SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Cultivation and characterization of adult stem cells from the human eye – relevance to physiology and disease

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Supervisor: Dr. Goran Petrovski MD, PhD

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF CLINICAL MEDICINE

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The Examination takes place at the library of Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Debrecen

at 11 a.m. on September 5, 2014

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen

at 1 p.m. on September 5, 2014

1. Introduction

Vision and visual perception are complex systems connecting the outside world to the visual cortex of the brain. Total of 285 million visually impaired people live worldwide (2012), out of which 39 million are blind. Despite concerted efforts, many diseases that impair vision have no definitive treatment yet.

The human eye is structured like a miniature camera placed in the anterior half of the orbital cavity: eyelids (shutter); pupil (aperture); cornea and lens (focusing); retina (film).

The human eye is composed of three concentric coats: 1) fibrous sclera and transparent cornea, 2) vascular coat consisting of iris, ciliary body (CB) and choroid, and 3) retina.

The cornea is a clear, dome-shaped tissue of the anterior part of the outer fibrous layer. It consists of 6 layers: cornea epithelium, Bowman's membrane, stroma, Dua's layer, Descemet's membrane and endothelium. The corneal epithelium is a stratified non-keratinized squamous epithelium which acts as a dynamic physical barrier for agents that are potentially harmful to the intraocular structures. The corneal stroma accounts for about 90% of the corneal thickness and is composed of several layers of collagen fibers giving the cornea transparency. Besides keratocytes which produce collagen and hydrated proteoglycans, fibroblasts, mesenchymal stem cells (MSCs) and immune cells can be found among the collagen layers. The corneal endothelial cells form a monolayer and help maintain corneal transparency by regulating hydration and nutrition of the cornea. The transparency of the cornea is due to its uniform structure, avascularity and appropriate function of endothelial cells.

The middle vascular layer of the eye is the uvea, which consists of the iris, CB and choroid. It is responsible for the blood supply of the retina. The CB and iris have similar structure: both consist of pigmented and non-pigmented epithelial cell layers which represent an anterior extension of the neuroretina and retinal pigment epithelium (RPE), as well as a stroma which contains vessels and muscles. The non-pigmented epithelial cells of the CB are responsible for producing the aqueous humor.

The lens is a biconvex, transparent structure with adjustable refraction of 3-20 Diopters.

The innermost layer of the eyeball is the retina which has a light sensitive and transducer function. It extends anteriorly to the CB, ending into a ragged edge called ora serrata. The retina consists of 10 layers out of which the inner 9 layers are known as neuroretina. The outermost layer is the RPE, a single layer of highly pigmented cells. Together with the underlying Bruch's membrane, this layer forms the blood-retina barrier. Furthermore, the

RPE plays a key role in supporting the photoreceptors' function by regulating the amount of light reaching them, as well as the rate of phagocytosis of photoreceptor outer segments, vitamin A turnover and secretion of growth factors and cytokines. The photoreceptor layer contains rods and cones. Müller cells which are found in the inner nuclear layer are the principal glial cells in the retina and are essential for the structural support and maintenance of balanced extracellular environment.

The choroid has 3 layers of blood vessels – with increasing lumen thickness dependent upon their depth.

Developmentally, the eye is an organ in which an architectural interplay of neural tube ectoderm, surface ectoderm, neural crest and mesoderm form "machinery" for achieving complex visual response.

One of the key molecular regulators in eye development is a member of the paired box (PAX) family, PAX6. Sonic hedgehog (SHH) expression induces the separation of the two optic vesicles; furthermore, it upregulates PAX2 and downregulates PAX6 genes in the centre of the eye field that leads to the development of the optic stalk. PAX6 plays role in the lens formation together with other transcription factors such as bone morphogenetic protein 4(BMP-4) (secreted by the optic vesicle), sex determining region Y-box 2 (SOX2) and neural retina leucine zipper (LMAF). Optic cup formation is regulated by fibroblast growth factors (FGF) and transforming growth factor β (TGFβ) beside other transcription factors like microphthalmia-associated transcription factor (MITF) and ceh-10 homeo domain containing homolog (CHX10).

Development of the cornea requires specific gene regulatory networks in which many transcription factors and molecular signals are involved. Detailed developmental networks are still not well defined.

Neuroprogenitor cells (NPCs) express a unique pattern of transcription factors due to their origin and developmental fate. The retinal progenitor cells (RPCs) can be further specified according to their final cell type.

In general, stem cells are unspecialized cells that have self-renewal capacity maintained by asymmetric division, forming a pool of cells and daughter progeny differentiating into special cell types. Three types of stem cells can be distinguished altogether: embryonic stem cells (ESCs), so-called induced pluripotent stem cells (iPSCs) and adult/progenitor stem cells (PCs). The PCs are multi-, oligo- or unipotent and have limited proliferation capacity. Although stem cell biology has improved greatly in the last decade, its translation to the clinic

is a much slower process. Only a few stem cell therapies are now available due to the several unsolved, yet important issues needed to develop effective stem-cell based therapies.

PCs can be found in many organs of the human body, such as bone marrow, skeletal muscle, heart, brain, skin and eye. They can only differentiate to the certain cell types of the organ/tissue they reside and can be cultured *in vitro*, but only for a limited time due to their limited proliferative capacity. The use of PCs is relatively safer thanks to the possibility of patient-specific autologous transplantation and the availability of ethical alternative sources.

PCs have been described in different parts of the eye, including the corneal epithelium and the retina. They exist in low numbers in so-called "stem cell niches" where they support tissue and organ turnover throughout life. Depending on the tissue origin they can be highly active (limbus) or relatively quiescent (retina).

Corneal epithelial cells are renewed or regenerated from the limbal epithelial stem cells (LESCs), while retinal injuries can lead to permanent damage. Therefore, the retina is considered to have limited regenerative capacity, although there is evidence for presence of PCs in the retina as well.

LESCs

The most successful stem-cell-based therapy so far is the use of LESCs to regenerate the corneal epithelium. The limbus is approximately 1.5-2 mm wide rim where the cornea becomes continuous with the sclera. It consists of crypts and so-called Vogt-palisades. Corneal epithelium is maintained and renewed from the limbus. Histological sectioning of the corneo-scleral junction confirmed the presence of PCs at the basal layer of the crypts. It is believed that LESCs give rise to transient amplifying cells (TACs) due to asymmetric division. TACs can further divide and their daughter cells become terminally differentiated while they migrate centrally and superficially until they finally shed off. Only limited proliferative capacity of corneal epithelium remains in the absence of limbus, while some groups have shown evidence for presence of oligopotent cells at the central cornea in mice.

Unfortunately, there are no definitive markers for identifying PCs in general or specifically in corneal stem cells. The presence or absence of various morphological features (small cell size, high nucleus/cytoplasm ratio), specific protein and gene expression pattern are used in combination to identify and distinguish stem cells from differentiated cells. Establishment of a comprehensive panel of these characteristics would let researchers compare their data and specify better the subpopulations of PCs.

