the risk allele (p = 0.0098), supporting the conclusion that the risk allele of this AMD-risk SNP reduces the activity of TRPM1 promoter in the macular

RPE, the affected tissue in AMD [9]. To perform allele-specific ChIP-seq and ATAC-seq analyses on additional samples, we will generate hES-RPE that is heterozygous for the risk alleles through CRISPR/Cas9 genome editing. Importantly we will generate from iPSC that carry multiple risk alleles and the isogenic iPSC with protective alleles the RPE-Choroid unit. This will allow to determine the impact of the genotype on gene expression and on cellular phenotype such as Burch's membrane integrity, the barrier function of the RPE and the deposition of Drusen [19].

Significance: The engineering of the RPE-vascular 3D tissue to model the outer-blood-retina barrier consisting of the RPE, Bruch's membrane, and choroid vasculature will allow generating the key tissue types involved in AMD and thus to model disease mechanism, identify the genomic regions involved in AMD as well as, in future studies platform to assess possible therapies.

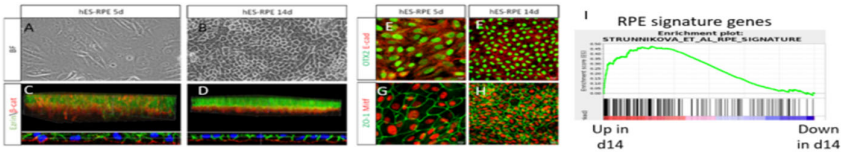

Fig. 1 Human RPE generated from human embryonic stem cells present key RPE features following differentiation in 2D cultures comparing dedifferentiated cells (5 days after splitting, d5) to differentiated RPE (d14) including accumulation of pigmented (A, B), cell polarity (beta-catenin and Ezrin (C, D), hexagonal morphology and formation of tight junctions (E-H) and detection of RPE transcription factors Otx2 (E,F green) and Mitf (G,H). (I) The gene set enrichment analyses (GSEA) of ~1000 differentially expressed genes present the significant enrichment for RPE signature genes among the upregulated genes following hES-RPE differentiation.

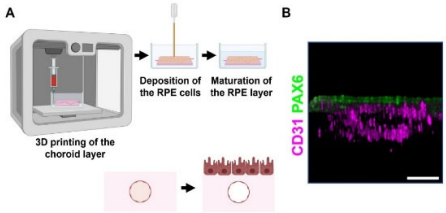

Fig. 2. Fabrication of the choroid-RPE interface. (A) Schematics of the process. 3D printing is used to engineer the blood vessel network. Following, iPSCs-derived RPE cells will be deposited and allowed to mature. (B) Immunostaining for detection of the RPE by detection of TF expressed in RPE (PAX6, green) and the underlying blood vessel (CD31, purple). bar= 150 µm.

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Budget – 100,000$
As this is a very ambitious project, we expect that it will take 2 years to accomplish the suggested goals.

Budget – in US dollars

	Ashery-Padan	**Dvir**
PhD fellowship	9,000	9,000
Sequencing,	5,000	
Regents for tissue culture, antibodies, biomaterials for printing	23,500	28,500
Total Budget	37,500	37,500
Matching	12,500	12,500

Matching: Salary for Laboratory technician.

Justification:
Salaries include a PhD student to be recruited once funding is available. One PhD for each laboratory will devote 100% of the time to engineering the RPE-Choroid interface. Laboratory technician will join the project, and the salary will be covered by other resources.
Antibodies will be used to assess endothelial cells (CD31), RPE cells (ZO-1), MITF, BEST-1, etc, Bruch's membrane (collagen I and IV, laminin).
Reagents for tissue culture include medium for iPSCs culture, molecules for differentiation, flasks, etc.

BIOGRAPHICAL SKETCH

NAME: Tal Dvir, Ph.D.

POSITION TITLE: Professor

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Ben Gurion University, Israel	B.Sc.	2003	Biotechnology Engineering
Ben Gurion University, Israel	Direct Ph.D. (Summa cum Laude)	2008	Tissue Engineering
MIT (Prof. Robert Langer's Lab), USA	Postdoctoral Fellow	2011	Biomaterials and Regenerative Medicine

A. Personal Statement

I have a broad background and strong track record in the fields of tissue engineering, biomaterials, and stem cells. My lab is focused on engineering tissues and organs such as the heart, spinal cord, brain, intestine, kidney, and retina. In regard to this proposal, my group developed several printing approaches, allowing to print large-scale, volumetric, vascularized patient-specific tissues. Several spin-off companies were formed based on the developed technologies

B. Positions and Honors

2011-2015- Senior Lecturer at the Department of Biotechnology, Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel.

2015-2019 – Associate Professor at the Department of Biotechnology, Faculty of Life Sciences, Materials Science and Engineering, Faculty of Engineering, Tel Aviv University, Tel Aviv, Israel.

2019- Present- Full Professor at the Department of Biotechnology, Faculty of Life Sciences, Department of Biomedical Engineering (since 2020), Faculty of Engineering, Tel Aviv University, Tel Aviv, Israel.

2017- Present- Founding Director of Sagol Center for Regenerative Biotechnology, Tel Aviv University, Tel Aviv, Israel.

2020 – Present – Director of Tel Aviv University's Center for Nanoscience and Nanotechnology, Tel Aviv University, Tel Aviv, Israel.

Selected Honors and Grants

2011-2015 Consul of higher education VATAT, Alon Fellowship
2021 – 2026 ERC Starter Award
2018 Juludan Research Prize
2018 The Rappaport Prize for Excellence in the Field of Biomedical Research
2021 – 2026 ERC Consolidator Award

Complete List of Published Work:

https://scholar.google.co.il/citations?user=ShkAj38AAAAJ&hl=en

BIOGRAPHICAL SKETCH

NAME: Ruth Ashery-Padan, Ph.D.

