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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Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
AIL	jacalin (Artocarpus integrifolia),
AMD	age-related macular degeneration
bmMSC	bone marrow-derived mesenchymal stem cells
BMP-4	bone morphogenetic protein 4
CAM	cell adhesion molecule
CB	ciliary body
CBE	ciliary body epithelium
CD	cluster of differentiation
CD104/Itg β4	integrin β4
CD105/Endoglin	endoglin
CD117/c-kit	c-kit
CD144/VE-cadherin	vascular endothelial-cadherin
CD146/MCAM	melanoma cell adhesion molecule
CD147/Neurothelin	neurothelin
CD166/ALCAM	activated leukocyte cell adhesion molecule
CD29/Itg β1	integrin β1
CD31/PECAM	platelet/endothelial cell adhesion molecule
CD44/HCAM	homing cell adhesion molecule
CD49a/Itg α1	integrin α1
CD49b/Itg α2	integrin α2
CD49f/Itg α6	integrin α6
CD56/NCAM	neural cell adhesion molecule
CD90/Thy-1	thymocyte differentiation antigen 1
CEC	corneal epithelial cell
CFU	colony forming unit
CHX10	ceh-10 homeo domain containing homolog
c-MYC	v-myc avian myelocytomatosis viral oncogene homolog
CMZ	ciliary marginal zone
CNS	central nervous system
ConA	concanavalin A (Canavalia ensiformis)
CRALBP	cellular retinaldehyde-binding protein 1
CXCR4	C-X-C chemokine receptor type 4
DAPI	4',6'-diamidino-2-phenylindole
DBA	horse gram lectin (Dolichos biflorus)
DMEM	Dulbecco modified Eagle’s medium
DNA	deoxyribonucleic acid
DSL	Datura stramonium lectin (Datura stramonium)
ECL	Erythrina cristagalli lectin (Erythrina cristagalli)
EDTA	ethylenediaminetetraacetic acid

EGF	epidermal growth factor
ESC	embryonic stem cell
FACS	fluorescence activated cell sorter
FC	fold change
FCS	fetal calf serum
FGF	fibroblast growth factor
FImed	fluorescence intensity median
FITC	fluorescein isothiocyanate
FN1	fibronectin 1
FOXC1	forkhead box transcription factor
GFAP	glial fibrillary acidic protein
GS	glutamine synthetase
GSL II	Griffonia (Bandeiraea) simplicifolia lectin II (Griffonia simplicifolia)
H&E	haematoxylin and eosin
HAM	human amniotic membrane
HLA	human leukocyte antigens
IOP	intraocular pressure
IPE	iris pigmented epithelium
iPSCs	induced pluripotent stem cells
ITG/Itg	integrin
KLF4	kruppel-like factor 4
KRT	keratin
KRT/CK	cytokeratin
LC	lens capsule
LEC	lens epithelial cell
LEL	tomato lectin (Lycopersicon esculentum)
LESC	limbal epithelial stem cell
LESCD	limbal epithelial stem cell deficiency
LMAF	neural retina leucine zipper
LMX1B	LIM homeobox transcription factor 1, beta
LR	laminated retina
MITF	microphthalmia-associated transcription factor
MKI67/Ki-67	antigen identified by monoclonal antibody ki-67/proliferation related
MSC	mesenchymal stem cells
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2h-tetrazolium, inner salt
NES/Nestin	nestin
NLR	non laminated retina
NPC	neuroprogenitor cell
OCT	optical coherence tomography

OCT4	POU class 5 homeobox 1
PAX/Pax	paired box
PC	adult/progenitor stem cell
Pcs	peripheral cysts
PDGF-Rβ	platelet-derived growth factor receptor β
PFA	paraformaldehyde
PI	propidium iodide
PITX2	paired-like homeodomain 2
Pla	pars plana
Pli	pars plicata
PNA	peanut agglutinin (Arachis hypogaea)
PNN	pinin, desmosome associated protein
PVR	proliferative vitreoretinopathy
qPCR	quantitative polymerase chain reaction
RCA	Ricinus communis Agglutinin (Ricinus communis)
RD	retinal detachment
RNA	ribonucleic acid
RP	retina periphery
RPC	retinal progenitor cell
RPE	retinal pigment epithelium
Rt	room temperature
RT	reverse transcription
S.D.	standard deviation
SBA	soy bean agglutinin (Glycine max)
SEMA3A	semaphorin 3A
SERPINA3	serpin peptidase inhibitor, clade A member 3
SHH	sonic hedgehog
SOX2/Sox2	sex determining region y-box 2
STL	potatoe lectin (Solanum tuberosum)
TAC	transient amplifying cell
TGF β	transforming growth factor β
TP63/p63	tumor protein p63
TYR	tyrosinase
UEA	Ulex europaeus agglutinin (Ulex europaeus)
VEGF	vascular endothelial growth factor
VEGFR2/KDR	vascular endothelial growth factor receptor 2/Kinase insert domain receptor
VIM/Vim	vimentin
VVA	hairy vetch agglutinin (Vicia villosa)
WGA	wheat germ agglutinin (Triticum vulgaris)

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Supplement I

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Supplement III**

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*R.Albert has shared contribution: Figures 1 and 2: clinical selection of donor and preparation of bulbus for immunohistochemistry (50% contribution); Figure 3: establishment of PVR induction in mice and microsurgical injection, OCT examination, preparation of bulbus for immunohistochemistry (70% contribution); overall manuscript preparation (20% contribution)

**R.Albert has shared contribution: Overall manuscript preparation (20% contribution)

1. Introduction

Vision is the ability to interpret the surrounding environment by processing information carried by the visible light. Visual perception starts with light entering the eye through the transparent cornea, which then continues its way through the lens and focuses on the photoreceptor layer of the retina. The light is then translated into neuronal signals and further transported and processed in the visual cortex of the brain.

According to the World Health Organization's database, there were 285 million visually impaired people worldwide in 2012, out of which 246 million had low vision and 39 million were blind. The leading causes for visual impairment were uncorrected refractive errors and cataract, while for blindness they were cataract, glaucoma, diabetic retinopathy and age-related macular degeneration (AMD). Despite concerted efforts, many diseases that impair vision have no definitive treatment yet. As quality of vision tightly correlates with quality of life, visual impairment and blindness have important socio-economic and healthcare implications. Therefore, many attempts are being made to improve the therapeutic possibilities for treating blinding diseases.

2. Theoretical Background

2.1. Anatomy of the Eye

The human eye is structured like a miniature camera placed in the anterior half of the orbital cavity. The eyelid is the shutter; the pupil controls the amount of light entering the eye; the cornea and the lens act as a camera, the lens being responsible for focusing the image onto the retina; the latter acts somewhat like a film in a camera.

The human eye is composed of three concentric coats: 1) fibrous coat consisting of a white, opaque, tough sclera and a transparent cornea, 2) vascular coat consisting of a heavily pigmented vascular layer including the iris, ciliary body (CB) and choroid, and 3) a retinal coat.

The cornea is a clear, dome-shaped tissue of the anterior part of the outer fibrous layer. It is responsible for protecting the eye against injuries and infections; furthermore, it provides two-thirds of the total refractive power of the eye. The cornea consists of 6 layers (Dua et al., 2013), which are from the surface to inside: 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 (Mantelli and Argueso, 2008). It consists of 1-3 layers of superficial squamous cells with surface-extending microvilli allowing close association with the tear film, 1-2 layers of central supra-basal cells and a single layer of columnar basal cells. The corneal stroma accounts for about 90% of the corneal thickness. It is composed of several layers of collagen fibers that run parallel to the surface, which thanks to the periodical and equal organization give 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. A pre-Descemet's layer (also called Dua's layer) has recently been discovered (Dua et al., 2013); its presence was noted using a big bubble technique during deep anterior lamellar keratoplasty and it consists of an acellular, approximately 5-8 lamellae thick, predominantly, type I collagen. The corneal endothelial cells form a monolayer on the inner surface of the cornea facing towards the anterior chamber of the eye. The crucial function of this layer is to maintain corneal transparency by regulating the hydration while allowing nutrition of the cornea. The

transparency of the cornea is due to its uniform structure, avascularity and appropriate function of endothelial cells.

The cornea continues into the fibrous sclera, which further goes into the dural sheath of the optic nerve. The conjunctiva starts from the corneo-scleral junction, also called limbal rim, and covers the anterior surface of the sclera and the posterior surface of the eyelids connecting with the skin at the lid margin.

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 iris controls the amount of light entering the eye ball by adjusting the diameter of the pupil. Circular fibers of the ciliary muscle affect the tension of the zonular fibers resulting in changes in the shape and refraction of the lens. The non-pigmented epithelial cells of the CB are responsible for producing the aqueous humor which circulates between the anterior and posterior chambers of the eye and exits through the trabecular meshwork near the limbus.

The lens is a biconvex, transparent structure with adjustable refraction of 3-20 Diopters. Anterior to the lens are the anterior and posterior chambers, while posterior is the vitreous.

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. The rods are light sensitive and responsible for vision in the dark. The cones are not that sensitive to light, but are responsible for color vision. Inner to the external limiting membrane is the outer nuclear layer which contains the photoreceptor cell bodies. The outer plexiform layer consists of the connections of the bipolar, horizontal and amacrine cells with the photoreceptors in it, while the inner nuclear layer contains the cell bodies. The inner plexiform layer consists of the connections of ganglion cells with the

amacrine and bipolar cells. Inner to the ganglion cell layer is the nerve fiber layer which contains the ganglion cell axons that continue towards the optic nerve. The internal limiting membrane is the innermost layer of the retina, between the nerve fiber layer and the vitreous. Müller cells are the principal glial cells in the retina and are essential for the structural support and maintenance of balanced extracellular environment. The cell bodies of these cells are found in the inner nuclear layer and they project irregularly thick and thin processes in either direction - to the outer limiting membrane and the inner limiting membrane.

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

2.2. Eye Development

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 (Chow and Lang, 2001; Fuhrmann, 2010; Graw, 2010; Zuber, 2010). Table 1 summarizes the embryonic origin of each part of the eye.

Neural tube ectoderm	All layers of the retina
	Epithelial lining of the ciliary body and iris
	Optic nerve
	Vitreous
Surface ectoderm	Lens
	Corneal epithelium
Neural crest	Sclera
	Corneal stroma and endothelium
	Connective tissue and bones of the orbit
Mesoderm	Extraocular muscles
	Vascular endothelial cells

Table 1. Summary of the eye development and tissue origin. The neuroectoderm differentiates into the retina, iris, and optic nerve; the surface ectoderm gives rise to lens and corneal epithelium; the neural crest cells become corneal stroma, sclera and corneal endothelium; the mesoderm differentiates into the extraocular muscles and the fibrous and vascular coats of the eye (modified from (Harada et al., 2007)).

Initially, the eyes develop as a pair of outpocketings called optic vesicles on each side of the forebrain at the end of the fourth week of pregnancy. The optic vesicles subsequently come into contact with the surface ectoderm and induce lens placode formation. The latter then starts invaginating until it pinches off from the ectoderm and becomes a lens vesicle. The lens vesicle acts as an inducer to the optic vesicle to transform into a double-walled optic cup and

also signals or induces the ectoderm to transfer it to the cornea. Periocular mesenchymal cells derived from mesoderm and neural crest then migrate into the space between the anterior surface of the lens vesicle and the surface ectoderm (Trainor and Tam, 1995). The migrated cells form fibroblasts and melanocytes of the anterior iris stroma, keratocytes of the corneal stroma and the corneal endothelium. The mesenchyme in front of the lens vesicle is split into an irido-pupillary membrane (inner layer) and substantia propria of the cornea (outer layer) by the appearance of anterior chamber through vacuolization. The anterior and posterior chambers communicate with each other through the pupil after the irido-pupillary membrane disappears. The surface ectoderm cells overlaying the mesenchymal cells become the corneal epithelium (Haustein, 1983).

The outer layer of the double-walled optic cup containing pigment granules develops into the pigmented layer of the retina - the RPE. The inner (neural) layer differentiates into light-receptive elements (rods and cones) and also gives rise to neurons (bipolar, amacrine, horizontal and ganglion cells) as well as supporting cells (Müller glia). The axons of the ganglion cells form a nerve fiber layer which converges into the optic stalk which later develops into the optic nerve. The anterior one fifth of the inner layer remains one cell layer thick (non-laminated retina, NLR) and later divides into the *pars ciliaris retinae* characteristic of its folding (pars plana (Pla) and pars plicata (Pli)), and *pars iridica retinae* which forms the posterior layer of the iris. The mesenchyme inside the optic cup forms the vitreous and the hyaloid vessels which supply the lens during the intrauterine life and also develop the vascular layer on the inner surface of the retina. By the end of the fifth week, loose mesenchyme completely surrounds the eye primordium, while its inner layer forms a highly vascularized and pigmented choroid, and the outer layer develops into sclera which continues with the dura mater around the optic nerve (Sadler, 2012).