LESC Deficiency (LESCD)

LESCD is a disease in which LESCs are lost or damaged due to hereditary, acquired or sometimes unknown (idiopathic) diseases. The most common genetic background associated with LESCD is aniridia, while common acquired causes include chemical and thermal burns, inflammatory diseases (such as Stevens-Johnson syndrome, ocular cicatricial pemphigoid and chronic limbitis), contact lens-associated toxicity or mechanical irritation and extensive cryotherapy, radiation or surgery. Definitive treatment requires replacement by healthy LESCs. Limbal allo-and autografts can be collected and transplanted, but due to lack of corneal donor tissues and decreased chance for graft survival, an autologous or homologous expansion of human LESCs has been proposed.

Although cell therapies should ideally be animal material-free, most standard protocols for culturing LESCs still use FCS, exogenous growth factors, hormones or cholera toxin in the growth media and/or mouse 3T3 fibroblasts as a feeder layer. The use of animal material carries a risk of transferring prions or as-of-yet unknown diseases.

Cultured cells can be transplanted with the help of carriers, such as synthetic sources like temperature responsive polymers, chitosan-gelatin biopolymer, contact lens and compressed collagen, to natural materials as fibrin matrix, human amniotic membrane (HAM) and human lens capsule (LC).

Most of the clinical case reports use intact or denuded HAM. It is thought to inhibit conjunctival overgrowth and provide a good substrate for normal epithelial migration, while having the advantage of containing growth, anti-angiogenic and anti-inflammatory factors that can prevent or decrease fibrosis in the healing tissue. The main disadvantage of using HAM is its lack of optical transparency.

Retinal Stem Cells

In humans, the neuroretina and other parts of the central nervous system (CNS) are considered to have limited capacity for regeneration once retinogenesis/neurogenesis is completed. Despite that, many attempts have been made to find NPCs in adult human retinas. CB epithelium (CBE) has been considered as one of the primary niches for NPCs based upon the fact that the neuroretina and CBE develop from the same neuroectoderm, and lower vertebrates have robust renewal and regenerative capacity within the retina. Stem cells at the ciliary marginal zone (CMZ) persist throughout lifetime of these animals giving rise to both neural retinal cells and RPE cells in these species. Generation of new retinal neurons at the retinal periphery (RP) in chicken has been shown after one month of hatching.

NPCs are present in the CBE (equal to the CMZ) during retinogenesis in humans, and provided they are present in the adult mammalian eyes, they should be able to react to retinal injuries and proliferate and migrate to the affected area. Due to the lack of definitive markers for NPCs, the combination of morphological analysis, stem and mature cell marker detection either at gene or protein level and differentiation studies should be used to confirm the NPC phenotype. Stem cells are thought to exist in niches where their properties are carefully regulated by their protective environment *in vivo*. Spheres are referred to "*in vitro* niches" which provide different stimuli and cues to the cells therein. NPCs are able to form spheres from singe cell suspension in media containing mitogens (neurosphere formation assay), while continued formation of neurospheres over many passages confirms their self-renewal and inducible proliferative capacity. Capability to differentiate into mature retinal cells is also required to fulfill the criteria of retinal stem cells. Furthermore, the desired tissue-specific function of the differentiated cells is crucial for the successful stem cell-based cell therapy.

Degenerative Retinal Diseases

Millions of people worldwide are affected by degenerative retinal diseases with varying degrees of irreversible vision loss. These diseases form a very diverse and large group (e.g. AMD, retinitis pigmentosa, glaucoma and retinal detachment (RD)) with progressive damage and eventual death of the retinal cells.

RD can appear due to rhegmatogenous and non-rhegmatogenous causes. Full-thickness defect in the neuroretina allows fluid to move through a hole or a break and separate the neuroretina from the RPE, leading to rhegmatogenous RD. Contraction of vitreoretinal membranes in absence of a retinal break(s) is the cause of tractional RD; alternatively, when RD involves separation of the retinal layers by subretinal fluid derived from vessels of the neuroretina or choroid, both types of RD have been collectively called exudative RD. Bleeding within the eye, increased inflammation due to trauma and previous RD surgery increase the risk of proliferative vitreoretinopathy (PVR). Early detection and treatment of RD can prevent PVR from occurring, so regular eyes can play an important preventative role. Signs of presence of NPCs would be expected in PVR, due to the damage of retinal cells.

2. Aims of the Studies

- To isolate cells derived from the stem cell niches of the human corneal limbus.
- To culture and expand the LESCs in media containing human serum as the only growth supplement.
- To characterize the *ex vivo* expanded human corneal limbus-derived cells by morphological analysis (light microscopy, histology) and molecular biology techniques (transcriptional profiling, flow cytometry, immunofluorescent staining, and colony formation assay).
- To investigate LESCs *in situ.*
- To examine human lens capsule (LC) as a possible biological carrier for LESC transplantation.

- To isolate cells derived from human stem cell niches of CB and RP in PVR.
- To culture CB/RP- derived cells into non-adherent spheres or adherent cultures.
- To characterize the *ex vivo* expanded CB/RP-derived cells by morphological analysis (light microscopy, transmission electron microscopy (TEM)) and molecular biology techniques (transcriptional profiling, immunofluorescent staining).
- To investigate the above mentioned stem cells *in situ.*

- To explicate the relevance of the above mentioned stem cells in physiology and disease.

3. Materials and Methods

Ethics

All human tissue collection complied with the Guidelines of the Helsinki Declaration and was approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen (DE OEC: 3094-2010) and by the Local Committees for Medical Research Ethics of Oslo, Norway. Animal experiments were performed according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation of LESCs

Limbal tissues were harvested from cadavers within 12 hours of biologic death. Male to female ratio was 4:3 and the average age of the cadavers was 70±9.3 years. After a profound povidone iodide eye wash, the conjunctiva was circumferentially incised and separated from the limbal junction. Two concentric circles were drawn 0.25 mm far from corneo-scleral junction, one in the clear cornea and the other in the sclera. Approximately 0.5 mm wide corneo-scleral rim was dissected with the help of lamellar knife placed tangential to the surface being cut and kept superficial within the epithelial layer. The limbal rim was then cut into 2x2x0.5 mm rectangular shaped grafts and placed with the epithelial side up on tissue culture plates, glass cover slips or denuded human LCs that were obtained from uneventful capsulorrhexis during cataract surgeries. LCs were pre-treated with 0.025% trypsin-ethylenediaminetetraacetic acid (EDTA) (20 minutes, 37^{o}C) to remove any remaining lens epithelial cells.

Isolation of Retinal and CBE Cells

After removal of the anterior segment of the enucleated human eyes, irrespective of known PVR, the CB and retinal tissue were mechanically separated and placed into Leibowitz-15 medium or trypsin-EDTA (0.05%, Invitrogen) for 10 min followed by extensive pipette trituration. The dissociated suspension was then passed through a 70 μm strainer and resuspended as single cells at a final density of 150 000 cells/mL.

Vitreous Samples

Vitreous samples were collected during vitrectomies for RD with or without confirmed PVR based upon evaluation of wide angle images. Cases where retinotomies, retinectomies or cutting of the retinal tear was performed were omitted from the study to exclude any retinal

contamination. The vitreous samples were centrifuged at 15000 rpm for 5 min and the resulting pellets were either fixed in 4% paraformaldehyde (PFA) directly and used for immunostaining or cultivated *in vitro*.