POSITION TITLE: Professor

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Hebrew University, Jerusalem, Israel	B.Sc.	1990	Biology and Psychology
Hebrew University, Jerusalem, Israel	M.Sc. (Summa cum Laude)	1991	Genetics
Hebrew University, Jerusalem, Israel	Ph.D.	1996	Genetics
Max Planck Institute, Goettingen, Germany	Postdoctoral training	2001	Neural Development

A. Personal Statement

I have a broad background and strong track record in the field of developmental neuroscience, focusing on developmental abnormalities leading to blindness. My studies have provided key insights into the processes that govern formation and maintenance of terminally differentiated retinal neurons and retinal pigmented epithelium (RPE) cells. Specifically, my group investigates the transcription factors, microRNAs (miRNAs) and signaling pathways that regulate organogenesis of the mammalian eye. My research team employs state-of-the-art genetic tools, including mouse models carrying mutations in transcription factors, miRNAs and signaling factors, as well as stem-cell technologies. My goal is to investigate the gene network controlling the development and survival of retinal cell types and to provide novel tools for retinal degeneration therapies.

B. Positions and Honors

2001-2007- Lecturer at the Department of Human Genetics and Molecular Medicine, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.

2008-2009 – Visiting Scientist, Laboratory of Don Zack, Wilmer Ophthalmological Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA**.**

2011-2017- Associate Professor at the Department of Human Genetics and Molecular Medicine, Sackler Faculty of Medicine and Sagol School of Neuroscience, Tel Aviv University, Tel Aviv, Israel.

2017-2018 – Visiting Scientist at NIH, NCI Laboratory of Gordon Hager, National Cancer Institute, NIH, Building 41, Bethesda, MD 20892, USA

2017 – current – Full Professor at the Department of Human Genetics and Molecular Medicine, Sackler Faculty of Medicine and Sagol School of Neuroscience, Tel Aviv University, Tel Aviv, Israel.

2021- current – Chair of the Department of Human Genetics and Molecular Medicine, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.

Other Experience and Professional Memberships

2006-2015 Member of the organizing committee, Israel Society of Developmental Biology
2013-2020 Member of the organizing committee, Israel Society of Eye Research

2015-2021 Head of the Switzerland Institute for Developmental Biology at Tel Aviv University
2021-current Head of the Yoran Institute for Genome Research at Tel Aviv University

Honors

1999 - British Genetical Society award for best presentation
2002-2005 - Consul of higher education VATAT, Alon Fellowship
2002 - "Dan David" fellowship for young investigators
2005 - "TEVA" fellowship, TEVA Prize for Research Achievements
2008 – 2011 The E. Matilda Ziegler Foundation for the Blind (award), Research Proposal in Vision Research

Complete List of Published Work: http://www.ncbi.nlm.nih.gov/pubmed/?term=Ashery-Padan

TEL AVIV UNIVERSITY

Department of Human Molecular Genetics & Biochemistry
School of Medicine
Faculty of Medicine
Tel Aviv University

Prof. Karen B. Avraham

Dean

החוג לגנטיקה מולקולרית של האדם ולביוכימיה
בית הספר לרפואה
הפקולטה לרפואה
אוניברסיטת תל אביב

פרופ' קרן אברהם

דקאן

January 29, 2023

Prof. Ashery-Padan & Prof. Dvir - Application for the Zimin Institute Research Grant: Dean's Endorsement Letter

As the Dean of the Faculty of Medicine, I hereby provide my strongest possible endorsement of **Prof. Ruth Ashery-Padan's** (**Faculty of Medicine**) proposal, in a joint submission with **Prof. Tal Dvir** (**Faculty of Life Sciences**).

The proposal is entitled "Modeling complex blinding human disease AMD in an engineered tissue to uncover disease mechanisms and develop personalized treatments."

This proposal focuses on 3D printing of the choroid-RPE interface in the retina to study age-related macular degeneration and for developing new therapies. The investigators have the necessary expertise and infrastructure to accomplish this project and advance AMD research and treatment successfully.

Based on their outstanding qualifications and expertise, Prof. Ashery-Padan, together with Prof. Dvir, are extremely likely to succeed in this exciting, innovative project. Therefore, I truly hope that you will favorably consider the application.

Sincerely,

Prof. Karen B. Avraham, Ph.D.
Drs. Sarah and Felix Dumont Chair for Research of Hearing Disorders
Dean, Faculty of Medicine
Tel Aviv University

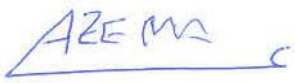
George S. Wise Faculty of Life Sciences
Prof. Abdussalam Azem
Dean
TEL AVIV UNIVERSITY
אוניברסיטת תל אביב
פרופ' עבדאלסלאם עאזם

To the Zimin Institute for Engineering Solutions Advancing Better Lives

I am writing in the strong support the application of Prof. Dvir and Prof. Ashery Padan for The Zimin Institute awards for research projects. The application titled: "***Modeling complex blinding human disease AMD in an engineered tissue to uncover disease mechanisms and develop personalized treatments.***" focuses on 3D printing of the choroid-RPE interface in the retina to study age-related macular degeneration and for developing new therapies.

Professors Dvir and Ashery Padan have all the necessary expertise and infrastructure to accomplish this project and advance AMD research and treatment successfully.

Sincerely,



Abdussalam Azem

Prof. Abdussalam Azem, Ph.D.
Dean of George S. Wise faculty of Life Sciences
School of Neurobiology, Biochemistry and Biophysics
Tel Aviv University