SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PH.D.)

Genomic and proteomic characterization of physiological and pathological angiogenesis in the human eye

Zoltán Veréb, MSc

Supervisor:

Dr.med.habil. Goran Petrovski, PhD

Department of Ophthalmology, Faculty of Medicine,
Doctoral School of Clinical Medicine,
University of Szeged
Szeged, Hungary

SZEGED
2014.

List of publication related to the dissertation:

I. **Veréb Z***, Albert R*, Póliska S, Olstad OK, Akhtar S, Moe MC, Petrovski G. Comparison of upstream regulators in human ex vivo cultured cornea limbal epithelial stem cells and differentiated corneal epithelial cells. *co-first author
BMC Genomics. 2013 Dec 17;14(1):900 **IF: 4.397** (2012)

II. **Veréb Z,** Lumi X, Andjelic S, Globocnik-Petrovic M, Urbancic M, Hawlina M, Facskó A, Petrovski G. Functional and molecular characterization of ex vivo cultured epiretinal membrane cells from human proliferative diabetic retinopathy.
Biomed Res Int. 2013;2013:492376. **IF: 2.880** (2012)

Total IF of publications related to the dissertation: 7.277

INTRODUCTION AND THEORETICAL BACKGROUND

The cornea

The eye is the most important sensory organ of our body. It can be divided into two large parts: the anterior segment covered mainly by the cornea, and the posterior segment wrapped up by the sclera. The cornea and the sclera are connected by a ring called the limbus. The protective and refractive function of the outermost surface of the eye is provided by the highly transparent and strong tissue - the cornea. During organogenesis this is the last part of the eye which is formed from the neural crest. The cornea has a rather complex structure compared to other body surface tissues. A distinct acellular Bowman's layer and acellular collagenous basement (Descemet's) membrane separate the single inner cell layer of the cornea – the cornea endothelial cells, from the stroma, which is the biggest middle thick layer of the tissue. The cornea endothelial cells play critical role in the maintenance of correct hydration of the corneal stroma. The latter contains collagen rich extracellular matrix with embedded, resting flattened stromal keratocytes and fibroblast-like cells. The surface of the cornea consists of stratified squamous epithelium acting as protective, impermeable barrier of the eye (**Figure 2**). Due to persistent physical, microbial, chemical and biological damage, the central corneal epithelial cells (CECs) need to be continuously replaced from the limbal epithelial stem cells (LESCs).

Limbal epithelial stem cells

Animal studies have shown that CECs can differentiate from approximately 100 progenitor cells, which means there is extremely low frequency of LESCs in the limbal tissue. Stem- and progenitors cells usually need special niches to maintain their multipotency and proliferation potential. In humans, only six limbal epithelial crypts have been identified as niches of the LESCs - they are located at the basal cell layer of the limbal epithelium at the corneo-scleral junction. The stem cell niches are important because they block cell division in both the corneal and conjunctival epithelium, therefore act as borderguards delineating the two distinct tissues. As mentioned before, the CECs differentiate from LESCs which can undergo asymmetric division and give rise to transient amplifying cells (TACs). TACs proliferate in the basal layer of the limbus before they differentiate and then migrate centripetally and superficially while becoming more and more differentiated and post-mitotic. During the differentiation from TACs into mature CECs, the cells lose their ability to proliferate.

LESC deficiency (LESCD) and angiogenesis in the cornea

Injuries - traumatic, chemical and iatrogenic, or diseases of the LESCs - inborn or acquired, can all lead to partial or total LESCD or corneal neovascularization accompanied by inflammation. In LESCD, the failure of corneal re-epithelization and the invasion of blood vessels cause a painful state which can lead to blindness. LESCD can be treated with autologous limbal graft transplantation from a healthy donor eye, if available, however, such treatment does not provide guarantee for the functionality of the graft itself.

Despite intense research in this field in the last couple of years, the real phenotype and biological behavior of LESCs is still not known in detail. The appearance of new vessels in the cornea during LESCD can be based upon immunological and angiogenic processes interplaying in the tissue affected.

Proliferative diabetic retinopathy (PDR)

Neovascularization plays a key role in wound healing and tissue regeneration, as well as various ischemic and inflammatory diseases. Pathological neovascularization is a hallmark of many ocular diseases such as age-related macular degeneration (AMD), diabetic macular edema (DME), neovascular glaucoma, corneal neovascularization, pterygium and PDR, which is the most advanced stage of diabetic retinopathy (DR) caused by diabetes mellitus.

The disease destroys the micro- and macro- vasculature in the posterior segment of the eye, and is frequent in patients having the disease for 10 or more years. Based upon the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR), the 10-year incidence of new retinopathy was 89% in the group diagnosed before age 30 years, 79% in the insulin-taking group of 30 years or older and 67% in the non-insulin-taking group. The disease itself affects those individuals who are in their most productive years, therefore, it poses great socio-economic burden on the society. The symptoms of the disease are visual field defects and blurred vision, while funduscopy can reveal fibrovascular membranes and neovascularizations with cotton wool spots, flame- and dot-blot hemorrhages as well as hard exudates. The pathophysiology of PDR is based upon the neovascularization formation in the retina, followed by formation of fibrovascular proliferations which can lead to tractional retinal detachment causing blindness.

The molecular background of DR remains still unclear. Several hypotheses regarding growth hormone involvement, sluggish platelet and erythrocyte circulation with consequent focal capillary occlusions and retinal ischemia, activation of aldose reductase pathway and consequent damage of intramural pericytes that altogether cause saccular outpunching of capillaries, ruptured microaneurysms, intra- and epi- retinal hemorrhages and exudation, have all been described as causes of DR.

The pro-inflammatory cytokines have been known stimulators of the production of angiogenic and vasoproliferative factors. Whenever pro-angiogenic factors are mixed with an ischemic environment, they can provoke neovascularizations in the retina, which can further lead to proliferations and penetration through the internal limiting membrane, the final product being fibrovascular epiretinal membranes (fvERMs) formation between the retina and the posterior hyaloid face. Strengthening this hypothesis are the high levels of pro-inflammatory cytokines such as interleukin 6 (IL- 6), IL-8 and tumor necrosis factor alpha (TNFα) that have been measured in samples from the vitreous body of patients with PDR. So far, the origin of the cells found in fvERMs has not been well understood. Although attempts to assess the presence of $CD34^+$ and $CD31^+$ vascular endothelial cells have been made using histological means in post-vitrectomy membranes, no such assessment has been made when the cells are cultivated *ex vivo* under adherent conditions.

AIMS OF THE STUDY

1. To compare the gene expression pattern of *ex vivo* cultured human LESCs and differentiated CECs to identify possible new putative LESC markers.