2.3. Molecular Regulation of Eye Development

One of the key regulators in eye development is a member of the paired box (PAX) family, PAX6, which expression begins before the neurulation starts, when a single eye field is presented at the anterior part of the neural plate. Other transcription factors expressed in the early eye-field are listed in Table 2.

Gene Symbol	Gene
RAX	retina and anterior neural fold homeobox
SIX3	sine oculis-related homeobox 3
SIX6	sine oculis-related homeobox 6
LHX2	LIM homeobox 2
MITF	microphthalmia-associated transcription factor
OTX2	orthodenticle homeobox 2
CHX10	ceh-10 homeo domain containing homolog
NES	nestin

Table 2: Early eye field genes (modified from (Sadler, 2012)).

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 is further expressed in the optic cup and the overlaying surface ectoderm, but it plays role only 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 (TGF β) beside other transcription factors like microphthalmia-associated transcription factor (MITF) and ceh-10 homeo domain containing homolog (CHX10). FGF is produced by the surface ectoderm and plays a role in the development of the neuroretina, while TGF β is produced by the surrounding mesenchyme and is important in the development of the pigmented layer of the retina.

Hence, the 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 as shown in Table 3.

Cell type	Gene Symbol	Gene Description
Ganglion	PAX6	paired box 6
	MATH3	neuronal differentiation 4
Amacrine	PAX6	paired box 6
	SIX3	sine oculis-related homeobox 3
	PROX1	prospero homeobox 1
	NEUROD	neuronal differentiation 1
	MATH3	neuronal differentiation 4

Horizontal	PAX6	paired box 6
	SIX3	sine oculis-related homeobox 3
	LIM1	LIM homeobox 1
	MATH3	neuronal differentiation 4
Bipolar	CHX10	ceh-10 homeo domain containing homolog
	MATH3	neuronal differentiation 4
	MASH1	achaete-scute complex homolog 1
Cone/rod	CRX	cone-rod homeobox
	NEUROD	neuronal differentiation 1
	MASH1	achaete-scute complex homolog 1
Müller	RAX	retina and anterior neural fold homeobox
	HES1	hairy and enhancer of split 1
	HES5	hairy and enhancer of split 5
	HESR2	hairy/enhancer-of-split related with YRPW motif 2

Table 3. Characteristic genes of retinal progenitor cells (modified from (Harada et al., 2007)).

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, but some contributing factors are known, such as PAX6 (Davis et al., 2003; Kroeber et al., 2010), paired-like homeodomain 2 (PITX2), Forkhead box transcription factor (FOXC1) (Cvekl and Tamm, 2004; Seo et al., 2012) , LIM homeobox transcription factor 1, beta (LMX1B) (Cvekl and Tamm, 2004), Kruppel-like factor 4 (Klf4) (Swamynathan et al., 2007) as well as pinin, desmosome associated protein (PNN) (Joo et al., 2010). Wnt/β-catenin (Arkell and Tam, 2012; Gage et al., 2008) and TGFβ pathways (Saika et al., 2001) are also involved in the anterior segment and cornea development in particular.

2.4. Stem Cells

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)(Alvarez et al., 2012; Ilic and Polak, 2011). The main differences among them are in their origin, self-renewal capacity and differentiation potential. The ESCs are pluripotent and show unlimited capacity for self-renewal (Semb, 2005). The iPSCs are somatic cells that have been

genetically reprogrammed to an ESC–like state by being forced to express genes, such as the POU class 5 homeobox 1 (OCT4), v-myc avian myelocytomatosis viral oncogene homolog (MYC), KLF4 and SOX2, important for maintaining the properties of ESCs (Takahashi and Yamanaka, 2006). Similar to the ESCs, they have high renewal capacity and pluripotency. The PCs are multi-, oligo- or unipotent and have limited proliferation capacity (Ilic and Polak, 2011).

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.

The fear of teratoma formation, immune-rejection issues and ethical concerns are also slowing down the progress of ESCs towards clinical trials (Deb et al., 2008; Lo and Parham, 2009). The iPSCs have major safety concerns about the involvement of retroviral or lentiviral vector integration and the possibility for genomic changes that can lead to tumor development (Lin et al., 2009; Stadtfeld et al., 2008; Zhou et al., 2009). Some reports highlight that the epigenetic memory from the somatic cell origin could limit the success of redifferentiation of these cells as well (Kim et al., 2010).

PCs can be found in many organs of the human body, such as bone marrow, skeletal muscle, heart, brain, skin and eye (Mimeault and Batra, 2008). As they have already undergone some critical developmental stages, they can only differentiate to the certain cell types of the organ/tissue they reside. PCs 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 (Power and Rasko, 2011; Snippert and Clevers, 2011). 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.

2.4.1. Limbal Epithelial Stem Cells

The most successful stem-cell-based therapy so far is the use of LESCs to regenerate the corneal epithelium (Pellegrini et al., 1997). 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. The latter are radially-oriented papillae-like fibrovascular ridges extending to the sub-epithelial connective tissue (Goldberg and Bron, 1982). Davanger and Evensen were the first in 1971 to suggest that corneal epithelium is maintained and renewed from the limbus (Davanger and Evensen, 1971). Histological sectioning of the corneo-scleral junction confirmed the presence of PCs at the basal layer of the crypts (Dua et al., 2005). The palisades are believed to provide protective environment for these PCs by hiding the cells from shear stress and supplying them with nutrients from the blood vessels (Boulton and Albon, 2004; Schlötzer-Schrehardt and Kruse, 2005). Dua *et al.* proposed that limbal crypts are most abundant in the nasal region (Shanmuganathan et al., 2007), while others found them preferentially located in the superior and inferior regions of the limbus (Shortt et al., 2007a; Wiley et al., 1991). 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 (Pellegrini et al., 1999; Schermer et al., 1986).

Huang and Tseng showed that only limited proliferative capacity of corneal epithelium remains in the absence of limbus (Huang and Tseng, 1991), which is in accordance with the findings of Majo *et al.* who showed some evidence for the presence of oligopotent cells at the central cornea in mice (Majo et al., 2008).

Unfortunately, there are no definitive markers for identifying PCs in general or specifically in corneal stem cells (Takács et al., 2009). 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.

2.4.1.1. Limbal Epithelial Stem Cell Deficiency

LESC deficiency (LESCD) is a disease in which LESCs are lost or damaged due to hereditary, acquired or sometimes unknown (idiopathic) diseases (Espana et al., 2002; Puangsricharern and Tseng, 1995). Maintenance of the corneal epithelium fails in LESCD,

thus the corneal epithelial defects appear and fail to heal properly. The barrier function of the limbus is also inappropriate, leading to corneal conjunctivization, loss of corneal clarity and visual impairment (Ahmad, 2012). The most common genetic background associated with LESCD is aniridia where, among several other abnormalities, developmental dysgenesis of the anterior segment including the limbus can be observed (Ramaesh et al., 2005). 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. LESCD can affect one eye (monocular) or both (binocular) which can be partial or diffuse. The diagnosis is mainly based on clinical symptoms (Ahmad et al., 2010) and can be confirmed by corneal impression cytology (Dart, 1997). In brief, filter paper is placed on the surface of the patient's previously anesthetized cornea and with the removal of the filter paper the superficial cells are peeled off. Normally, only corneal epithelial cells can be found on the paper; however, when corneal and conjunctival epithelial cells are mixed or only conjunctival cells can be found, LESCD is confirmed.

Definitive treatment requires the replacement of healthy LESCs. Due to lack of corneal donor tissues and decreased chance for graft survival, an autologous or homologous expansion of human LESCs has been proposed, which can be carried out directly via a piece of limbal tissue harvested from the contralateral healthy eye (autologous graft) or dissected from living tissue-matched eyes or non-matched cadaver eyes (allogeneic graft) (Kenyon and Tseng, 1989; O'Sullivan and Clynes, 2007). These procedures of harvesting relatively large limbal tissue carry the risk of serious complications like insufficient wound healing or damage of vision in the healthy eye, while side effects of the immunosuppressive drugs used to prevent rejection of the transplanted vascularized limbal tissue can cause other complications (Secker and Daniels, 2008). Kenyon and Tseng were the first to use limbal transplants from the healthy eye to treat the other eye of a patient with unilateral total LESCD (Kenyon and Tseng, 1989). Since then, the techniques of transplantation have been modified and advanced. Transplantation of small biopsies or *ex vivo* expanded cells from these biopsies either from living or cadaver donors can be an alternative for harvesting limbal grafts. The biopsy needs to be taken from a stem cell-rich region, yet, it should be small enough not to deplete the population of stem cells in the living donor eye (O'Callaghan and Daniels, 2011).

Pellegrini *et al.* were the first to take a biopsy from the limbus of a patient's healthy eye and culture LESCs from the graft to form a transplantable epithelial cell sheet. They used growth-

arrested 3T3 fibroblasts as feeder layer and therapeutic contact lens to place the epithelial cell sheet on the surface of the eye (Pellegrini et al., 1997).

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 (Baylis et al., 2011; Shahdadfar et al., 2012; Shortt et al., 2007b) and/or mouse 3T3 fibroblasts as a feeder layer (Notara et al., 2010). The use of animal material carries a risk of transferring prions or as-of-yet unknown diseases (Shahdadfar et al., 2012; Tekkatte et al., 2011). Therefore elimination of the animal materials would be a great advantage for preventing safety issues.

Different carriers have been proposed for supporting the growth of LESCs ranging from synthetic sources like temperature responsive polymers (Nishida et al., 2004a), chitosan-gelatin biopolymer (e la Mata et al., 2013), contact lens (Di Girolamo et al., 2009; Gore et al., 2013) and compressed collagen (Mi and Connon, 2013), to natural materials as fibrin matrix (Forni et al., 2013; Rama et al., 2001), human amniotic membrane (HAM) (Grueterich et al., 2003; Koizumi et al., 2007a; Pauklin et al., 2010) and LC (Galal et al., 2007).

Most of the clinical case reports use intact or denuded HAM either as a biologic drape to cover the bare stroma after the removal of abnormal conjunctival tissue (Gomes et al., 2005) or as a carrier for transplanting the LESCs (Grueterich et al., 2003; Koizumi et al., 2007a; Pauklin et al., 2010). HAM 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 (Plummer, 2009). The use of denuded HAM has been found superior to the intact form as a carrier for LESCs (Chen et al., 2010; Koizumi et al., 2007b). Although Pauklin *et al.* could successfully transplant cultured LESCs on intact HAM and restore the corneal surface, thus providing significant increase in visual acuity after 14-28 months (Pauklin et al., 2010), (Grueterich et al., 2003; Koizumi et al., 2007a; Pauklin et al., 2010), the main disadvantage of using HAM is its lack of optical transparency.

2.4.2. 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 (Ahmad et al.,

2000a; Tropepe et al., 2000) 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 (Perron and Harris, 2000). Stem cells at the ciliary marginal zone (CMZ) persist throughout lifetime of these animals, adding new cells as the eye grows and replacing damaged or lost retinal cells. These stem cells give rise to both neural retinal cells and RPE cells in these species (Fischer and Bongini, 2010; Karl and Reh, 2010; Locker et al., 2009; Moshiri et al., 2004). In addition to that, Fischer at al. showed generation of new retinal neurons at the retinal periphery (RP) in chicken even after one month of hatching (Fischer and Reh, 2000).

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.

Most studies regarding isolation and characterization of NPCs have been carried out in lower vertebrates and rodents, not in humans. Although, rodents represent ideal animal models for studies of the CNS due to their ease of maintenance, low cost, short breeding times and also due to the shared similarities in anatomy and physiology of the CNS with their human counterparts, not all the results of the experiments performed on rodents can be reflected in humans. 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 (Froen et al., 2011). 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. Due to the physical and geometrical nature of the spheres the nutrient and oxygen supply varies within the structure (Pastrana et al., 2011) may lead to differentiation of the cells (Arsenijevic et al., 2001). 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 (Liu et al., 2013).

2.4.2.1. 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.

The most common cause of blindness in people over the age of fifty in developed countries is AMD, which is characterized by central loss of vision. Although the exact cause of AMD is not known, age is considered to be the major risk factor for its development. AMD has two forms: dry and wet type, based on the integrity of the blood-retinal barrier. There is no treatment for the dry type of AMD, but repeated intraocular injections of anti-vascular endothelial growth factor (VEGF) can slow down the progression of the wet type of AMD (Rofagha et al., 2013).

Retinitis pigmentosa is an inherited genetic disease with primary photoreceptors (rods) degeneration leading to gradual loss of night and peripheral vision, causing tunnel vision. Later, loss of central vision leads to total blindness for which, unfortunately, no treatment is available to stop the progression.

Glaucoma leads to a progressive damage of retinal ganglion cells with subsequent visual field loss. In most of the cases, increased intraocular pressure (IOP) is a key modified factor, but glaucoma can also occur with normal IOP. Drugs and surgical procedures can only slow down the progression of glaucoma, but as of yet there is no definitive cure.