Mice Samples

To characterize the pathological changes in PVR formation, intravitreal injection of the proteolytic enzyme dispase was given to mice which induce glial activation as well as epi- and subretinal membrane formation. Four to six months old, female wild type mice (C57/BL6, n=6) were anesthetized with pentobarbital (90 mg/kg i.p.) followed by one drop of 1% procaine hydrochloride (Novocain) for local anesthesia and one drop of tropicamide (Mydrum) for iris dilatation. Under microscopical control, 4 µL of dispase (Sigma; 0.4 U/mL, dissolved in sterile physiological saline) was injected intravitreally in the right eyes using an automatic pipette fitted with 30G 1/6 needle. Control animals received 4 µL of sterile physiological saline solution in their right eyes. All left eyes remained untreated. Stratus Optical Coherence Tomography images were taken regularly after injections to monitor the progression of PVR. One to two weeks after treatment, when signs of PVR formation were evident, control and dispase treated mice were sacrificed and their eyeballs enucleated and used for immunostaining.

In Vitro Culturing of Cells

Limbal grafts were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 20% human AB serum, 200 mM/mL L-glutamine, 10,000 U/mL penicillin-10 mg/mL streptomycin at 37ºC in 5% CO_2. To grow LESCs on LC, a drop of medium was used to smooth-out the capsule first and then the limbal tissue was placed in the middle of the capsule. Following adherence to the LC and/or the culture plate, the graft was cultivated in total of 1 mL medium. Media was changed on every alternate day. Cell growth was monitored under phase contrast microscope regularly. Only grafts which had cell outgrowth within 24 hours were used further to decrease the chance of fibroblast contamination.

Human adherent retinal culture cells and spheres derived from CBE and isolated from the vitreous of patients with PVR were cultured in DMEM/F12 containing B27 supplement (2%), epidermal growth factor (EGF) (20 ng/ml), bFGF (10 ng/ml), 1% fetal calf serum (FCS), Heparin (2.5 mg/ml) and Penicillin/Streptomycin (100 U/ml) at 37ºC in 5% CO_2. The formed spheres were supplemented with bFGF and EGF twice a week and passaged every 2-3 weeks by incubation in trypsin-EDTA (0.05%) for 2-4 minutes.

LESCs

Assay for Cell Death Analysis of In Vitro Cultured LESCs

Cell death was assessed by the Annexin-V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (MBL, Woburn, MA, USA) according to manufacturer's recommendations. Proportion of stained Annexin-V^+ and Annexin-V^+/Propidium iodide $(PI)^+$ cells was determined by fluorescence activated cell sorter (FACS) analysis on FACSCalibur flow cytometer and data was analyzed using WinMDI freeware. Alternatively, cell viability was assessed using3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTT) assay according to manufacturer's recommendations.

Histological and Immunofluorescent Analysis of LESCs

LESCs grown on the surface of glass-cover slips or denuded human LCs underwent standard 4% PFA fixation and paraffin embedding; consequently, Hematoxylin and Eosin (H&E) staining of the sections was performed. LESCs grown on glass-cover slips were investigated by anti-p63alpha (TP63/p63), ATP-binding cassette sub-family G member 2 (ABCG2), cytokeratin (CK/KRT) 19, CK8/18, vimentin (VIM/Vim) and antigen identified by monoclonal antibody Ki 67 (MKI67/Ki-67) primary antibodies followed by incubation with fluorescently labelled secondary antibodies and 4',6'-Diamidino-2-Phenylindole (DAPI) for nuclear staining. Sections were visualized under a ZEISS Axio Observer.Z1 (ZEISS, Oberkochen, Germany)

Detection of Cell Surface Markers of LESCs by Flow Cytometry

The phenotype of the cultured LESCs was analyzed by multicolor flow cytometry using FITC, R-phycoerythrin (PE) and allophycocyanin (APC) conjugated antibodies. The expression of CD34, CD44/homing cell adhesion molecule (HCAM), CD45, CD49f/Integrin (ITG/Itg)α6, CD73, CD144/Vascular endothelial (VE)-Cadherin, CD147/Neurothelin (all from BD Biosciences, San Jose, CA, USA); CD49a/Itgα1 (Biolegend, San Diego, CA, USA), CD14, CD29/Itgβ1, CD31/platelet/endothelial cell adhesion molecule (PECAM), CD47, CD49b/Itgα2, CD56/neural cell adhesion molecule (NCAM), CD90/thymocyte differentiation antigen 1 (Thy-1), CD104/Itg β4, CD105/Endoglin, CD117/c-kit, CD146/melanoma cell adhesion molecule (MCAM), CD166/activated leukocyte cell adhesion molecule (ALCAM), C-X-C chemokine receptor type 4(CXCR4), human leukocyte antigens (HLA)-DR, platelet-derived growth factor receptor β (PDGF-Rβ), vascular endothelial growth factor receptor 2 (VEGFR2)/Kinase insert domain receptor (KDF) and CD133 molecules were all measured.

For comparison, a well characterized mesenchymal origin adult stem cell type - bone marrow derived mesenchymal stem cells (bmMSCs) were used.

Lectin analysis kit from Vector Labs (Burlingame, CA) was used for detecting specific carbohydrate structures on the surface of cultured LESCs. The following lectins were tested: sialic acid (WGA: Wheat germ agglutinin (Triticum vulgaris)); N-acetylglucosamines (STL: Potatoe lectin (Solanum tuberosum), DSL: Datura stramonium lectin (Datura stramonium), ECL: Erythrina cristagalli lectin (Erythrina cristagalli), LEL: Tomato lectin (Lycopersicon esculentum), GSL II: Griffonia (Bandeiraea) simplicifolia lectin II (Griffonia simplicifolia)); mannose (ConA: Concanavalin A (Canavalia ensiformis)); galactose N-acetylgalactosamines (RCA: Ricinus communis Agglutinin (Ricinus communis), PNA: Peanut agglutinin (Arachis hypogaea), AIL: Jacalin (Artocarpus integrifolia), VVA: Hairy vetch agglutinin (Vicia villosa), DBA: Horse gram lectin (Dolichos biflorus), SBA: Soy bean agglutinin (Glycine max)) and fucose (UEA: Ulex europaeus agglutinin (Ulex europaeus)).

Samples were measured by FACSCalibur flow cytometer and data were analyzed using WinMDI freeware.

Microarray and Data Analysis of LESCs

Affymetrix GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) containing 28869 gene transcripts was used for the microarray analysis. For whole genome gene expression analysis, 150 ng of total ribonucleic acid (RNA) isolated from cultured LESC and control samples (differentiated cornea epithelial cells scraped from the central part of the cornea) were subjected to Ambion WT Expression Kit and GeneChip WT Terminal Labeling Kit according to the manufacturers' protocols. Robust microarray analysis was applied for normalization. Differentially expressed genes between groups were identified using one-way ANOVA analysis in Partek Genomics Suite Software. Clustering analysis was made using the clustering analysis module in Partek Genomics Suite Software.

Colony Forming Assay of LESCs

To investigate the colony forming properties of LESCs, cells grown on either culture plate or human LC (n= 4) were dissociated and seeded at 3000 cells/cm^2 density onto 0.1% Gelatine, 10 ng/mL Fibronectin or MethoCult coated 6 well plates. Samples were fixed in 4% PFA prior to staining with crystal violet (0.5% w/v) or labelling the cytoskeletal actin (phalloidin-FITC) and nuclei (Hoechst 33342) of colonies formed at day 7. Examination was carried out under an Olympus IX81 inverted microscope with MT20 station, acquired and analyzed by ScanR (Olympus) software.