2. To discover new biological function or signaling-related pathways with custom made gene expression selection focusing on stemness and proliferation-, epithelial differentiation-, tissue development and growth-, immunological and angiogenic factors-related genes, based upon the differences found between LESCs and CECs.

3. To determine and compare the expression of mesenchymal stem cell (MSC)-related surface markers and compare it to the expression results obtained from the gene array of *ex vivo* cultured LESCs and CECs.

4. To identify angiogenesis related signaling pathways and networks related to the IL-6 and IL-8 gene expression and protein secretion of LESCs.

5. To isolate and cultivate cells from fvERMs (collected from patients with DR) and investigate their phenotype using cell surface markers for hematological-, endothelial-, MSCs- and cell adhesion molecules (CAMs) to determine the possible origin of these cells.

6. To compare the phenotype of fvERM outgrowing cells to non-DR primary human retinal pigment epithelial (hRPE) cells.

7. To investigate the angiogenic potential of the fvERM outgrowing cells under presence or absence of pro-inflammatory factor TNFα using high-throughput screening by angiogenic protein array.

8. To measure the intracellular calcium dynamics in fvERM outgrowing cells in response to mechano-stimulation to prove the viability and functionality of these cells and to mimic the tractional forces appearing due to presence of fvERMs in PDR.

MATERIALS AND METHODS

Tissue collection and isolation of cells

All tissue collection complied with the Guidelines of the Helsinki Declaration (1964) and was approved by the National Medical Ethics Committee of Hungary (14415/2013/EKU – 183/2013 and DEOEC RKEB/IKEB 3094/2010) and the National Medical Ethics Committee of the Republic of Slovenia. Limbal tissue and CEC collection was done within 12 hours of biologic death from cadavers. Following enucleation, the bulbus was washed with 5% povidone iodine (Betadine; Egis Pharmaceuticals PLC, Budapest Hungary), then the conjunctiva was incised and separated from the limbal junction; consequently, a 2 x 1 mm rectangular-shaped limbal graft was dissected away and towards the cornea. The depth of the graft was kept superficial or within the epithelial layer. Multiple grafts were collected from a single eye and tested for growth potential. The graft dissection was performed using a lamellar knife placed tangential to the surface being cut. LESCs were cultured in a high-glucose Dulbecco-modified Eagle's medium (DMEM-HG, Sigma-Aldrich, Budapest, Hungary) supplemented with 20% human AB serum, 200 mM/mL L-glutamine, 10,000 U/mL penicillin- 10 mg/mL streptomycin (all from Sigma-Aldrich) at 37^{o}C, 5% CO_2, the medium being changed every alternate day. The growth of the cells was monitored under phase contrast microscope regularly. Only grafts which had cell outgrowth within 24 hours were processed further to decrease the chance of fibroblast contamination. Differentiated CECs were scraped from the central part of the cornea of cadavers and were used as a positive control.

FvERMs were obtained from patients (Mean Age: 62.7$\pm$9.0 years) undergoing vitrectomy due to intravitreal hemorrhage in PDR. After the isolation, fvERMs were transported and cultivated *ex vivo* under adherent conditions in L-glutamine containing DMEM:F12 (Sigma-Aldrich, Ljubljana, Slovenia) supplemented with 10% fetal calf serum (FCS), antibiotics and antimycotics (PAA Laboratories GmbH, Pasching, Austria) and kept until reaching confluence. For control experiments, hRPE cells were used, which were isolated from cadavers with mechanical isolation technique and cultivated *ex vivo*.

Surface protein expression analysis by flow cytometry

The phenotype of the isolated cells was determined by multicolor flow cytometry using FITC-, PE- and APC- conjugated antibodies with the matching isotype controls as well against CD29/Integrin β1, CD44/HCAM, CD45, CD54/ICAM1, CD73, CD90/Thy-1, CD117/c-kit and CD146/MCAM in the case of LESCs and CECs, CD11a/LFA-1, CD14, CD18/Integrin β2, CD29/Integrin β1, CD34, CD44/HCAM, CD47, CD49a, CD49d, CD51/Integrin αV, CD54, CD73, CD90/Thy-1, CD338/ABCG2, CD105, CD106/VCAM-1, CD166/ALCAM, PDGFRβ, HLA-G, CXCR4, CD146/MCAM, HLA-DR, VEGFR2, CD45, CD146, CD117 and CD31 were used in the case of fvERM outgrowing cells and hRPEs. Measurement of the samples was carried out with FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) and the data were analyzed using FlowJo (TreeStar, Ashland, OR, USA) software. The results were expressed as means of positive cells (%)$\pm$SD in the case of LESCs and CECs and means of positive cells (%)$\pm$SEM in the case of hRPE and fvERM, respectively. Hierarchical clustering was performed by the R software.

Microarray and data analysis

Total RNA from each analyzed sample was extracted by miRNeasy Mini Kit (QIAGEN, QIAGEN Inc.,Valencia, CA, USA) and quantified with NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity was confirmed with Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). For microarray-based gene expression profile analysis Affymetrix GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) was used. The array contained 28,869 gene transcripts. For the whole genome gene expression analysis 150 ng of total RNA was subjected to Ambion WT Expression Kit (Ambion, Life Technologies, Carlsbad, CA, USA) and GeneChip WT Terminal Labeling Kit (Affymetrix) according to the manufacturers' protocols. After washing, the arrays were stained using the FS-450 fluidics station (Affymetrix) and signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA, USA). The scanned images were processed using GeneChip Command Console Software (AGCC) (Affymetrix) and the CEL files were imported into Partek Genomics Suite software (Partek, Inc. MO, USA). Robust microarray analysis (RMA) was applied for normalization. Gene transcripts with a maximal signal values less than 32 across all arrays were removed to filter for low and non-expressed genes, reducing the number of gene transcripts to 23,190. Differentially expressed genes between groups were identified using one-way ANOVA analysis in Partek Genomics Suite Software. Clustering analysis was made using the same name module in a Partek Genomics Suite Software.

To identify and visualize the relationships between selected genes, the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) was used. The software modules provided all known functions and interactions which were published in the literature. ANOVA was used to calculate a p-value to determine the probability that each biologic function or canonical pathway assigned to the data set was due to chance alone.