RD can appear due to rhegmatogenous and non-rhegmatogenous causes. Full-thickness defect in the neuroretina allows fluid to move throw 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). Many of the factors that increase the risk of PVR are the same as those of RD, such as high degree of myopia (short-sightedness), family history and previous cataract surgery. RD can also be caused by other diseases in the eye such as tumors, severe inflammation or complications from diabetes. Early

detection and treatment of RD can prevent PVR from occurring, so regular eye examinations can play an important preventative role. Signs of presence of NPCs would be expected in PVR, due to the damage of retinal cells.

3. 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, colony formation assay).
- 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.

4. Materials and Methods

4.1. 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. Samples obtained from patients (LCs, vitreous) were collected after written informed consents had been signed. 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.

4.2. Sample Dissection

4.2.1. Human Samples

4.2.1.1. Isolation of Limbal Epithelial Stem Cells

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) (PAA, Pasching, Austria) (20 minutes, 37^{o}C) to remove any remaining lens epithelial cells.

4.2.1.2. Isolation of Retinal and Ciliary Body Epithelial 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 (L15, Invitrogen, Carlsbad, CA) containing Dispase (1.2 U/ml, Roche Diagnostics, Basel, Switzerland) 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 (BD Biosciences, San Diego, CA) and resuspended as single cells at a final density of 150 000 cells/mL.

4.2.1.3. Vitreous Samples

Vitreous samples were collected during vitrectomies for RD with (10 samples) or without (15 samples) confirmed PVR based upon evaluation of wide angle images (Optomap P200Tx, Optos, Dunfermline, UK). 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*.

4.2.2. Mice Samples

To characterize the pathological changes in PVR formation, intravitreal injection of the proteolytic enzyme dispase was given to mice. Dispase is known to induce glial activation as well as both epi - and subretinal membrane formation (Frenzel et al., 1998; Soler et al., 2002).

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 (Novocaine) 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, as previously described (Soler et al., 2002). Control animals received 4 µL of sterile physiological saline solution in their right eyes. All left eyes remained untreated. Stratus Optical Coherence Tomography images (OCT, Zarl Zeiss Meditec, DublinCA) 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.

4.3. In Vitro Culturing of Cells

Limbal grafts were cultured in Dulbecco modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% human AB serum (Human serum Type AB, PAA, Pasching, Austria), 200 mM/mL L-glutamine (Sigma-Aldrich), 10,000 U/mL penicillin-10 mg/mL streptomycin (Sigma-Aldrich) at 37^{o}C 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%, Invitrogen), epidermal growth factor (EGF) (20 ng/ml, R&D Systems), bFGF (10 ng/ml, R&D Systems, MN), 1% fetal calf serum (FCS, Sigma-Aldrich), Heparin (2.5 mg/ml, LEO Pharma, Denmark) and Penicillin/Streptomycin (100 U/ml, Sigma-Aldrich, St. Louis, MO) at 37^{o}C 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%, Invitrogen) for 2-4 minutes.

4.4. Characterization of In Vitro Cultured Cells

4.4.1. Cultured Limbal Epithelial Stem Cells

4.4.1.1. Assay for Cell Death Analysis

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 (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) and data was analyzed using WinMDI freeware (Joseph Trotter, La Jolla, CA,USA). Alternatively, cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA) according to manufacturer's recommendations. Index of cell viability was calculated by measuring the absorbance of the formazan product resulting from reduction of the MTS tetrazolium compound at 490 nm by Wallac 1420 VICTOR2™ Counter (Perkin Elmer, Turku, Finland)).

4.4.1.2. Histological and Immunofluorescent Analysis

LESCs grown on the surface of glass-cover slips or denuded human LCs were fixed in 4% PFA for 20 min at room temperature (Rt).

The LC-grown samples were embedded in paraffin; consequently, 3 µm thick longitudinal sections were made for staining with Hematoxylin and Eosin (H&E) according to standard laboratory protocols.

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) fluorescent microscope (list of antibodies used for characterization is provided in Table 4).

Antibody	**Clone**	**Cat.No.**	**Company**
ABCG2	BXP-21	b7059	Sigma
CD14	134620	FAB3832P	R&D
CD29/Itg β1	P5D2	FAB17781P	R&D
CD31/PECAM	9G11	FAB3567P	R&D
CD34	581	555821	BD
CD44/HCAM	G44-26	555478	BD
CD45	HI30	555485	BD
CD47	472603	FAB4670A	R&D
CD49a/Itg α1	TS2/7	328304	Biolegend
CD49b/Itg α2	HAS3	FAB1233P	R&D
CD49f/Itg α6	GoH3	555735	BD
CD56/NCAM	301040	FAB2408A	R&D
CD73	AD2	550257	BD
CD90/Thy-1	50000000000	555595	BD
CD104/Itg β4	-	555720	BD
CD105/Endoglin	166707	FAB10971F	R&D
CD117/c-kit	47233	FAB332P	R&D
CD133	AC133	130-090-826	Miltényi
CD144/VE-Cadherine	55-7H1	560410	BD
CD146/MCAM	128018	FAB932A	R&D
CD147/Neurothelin	HIM6	555962	BD
CD166/ALCAM	105902	FAB6561P	R&D

CXCR4	44717	FAB173A	R&D
CK 8/18	5D3	ab 17139	abcam
CK 19	-	rb-9021-P	NeoMarkers
HLA-DR	L203	FAB4869F	R&D
Ki-67	-	RM-9106-S	NeoMarkers
p63α	Ab-4	Ms-1084-P	NeoMarkers
PDGF Rβ	PR7212	FAB1263P	R&D
VEGFR2/KDR	89106	FAB357P	R&D
Vim	SP20	RM-9120-S	NeoMarkers

Table 4. Details of the antibodies used for immunohistochemistry and/or flow cytometry analysis of limbal epithelial stem cell.

4.4.1.3. Detection of Cell Surface Markers by Flow Cytometry

To analyze the phenotype of the cultured LESCs, multicolor flow cytometric analysis was performed. FITC, R-phycoerythrin (PE) and allophycocyanin (APC) conjugated antibodies were used to measure 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/Itg α1 (Biolegend, San Diego, CA, USA), CD14, CD29/Itg β1, CD31/platelet/endothelial cell adhesion molecule (PECAM), CD47, CD49b/Itg α2, 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) (all from R&D Systems, Minneapolis, MN, USA) and CD133 molecules (Miltenyi Biotech, Gladbach, Germany) (for further details refer to Table 4). 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 (BD Biosciences Immunocytometry Systems) and data were analyzed using WinMDI freeware (Joseph Trotter, La Jolla, CA, USA).

4.4.1.4. Microarray and Data Analysis

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 (Ambion) and GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturers' protocols. After washing, the arrays were stained using FS-450 fluidics station (Affymetrix). 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 was applied for normalization. Gene transcripts with maximal signal values of less than 32 across all arrays were removed to filter for low and non-expressed genes, reducing the number of gene transcripts to 23190. 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.

4.4.1.5. Colony Forming Assay

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/cm2 density onto 0.1% Gelatine (Sigma-Aldrich), 10 ng/mL Fibronectin (BD Biosciences) or MethoCult (Stem Cell Technologies, Vancouver, Canada) coated 6 well plates. Standard growth medium for the LESCs was used and changed every other day. 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 (Olympus, Münster, Germany), acquired and analysed by ScanR (Olympus) software.

4.4.2. Cultured Retinal, Ciliary Body Epithelial and Vitreous Samples

4.4.2.1. Histological and Immunofluorescent Analysis

Before fixation in 4% PFA and paraffin embedment, a mixture of human plasma and thrombin (Sigma Aldrich) was used to clot the spheres. 3µm sections were cut and stained subsequently. H&E staining according to standard laboratory protocols was performed for morphological examination. Spheres were investigated by N-cadherin, Claudin1, glial fibrillary acidic protein (GFAP), Nestin, β-III-tubulin, Rhodopsin, Ki-67, Sox2, Pax6, RPE65 and ABCG2 primary antibodies followed by the incubation with fluorescently labelled secondary antibodies and Hoechst 33342 for nuclear staining. Sections were analyzed using an Olympus BV 61 FluoView confocal microscope (Olympus, Hamburg, Germany) and a ZEISS Axio Observer.Z1 fluorescence microscope (ZEISS, Oberkochen, Germany). List of antibodies used for characterization is provided in Table 5.

Antibody	**Company**
ABCG2	Sigma-Aldrich
Claudin1	LabVision
GFAP	Santa Cruz Biotechnology
Ki-67	Neo Markers
N-Cadherin	DAKO
Nestin	Sigma-Aldrich
Nestin	Santa Cruz Biotechnology
Pax6	Chemicon
Rhodopsin	Sigma-Aldrich
RPE65	Millipore
Sox2	Chemicon
β-III tubulin	Sigma-Aldrich

Table 5. Details of the antibodies used for immunohistochemical analysis of cultured retinal, ciliary body epithelial and vitreous samples

4.4.2.2. Transmission Electron Microscopy

Freshly prepared aldehyde-fixative containing 0.1 M 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 4^{o}C, post fixed 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 (Electron Microscopy Sciences, Hatfield, PA). Ultra-thin sections (60-70 nm thick) were cut on a Leica Ultracut Ultramicrotome UCT (Leica, Wetzlar, Germany), stained with uranyl acetate and lead citrate, and followed by examination using a Tecnai12 transmission electron microscope (Phillips, Amsterdam, the Netherlands).

4.4.2.3. 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 (Bharti et al., 2011; Lawrence et al., 2007) and CBE cells forming pigmented spheres *in vitro* (Coles et al., 2004; Moe et al., 2009). Total RNA was extracted using TRIzol Reagent according to the manufacturer's protocol, RNA concentration and purity was measured using Nanodrop (Wilmington, DE). Reverse transcription (RT) was performed using the High Capacity cDNA Archive Kit (Applied Biosystems, Abingdon, U.K.) with 200 ng total RNA per 20 µL RT reaction. The qPCR was performed using the StepOnePlus RT-PCR system (Applied Biosystems) and Taqman Gene Expression assays following protocols from the manufacturer (Applied Biosystems). The thermo cycling conditions were 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15 sec and 60^{o}C for 1 min. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method as the fold change in gene expression relative to CBE spheres which was arbitrarily chosen as calibrator and equaled one. All samples were run in duplicates (each reaction: 2.0 µL copy-deoxyribonucleic acid (cDNA), total volume 15 µL). Primers used for qPCR are listed in Table 6.

Gene Symbol	**Assay ID**
GAPDH	Hs99999905_m1
KLF4	Hs00358836_m1
OCT4	Hs03005111_g1
Nanog	Hs02387400_g1
SOX2	Hs01053049_s1
c-MYC	Hs00905030_m1
GFAP	Hs00157674_m1
PAX6	Hs01088112_m1
RAX	Hs00429459_m1
SIX3	Hs00193667_m1

LHX2	Hs00180351_m1
MITF	Hs01117294_m1
CHX10	Hs01584046_m1
OTX2	Hs00222238_m1
NES	Hs00707120_s1
TYR	Hs00165976_m1
Notch1	Hs01062011_m1
CRALBP	Hs00165632_m1
MKI67	Hs01032443_m1
GS	Hs00365928_g1

Table 6. List of primers used for qPCR.

4.5. 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. Longitudinal sections (3-7µm thick) perpendicular to the surface of the corneo-scleral tissue, were obtained for H&E staining and immunofluorescent labelling with anti-CD34, CD45, CD144/VE-Cadherin, CD144/HCAM, CD146/MCAM and CD166/ALCAM antibodies (list of primary antibodies is provided in Table 4).

The posterior segments of the human cadaveric eyes with or without PVR were embedded into paraffin after fixation in 4% PFA. Axial sections (3-10 µm thick) were cut from the iris to the mid-peripheral retina and stained for N-cadherin, Claudin1, GFAP, Nestin, Rhodopsin, Sox2, Pax6 and ABCG2 using LabVision Autostainer360 (Lab Vision Corporation, VT).

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

4.6. Statistics

Each experiment was performed at least three times and each sample was tested in triplicate (exceptions were marked accordingly). 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.

5. Results

5.1. Cultured Limbal Epithelial Stem Cells

5.1.1. 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 (Figure 1A2 and 1A4), and confluent cultures were obtained after 14 days. Furthermore, grafts grown on denuded human LC produced stratified epithelial layers within 7 days of cultivation (Figure 1B).