Characterization of Cultured Retinal, CBE and Vitreous derived Samples

Histological and Immunofluorescent Analysis

Before fixation in 4% PFA and paraffin embedment, a mixture of human plasma and thrombin was used to clot the spheres. H&E staining of the sections was performed according to standard laboratory protocols. Spheres were investigated for expression of N-cadherin, Claudin1, glial fibrillary acidic protein (GFAP), Nestin, β-III-tubulin, Rhodopsin, Ki-67, Sox2, Pax6, RPE65 and ABCG2 and the nuclei counterstained by Hoechst 33342. Imaging was performed using Olympus BV 61 FluoView confocal microscope and a ZEISS Axio Observer.Z1 fluorescence microscope.

Transmission Electron Microscopy

Freshly prepared aldehyde-fixative containing 0.1M sodium cacodylate buffer, 2% glutaraldehyde, 2% PFA and 0.025% calcium chloride (pH 7.4) were used for 30-60 min at Rt to fix the spheres, then fixation was continued overnight at 4ºC, postfixed in 1% osmium tetroxide and dehydrated through a graded series of ethanol washes up to 100%. After immersion in propylene oxide for 20 min, the spheres were embedded in Epon. Ultra-thin sections (60-70 nm thick) were cut on a Leica Ultracut Ultramicrotome UCT, stained with uranyl acetate and lead citrate, and followed by examination using a Tecnai12 transmission electron microscope.

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) was performed to compare spheres at P1 formed from vitreous cells of patients with PVR to two well-characterized cell populations of the adult human eye that previously have been thought to have NPCs properties: cultures of retinal cells with a Müller glia phenotype and CBE cells forming pigmented spheres *in vitro*. Total RNA was extracted using TRIzol Reagent according to the manufacturer's protocol, while reverse transcription (RT) was performed using the High Capacity cDNA Archive Kit with 200 ng total RNA per 20 μL RT reaction. The qPCR was performed using the StepOnePlus RT-PCR system and Taqman Gene Expression assays following protocols from the manufacturer. The thermo cycling conditions were 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method as the fold change (FC) in gene expression relative to CBE spheres which was arbitrarily chosen as calibrator and equaled one.

In Situ Analysis

To localize and show the characteristic protein expression pattern of the stem cells of interest *in situ,* and to compare it to the cultured LESCs, cross-sections of the human limbus and axial sections of the posterior segment of cadaveric and mice eyes with or without PVR formation were used.

The full thickness cadaveric limbus grafts were fixed in 4% PFA and embedded into paraffin. H&E staining and immunofluorescent labelling were carried out with anti-CD34, CD45, CD144/VE-Cadherin, CD144/HCAM, CD146/MCAM and CD166/ALCAM antibodies.

The posterior segments of the human cadaveric eyes with or without PVR were embedded into paraffin after fixation in 4% PFA. Stained for N-cadherin, Claudin1, GFAP, Nestin, Rhodopsin, Sox2, Pax6 and ABCG2 was used here.

The entire mice eyes (controls and eyes treated with dispase for PVR induction) were fixed in 4% PFA, embedded in freezing medium, then 10 μm frozen sections were thawed onto Super Frost/Plus glasses and stored at -20^{o}C before immunohistochemical analysis for Claudin1, GFAP, Nestin and Sox2 was performed.

Statistics

Each experiment was performed at least three times and each sample was tested in triplicate. The percentage of cells positive for the immunofluorescent markers was calculated from counting 100 cells from 5 different sections, and the expression pattern was evaluated by two independent investigators. Results are presented as mean$\pm$standard deviation (S.D.). Differences between groups were tested by the two-tailed independent sample t-tests. The significance level was set to $p<0.05$=*, $p<0.01$=**, $p<0.001$=***. Data were analyzed using SPSS Version 18.0.

4. Results

Cultured LESCs

Cultivation and Viability Testing

Migration and proliferation of cells from the harvested limbal tissue were observed within 24 hours of cultivation. The outgrowing cells showed epithelial morphology with intact actin cytoskeleton, and confluent cultures were obtained after 14 days. Furthermore, grafts grown on denuded human LC produced stratified epithelial layers within 7 days of cultivation. According to the MTT assay, the cell viability of the outgrowing LESCs under both growth conditions was more than 97% at the two checkpoints (day 7 and 14) (Figure 2A), while the number of apoptotic cells investigated by Annexin-V-FITC Apoptosis Detection Kit (early apoptotic: annexin V-FITC$^+$ <2%; late apoptotic: annexin V-FITC$^+$/PI$^+$ <1%) remained low up to day 14, respectively.

Histological and Immunofluorescent Analysis

Although there are no definitive markers for identifying either adult stem cells or corneal stem cells, characterization by a consensus-based panel of different markers combined with some, previously, not described putative stem cell markers were used. Immunohistochemistry was performed to detect CK8/18/Ki-67, CK19, ABCG2, p63α/Vim on LESCs grown on human LC. Strong staining of CK8/18 was present and co-localization with the proliferation marker Ki-67 could be observed in some cells, confirming the proliferating and transition potential of these cells towards differentiated corneal epithelium. Putative stem cell markers such as CK19 showed a scattered cytoplasmic staining throughout the outgrowing cell sheet; ABCG2 was observed in both the cell membrane and cytoplasm of LESCs; the nuclear protein p63α and the cytoplasmic Vim showed co-localization and positivity in most of the LESCs grown on human LCs, confirming their progenitor nature. *In situ* immunostaining of human limbal sections revealed the non-hematopoietic, -endothelial and -mesenchymal stem cell phenotype of the LESCs and the localization of specific markers such as CD44/HCAM at the apical-, CD146/MCAM and CD166/ALCAM in the basal cell layer of the human limbal epithelium.

Phenotyping of Cell Surface Markers by Flow Cytometry

LESCs grown on LC were investigated by flow cytometry with well-known cell surface markers corresponding to hematopoietic-, endothelial- and mesenchymal lineages and also compared to bmMSCs. LESCs showed absence of common hematopoietic cell surface

markers such as CD45, CD34, CD133 and HLA-DR. The expression of CD14, CD117/c-kit (an early progenitor/pluripotent stem cell marker) and CXCR4 (characteristic for migration) were slightly, but significantly higher in LESCs than bmMSCs ($p<0.05$, $p<0.01$). High expression of CD47 refers to the viability and immunocompetence of both cell types. No common endothelial cell markers such as CD31/PECAM or VEGFR2/KDR were detectable in LESC cultures, excluding any endothelial cell contamination, while significantly more LESC expressed CD144/VE-Cadherin ($p=0.0321$) and CD104/Itgβ4 ($p=0.0458$) when compared to bmMSCs. Significant differences ($p<0.001$) were found regarding the most important MSC-markers: only 12% of LESCs expressed CD90/Thy-1 ($p=0.000032$) and less than 50% were CD105/Endoglin positive ($p=0.0006$). In contrast to that, no significant difference in the expression of CD73, CD147/Neurothelin and PDGF-R β was found between LESCs and bmMSCs. The presence of cell adhesion molecules (CAMs) and Itgs were also tested, due to their importance in cellular attachment to the extracellular matrix and maintenance of growth supporting environment. The expression of CD29/Itgβ1, CD49a/Itgα1, CD56/NCAM, CD146/MCAM and CD166/ALCAM were similar, while LESCs expressed CD44/HCAM at lower ($p=0.00052$), and CD49b/Itgα2 ($p=0.038$) and CD49f/Itgα6 ($p=0.008$) at higher levels compared to bmMSCs. Characterization of the lens epithelial cells (LECs) by flow cytometry was carried out to exclude a possible LEC origin of the significantly expressed markers on LESCs grown on denuded human LC compared to bmMSCs. The LECs expressed no CD34, CD45 and CD144/VE-Cadherin, while they showed lower expression of CD166/ALCAM (74.19$\pm$46.07%), similar CD146/MCAM (74.22$\pm$2.23%) and higher CD44/HCAM (50.43$\pm$29.28%) expression compared to the cultured LESCs (n =3).