Transmission electron microscopy

Human corneal tissue procurement and use were conducted in accordance with local regulations and approved by the Research Ethics Committee of King Saud University. Unless specified otherwise, reagents were obtained from TAAB Laboratories Equipment Ltd. (Aldermaston, UK). Pieces of LESCs grown on lens capsules were fixed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate for 2hrs at 4°C. Tissues were processed at low temperatures and were embedded in LR White resin (Sigma-Aldrich) at −20°C for 48hrs under ultraviolet light. Ultrathin sections were collected on 200 mesh formvar-coated carbon nickel grids and examined in a Jeol 1400 transmission electron microscope (Jeol Ltd, Tokyo, Japan).

Treatment and supernatant collection

The expanded fvERM cells were plated onto 6 well plates at a density of $2x10^5$ cells per well in triplicates. After 24hrs, the medium was changed and the cells were treated with 100ng/mL recombinant human TNFα (Preprotech, Rocky Hill, NJ, USA) for additional 24hrs. The cells were then collected for analysis of the expression of cell surface markers and their supernatants collected and centrifuged at 1400rpm for 10 minutes. The centrifuged supernatants were then pooled into one stock pre-treated by 0.025N hydrochloric acid for 15 minutes at room temperature. The secreted factors were consequently analyzed by Human Angiogenesis Array (Proteome Profiler, R&D Systems, Minneapolis, MN, USA) and ELISA.

LESCs were seeded on 24-well plates at a $5x10^4$ cell/mL density. Cells were cultured for 9 to 13 days. At the end of the culturing period, the supernatants were harvested and centrifuged at 1400rpm for 10 minutes to eliminate cellular debris from the samples. All the samples were then kept at -20°C until measurement took place.

Measurement of secreted cytokine concentrations by ELISA

BD OptEIA ELISA (BD Pharmingen, San Diego, CA, USA) assay kits were used following the supplier's instructions to measure the concentration of secreted IL-6 and IL-8 cytokines. Each experiment was performed at least three times and each sample was tested in triplicates. Statistically significant differences were determined by the paired Student's t-test.

Measurement of secreted angiogenesis related cytokines by Angiogenesis Protein array

The Proteome Profiler Human Angiogenesis Array Kit (R&D Systems) provides a platform for detection of 55 different angiogenesis related cytokine in one run. Measurements were carried out according to the manufacturer's protocol. The membranes were exposed to X-ray films and developed with KODAK Medical X-Ray Processor 101. Data analysis was performed according to the manufacturer's recommendation with ImageJ software.

Calcium dynamics in the fvERM outgrowing cells

The cultured fvERM outgrowing cells were loaded with acetoxymethyl (AM) ester of Fura-2 (Fura-2 AM; Invitrogen – Molecular Probes, Carlsbad, CA, USA), a free cytosolic calcium (Ca^{2+}) sensitive dye, which was dissolved in DMSO and suspended in 1.5 mL of culture medium (final working concentration: 8 μM). The Fura-2 AM loading was carried out at 37^{o}C, 5% CO_2 for 40 minutes. After loading, the cultures were washed twice for 7 minutes with 3 mL of the physiological saline with (in mM): NaCl (131.8), KCl (5), $MgCl_2$ (2), NaH_2PO_4 (0.5), $NaHCO_3$ (2), $CaCl_2$ (1.8), HEPES (10), glucose (10)), pH 7.24. The Petri dish was then mounted onto inverted microscope, Zeiss Axiovert S 100 (Carl Zeiss, AG, Oberkochen, Germany). To test responses to mechanical stimuli, the mechano-stimulation with a tip of a glass micropipette mounted on a MP-285 micromanipulator (Sutter, Novato, CA, USA) was used. Image acquisition was done with the 12-bit cooled CCD camera SensiCam (PCO Imaging AG, Kelheim, Germany). The software used for the acquisition was WinFluor (written by J. Dempster, University of Strathclyde, Glasgow, UK). Microscope objectives used were: 10x/ 0.30 Plan-NeoFluar and 63x/1.25 oil Plan-NeoFluar (Zeiss). The light source used was XBO-75W (Zeiss) Xe arc lamp. The excitation filters used, mounted on a Lambda LS-10 filter wheel (Sutter Instruments Co.) were 360 and 380 nm (Chroma). Excitation with the 360 nm filter (close to the Fura-2 isosbestic point) allowed observation of the cells' morphology and of the changes in the concentration of the dye, irrespective of changes in free cytosolic Ca^{2+} concentrations ($[Ca^{2+}]i$), while the 360/380 nm ratio allowed visualization of the $[Ca^{2+}]i$ changes in the cytoplasm. Image acquisition, timing and filterwheel operation were all controlled by WinFluor software via a PCI6229 interface card (National Instruments, Austin, TX, USA). Individual image frames were acquired every 500 ms resulting in frame cycles being 1 second long (two wavelengths).

Statistical analysis

Each experiment was performed at least three times and each sample was tested in triplicates. Statistica 7.0 software (StatSoft Inc., USA) was used for the statistical analyses. Statistically significant difference between the two groups (fvERM cells vs. primary hRPE) was determined with paired Student's t-test and a value of $p<0.05$ was considered significant. Data are expressed as mean$\pm$SD or SEM.

RESULTS

Gene expression profiling of LESCs and CECs

Comparing the transcriptional profiles of LESCs to differentiated CECs and based upon the intensity profiles of the $\log_2$ transformed signal values of the 28,869 transcripts, 955 and 875 transcripts were found to have a more than 2 fold change (FC) increase and decrease in expression between the two cell types, respectively ($n=3$, $p<0.01$). Based on the transcriptional data, the affected canonical pathways and biological functions were calculated, as well as top up- or down- regulated genes were identified with the IPA software. The top canonical pathways included genes involved in hepatic fibrosis, angiogenesis inhibition by thrombospondin 1 (TSP-1), retinoic acid receptor (RAR) activation, antigen presentation and axonal guidance signaling. Some of the signaling pathways were also related to diseases or toxicological pathways such as induction of reactive metabolites, renal ischemia and renal proliferation.