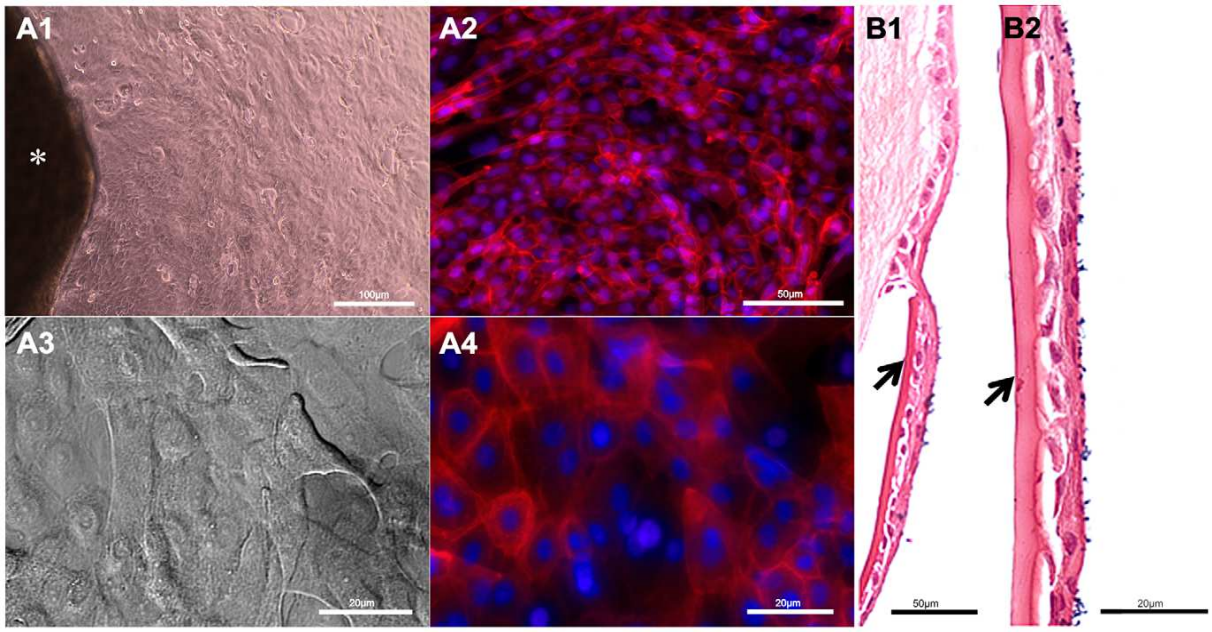

Figure 1. Limbal graft cultured on cell culture plate or denuded human lens capsule. Limbal graft (*) cultured on cell culture plate (A) or human lens capsule (LC) (B) shows epithelial cell outgrowth. (A) representative pictures of 3 days cultivation on culture plates (A1 and A3 are bright field images, A2 and A4 show fluorescent labelling against actin cytoskeleton (red) and nucleus (DAPI, blue)); (B) Hematoxylin and Eosin staining of limbal graft-derived stratified epithelial layers grown on LC after 7 days of cultivation. (Scale bars: A1: 100 µm; A2, B1: 50 µm; A3, A4, B2: 20 µm).

According to the MTS 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 (Figure 2B), respectively.

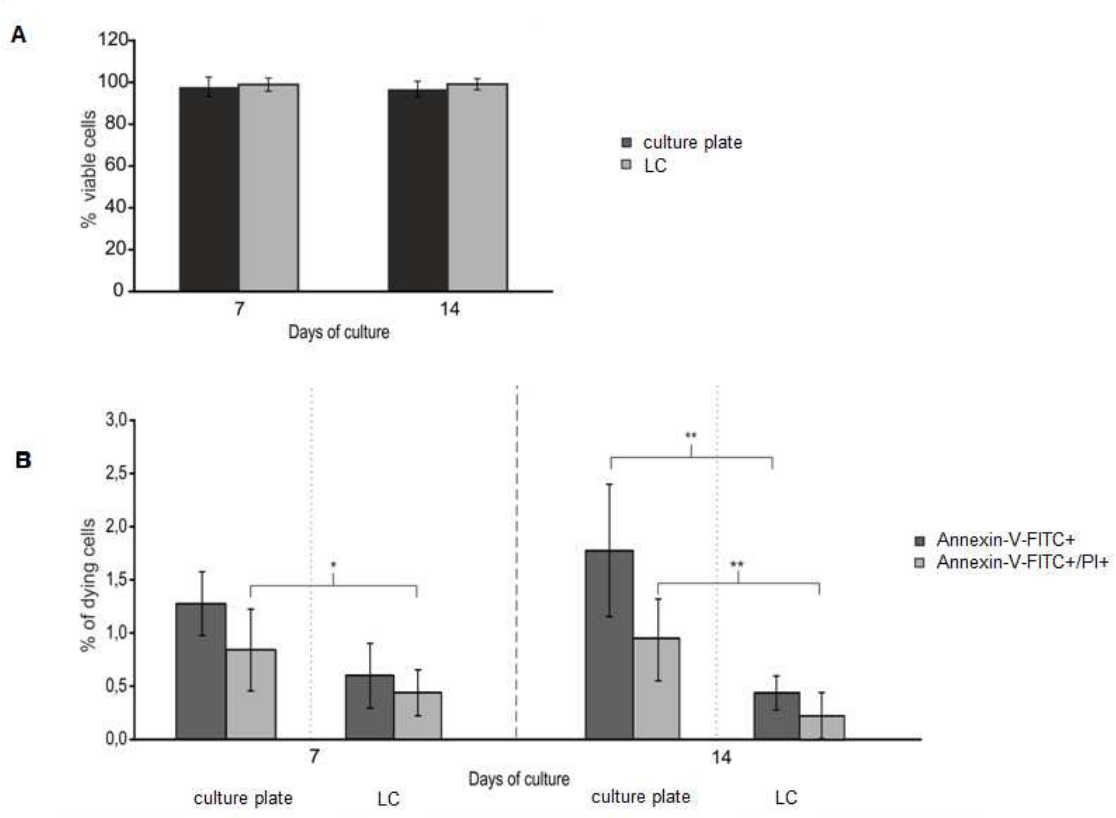

Figure 2. Cell viability and death of the cultured limbal epithelial stem cells. (A) Under both growth conditions (culture plate: black bar, lens capsule (LC): grey bar) the outgrowing cells showed more than 97% viability after 7 or 14 days of cultivation (n=4), (B) Annexin-V-FITC$^+$ cells (early apoptotic cells, dark grey bar) and Annexin-V-FITC$^+$/Propidium iodide$^+$ cells (late apoptotic cells, light grey bar) remained less than 2% at the two check points (n=3). Data shown are mean $\pm$S.D, * =$p<0.05$, **= $p<0.01$.

5.1.2. 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 (Schlötzer-Schrehardt and Kruse, 2005) 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 (Figure 3). Strong staining of CK8/18 was present and co-localization with the proliferation marker Ki-67 could be observed in some cells (Figure 3A insert), 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 (negative data are not shown) 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 (Figure 4).

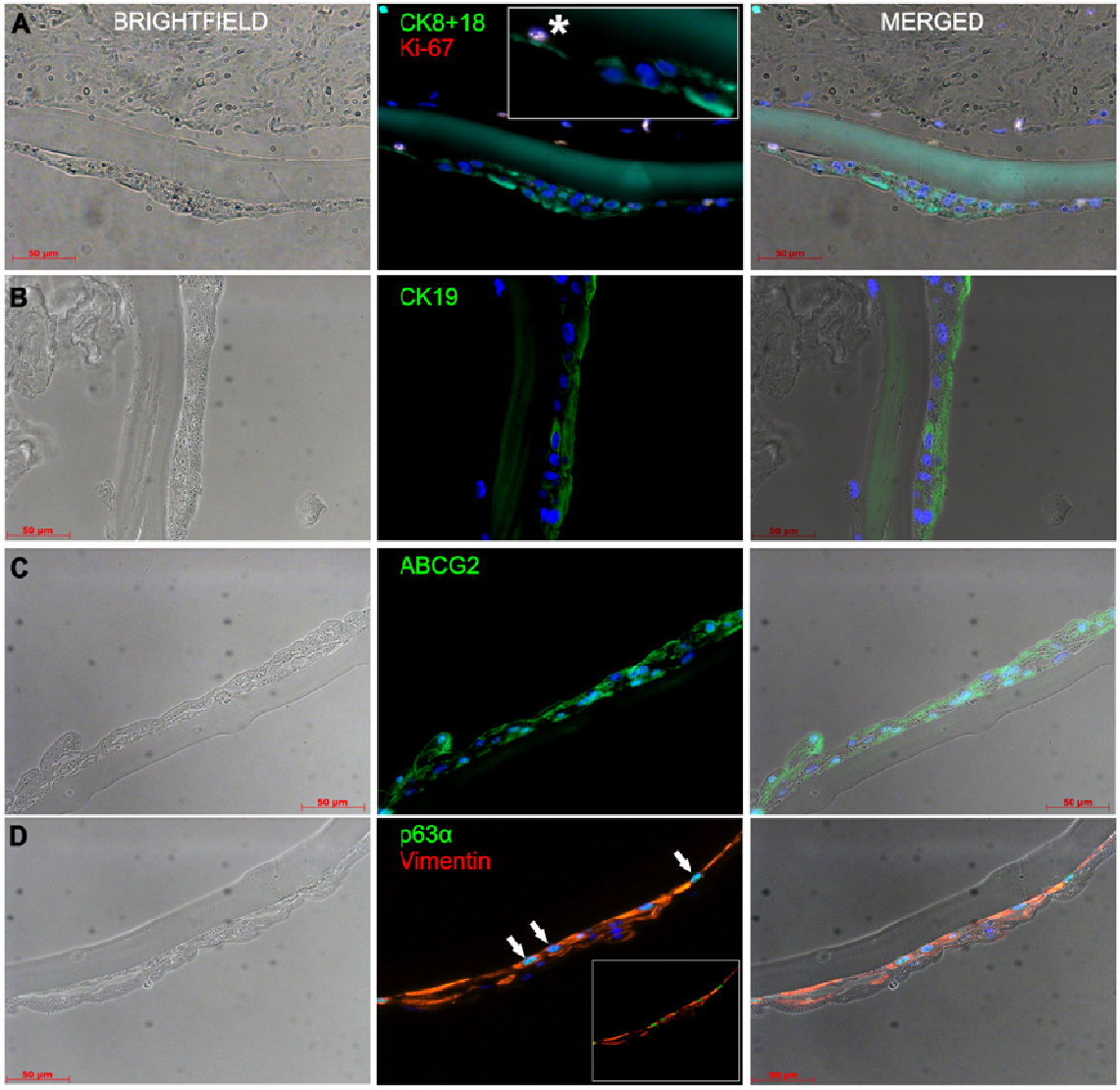

Figure 3. Immunofluorescence staining of epithelial-, stemness- and proliferation specific markers in limbal epithelial stem cells grown on human lens capsule.
Immunohistochemistry was performed to detect and localize the (co)-expression of CK8/18/Ki-67, CK19, ABCG2, Vim/p63α in the limbal epithelial stem cells grown on human lens capsule. Left column: bright field-; Center: immunofluorescent; Right column: merged image. Colors of the text correspond to the colors of the markers examined, while all nuclei are stained blue with DAPI. *: refers to the co-localization of $CK8^{+}18$ and Ki-67; arrows: show expression of vimentin in the basal cells. The images are representative of at least 3 independent experiments, scale bar: 50 µm.

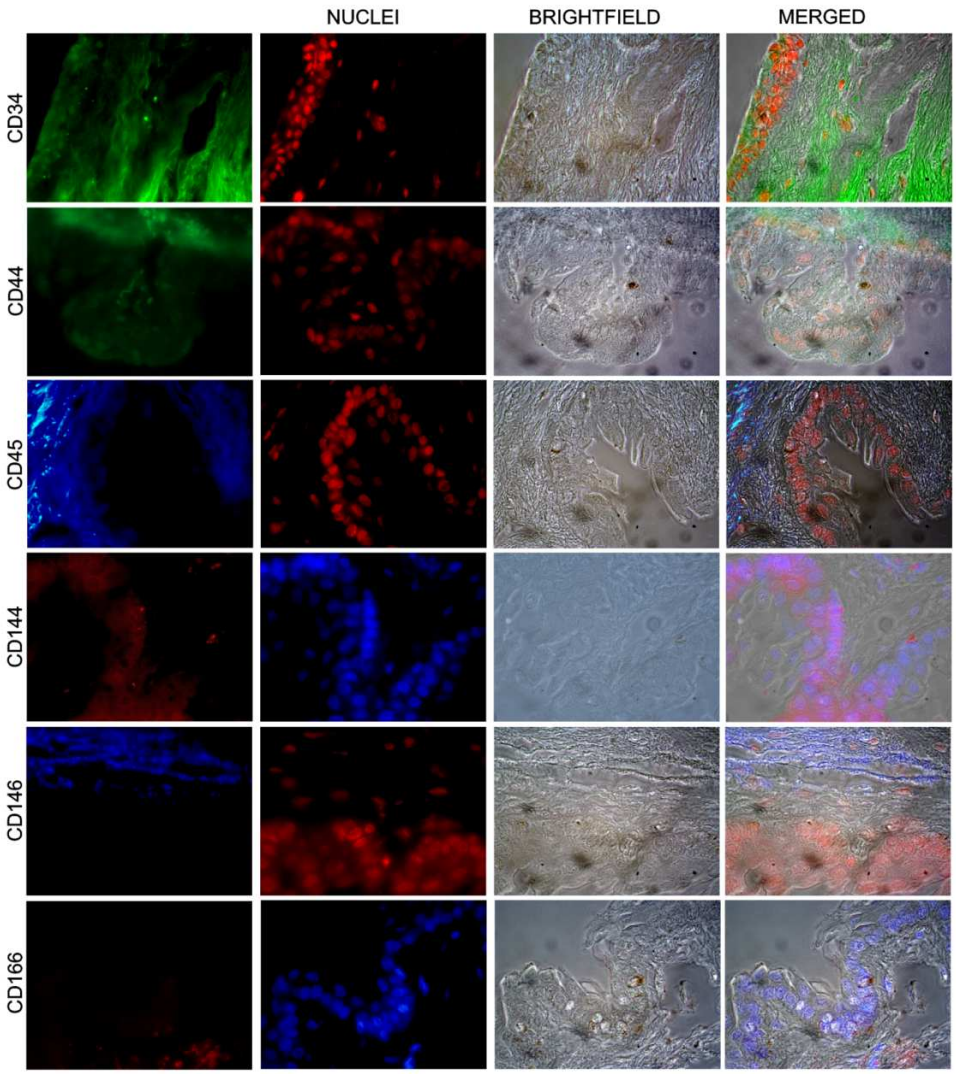

Figure 4. In situ immunohistochemical analysis of human limbal epithelium. Yet uncharacterized putative markers of limbal epithelial stem cells could be localized: CD44/HCAM in the apical layer, and CD146/MCAM and CD166/ALCAM in the basal layer. Magnification: 600x.