It is known that stem cells can be characterized by their unique surface carbohydrate pattern which shows changes during differentiation. Lectin-based screening of the most common terminal cell-surface glycolipids and glycoproteins was carried out on LESCs and revealed presence of subpopulations within the cell culture. Staining with WGA showed high sialic acid content, while more than 50% of the cells (51.59$\pm$3.1%) showed strong ConA (Fluorescence Intensity Median (FImed)= 2125.02$\pm$25.99) positivity due to the presence of branched a-mannosidic structures on the surface. LESCs were also positive for the galactose and/or N-acetylgalactosamine binding lectins (RCA, AIL and PNA), although lower fluorescence intensity could be detected by PNA (FImed = 185.75$\pm$1.06) regarding low T-antigen expression. UEA-lectin exhibited moderate fluorescence intensity on 61.1$\pm$1.97% of the cells, indicating low levels of detectable fucose molecules on LESCs.

Transcriptional Profiling

Microarray analysis was performed to compare the transcriptional profile of cultured LESCs and differentiated corneal epithelial cells (CECs). The intensity profiles of the log_2 transformed signal values of the 28869 transcripts were obtained, out of which 1830 transcripts had more than 2 FC increase or decrease in expression (955 and 875 transcripts), indicating a relatively high transcriptional difference between the two cell types ($n=3$, $p<0.01$). The heatmap and the functional clustering of 67 selected genes based on their high or low FC or previously documented relation to LESCs ($n=3$, $p<0.01$) showed that these genes were mostly involved in ion-, nucleotide- or protein binding, as well as receptor- or enzyme activities. Among the general epithelial markers, limbal epithelium recognizing markers (KRT8/KRT18 and KRT14) could be distinguished, along the ones specific for differentiated corneal epithelium (KRT3/12). KRT8 and KRT14 showed similar or slightly higher expression levels in the limbal tissue-derived cells compared to the differentiated CECs (FC: 4.0 and 1.9, respectively) indicating the commitment of LESCs towards the corneal epithelial lineage. Meanwhile, the specific differentiated CEC markers KRT3 and KRT12 showed decreased expression (FC: 231.0 and 25.8, respectively), probably due to an earlier differentiation state or preserved multipotency of the LESCs. Higher expression of putative stem cell markers (KRT19 (FC: 6.0) and VIM (FC: 4.4) was found in the LESCs compared to the differentiated CECs, strengthening their stem-like character. The high proliferation capacity of the cultured LESCs was also confirmed by higher expression of the proliferation-specific marker MKI67 (FC: 3.0).

Colony Forming Assay

Colony forming potential was tested to confirm the pluripotency signature of cultured LESCs supposed by the previously shown gene and protein expression levels of putative stem cell markers. All the tested epithelial sheets ($n=3$) were capable of forming epithelial holoclone-like colonies on Gelatin and Fibronectin within 7 days of cultivation, but not on MethoCult coated plates.

Retinal, CBE and Vitreous Samples

Histological and Immunofluorescent Analysis

In control human eyes, Nestin positivity was found at the inner surface of the retina and in a few cells with Müller glia morphology at the central laminated retina (LR). Nestin was not found at the peripheral Pla or the Pli regions, except for cells lining the wall of peripheral

cysts (Pc). Cystic degeneration of RP is a common finding in elderly; however, the pathological consequences remain unknown. Cells lining the cyst wall were also positive for Pax6 and Sox2, two central transcription factors controlling eye development. Furthermore, positivity for ABCG2 in the Pc and N-cadherin in the Pli was observed, while GFAP expression was only detected around the Pc of the RP. The CBE of normal eyes expressed Claudin and N-cadherin, markers of differentiated epithelial cells, whilst no NPC markers or Rhodopsin could be detected in that part of the eye. Rhodopsin was only expressed by differentiated photoreceptors at the LR and by few cells at the periphery of NLR.

In eyes with PVR, Nestin staining extended to the proximal Pla. Around the ora serrata, cells were positive not only for Nestin, but also for Sox2 and GFAP. $Nestin^{+}$ and $GFAP^{-}$ cells could be detected at the proximal Pla. No positivity for Nestin, GFAP or Sox2 could be found in either peripheral Pla, Pli or iris pigment epithelium (IPE). In response to PVR formation, remarkable proliferation of non-pigmented CBE was observed mainly at the transitional zone between Pla and Pli as confirmed by Ki67 positivity. Clusters of Rhodopsin positive cells were found in the NLR close to the area of photoreceptor loss, but no such positivity was detected in either peripheral Pla, Pli or IPE even in eyes with extensive retinal damage.

Immunohistological Analysis of the CBE and PR of Mice Eyes with PVR

In control mice eyes, only a few Nestin positive cells were found in the CBE with no Pax6 or Sox2, but robust Claudin positivity. Similar to our findings in humans, gliotic reaction of the retina was marked by an increased GFAP staining accompanied by nuclear hyperplasia and adenomatous-like proliferation of the CBE following PVR induction. In contrast to humans, Nestin was upregulated in CBE upon PVR formation. $Sox2^{+}$/$Nestin^{+}$ and $Nestin^{+}$/$GFAP^{+}$ clusters were found in the peripheral vitreous probably due to development of PVR.

Characterization of the Sphere-Like Structures Isolated from Vitreous of Patients with Retinal Detachment

Sphere-like structures were isolated from the vitreous of patients undergoing vitrectomy for RD with and without confirmed PVR development preoperatively. Immunohistochemical characterization of their content was aimed to detect NPCs. The theory behind is that these cells should be able to sense and migrate towards CNS lesion such as those in RD, thus, while performing vitrectomy for RD with PVR, the surgeons would recognize sphere-like structures in the far periphery close to the vitreous base.

Most of the cells inside the isolated sphere-like structures showed Nestin and GFAP positivity, some cells were positive for Sox2 and for Pax6. No cells were positive for the

photoreceptor marker Rhodopsin or RPE65, but a few cells revealed to be positive for the immature neuronal marker β-III-tubulin.