With further deep-analysis, more biological functions and diseases from the significantly changed expression levels of groups of genes were determined. 733 molecules were found to be involved in cancer development, 344 in reproductive system diseases, 282 in dermatological diseases, 402 in gastrointestinal diseases and 257 in endocrine system related disorders. Within the cellular functions' group of genes, 567 were involved in cellular growth and proliferation, 552 in cellular development, 520 in cell death, 381 in cellular movement and 290 in cell-to-cell signaling. In system development, the IPA software found 371 genes involved in organogenesis and 350 in tissue development. High number of genes belonged to the cardiovascular development (271) and tumor morphogenesis (140) group. Only a small number of molecules related to visual system development and function (98) could be detected. Furthermore, only 42 genes could be identified as related to clinical chemistry and hematology, 142 genes to be involved in cardiotoxicity, 205 in hepatotoxicity and 195 in nephrotoxicity, respectively**.**

Customized gene array data and gene networks – upstream regulators in LESCs

Groups from the significantly and differentially expressed upstream regulators in LESCs were generated, in particular, those which were extensively related to physiological maintenance of the cells. 257 genes collected and divided into subgroups of interest such as stemness and proliferation, differentiation, cytokines and growth factors, angiogenesis and cell adhesion. The genes that were mostly affected were involved in ion-, nucleotide- or protein binding, protein secretion as well as receptor or enzyme activities. Most of the upregulated genes were involved in ECM re-organization or ECM-to-cell connection such as *FN1* (Fibronectin 1 with 74.934 fold change), *MMP9* (type IV collagenase with 14.243 FC), *MMP1* (interstitial collagenase, 13.875 FC), *ICAM1* (Intercellular adhesion molecule 1, 13.681 FC) and *ITGA5* (fibronectin receptor with 13.455 FC respectively).

Stemness and proliferation

122 upstream regulators were found to be related to stemness and proliferation in LESCs, in particular, related to MSCs, the transcriptional pattern of the genes being able to distinguish between LESCs from CECs. These differential genes coded for transcriptional factors, surface molecules, cytokines and growth factors, all playing a key role in the maintenance of multipotency and proliferation capacity of stem cells. Up- and down-regulation was also found in 66 and 56 genes, respectively, and within the custom selected gene cluster. The 10 most highly expressed upstream regulators were: *CCNA1* (27.199 fold), *IL1B* (24.948*), GDF15* (16.924), *ICAM1* (13.681), *TGFB* (16.745), *SOX9* (4.859 fold), *VIM* (4.368), *NT5E* (4.009), *TGFBR2* (3.772) and *BMP6* (3.494), while the 10 most down-

regulated regulators were: *BMP7* (-6.436 fold), *LEF1* (-4.441), *GJA1* (-3.94), *KAT2B* (-3.829), *KLF4* (-3.041), *EGF* (-2.563), *FOXN1* (-2.11), *SOX6* (-1.984), *GDF9* (-1.865) and *HSPA9* (-1.838). The expression pattern of the selected genes confirmed that the LESCs have a higher proliferation potential and stemness-related gene expression than differentiated CECs. The SRY related HMG-box family members *SOX9* and *SOX6*, both involved in chondrogenesis and proliferation, were down-regulated in the LESCs.

Differentiation

This group of upstream regulators consisted of growth factors, cytokines, adhesion molecules, transcription regulators and enzymes. Transcriptional regulators such as *FOXA1* and *PMEL* were up-regulated in contrast to *FOXG1* (-1.165), *FOXD3* (-1.1), *MYOD1* and *OSGIN1* (-1.109) which were among the downregulated genes. Within the collection of cytokines and growth factors which play a role in epithelial differentiation, *IL1B* (24.948), *INHBA* (21.815), *IL1A* (7.853), *TGFB1* (6.745), *EREG* (3.836), *BMP6* (3.494) and *DKK1* (2.88) were up-regulated, while *BMP7* (-6.436 fold), *FGF1* (-2.96), *FGF7* (-1.473), *IL18* (-1.152) and *IGF2* (-1.126) were down-regulated

Cytokines and growth factors

Cytokines and growth factors have an important function in differentiation, maturation of cells, cell-cell communication, as well as controlling immunogenicity. Their expression level was different in LESCs compared to CECs. The most up-regulated gene in this group was *IL1B* (24.948 FC), followed by *CXCL10* (15.171), *IL1A* (7.853), *IL8* (5.849), *EDN1* (5.504), *IFNE* (4.601), *IL6* (2.57), *SPP1* (2.077) and *CCL5* (1.973). These genes code for pro-inflammatory cytokines, while other cytokine family genes which have similar pro-inflammatory properties, such as *IL17* (-1.129), IL-1 superfamily members *IL18* (-1.152) and *IL36RN* (-1.059), were down-regulated. Human *EDA* (-1.113) which belongs to the TNF family was within the most down-regulated genes, while the top down-regulated gene was *FAM3B* (-3.900). The detected genes in the growth factor subgroup were related to epidermal growth factor (EGF) and TGF beta (TGFβ) superfamily. In particular, *INHBA* (21.815 fold), *GDF15* (16.924), *TGFB1* (6.745) and *BMP6* (3.494) – all members of the TGFβ family, were among the most up-regulated genes. Other genes such as epiregulin - *EREG* (3.836), amphiregulin - *AREG* (4.047) and connective tissue growth factor *CTGF* (6.513) were in the top up-regulated genes as well, while other TGFβ superfamily members such as *BMP7* (-6.436) and *GDF9* (-1.865), acidic fibroblast growth factor, *FGF1* (-2.96) and *FGF7* (-1.473), were all down-regulated. Similarly, NOV-like CTGF- member of the CCN protein family: nephroblastoma overexpressed/*NOV* (-3.149) and the EGF (-2.563) gene expression responsible for regulation of cell division and proliferation were both down-regulated.

Cell adhesion

The upstream regulators of 54 genes coding for molecules involved in cell adhesion were analyzed. In the first subgroup, highly expressed transcriptional factors and transmembrane receptors were collected. This group contained *MAP2K* (12.088), *AKT3* (11.843), *TGFBI* (6.745), *CTGF* (6.513), *SPP1* (2.077), *CDH1* (1.536) and *SRC* (1.931) as up-, and *AKT1* (-1.026), *ROCK2* (-1.076), *PRKCA* (-1.154), *HRAS* (-1.5), *PRKCB* (-1.583) and *NOV* (-3.149) as down-regulated genes**.** *MMP9* (14.243), *MMP1* (13.875), and *MMP14* (1.836) were found to be the most up-regulated genes, while the other family member *MMP3* was down-regulated (-1.105). Laminins, which encode important proteins of the basal membrane, were all up-regulated - *LAMA3* (3.289), *LAMC1* (1.724) and *LAMA1* (1.428), in the LESCs. Within CAMs *ICAM-1* (13.681), *CAV1* (1.608) and *CLDN7* (3.056), while *GJA1* (-3.94) was down- regulated.