5.1.3. 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. The results are summarized in Table 7, while the representative histograms are presented in Figure 5.

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/Itg β4 ($p=0.0458$) 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-Rβ 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/Itg β1, CD49a/Itg α1, CD56/NCAM, CD146/MCAM and CD166/ALCAM were similar, while LESCs expressed CD44/HCAM at lower ($p=0.00052$), and CD49b/Itg α2 ($p=0.038$) and CD49f/Itg α6 ($p=0.008$) at higher levels compared to bmMSCs.

		LESC	bmMSC	
Hematopoietic and Monocyte markers	CD14	12.13±4.85	0.38±0.1	*
	CD34	0±0	0±0	
	CD45	0±0	0±0	
	CD47	98.98±0.10	96.97±0.81	
	CD133	0±0	0±0	
	CD117/c-kit	17.98±4.53	0±0	*
	CXCR4	27.81±4.41	0±0	**
	HLA-DR	0±0	0±0	
Endothelial markers	CD31/PECAM	0±0	0±0	
	CD144/VE-Cadherin	81.92±3.57	41.55±9.57	*
	VEGFR2/KDR	0±0	0±0	
	CD104/Itg β4	75.87±5.18	38.49±10.31	*
MSC Fibroblast markers	CD73	87.91±1.24	90.59±1.80	
	CD90/Thy-1	12.25±4.29	90.13±0.96	***
	CD105/Endoglin	42.09±4.91	81.90±1.96	***
	CD147/Neurothelin	97.13±0.33	75.21±7.81	
	PDGF Rβ	54.93±1.68	75.36±7.80	
Integrins and CAMs	CD29/Itg β1	97.01±0.40	92.77±1.65	
	CD44/H-CAM	16.55±4.95	87.90±2.48	***
	CD49a/Itg α1	71.73±6.09	71.42±7.15	
	CD49b/Itg α2	91.16±1.27	60.55±7.19	*
	CD49f/Itg α6	68.38±8.18	0±0	***
	CD56/NCAM	2.17±1.03	24.68±7.57	
	CD146/MCAM	82.40±3.11	87.28±2.18	
	CD166/ALCAM	98.02±0.20	86.57±6.26	

Table 7. Expression of hematopoietic-, endothelial-, mesenchymal stem cell markers and adhesion molecules on limbal epithelial stem cells. The expression pattern of different groups of cell surface markers was compared between limbal epithelial stem cells (LESCs) and bone marrow derived mesenchymal stem cells (bmMSCs). Significant differences were found in the expression of CD14, CD117/c-kit, CXCR4, which are markers of special progenitor cell types; CD90/Thy-1 and CD105 which determine the MSC phenotype; CD44/HCAM, CD49b/Itg α2 and CD49f/Itg α6. Data represent percentage of positive cells within the total LESC culture shown as mean±S.D. (n=7; $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***)

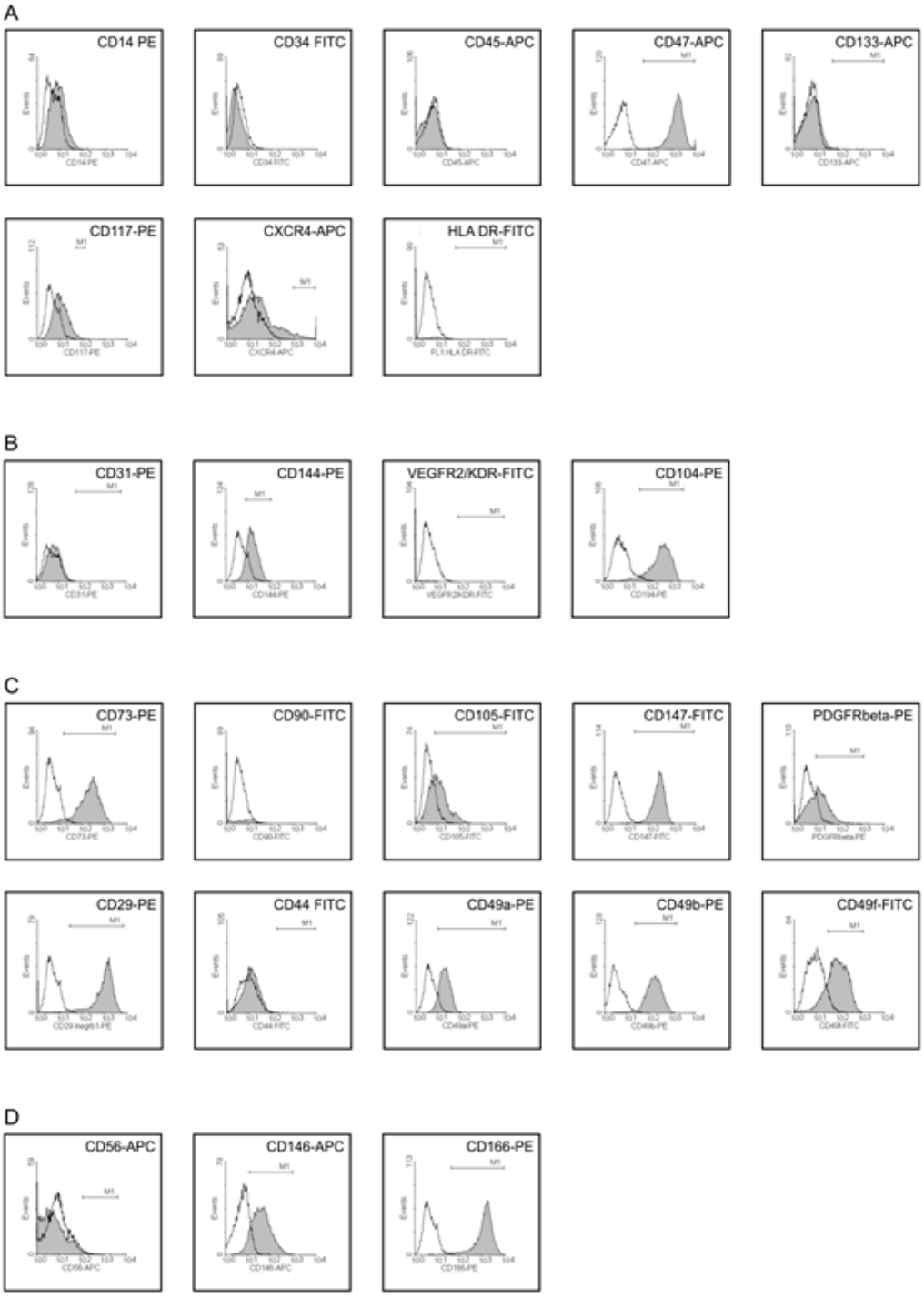

Figure 5. Representative histograms of the expression of hematopoietic-, endothelial-, mesenchymal stem cell markers and adhesion molecules. (A) hematopoietic (CD14, CD34, CD45, CD47, CD133, CD117/c-kit, CXCR4, HLA-DR), (B) endothelial (CD31/PECAM, CD144/VE-Cadherin, VEGFR2/KDR, CD104/Itg β4), (C) mesenchymal stem cell markers (CD73, CD90/Thy-1, CD105/Endoglin, CD147/Neurothelin, PDGF-Rβ) and (D) adhesion molecules (CD29/Itg β1, CD44/HCAM, CD49a/Itg α1, CD49b/Itg α2, CD49f/Itg α6, CD56/NCAM, CD146/MCAM, CD166/ ALCAM) on limbal epithelial stem cells.

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±46.07%), similar CD146/MCAM (74.22±2.23%) and higher CD44/HCAM (50.43±29.28%) expression compared to the cultured LESCs (n =3). The LESCs are therefore a distinct cell population from the LECs originally found on LC, further supporting the fact that LCs were denuded of LECs before use.

It is known that stem cells can be characterized by their unique surface carbohydrate pattern which shows changes during differentiation (Dodla et al., 2011; Wearne et al., 2008). Lectin-based screening of the most common terminal cell-surface glycolipids and glycoproteins was carried out on LESCs (Figure 6 and Table 8) and revealed presence of subpopulations within the cell culture. Staining with WGA showed high sialic acid content (Median = 1423.19±8.08), while more than 50% of the cells (51.59±3.1%) showed strong ConA (Fluorescence Intensity Median (FImed) = 2125.02±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±1.06) regarding low T-antigen expression (FImed =850.79±14.96). UEA-lectin exhibited moderate fluorescence intensity on 61.1±1.97% of the cells, indicating low levels of detectable fucose molecules on LESCs.

	Lectin	Cells (%)	Median of FI	Affinity
Sialic acid	WGA	97.37±0.33	1423.19±8.08	GlcNAcβ1-4GlcNAcβ1-4GlcNAc. Neu5Ac (sialic acid)
N-acetylglucosamine binding lectins	STL	97.68±1.64	335.38±35.63	GlcNAc oligomers
	DSL	98.94±0.23	190.04±1.23	(b-1.4) linked N-acetylglucosamine oligomers
	ECL	95.64±2.79	59.45±0.16	galactosyl (b -1.4) N-acetylglucosamine
	LEL	83.39±14.23	38.15±4.22	N-acetylglucosamine oligomers
	GSL II	82.52±2.66	24.63±0.27	alpha- or beta-linked N-acetylglucosamine
Mannose binding lectins	ConA	51.59±3.10	2125.02±26.00	high-mannose type. hybrid type and biantennary complex type N-Glycans
Galactose N-acetylgalactosamine binding lectins	RCA	98.28±0.35	850.79±14.96	Galβ1-4GlcNAcβ1-R
	PNA	97.92±0.51	185.75±1.06	Galβ1-3GalNAcα1-Ser/Thr (T-Antigen)
	AIL	98.99±0.01	687.85±7.61	(Sia)Galβ1-3GalNAcα1-Ser/Thr (T-Antigen)
	VVA	94.49±0.38	61.49±5.32	alpha- or beta-linked terminal N-acetylgalactosamine
	DBA	89.93±2.54	12.24±0.10	N-acetylgalactosamine
	SBA	97.11±0.60	211.11±1.32	a- or b-linked N-acetylgalactosamin
Fucose binding lectins	UEA	61.10±1.97	148.39±5.91	Fucα1-2Gal-R

Table 8. Lectin-based staining of surface carbohydrate molecules on limbal epithelial stem cells. The surface of limbal epithelial stem cells (LESCs) contained high amount of sialic acid, N-acetylglucoseamine and galactose molecules. Approximately 50% of the cells contained mannose and two-thirds contained fucose molecules on their surface. WGA: Wheat germ agglutinin (Triticum vulgaris), 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), ConA: Concanavalin A (Canavalia ensiformis), 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), UEA: Ulex europaeus agglutinin (Ulex europaeus) (Data shown are mean±S.D., n=3).