Characterization of *In Vitro* Cultured Retinal, CBE and Vitreous Samples

Single cells obtained from vitrectomies for RD in patients with preoperatively confirmed PVR formed spheres in more cases than cells isolated from patients with no PVR. Spheres could be passaged up to P2 (no attempts were made for further passages). Transmission electron microscopy revealed that spheres are composed of elongated and polarized cells at the periphery and pigmented and non-pigmented polymorphic cells with high nuclear/cytoplasmic ratio in the central areas. Robust Nestin and GFAP positivity were detected inside the spheres, while some cells at the periphery expressed β-III-tubulin. QPCR was performed to compare the spheres at P1 from the vitreous cells of patients with PVR to cultures of retinal cells with a Müller glia phenotype and CBE cells forming pigmented spheres *in vitro* - the last two cell types being well-characterized cell populations of the adult human eye and previously thought to have NPC properties. Out of the examined nine early eye-field transcription factors, only OTX2 and MITF showed significant ($p<0.05$) difference between the Ct values of retina and CBE. OTX2 was found to be more expressed in retinal cultures, while MITF was more expressed in CBE cultures (n=3). CHX10 was highly expressed in retinal and PVR samples as well, but no statistical difference could be detected. Although immunofluorescent staining revealed that only the cells around Pc are Nestin and Pax6 positive, the mRNA expression was comparable in all groups after *in vitro* cultures. In agreement with the immunofluorescent staining of the sphere-like structures derived from PVR eyes, the differentiation marker GFAP showed a 40.4 times higher expression in PVR cultures compared to CBE cultures. No difference in glutamine synthesizes (GS) expression could be detected. Tyrosinase (TYR) which was previously found in differentiating RPE cells showed comparable expression in CBE and PVR spheres (FC=1.34). These findings further support the previous observation that markers found in NPCs may be upregulated in epithelial cells of CBE origin during sphere-promoting cultivation.

5. Discussion

At present, the promise of regenerative medicine seems more realistic when it comes to treating injuries or degenerative diseases. Limitations of the use of ESCs or iPSCs in the clinical practice naturally force research towards the use of adult stem cell therapy. Adult stem cells are ideal target for achieving significant improvements in the visual acuity and discomfort in LESCD as well as degenerative retinal diseases.

Our key aims were to isolate and culture adult stem cells from the limbus or the CBE of human eyes with the purpose of future transplantation. The replacement of lost or damaged cells by stem or progenitors can be achieved either directly after isolation, after an *ex vivo* expansion or following full or partial *in vitro* differentiation. The theory behind the first two is to achieve a paracrine effect, whereby the transplanted stem cells would secrete trophic factors which can induce the recipient tissue to self-renewal and proliferation, while the latter case would allow transplanted differentiated cells to integrate and restore function.

LESCs

LESCD is a disease in which the stem cell- and the barrier function of the limbus fail due to genetic causes or acquired insults. The loss or absence of LESCs lead to repeated and persistent surface breakdown and repair by conjunctival epithelium - the latter results in neovascularization, chronic inflammation and scaring with significant decrease in visual acuity and severe discomfort. Replacement of defective or deficient LESCs by healthy ones can rescue vision. *Ex vivo* expanded LESCs can be transplanted with the help of carriers, such as denuded HAM, human lens capsule (LC) or bioengineered membranes.

In a recent study, we took advantage of using human denuded LC to develop a transplantable graft of *ex vivo* expanded LESCs, as one group has previously introduced the advantages of this material. LC is easily available and can be obtained during one of the most common ophthalmological procedures (cataract surgery) or alternatively, it can be obtained from enucleated human cadaveric eyes. It has a limited size and usually is not completely flat, yet the main advantages of LC over HAM are its transparency and thinness – a property which is superior to any other biomaterials as well. We combined the use of human denuded LC as a carrier and the use of human serum as the only growth supplement for culturing LESCs excluding any animal material from the culture system.

Since no single, reliable marker has been found that is capable of discriminating stem cells that maintain the corneal epithelium from the surrounding cells or tissue, a consensus-based panel of LESC markers was used in our study, with some additional, previously not described

markers to confirm the LESC features of the *ex vivo* cultured cells. The expression of known putative stem cell markers such as TP63/Δp63α, ABCG2, CK19 and VIM/vimentin were confirmed at both gene and protein level proposing the presence of stem cells in the culture. The percentage of intensely stained p63α positive cells can be used to predict the outcome of transplantation. More than 3% p63α-bright cells in the culture have been associated with better success rate of transplantation. Therefore, only transplants prepared from cultures containing more than 3% of the p63α-bright cells should be used, while the percentage of p63α-bright cells can be used to evaluate the purity of the culture.

Based on the ability of the LESCs to efflux Hoechst 33342 dye via the ABCG2 transporter, a so called 'side population' of cells can be distinguished. It has been shown that the side population of cells which displayed stronger ABCG2 expression also expressed higher p63α and showed greater colony forming efficiency.

Regarding CK19 expression, there are controversial data in the literature - some groups describe CK19 as marker for conjunctival epithelium, while others use it as LESC marker. Our gene array analysis revealed an upregulation of CK19 in the *ex vivo* cultured LESCs compared to differentiated corneal epithelium. Beside the stem cell marker positivity, our LESCs showed proliferative potential (higher MKI67 expression/Ki67^{+}) and formed small and large colonies on different extracellular matrix-coated plates, which strengthened the pluripotent nature of these cells. Knowing the fact that stem cells are slowly proliferating cells, it should be noted that our *ex vivo* expanded LESCs are a mixture of stem cells and highly proliferative TACs; indeed, some of the cells express stem cell markers and show slow proliferation rate, while others are committed to the corneal epithelial cell fate and are highly proliferative, showing remarkable migratory potential.

Immunofluorescent staining of the cultured LESCs showed presence of CK14 and CK8/18, confirming the corneo-conjunctival origin of these cells, which together with the low expression of terminally differentiated cornea epithelial markers confirm the undifferentiated limbal epithelial cell character and their commitment to become corneal epithelium. The migratory capacity of these cells is reflected in the expression of ITGα9, CK8 and CXCR4. During characterization of the cultured LESCs, some yet not described markers were found to be expressed, such as CD44/HCAM, CD144/VE-Cadherin, CD146/MCAM and CD166/ALCAM. These markers could also be localized in the limbal epithelium *in situ*: CD44/HCAM was found expressed in the cells of the apical layer, while CD146/MCAM and CD166/ALCAM were detected at the basal layer of the limbus.

The expression pattern of terminal carbohydrates on the cell surface in the form of glycolipids and glycoproteins has not yet been investigated in a larger extent before. We examined 14 carbohydrates: 1 sialic acid, 5 N-acetylglucosamines, 1 mannose, 6 galactoses and 1 fucose on the surface of LESCs. Although previously LESCs have been shown to express unsialynated galactose and had a lack of α-2,3-bound sialic acid, our LESCs had lower FI_{med} of PNA compared to WGA, ConA, RCA or AIL, while 98% of the LESCs were PNA positive.