Angiogenesis

The fibronectin (*FN1)* gene, which is important in new vessel sprout formation, had the highest up-regulation (74.934), followed by *SERPINE1* (18.854) and *MMP9* (14.243) in this custom selected group. The coagulation factor III (thromboplastin) gene *F3* (7.054) was also highly expressed in the LESCs. The most down-regulated genes were *PLG* (-2.521), *TIMP1* (-1.658), *FOXO4* (-1.213), *TGFBR1* (-1.179). Certain cytokines and growth factors which are also important in angiogenesis were up-regulated in the LESCs: *ILB1* (24.948), C-X-C motif chemokine 10, *CXCL10* (15.171), *TGFB1* (6.745) and *VEGFA* (2.742). In addition, IL-6 and IL-8, two very potent angiogenic cytokines, were also up-regulated in these cells: *IL-6* (2.57) and *IL-8* (5.849), similar to *EDN1* (5.504), *EREG* (3.836) and *BMP2* (2.686). Only four of the angiogenic cytokines were down-regulated in the LESCs: acidic FGF - *FGF1* (-2.96), *IL17F* (-1.129), *TGFB2* (-1.106) and *KITLG* (-1.015).

IL-6 and IL-8 signaling pathway analysis by IPA

IL-6 and IL-8 are important cytokines not only in the inflammatory processes, but play a key role in maintaining stemness, differentiation and immunosuppression of stem cells, as well as epithelial differentiation and angiogenesis. Their gene expressions were up-regulated in LESCs: *IL-6* (2.570) and *IL-8* (5.849), respectively.

Secretion of IL-6 depends on many stimulatory signals; hallmark pathway is the response of activated cells to pro-inflammatory stimuli, such as IL-1β- and TNFα-mediated activation. This signaling is further mediated by the NFκB and JNK (JUN, C-Fos) transcriptional factors and can lead to IL-6 and IL-8 release in parallel to collagen type I production (COL1A1), which is the major component of connective tissue. The second network affected is the autocrine or IL-6-mediated-IL-6-secretion through RAF1, MAP2K and ERK1/2. This process needs to be initiated by the IL-6 receptor (IL6R), however, the JAK-STAT pathway (STAT3) can also induce release of angiogenic factors such as VEGF and activation of SOX3. As shown before in our dataset, *IL1B* was highly up-regulated with a 24.948 fold change hand-in-hand with its receptor *IL1R1* (13.972) and *IL1A* (7.853). Although a subunit of the receptor for IL-6 coding gene was down-regulated - *IL6R* (-2.640), a member of the type I cytokine receptor family - oncostatin M receptor (*OSMR*), was found to be highly up-regulated (15.366) in the LESCs. In our dataset, *SOCS3* was up-regulated (1.397), while *SOCS1* was down-regulated (-1.120). Four MAPKs were slightly up-regulated in the LESCs: *MAP2K1* (2.088), *MAPK1* (1.339), *MAPK14* (1.011) and *MAPK3* (1.163), while the members of the NFκB pathway were down-regulated: *NFKB1* (-1.178) and *NFKBIA* (-1.193). CXCL10 in presence of high amount of IL-6 has been shown to induce migration of trophoblasts through activation of the CXCR3 receptor. Interestingly, *CXCL10* was among the highest up-regulated genes (15.171) in the LESCs compared to the CECs.

The pathways in which IL-8 participates are in general more complex than for other cytokines, due to its multipotent effect on the different cell types. IL-8 can act as chemotactic factor for neutrophils, can activate immune cells, stem cells and has been also described as potent pro-angiogenic cytokine, especially in the eye. The molecular background of such angiogenic processes has not been well described, but IL-8 seems to be an important activator of circulating EPCs with myeloid origin. IL-8 can bind to G protein-coupled serpentine receptors such as CXCR1 and CXCR2 and beside immunological activation, it can induce rearrangement of cytoskeletal proteins, increase the expression of VCAM and ICAM1, and the migration as well as vessel formation of endothelial cells and stem cell-like EPCs, in parallel with increase in vascular permeability.

IL-6 and IL-8 secretion by LESCs

The level of IL-6 and IL-8 cytokines in the LESC culture supernatants were measured by ELISA and were continuously high and correlated well with the gene expression data. At day 9, the level of IL-6 was 5885.24±685.6 pg/mL (Data are Mean±SEM), and at day 13 it was 6147.14±530.21 pg/mL, with no significant difference at both time points (p=0.778). The same pattern could be observed in the IL-8 levels, 6665.00±312.41 pg/mL could be detected at day 9 and 7429.52±352.43 pg/mL at day 13, without significant difference between the two (p=0.14).

Phenotype analysis of LESCs and CECs

Genes coding MSC-related surface markers were measured at the protein level as well. Interestingly, no difference was found in the positivity for CD90 and CD73 between the two cell types. In the LESC cell cultures, more cells expressed the stem cell factor receptor CD117/c-kit compared to the CECs. CAMs and integrins showed more difference between the two cell types. At a protein level, CD146/MCAM was found not to be expressed on the surface of CECs compared to LESCs and higher number of positive cells for ICAM1 in CECs than in LESCs could be observed. No difference could be found between LESCs and CECs in the expression of CD29/Integrin β1 and CD44/HCAM. The percentage of CD47 positive cells, which plays a role in cell viability and immunoregulation, was significantly higher in LESCs compared to CECs, showing higher viability and inhibition of phagocytosis in the LESCs. Cluster analysis of the results showed different surface protein pattern in LESCs cultures compared to CECs.

Surface marker expression of fvERM and hRPE

The fvERM outgrowing cells assumed an elongated, fibroblastoid-like morphology when cultivated under adherent conditions *ex vivo*. They showed no purely common hematopoietic or monocyte phenotype. Similarly, these cells expressed no CD45, CD11a (LFA-1) and HLA-G, like the primary hRPE cells (an exception being the very low CD11a expression in one of the hRPE donors). Higher percentage of the primary hRPE cells were positive for CD14 compared to the fvERM, while inversely, higher CD47 expression was observed on the fvERM than the primary hRPE cells. Both cell types had a low surface expression of HLA-DR, while the percentage of CD117/c-kit, CXCR4 and CD338/ABCG2 cells was, in general, lower in the fvERM compared to the primary hRPE, respectively. Only the expression of CD34 was more abundant in the fvERM cultures compared to the primary hRPE, however, this difference was not statistically significant. Similarly, the expression of CD73, CD105 and PDGFRβ was not significantly different between the fvERM cells and the primary hRPEs, while a significant difference in the CD90 expression was measured between the two cell types. Lower expression of CD18/Integrin β2 (p=0.01) and CD51/Integrin αV (p= 0.004) was found on fvERM cells compared to primary hRPE cells. The expression of CD29/integrin β1, CD49a/integrin α1, CD49d/ integrin α4, CD54/Intercellular adhesion molecule 1 (ICAM-1), CD106/V-CAM 1, CD146/MCAM and CD166/ALCAM on fvERM cells were similar to those detected on primary hRPEs.