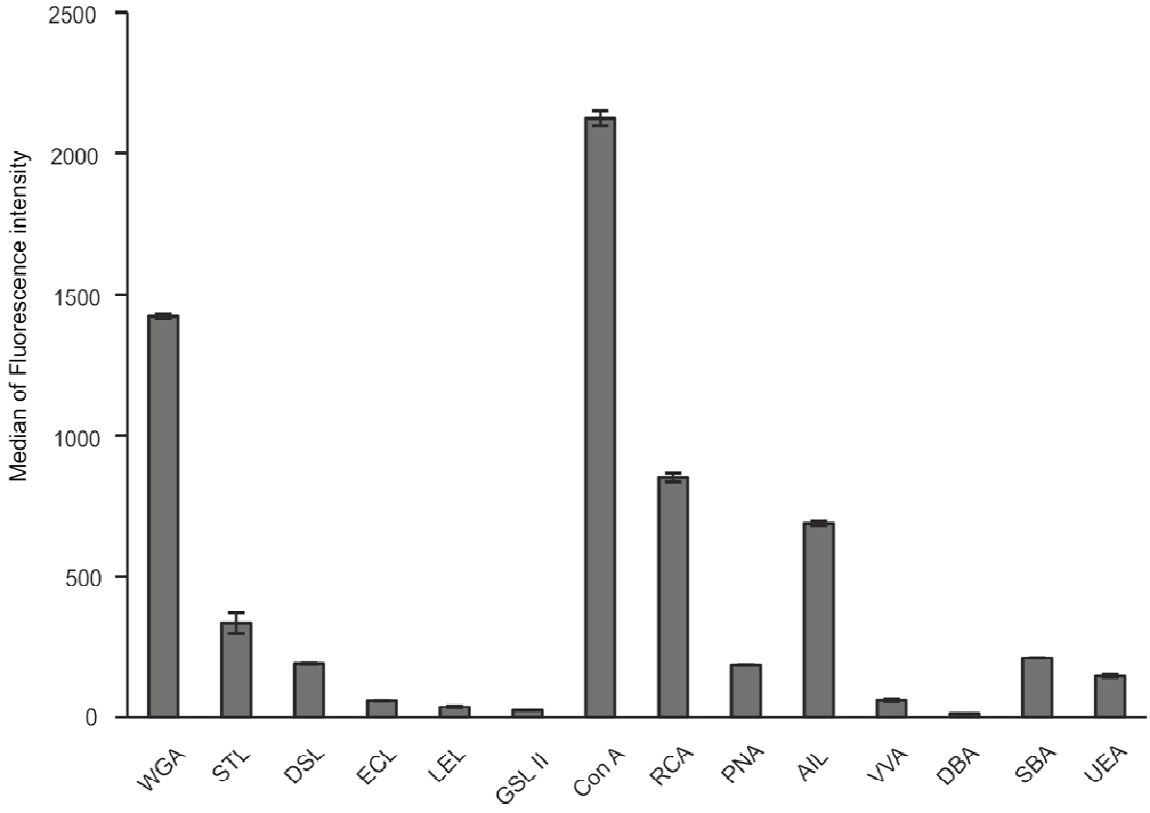

Figure 6. Expression pattern of carbohydrate molecules on the surface of limbal epithelial stem cells. Lectin-based staining of carbohydrate specific molecules on the surface of limbal epithelial stem cells (LESCs). WGA: Wheat germ agglutinin (Triticum vulgaris), STL: Potatoe lectin (Solanum tuberosum), 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), ConA: Concanavalin A (Canavalia ensiformis), 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), UEA: Ulex europaeus agglutinin (Ulex europaeus) (Data shown are mean$\pm$S.D. of the median of fluorescence intensity, n=3).

5.1.4. 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 fold change (FC) increase or decrease in expression (955 and 875 transcripts), indicating a relatively high transcriptional difference between the two cell types ($n=3$, $p<0.01$).

Figure 7 and Table 9 show the heatmap and the functional clustering of 67 selected genes based on their high or low FC or previously documented relation to LESCs ($n=3$, $p<0.01$). 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 (Table 9). 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) (Table 9).

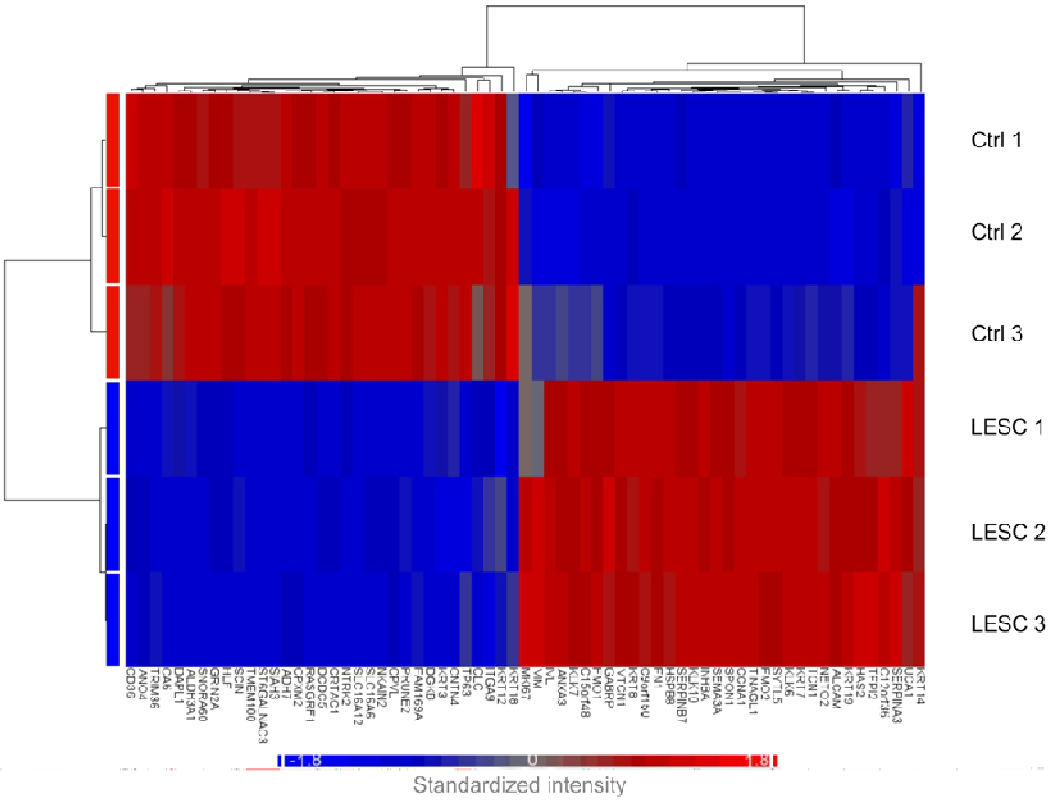

Figure 7. Transcriptional profiling in the limbal epithelial stem cells. Heatmap of the transcripts and functional clustering of 67 genes selected on the basis of their high or low fold change or previously documented relation to limbal epithelial stem cells (n=3, $p<0.01$). Red and blue colors indicate high and low expression, respectively.

Gene symbol	Gene description	FC	Regulation	Molecular function
KRT14	Keratin 14	2	Up	Structural constituent of cytoskeleton
SERPINA3	Serpin peptidase inhibitor, clade A member 3	21	Up	DNA binding
KRT19	Keratin 19	6	Up	Structural constituent of cytoskeleton
ALCAM	Activated leukocyte cell adhesion molecule	20	Up	Receptor binding
KRT7	Keratin 7	31	Up	Structural molecule activity
KLK6	Kallikrein-related peptidase 6	71	Up	Serine-type endopeptidase activity
FMO2	Flavin containing monooxygenase 2	75	Up	Monooxygenase activity
SEMA3A	Semaphorin 3A	40	Up	Receptor activity

KLK10	Kallikrein-related peptidase 10	29	Up	Serine-type endopeptidase activity
SERPINB7	Serpin peptidase inhibitor, clade B member 7	29	Up	Serine-type endopeptidase inhibitor activity
FN1	Fibronectin 1	75	Up	Extracellular matrix structural constituent
KRT8	Keratin 8	4	Up	Structural molecule activity
KLK7	Kallikrein-related peptidase 7	57	Up	Serine-type endopeptidase activity
VIM	Vimentin	4	Up	Structural constituent of cytoskeleton
MKI67	Antigen identified by monoclonal antibody Ki-67	3	Up	Nucleotide binding
KRT18	Keratin 18	1	Down	Structural molecule activity
KRT12	Keratin 12	6	Down	Structural molecule activity
ITGA9	Integrin, alpha 9	1	Down	Receptor activity
TP63	Tumor protein p63	1	Down	DNA binding
KRT3	Keratin 3	31	Down	Structural molecule activity
NTRK2	Neurotrophic tyrosine kinase, receptor, type 2	30	Down	Nucleotide binding
CRTAC1	Cartilage acidic protein 1	72	Down	Calcium ion binding
DCDC5	Doublecortin domain containing 5	43	Down	Tubulin binding
RASGRF1	Ras protein-specific guanine nucleotide-releasing factor 1	20	Down	Guanyl-nucleotide exchange factor activity
CPXM2	Carboxypeptidase X, member 2	25	Down	Metallocarboxypeptidase activity
ADH7	Alcohol dehydrogenase 7	64	Down	Alcohol dehydrogenase (NAD) activity
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1	30	Down	Aldehyde dehydrogenase (NAD) activity
DAPL1	Death associated protein-like 1	33	Down	Epithelial differentiation or apoptosis
CA6	Carbonic anhydrase VI	33	Down	Carbonate dehydratase activity

Table 9. Transcripts and functional clustering of selected genes in limbal epithelial stem cells compared to differentiated corneal epithelial cells (n=3, $p < 0.01$).

5.1.5. 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 (Table 9, Figure 3). All the tested epithelial sheets (n=3) were capable of forming epithelial holoclone-like colonies on Gelatin and Fibronectin within 7 days of cultivation as previously described (Kolli et al., 2008; Pellegrini et al., 1999) (Figure 8), but not on MethoCult coated plates (Figure 8C).

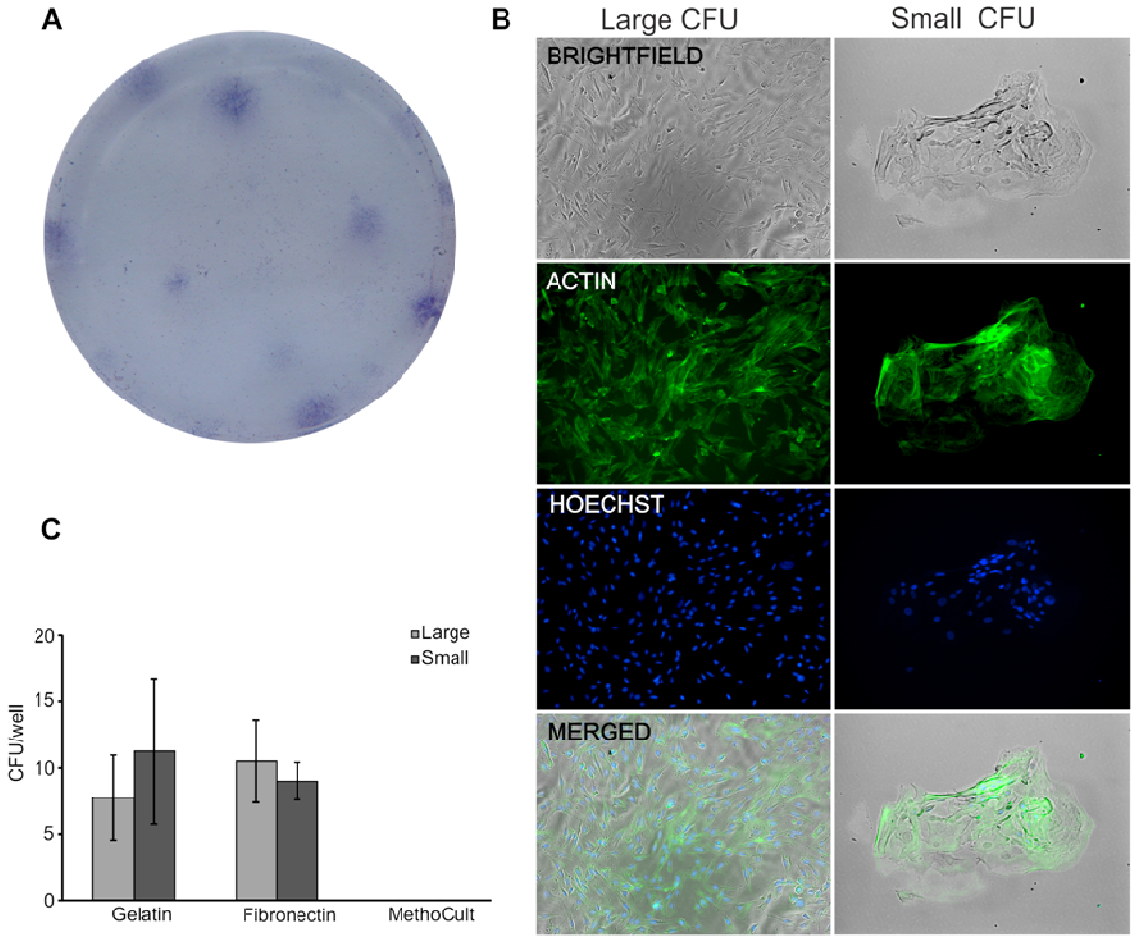

Figure 8. Colony-forming potential of limbal epithelial stem cells. The limbal epithelial stem cells (LESCs) were cultured at 3000 cells/cm^2 density and early epithelial holoclone-like colony formation was read after 7 days of cultivation. (A) Representative picture of LESC colonies on Gelatin-coated plates after crystal violet (0.5% w/v) staining. Two types of colony forming units (CFUs) could be distinguished: large CFUs containing >50 cells, and small CFUs containing <50 LESCs. (B) The CFUs are shown as stained for actin (phalloidin-FITC, green) and nuclei (Hoechst 33342, blue). (C) The LESCs formed no colonies when seeded on MethoCult. No significant difference of CFU types or numbers could be detected between LESCs seeded on Gelatin or Fibronectin matrices (Data shown are mean±S.D., n=3).