Genome-wide profiling also revealed some genes which function has not been defined yet in LESCs: Serpin peptidase inhibitor, clade A member 3 (SERPINA3) (FC: 21.1) that has been investigated previously for its anti-angiogenic and anti-inflammatory effects during corneal injury; Semaphorin 3A (SEMA3A) (FC: 40.2), which has been shown to be involved in the development of mouse cornea and differentiation of corneal epithelial cells and Fibronectin1 (FN1) (FC: 74.9), which is involved in cell adhesion and migration processes during wound healing, were found to be overexpressed in the LESCs. Altogether, these markers can be added to the LESC 'fingerprint' and be used to better identify these cells within the basal limbal epithelium. Flow cytometry showed that LESCs are of non-hematopoetic origin, express lower levels of MSC markers than bmMSCs and express no CD31, which would assess the endothelial characteristic. CD49a/Itgα1, CD49b/Itg α2, CD49f/Itg α6 and CD29/Itg β1 have been previously reported as basal limbal and basal corneal cell markers - we found high expression of these markers *in situ* as well in the cultured LESCs.

In conclusion, our data suggest that our limbal outgrowing cells are a mixture of slowly proliferating LESCs and highly proliferating, migrating and potentially differentiating TACs, based upon the distinct surface marker fingerprint including positive and negative markers together. From practical point of view, the transplantation of a mixed population of stem cells and pre-committed cells would be a highly desirable condition toward successful corneal transplantation. Furthermore, the use of LC as a carrier for *ex vivo* expanded LESCs can be a good and prominent alternative to HAM due to its transparency and protective effect on the cultured cells. Alternatively, transplantation of *ex vivo* expanded oral mucosal sheets and transdifferentiated bone marrow-derived stem cells are a possible substitute for corneal epithelial cells in bilateral LESCD. Transdifferentiation of hair follicle stem cells to substitute corneal epithelial cells may be a more promising process due to the resemblance of the two lineages, however, such experiments are yet to go beyond the *in vitro* stage.

Progenitor Cells of the Posterior Segment

Stem cell therapy holds great promise for retinal diseases. The proof of this are the several ongoing clinical trials using human embryonic-, fetal-, umbilical cord- and bone marrow-derived stem cells for treating visual disorders such as AMD and retinitis pigmentosa. The first to proposition that human CBE contains NPCs which are able to make new neural cell types was in the year 2000. Due to its easy accessibility, CBE would be an ideal source of NPCs. Retinal NPCs by definition should have the ability of self-renewal, ability to respond to injuries by targeted migration into the lesion, similar to how NPCs do in other parts of the CNS, and should be able to differentiate into functioning cells. Recently several studies have questioned the presence of NPCs in the CBE of the adult human eye. Relatively common CNS lesions in ophthalmology are retinal breaks and holes with the consequent RD and PVR development. Hence, if NPCs are present in the adult human eye, they should be able to respond to RD and PVR formation as well. In order to clarify whether NPCs exist in the adult human eye, we carefully investigated the CBE and RP for NPC markers in enucleated eyes with or without previously confirmed PVR. The expression of NPC markers were analyzed in a mice model of PVR as well. Finally, we looked for signs of targeted migration of NPC-like cells to the vitreous of patients in samples obtained during vitrectomy due to PVR.

In situ staining of control, non-PVR human cadaveric eyes revealed the presence of both neural stem cells markers (Pax6, Sox2, Nestin) and epithelial stem cell markers (ABCG2, N-Cadherin), but only within the wall of peripheral cysts at the most proximal Pla and RP, while GFAP positivity was found only in cells at the RP cysts. Cystic degenerations are common in the uninjured human eye and are frequently found in elderly, but with no known pathological consequence. In agreement with previous findings, we could not detect any cell division or Ki67 positivity in uninjured human retina *in situ*. In response to PVR formation, proliferation was observed in either the $GFAP^-$ or the $GFAP^+$ cells at the proximal Pla and RP. These findings partially correspond to the results of another group, although they described a few $Rhodopsin^+$ cells next to the $GFAP^-$ pigmented cells in eyes with extensive PVR formation. Since no neuroprogenitor marker or Rhodopsin positivity could be detected within the CBE of control or PVR eyes, the probability of new photoreceptor production by NPCs at the CBE is therefore low.

In contrast to the human findings, CBE of mice showed not just nuclear hyperplasia, but increased Nestin expression after PVR induction by intravitreal injection of dispase, raising awareness to other groups' findings from other species and the need for careful comparison to humans.

During neurosphere formation assay, free-floating clusters developed from single cell suspension under specific culture conditions. This is a widely used method to confirm NPC properties such as self-renewal and inducible proliferative capacity, although the method is not specific for stem cells as some other cell types like epithelial cells can also form spheres. There is also evidence that small spheres are not derived from single cells but develop as aggregation of cells. Therefore, self-renewal must be demonstrated by extended time of cultivation and for several passages. On the other hand, it is hard to distinguish the cell types within a sphere, due to its physical and geometric nature, which also affects the nutrients, growth factors and oxygen supply within the sphere. It also means that the population of cells within the sphere is heterogeneous in terms of their differentiation stage and commitment. The lack of a standardized protocol for neurosphere formation assay and the use of different cell density and media, different concentration of mitogens, hormones and additional supplements make the comparison of the results obtained from different laboratories difficult.

The capability for self-renewal and proliferation of CBE-derived cells has been confirmed by several research groups. They found that spheres formed only from the CB and iris, such spheres or cells could not be isolated from the neuroretina or RPE; they could isolate spheres from the neuroretina and CB regardless of sex, age or post-mortem time; the spheres derived from CB which consist of proliferating cells expressing immature neuronal and glial markers could be characterized. Our findings indicate that more spheres form when cells are isolated from the vitreous of human eyes with known PVR compared to non-PVR eyes. These spheres could be passaged up to two times, although no attempt was made to continue passaging further than this.

Stem cells can be identified by a specific marker pattern, including markers expressed exclusively by the stem cell (positive markers) or those absent on stem cells but expressed by their progenitors (negative markers). In most of the cases, due to the lack of one definitive marker, combinations of positive and negative markers are used. CBE is derived from the neural tube ectoderm, so it should be investigated whether it keeps the epithelial phenotype in spheres, in parallel to checking its neural and glial marker expression. Our collaborators have demonstrated that CBE spheres contain proliferating epithelial-like cells with decreased expression of NPC markers compared to CNS neurospheres, which is in agreement with other findings that CBE-derived spheres are pigmented and display more epithelial characteristics. Cells derived from CBE express significant amount of NPC markers, but fail to differentiate to photoreceptors. The pigmented and non-pigmented epithelial cells of the CBE can be separated; only the non-pigmented CBE proliferate to form spheres in culture, expressing high

level of epithelial markers and limited level of NPC markers. Spheres derived from the vitreous of patients with PVR showed GFAP and Nestin positivity at their central part and β-III-tubulin positivity at the periphery in either *in situ* or *in vitro* conditions. These spheres comprised of pigmented and non-pigmented cells, while the RPE origin of the pigmented cells could be excluded by showing RPE65 negativity.