Detection of angiogenic factors secreted by the fvERM outgrowing cells

Altogether 55 angiogenesis-related markers were screened from the supernatants of the fvERM outgrowing cells under presence or absence of TNFα treatment. Unstimulated fvERM cells expressed high amount of serine protease inhibitor E1 (Serpin E1), also known as endothelial plasminogen activator inhibitor 1 (PAI-1) and tissue inhibitor of metalloproteinase 1 (TIMP-1). After 24 hours of pro-inflammatory stimulation by TNFα, both factors increased further in the supernatants of the treated compared to the control untreated cells. More

importantly, 11 secreted angiogenesis-related factors could be detected in the cell culture media of fvERM cells treated by TNFα that were otherwise absent in the untreated controls. Besides Serpine E1, the anti-angiogenic and tumorigenic pigment epithelium-derived factor (PEDF, also known as Serpin F1) was induced and secreted. Endothelin 1 (ET-1), a molecule that has been implicated in the development and progression of vascular disorders and usually secreted by endothelial cells upon stimulation by pro-inflammatory cytokines or hypoxia, could also be detected upon TNFα treatment. In addition, TNFα stimulation caused expression of pentraxin-related protein 3 (PTX3), monocyte chemotactic protein-1 (MCP-1 or CCL2;), IL-8 (also referred as CXCL8), thrombospondin 1 (TSP-1), endocrine-gland-derived vascular endothelial growth factor (EG-VEGF)/prokineticin (PK), dipeptidyl peptidase-4 **(**DPPIV, also known as CD26), and matrix metallopeptidase 9 (MMP-9). Two members of the insulin-like growth factor-binding proteins (IGFBPs) appeared upon the TNFα pro-inflammatory stimulus: IGFBP-2 and IGFBP-3 while it was absent in the supernatants from the control fvERM outgrowing cells.

Functionality and viability of the fvERM outgrowing cells

The dynamics of $[Ca2^{+}]_i$ upon mechanical stimulation reflects well upon the functionality and viability of the outgrowing fvERM outgrowing cells, and such mechanical tractional forces can be common in fvERMs in late stages of PDR. Mechano-stimulation was induced by a glass micropipette applied to a single cell, which caused and intracellular calcium propagation that could be followed from the cell body to the periphery. The fvERM outgrowing cells responded to mechano-stimulation by increasing their $[Ca2^{+}]_i$ in a monophasic manner.

DISCUSSION

The importance of the LESCs in corneal biology and regeneration is well known, the absence or removal of which can cause defective corneal regeneration. In humans, several diseases are known with acquired or genetic loss of LESCs. Inflammatory processes with uncontrolled neovascularization very likely change the environment in such a way, that the small niches of stem cells become non-functional, which process leads to decreased epithelial differentiation and transparency of the cornea, resulting in a serious and painful disease with subsequent loss of vision. The treatment of LESCD is based upon the replacement of stem or progenitor cells in the limbus, with the more appropriate *ex vivo* culturing and proof-of-functionality of the LESCs becoming widely accepted today. Other cells such as embryonic stem cells, bone-marrow and Wharton jelly-derived stem cells have been attempted for LESCD treatment in animal models with relatively good outcomes as well. Although the overall success rate of the cell therapies has been reported to be 76% with replacement of corneal epithelial cells, the recovery of stemness is still unknown. According to the new directives and guidelines for quality, safety and efficacy of advanced-therapy medicinal products (ATMPs), animal material-free methods would be more plausible and accurate way to go, however, the right quantity and phenotype of the cells needed for it has not been determined yet. The latter is crucial in stem cell-based therapies, since the purity of the product (the percentage of stem cells within the cell culture) determines the outcome.

In the maintance of stemness, the SRY-related HMG-box family member SOX plays important role. In our LESC cultures, SOX9 expression was up-regulated, while SOX6 was down-regulated, indicating no chondrogenic differentiation but high proliferative capacity of the LESCs. Similarly, Wnt2, Wnt6, Wnt11 and Wnt16b have been reported to be typically expressed in the limbal region and to be important for the LESCs proliferation. We could confirm that the expression of *WNT1* and *WNT5A* was up-, while *WNT3A* was down-regulated in our LESCs, along the wider lines of the results mentioned above. The limbal epithelial crypt cells express S100A4 and A9 proteins; our LESCs, the *S100A4* was down-regulated, indicating they are not crypt cells. We could not confirm a difference between the LESCs and differentiated CECs in the other known or putative LESC markers, such as *CXCL12*, *COL2A*, *ISL1*, *FOXA2*, *NCAM1*, *ACAN*, *GJB1* and *MSX1*. In our dataset, only *FOXA1* was up-regulated and *GJA1* was down-regulated, the latter also being known as negative marker for LESCs. At protein level, the LESCs were different from the CECs in their higher positivity for CD146/MCAM, CD47 and CD117/c-kit, showing a pattern typical for stem cells and higher multipotency. The phenotype analysis demonstrated that the classical MSC markers, such as CD90/Thy-1 and CD73 are not good enough to differentiate between LESCs and CECs. The CAMs and integrins are important for attachment to ECM proteins, and their expression pattern is typical for the tissue of origin. Indeed, the integrin expression is able to define the cell phenotype and seems to be useful in classifying MSCs from various tissues besides the well-known MSC markers we have reported before. The results of our gene array data analysis strengthen the fact that the LESCs cultured in medium containing human serum as the only growth supplement can keep their integrin and CAM pattern that relates them to their limbal tissue phenotype.

Not only the attachment, but the ECM breakdown is important in the initial steps of tissue healing and remodeling MMPs play a key role in this process, which were up-regulated in the LESCs, and their pattern implicates a preferred degradation of collagens to rebuild the ECM. Along the upregulated MMP genes, the limbal basal membrane's laminin and vitronectin and their genes were also up-regulated in the LESCs. Wound healing can often lead into angiogenesis, which can have a very important and controllable pathological role in the limbus. Fibronectin plays a key role in the *de novo* formation of new vessels, sprouting

and differentiation of vessel wall resident and endothelial progenitor/stem cells into endothelial cells. Beside the most upregulated fibronectin gene, several possible members of angiogenesis were also detected.