5.2. Retinal, Ciliary Body Epithelium and Vitreous Samples

5.2.1. Histological and Immunofluorescent Analysis

5.2.1.1. In Situ Characterization of Peripheral Retina and Ciliary Body Epithelium of Control Human Eyes and Eyes with Proliferative Vitreoretinopathy

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) (Figure 9B-E). Nestin was not found at the peripheral Pla or the Pli regions (Figure 9A, 9D and 9F), except for cells lining the wall of peripheral cysts (Pcs) (Figure 9C). 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 (Figure 9G) and Sox2 (Figure 9I), two central transcription factors controlling eye development. Furthermore, positivity for ABCG2 (Figure 9H) in the Pc and N-cadherin in the Pli (Figure 9F) was observed, while GFAP (Figure 9C) expression was only detected around the Pc of the RP. The CBE of normal eyes expressed Claudin (Figure 9D) and N-cadherin (Figure 9F), 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 (Figure 9E) and by few cells at the periphery of NLR (Figure 9F insert).

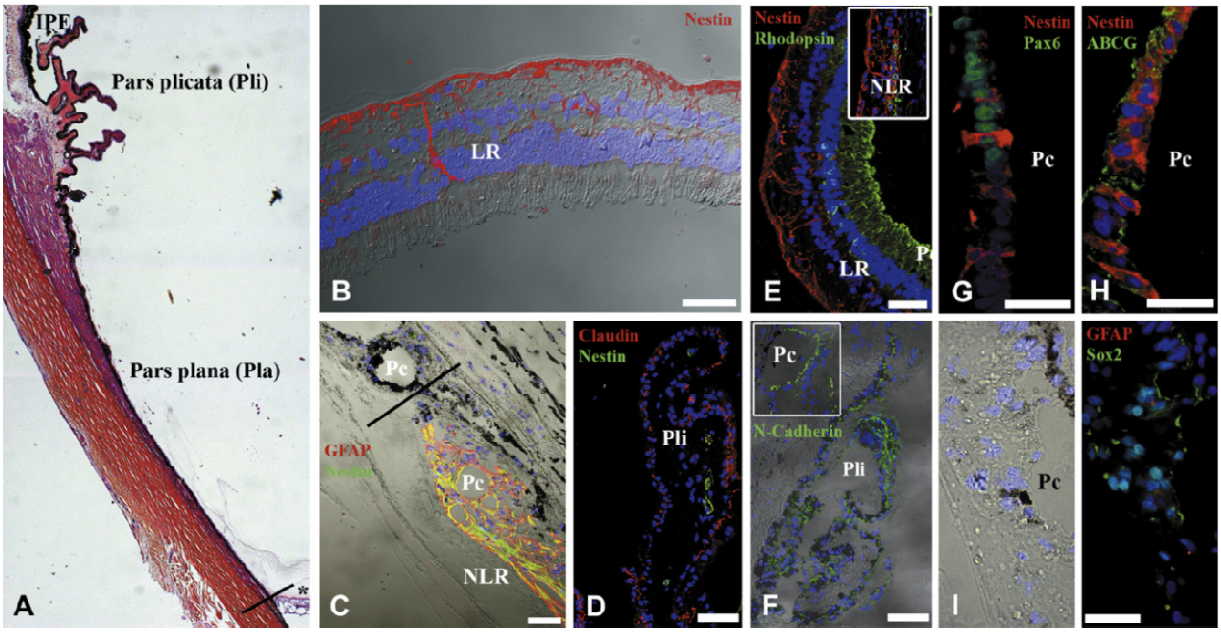

Figure 9. In situ characterization of the retina and ciliary body epithelium of the adult human eye. (A) Representative light microscopic image of the most peripheral retina, ciliary body epithelium (pars plana (Pla) and pars plicata (Pli)), iris pigmented epithelium (IPE) and peripheral cysts (Pc, *). Solid line represents the ora serrata. (B) Nestin staining of the laminated retina (LR) (C) Nestin and GFAP expression in the non-laminated (NLR) of the far peripheral retina with Pc. (D) Nestin and Claudin expression in the Pli. (E) Rhodopsin and Nestin expression in LR and NLR (insert). (F) N-Cadherin staining in the Pli and Pc (insert). (G) Nestin and Pax6 staining of cells lining the wall of Pc of the proximal Pla. (H) Nestin and ABCG2 staining of cells lining the wall of Pc of the Pla. (I) GFAP and Sox2 staining of the proximal Pli with Pc. Nuclear staining with Hoechst33342 (blue). Scale bars: 50 µm. *Author's contribution: clinical selection of donor and preparation of bulbus for immunohistochemistry (50% contribution).*

In eyes with PVR, Nestin staining extended to the proximal Pla (Figure 10C). Around the ora serrata, cells were positive not only for Nestin, but also for Sox2 and GFAP (Figure 10C). $Nestin^{+}$ and $GFAP^{-}$ cells could be detected at the proximal Pla (Figure10C and 10D). No positivity for Nestin, GFAP or Sox2 could be found in either peripheral Pla, Pli or iris pigment epithelium (IPE) (Figure 10B, 10D and 10E). 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 (Figure 10D, 10E and 10F). Clusters of Rhodopsin positive cells were found in the NLR close to the area of photoreceptor loss (Figure 10G), but no such positivity was detected in either peripheral Pla, Pli or IPE even in eyes with extensive retinal damage (Figure 10F).

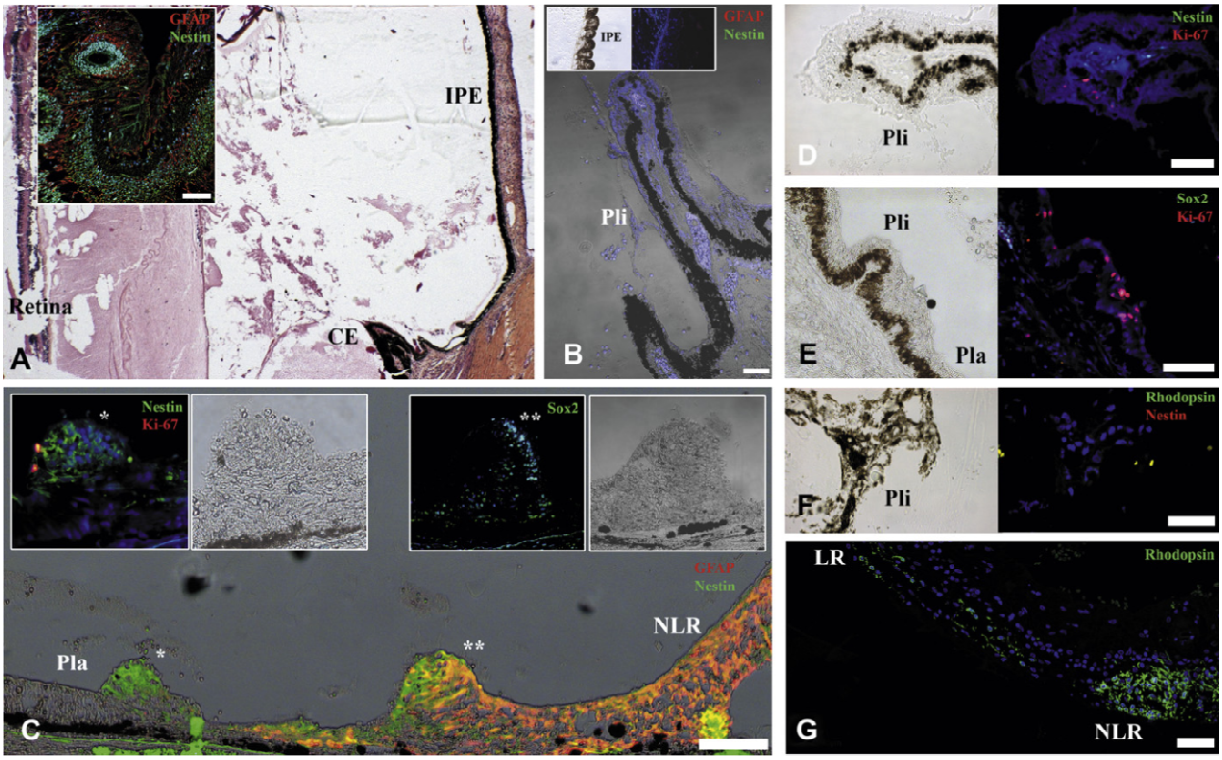

Figure 10. In situ characterization of the most peripheral neural retina and ciliary body epithelium of the adult human eye with proliferative vitreoretinopathy formation. (A) Light microscopic overview showing detached retina, exudates and adenomatous-like extension of the pars plicata (Pli) CBE, two images are merged. Insert: Thick central retina with PVR scar formation stained with GFAP and Nestin. (B) GFAP and Nestin staining of the Pli CBE and iris pigment epithelium (IPE, insert). (C) GFAP and Nestin staining of the peripheral non-laminated retina (NLR), ora serrata (line) and pars plana (Pla). The left rounded elevation of the surface (*) is also stained with Nestin and Ki-67 (left insert), while the right elevation (**) is stained with Sox2 (right insert). Ki-67 and Nestin (D), Sox2 and Ki-67 (E) and Rhodopsin and Nestin (F) staining of the CBE Pli, respectively. (G) Rhodopsin staining of the peripheral laminated retina (LR) and NLR. Nuclear staining with Hoechst33342 (blue). Scale bars: 50 µm. *Author's contribution: clinical selection of donor and preparation of bulbus for immunohistochemistry (50% contribution).*

5.2.1.2. Immunohistological Analysis of the Ciliary Body Epithelium and Peripheral Retina of Mice Eyes with Proliferative Vitreoretinopathy

In control mice eyes, only a few Nestin positive cells were found in the CBE (Figure 11E) with no Pax6 or Sox2 (negative data are not shown), but robust Claudin positivity (Figure 11F).

Similar to our findings in humans, gliotic reaction of the retina was marked by an increased GFAP staining (Figure 11G) accompanied by nuclear hyperplasia and adenomatous-like proliferation of the CBE following PVR induction (Figure 11C and 11D). In contrast to humans, Nestin was upregulated in CBE upon PVR formation (Figure 11G). $Sox2^{+}$/$Nestin^{+}$

(Figure 11H) and Nestin^{+}/GFAP^{+} (Fig 11H insert) clusters were found in the peripheral vitreous probably due to development of PVR.

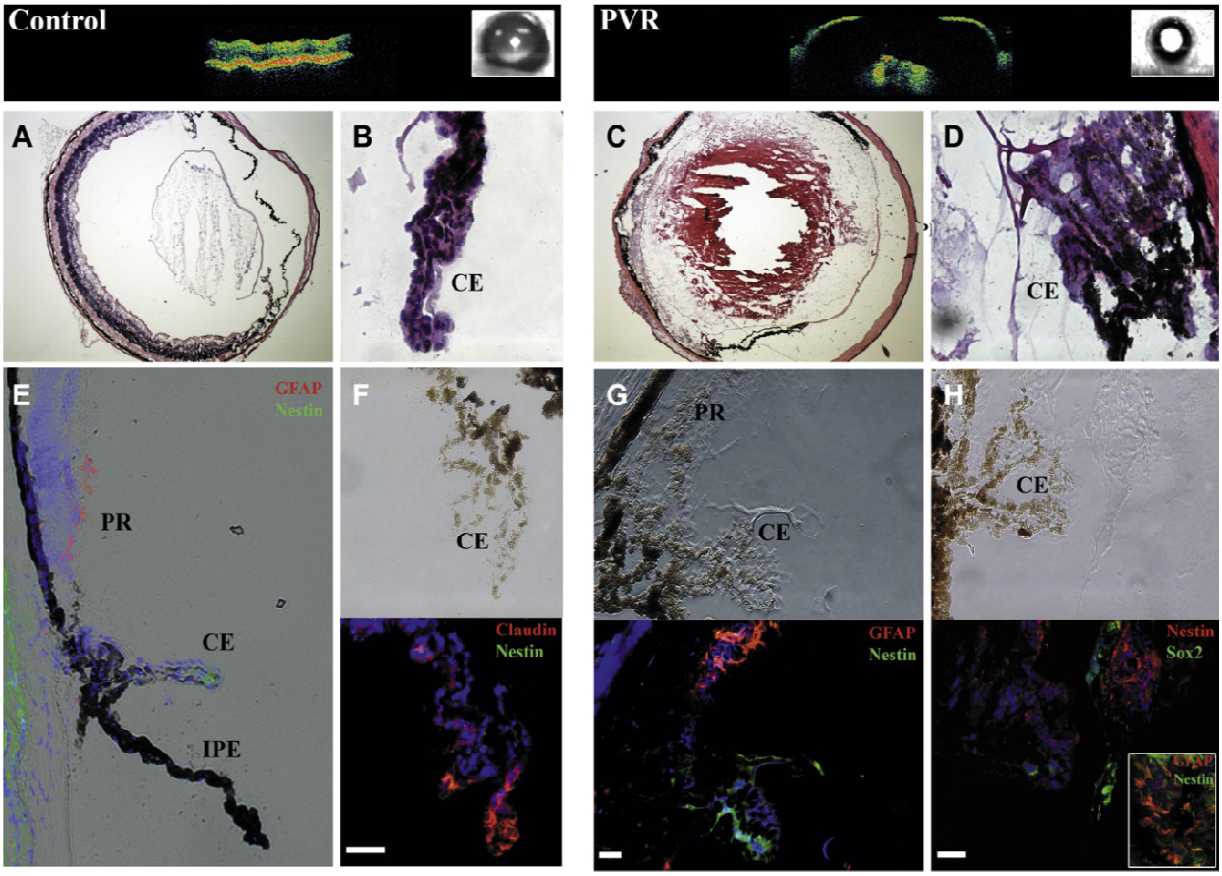

Figure 11. In situ characterization of peripheral retina and ciliary body epithelium of control mice and mice with proliferative vitreoretinopathy formation. Optical coherence tomography (OCT) images of control retina (left panel) and retina with dispase-induced proliferative vitreoretinopathy (PVR) (right panel). (A) Light microscopic appearance of a control eye with Hematoxylin and Eosin (H&E) staining, and (B) close-up of ciliary body epithelium (CBE). (C) Light microscopic appearance of a PVR eye with H&E staining, and (D) close-up of the CBE, showing nuclear hyperplasia and adenomatous-like proliferations. (E) GFAP and Nestin staining of the peripheral retina (PR) and CBE of control eye. (F) Claudin and Nestin staining of CBE in control eye. (G) GFAP and Nestin staining of PR and CBE of PVR eye. (H) Nestin and Sox2, and Nestin and GFAP (H insert) staining of CBE and peripheral vitreous in PVR eye. Nuclear staining with Hoechst 33342 (blue). Scale bars: E: 100 µm; F, G, H: 50 µm. *Author's contribution: establishment of PVR induction in mice and microsurgical injection, OCT examination, preparation of bulbus for immunohistochemistry (70% contribution).*