ESC markers like OCT4, SOX2 and Nanog have been detected in several different PCs before, although their role has yet to be fully determined. The ESC markers are considered as essential for showing pluripotency, and we could demonstrate varying, low levels of all three markers in the spheres obtained from vitrectomy of eyes with PRV using RT-PCR. The presence of Pax6 and Sox2 could further be confirmed at a protein level by immunofluorescent staining. The most common NPC marker is Nestin, which is an intermediate filament protein and can be upregulated in pathological conditions like gliosis. During neuro-and gliogenesis, Nestin is replaced by tissue-specific intermediate filaments like neurofilaments or GFAP. GFAP plays a role in the formation of glial scars in the CNS. ABCG2 is a widely used general stem cell marker, which functions as a xenobiotic transporter and plays a major role in multi-drug resistance. Based on the ability to efflux the DNA binding dye (Hoechst 33342), a so called 'side population' can be isolated which is considered to be a heterogeneous population of progenitor-like cells as mentioned before. Claudin is a tight junction protein which controls the flow of molecules in the intercellular space between epithelial cells, while N-Cadherin is a calcium dependent cell-cell adhesion glycoprotein which plays key role in the development of the neural plate. N-Cadherin and ABCG2 have both been found to be expressed by the epithelial and neuroepithelial stem/progenitor cells. Our collaborators have shown Nestin and Claudin positivity, but not double positivity of cells in spheres derived from CBE, which suggest that the spheres are a mixture of two different cell types: epithelial and neural progenitor-like cells. In contrast to that, others have shown homogeneous population of epithelial cells in the spheres of CBE.

We show here that sphere-like structures isolated from the vitreous of patients with PVR show high GFAP positivity as well. PCR could also reveal that GFAP is expressed significantly higher in PVR spheres than in CBE spheres. No RPE65 (RPE marker) could be detected in the spheres, which rules out any contamination of RPE cells.

$GFAP^+$ Müller glia cells are suggested to have latent neuroregenerative capacity in humans. Furthermore, it has been recently shown that Müller glia isolated from the human RP during vitrectomies can be an efficient source for producing photoreceptors which correlates well with our findings: we detected $Rhodopsin^+$ cells in the NLR peripheral to the areas of

photoreceptor loss in human eyes with PVR, although this cannot be concluded as sign of active neurogenesis yet. Some additional investigations as clonal expansion, differentiation studies of NPCs isolated from the vitreous are needed to further support these findings.

Based on the previously stated findings, we support the hypothesis that the adult human eye may contain two different populations of neuroepithelial PCs: non-glial ($GFAP^-$) population located close to Pc in the proximal Pla and another population with Müller glia characteristics ($GFAP^+$). So far, we only found evidence that the glial population is able to respond to retinal injury by targeted migration into the vitreous.

Protein and gene expression of mature markers within the spheres derived from CBE is not a definitive proof for functional differentiation. Furthermore, it would be necessary to demonstrate the morphological and functional identity of differentiated cells like post-mitotic action potential firing and neurotransmitter release.

6. Keywords

Stem Cells, Limbal Epithelial Stem Cells, Retinal Stem Cells, Proliferative Vitreoretinopathy

7. Summary

Stem cell therapy holds great promise for treating diseases arising from sight-threatening injuries and disorders such as chemical and thermal burns, AMD or retinitis pigmentosa. Due to ethical issues or the fear of cancer development the use of adult stem cells seems more plausible compared to ESCs or iPSCs.

We isolated, cultured and characterized successfully LESCs which are responsible for corneal epithelial regeneration, and confirmed the plausibility of using thin, transparent LCs as biological carriers for future LESC-transplantation. The main benefit of our work is the exclusion of animal materials during cultivation, the use of human serum as the only growth supplement and the extension of the characteristic marker pattern of LESCs with previously not identified markers: CAMs (CD44/HCAM, CD146/MCAM, CD166/ALCAM) and surface glycoproteins. Limbal grafts showed viable cell outgrowth which formed stratified epithelium on LCs within two weeks. The outgrowing cells showed stem cell characteristics (ABCG2, CK19, TP63/p63, VIM/Vim), high proliferation capacity (MKI67/Ki67) and positivity for markers related to the corneal epithelial commitment (CK8/CK18, low CK3/CK12), giving the benefit of having a mixed population of stem cells and TACs.

CBE is thought to contain NPCs which can identify retinal injuries like those in RD and consequent PVR, and respond by targeted migration and functional differentiation. In our experiments, no NPC markers in human eyes could be detected, except within peripheral cysts of the proximal Pla and RP; furthermore, $GFAP^+$ cells could only be detected at the RP. Our findings confirm proliferation of $GFAP^-$ CBE cells in response to PVR formation, but show no evidence for stemness or photoreceptor differentiation. Only some clusters of $Rhodopsin^+$ cells in the NLR could be found peripheral to the large areas of photoreceptor loss after PVR. In contrast to that, CBE of mice showed hyperplasia and increased Nestin positivity in PVR.

Surgeons can often visualize sphere-like structures at the far periphery of the vitreous during vitrectomy for RD with PVR. To identify the origin and characteristics of these spheres, direct isolation from the vitreous and staining before and after cultivation were performed. Immunostaining and TEM of the spheres revealed presence of pigmented and non-pigmented cells, Nestin and GFAP positivity at the central part, β-III tubulin positivity at the periphery and RPE65 negativity excluding contamination with RPE cells. The non-glial and glial response to PVR formation could be confirmed, yet, no evidence for targeted migration of glial cells into the vitreous could be confirmed.

8. Publications

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Register Number: DEENKÉTK/1/2014.
Item Number:
Subject: Ph.D. List of Publications

Candidate: Réka Albert

Neptun ID: CA5WC0

Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Albert, R.**, Veréb, Z., Csomós, K., Moe, M.C., Johnsen, E.O., Olstad, O.K., Nicolaissen, B., Rajnavölgyi, É., Fésüs, L., Berta, A., Petrovski, G.: Cultivation and characterization of cornea limbal epithelial stem cells on lens capsule in animal material-free medium.
PLoS ONE. 7 (10), e47187, 2012.
DOI: http://dx.doi.org/10.1371/journal.pone.0047187
IF:3.73

2. Johnsen, E.O., Froen, R.C., **Albert, R.**, Omdal, B.K., Sarang, Z., Berta, A., Nicolaissen, B., Petrovski, G., Moe, M.C.: Activation of neural progenitor cells in human eyes with proliferative vitreoretinopathy.
Exp. Eye Res. 98, 28-36, 2012.
DOI: http://dx.doi.org/10.1016/j.exer.2012.03.008
IF:3.026

List of other publications

3. Veréb, Z., **Albert, R.**, Póliska, S., Olstad, O.K., Akhtar, S., Moe, M.C., Petrovski, G.: Comparison of upstream regulators in human ex vivo cultured cornea limbal epithelial stem cells and differentiated corneal epithelial cells.
BMC Genomics. 14 (1), 1-33, 2013.
DOI: http://dx.doi.org/10.1186/1471-2164-14-900
IF:4.397 (2012)

H-4032 Debrecen, Egyetem tér 1. e-mail: publikaciok@lib.unideb.hu

4. **Albert, R.**, Vásárhelyi, G., Bodó, G., Kenyeres, A., Wolf, E., Papp, T., Terdik, T., Módis, L., Felszeghy, S.: Computer-assisted microscopic analysis of bone tissue developed inside a polyactive polymer implanted into an equine articular surface.
Histol. Histopath. 27 (9), 1203-1209, 2012.
IF:2.281

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In: Autophagy: Principles, Regulation and Roles in Disease. Szerk.: Nikolai Gorbunov, Nova Science Publishers, Hauppauge, New York, 157-180, 2012.

Total IF of journals (all publications): 13.434

Total IF of journals (publications related to the dissertation): 6.756

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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