The two highest up-regulated gene products found in our LESCs seem to have an opposite effect on the angiogenesis pathway: IL-1β can induce-, while CXCL10 can inhibit the formation of new vessels. Furthermore, CXCL10 has been found to be expressed by human limbal epithelial progenitor cells, while its absence decreased the level of IL-6 in mice corneas. During wound healing, *TGFB1* is highly expressed and it can induce *VEGF* expression which was also up-regulated in the LESCs, capable of provoking angiogenesis in a damaged tissue. Similar to the VEGF, ET-1 has many direct and indirect angiogenic effects. IL-6 and IL-8 play a role in inflammation, angiogenesis and MSC differentiation. Our results indicate that both pro- and anti-angiogenic genes are expressed at the same time or in a balanced fashion in LESCs, maintaining an avascular state in the normal cornea. Loss of this control can be initiated by either a decreased production of anti-angiogenic molecules or increased production of pro-angiogenic and inflammatory factors. Although transplantation of LESCs has been known to suppress corneal inflammation and angiogenesis, the molecular mechanism how LESCs participate in the processes has not yet been fully understood. Limbal niche cells have been found to have a differentiating ability towards angiogenic progenitors and inhibition of vascular endothelial differentiation of LESCs.

Overall, our gene selection and networks are somewhat different from the well-known canonical pathways described so far, because they were generated *de novo* and were based on own and already published data from networks in the literature. It remains to be further investigated and confirmed whether these pathways reflect in the same manner at protein level both *ex vivo* and *in situ*, thus providing an alternate or more specific way to select for LESCs and, at the same time, standardize the cell cultures used as ATMP in the near future.

Neovascularization is a key mediator of corneal diseases, but it is also an important player in PDR. Many cell types have been shown to have a direct or indirect function in angiogenesis. In the retina, the EPCs have been described as key members of retinal neovascularization formation as well as the pathology of DR. Nevertheless, the real phenotype of the cells found in fvERMs and capable of growing out of the membranes is not clear; in particular, it is not known what type of cells initiate angiogenesis in PDR. The EPCs express hematopoietic stem cell- and monocyte- surface markers such as CD31, CD34, CD45, CD14 and VEGFR2. Our *ex vivo* cultured fvERM and primary hRPE cells did not show an EPC - or retinal endothelial cell phenotype, although they expressed CD14, CD31 and CD34 to certain levels. Interestingly, the surface marker expression pattern of the primary hRPE cells appeared to be the closest to an EPC phenotype, although no CD45 positivity could be detected on their surface, meaning no hematopoietic origin of these cells. The CD117/c-kit marker is characteristic of retinal progenitor cells and angiogenic cells, which to a certain extent was the case with our primary hRPE, but it was absent from the fvERM outgrowing cells. CD73, CD90 and CD105 are known as putative MSC (fibroblast-like) markers; while CD73 and CD90 were present on both cell types, CD105 did not reach a sufficient expression level to qualify the cells as MSCs. CD73 has been described as a retinal photoreceptor progenitor marker in mice and found on human RPE cells as well. ABCG2, often referred as a stem cells- and retinal progenitor cells marker, was expressed at a low level on the fvERM outgrowing cells, while it was much higher on the primary hRPE cells. The immune status of the cells is also very important - no HLA-G expression of this immunosuppressive marker could be detected on both cell types, while HLA-DR expression was very low on these cells.

CAMs and integrins play a key role in the cell-cell and cell-ECM contacts during immune response. In PDR, the cellular and soluble forms of ICAM-1 have been frequently shown in epiretinal membranes, serum or vitreous and diabetic fibrovascular membranes of

patients. Our fvERM outgrowing cells increased the ICAM-1 expression upon TNFα pro-inflammatory stimulus, similar to the primary hRPE cells, meaning that these cells function as activated epithelial cells for leukocyte adhesion. CD11a (LFA-1) interacts to ICAM-1 and plays a key role in lymphocyte co-stimulation. No CD11a (LFA-1) positivity was observed in both cell types under basal conditions. Previously, CD29/ integrin β1, CD51/integrin αV and CD44/HCAM have been described to be present on the surface of hRPE cells, but we detected more positivity for integrin α1 and integrin α2 in the primary hRPE cell cultures compared to the studies published to date. The integrin pattern of our cells differed in their CD18/integrin β2 and CD51/integrin αV expression. The fvERM outgrowing cells expressed low levels of the α-subunit-containing integrins, besides a reported expression of integrin α4 in DR. Interestingly; the presence of integrin β2 subunit has been considered an important factor in RPE-T cell interactions.

The CD146 expression on fvERM outgrowing- or primary hRPE cells was described the first time by our group. The cells expressed low levels of CD146 of this generally considered MSC marker, but also a novel endothelial biomarker, which plays an essential role in the angiogenesis by interacting directly with VEGFR2 found on endothelial cells. In addition, CD146 has been accepted as a marker of a new EPC subset as well. Although no data exists about positivity of fvERM outgrowing- and primary hRPE cells for CD166, cancer-, stem- and retinal endothelial- cells have been shown to express it.

The PDGF receptor and its subunits have already been detected on RPE cells and investigated in ERMs of patients with proliferative vitreoretinopathy (PVR). The PDGFRβ subunit has been found to be less frequent than the PDGFRα subunit. The PDGF produced by hRPE cells can act as autocrine growth factor and influence wound healing and migration of these cells towards wounds; the interaction of the ligand with its receptor seems to be exaggerated during wound repair and, therefore, ERM formation. Indeed, cells isolated from ERMs removed during vitrectomy for PVR show expression of PDGF receptor and are usually identified as RPE cells.

The presence of TNFα changed the CXCR4 expression in fvERM outgrowing cells, similar to how it acts upon HUVEC or Langerhans cells. IL-1β or TNF-α can increase the CXCR4 mRNA expression in RPE cells, which indicates that fvERM outgrowing cells are not the migrating cells in DR in response to increased local SDF1α, nor that cell migration occurs via the well-known SDF1α-CXCR4 axis. VEGFR2 plays a crucial role in endothelial differentiation of progenitors mediated by pro-angiogenic or inflammatory signals. We investigated the possible expression of VEGFR2 on fvERM outgrowing cells upon stimulation with TNFα and found no expression of this receptor under such treatment. Despite this finding, cells that are not directly involved in the angiogenesis can secrete pro- or anti-angiogenic factors during cell differentiation to either support- or inhibit- vessel formation in their microenvironment, respectively. In PDR the presence of TNFα in the vitreous is an important marker of the disease.

In vitro, the fvERM cells secreted MCP-1/CCL-2 and IL-8/CXCL-8 upon TNFα treatment - phenomenon which has been observed in ARPE -19 cells and primary hRPE cells also. Besides TNFα, IL-1β and co-culturing with T lymphocytes have all been shown to induce IL-6, IL-8 and MCP-1 secretion in ARPE-19 cells in addition, TLR3 ligands have been shown to have the same effect on hRPE cells Furthermore, MCP-1 has been detected in the aqueous humor of patients with diabetic retinopathy and its level was higher in a rodent model of this disease. MMPs play a key role in the first stage of cell migration, connective tissue remodeling and degradation of basal lamina and surrounding extracellular matrix (ECM) during neovascularization. MMP-9 appeared, while TIMP-1 levels increased upon TNFα treatment of fvERM outgrowing cells. Although the MMP-9 level has been previously determined in PDR, nothing is known about its level in healthy retina. TIMP-1 and TIMP-2

have both been measured in the vitreous of patients with PDR - increased levels of TIMP-1 have been associated with the disease. The fvERM outgrowing cells expressed and secreted a basal level of TIMP-1, probably serving as a counter-coup to angiogenesis via inhibiting MMPs. PTX3 has many immunological activities in innate immunity, mainly in the inhibition of the uptake of apoptotic cells by antigen presenting cells (APCs). TNFα could induce PTX3 in ARPE-19 cells, but no such data have been shown in primary hRPE and fvERM outgrowing cells. EG-VEGF/PK-1 is a novel selective angiogenic mitogen with a special pro-survival effect in different cell types, including endothelial cells, dendritic cells (DCs), monocytes and neutrophils; no reports have been made about its presence in primary hRPE and fvERM outgrowing cells. Interestingly, PK1 and PK2 have been shown to induce monocyte differentiation and activation, suggesting an immunomodulatory function at local sites of inflammation. Hypoxic conditions stimulate Serpin E1/PAI-1 by hRPE. Serpin E1/PAI-1 is a principal inhibitor of tissue plasminogen activator (tPA) and urokinase. PAI-1 could facilitate retinal angiogenesis in a model of oxygen-induced retinopathy. Serpin F1/PEDF plays a very important anti-angiogenic and anti-oxidant role in the eye. It has been found in the vitreous and serum of patients with PDR. IGFBPs have been detected in the vitreous of patients suffering from neovascular ocular diseases. In particular, IGFBP-2 and IGFBP-3 appear to be potent inhibitors of IGF-I and IGF-II activity in Muller cells and inhibitors of cell growth of retinal endothelial cells. Overexpression of ET-1 can lead to retinal edema, degeneration and neuronal death in animal models. Dipeptidyl peptidase-4 (DPP4) is an important player in glucose metabolism, but it has also been described for its role in immunoregulation and apoptosis. Thrombospondin-1 (TSP-1) is a natural inhibitor of angiogenesis. High levels of TSP-1 have been detected in drusen found in early stage age-related macular degeneration. The existence of TSP-1 in the vitreous has been controversial and sometimes reported as present or absent in PDR patients.

So far, the origin of the cells found in the fvERMs has not been well understood. There is also lack of knowledge regarding the fvERMs cells specific features, including the Ca^{2+}-signaling pathways. The knowledge of Ca^{2+} dynamics is important toward understanding cell biology. Mechanical force modulates a wide array of cell physiological processes. Cells from diverse tissues detect mechanical load signals by similar mechanisms, but respond differently. The diversity of responses reflects the genotype of the cell and the mechanical demands of the resident tissue. It has been shown that hyperglycemia alters the tight control of $[Ca2^{+}]_i$ dynamics in retinal cells and may lead to the development of DR. Calcium signaling upon mechanical stimulation has been previously demonstrated in human corneal endothelial-, RPE- cells, rat retinal astrocytes and Müller cells. Mechanosensory function of Müller cells in the retina upon application of tractional force to the living retina showed that Müller cells respond to retinal stretch both with fast changes, as evidenced by transient $[Ca2^{+}]_i$ increases, and with slower changes in protein expression. A model for Ca^{2+} waves in networks of glial cells has been studied which confirms the importance of Ca^{2+} signaling measurements. It has also been shown that calcium signaling mediates mechanically induced human MSC proliferation. The calcium signaling has been also recognized as a regulator of hematopoiesis - the application of shear stress to hematopoietic progenitor cells has been shown to stimulate hematopoiesis. Store-operated Ca^{2+} entry has been shown to be expressed in human endothelial progenitor cells. $[Ca^{2+}]_i$ stores are essential for injury-induced Ca^{2+} signaling and re-endothelialization.

An unwanted consequence of the appearance of fibrovascular membranes in PDR are the tractional forces these membranes can impose upon the retina, which can eventually lead to retinal detachment. Mechanical stimulation of the cells in the membranes or the retina can result in $[Ca^{2+}]_i$ changes that can propagate through the cells as intercellular waves. Generation of such calcium waves occurs by the release of calcium from internal stores. The

waves do not evoke changes in the cell membrane potential, but may constitute a pathway for extraneuronal signaling. Along that line, isolated retinal ganglion cells can respond directly to mechanical deformation with pannexin-mediated ATP release and auto-stimulation of the $P2X_7$ receptors. Furthermore, stimulation of RPE cells with mechanical stress can upregulate MMP-2 and fibronectin (FN) expression through activation of the p38 pathway.
Overall, a method for isolating and cultivating cells coming from fvERMs from PDR could be established and characterization of their origin as mixed or not pure hematopoietic, mesenchymal or epithelial could be achieved. The fvERM outgrowing cells had a clear proliferative potential, were capable of releasing pro-inflammatory and angiogenic molecules upon stimulation with TNFα and showed functionality and viability reflected through calcium dynamics upon mechano-stimulation, which can serve as a study model for tractional forces present in fvERMs in PDR ex vivo.

ACKNOWLEDGEMENT

I would like to thank my supervisor, ***Dr.med.habil. Goran Petrovski,*** for his continuous support, excellent professional mentorship and inspiration during my work and in my personal life. I am grateful for the opportunity to train and work in his team and his faith in me from the first time when we met. After many unsuccessful hard years, he showed to me the professionalism and prosperous research style and switched me back to the right track of science.

I also want to thank ***Professor Andrea Facskó, MD, PhD***, Head of the Department of Ophthalmology (University of Szeged) for giving me the chance to work in her department of scientific excellence.

Special thanks go to ***Professor László Fésüs, MD, PhD, MHASc***, former Head of the Department of Biochemistry and Molecular Biology (University of Debrecen, MHSC) for the pleasure and priviledge of working in his department. I am grateful for his support and guidance.

I would like to thank all the past and current members of the department and the Stem Cells and Eye Research Laboratory for their help and kindness. Special thanks go to ***Irén Mező***, ***Dr. Réka Albert***, ***Mária Tóth*** and ***Zoltán Doró*** for the excellent technical and laboratory assistance, and the friendly environment as well. I am thankful to our collaborators for their excellent work and helping me during the co-operations.

And last, but not the least, I am sincerely grateful to my whole family and to all of my dear friends helping me get through the difficult times, and for their patience, understanding, emotional support and encouragement during my PhD years.