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

Sphere-like structures were isolated from vitreous of patients undergoing vitrectomy for RD with and without confirmed PVR development preoperatively (Fig12A, 12B and 12C). 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 (Figure 12D).

Most of the cells inside the isolated sphere-like structures showed Nestin (Figure 12F) and GFAP (Figure 12F insert) positivity, some cells were positive for Sox2 (Figure 12G) and for Pax6 (Figure 12H insert). 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 (Figure 12H).

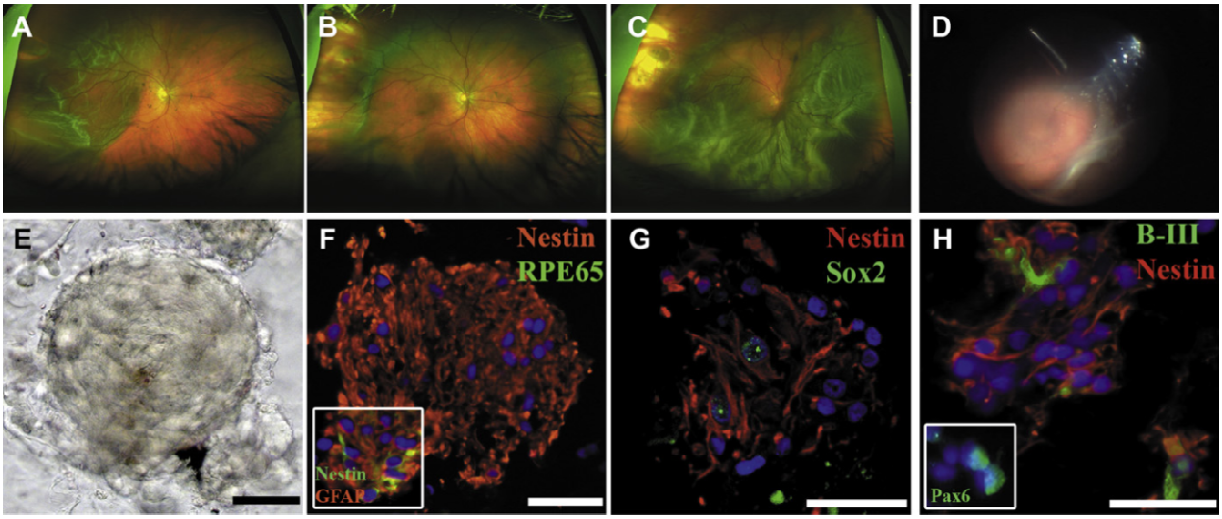

Figure 12. Characterization of sphere-like structures isolated from the vitreous of patients with proliferative vitreoretinopathy. (A) Primary retinal detachment (RD) with retinal tear in upper temporal quadrant. (B) Postoperative appearance after initial successful primary buckling surgery. (C) Three months postoperatively extensive proliferative vitreoretinopathy (PVR) formation has occurred. (D) During vitrectomy of PVR RDs, sphere-like structures can be visualized close to the vitreous base. (E) Light microscopic appearance of isolated sphere like structure of eye with PVR formation. (F) Nestin and RPE65, (F insert) Nestin and GFAP, (G) Nestin and Sox2, (H) β-III-tubulin and Nestin and (H insert) Pax6 staining of sphere-like structures isolated from the vitreous of patients with PVR. Nuclear staining with Hoechst 33342 (blue). Scale bars: E, F, G, H: 50 µm.

5.2.2. Characterization of *In Vitro* Cultured Retinal, Ciliary Body Epithelial 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 (Table 10).

Retinal detachment	Numbers of patients	Age mean (range)	Sex female/male	Sphere formation
with PVR	10	56 (17-82)	1/9	7/10
with no PVR	15	62 (45-87)	7/8	2/15

Table 10. Clinical information and sphere-forming capacity of cells isolated from the vitreous of patients with retinal detachment with/without proliferative vitreoretinopathy.

Spheres could be passaged up to P2 (no attempts were made for further passages) (Figure 13A). Transmission electron microscopy revealed that spheres are composed of elongated and polarized cells at the periphery (Figure 13B) and pigmented and non-pigmented polymorphic cells with high nuclear/cytoplasmic ratio in the central areas (Figure 13B insert). Robust Nestin (Figure 13C and 13D) and GFAP (Figure 13D) positivity were detected inside the spheres, while some cells at the periphery expressed β-III-tubulin (Figure 13C).

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 (Figure 13F) and CBE cells forming pigmented spheres *in vitro* (Coles et al., 2004; Moe et al., 2009) (Figure 13E) - 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, Table 11). 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 Pcs are Nestin and Pax6 positive (Figure 9G), the mRNA expression was comparable in all three groups after *in vitro* culturing (Table 11).

In agreement with the immunofluorescent staining of the sphere-like structures derived from PVR eyes (Figure 13D), the differentiation marker GFAP showed a 40.4 times higher expression in PVR cultures compared to CBE cultures. No difference in glutamine synthetase

(GS) expression could be detected. Tyrosinase (TYR) which was previously found in differentiating RPE cells (Martinez-Morales et al., 2003; Nakayama et al., 1998), showed comparable expression in CBE and PVR spheres (FC=1.34) (Table 11).

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 (Bhatia et al., 2011; Cicero et al., 2009; Kohno et al., 2006; Moe et al., 2009) (Table 11).

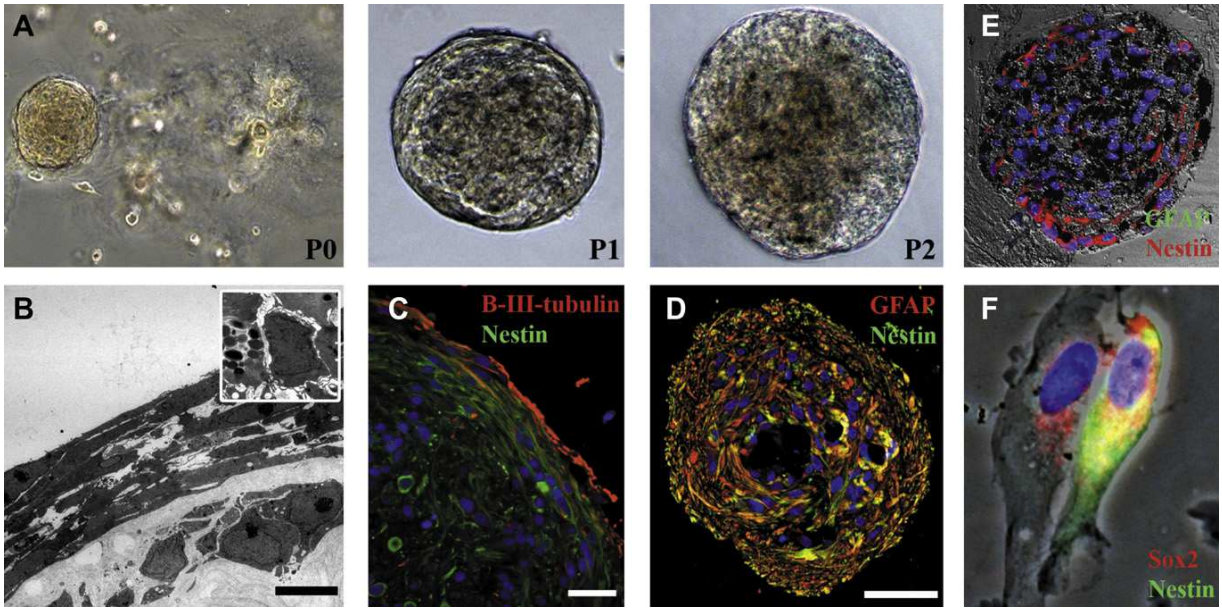

Figure 13. Sphere-forming capacity and expression of neuroprogenitor markers in cells isolated from the vitreous of patients with proliferative vitreoretinopathy. (A) Appearance of spheres isolated from eyes with proliferative vitreoretinopathy (PVR) at P0, P1 and P2. (B) Transmission electron microscopic appearance of peripheral (B insert) and central P0 PVR spheres. (C) Nestin and β-III-tubulin, and (D) Nestin and GFAP staining of PVR sphere at P1. (E) Pigmented ciliary body epithelium (CBE)-derived sphere stained with Nestin and GFAP and (F) adherent retinal cells stained with Sox2 and Nestin at P1. Nuclear staining with Hoechst 33342 (blue). Scale bars: B: 10 µm; C: 50 µm, D: 100 µm.

Gene symbol (fold change)	Up/downregulation	
	Retinal cultures	PVR-derived spheres
Pluripotency		
OCT4_	2.65	-1.35
SOX2	4.25	7.28
MYC	1.39	-1.44
KLF4	-1.36	-1.62
Nanog	3.46	-1.11
Notch1	1.74	1
Early eye-field		
PAX6	-1.85	-1.85
RAX	1.44	1.75
SIX3	-1.16	-1.21
SIX6	1.54	2.17
LHX2	1.86	1.99
MITF	-3.32	1.57*
OTX2	6.57	2.66
CHX10	85.97	8.3
NES	-1.02	1.77
Differentiation		
GFAP	26.18	40.42
CRALBP	31.37	7.51
MKI67	6.88	3.21
GS	9.3	2.52
NRL	77.42	3.54*
TYR	-11.25	1.34

Table 11. Comparative mRNA expression of genes in adherent retinal cultures and spheres isolated from vitreous of patients with proliferative vitreoretinopathy normalized to the human ciliary body epithelium derived spheres.

6. 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 (Bi et al., 2009; Dahlmann-Noor et al., 2010) or following full or partial *in vitro* differentiation (Meyer-Blazejewska et al., 2011; Wang et al., 2010). 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 (Baglio et al., 2012).

6.1. Limbal Epithelial Stem Cells

LESCD is a disease in which the stem cell- and the barrier function of the limbus fail (Ahmad et al., 2010) 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 (Ahmad, 2012; Holland and Schwartz, 1996; Tseng, 1995). Replacement of defective or deficient LESCs by healthy ones can rescue vision (Secker and Daniels, 2008).

Ex vivo expanded LESCs can be transplanted with the help of carriers, such as denuded HAM (Grueterich and Tseng, 2002; Lekhanont et al., 2009), human lens capsule (LC) (Galal et al., 2007) or bioengineered membranes (Nishida et al., 2004a; Rama et al., 2001; Sangwan et al., 2011).

In a recent study, we took advantage of using human denuded LC to develop a transplantable graft of *ex vivo* expanded LESCs. Galal *et al.* have previously introduced the advantages of

this material (Galal et al., 2007) . 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 (Mort et al., 2012), a consensus-based panel of LESC markers was used in our study (Schlötzer-Schrehardt and Kruse, 2005), 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 (Arpitha et al., 2005; Parsa et al., 1999), ABCG2 (Chen et al., 2004; Zhou et al., 2001), CK19 (Sacchetti et al., 2005; Schlötzer-Schrehardt and Kruse, 2005) and VIM/vimentin (Sacchetti et al., 2005; Schlötzer-Schrehardt and Kruse, 2005; Stepp et al., 1995) were confirmed at both gene and protein level proposing the presence of stem cells in the culture.

Interestingly, Rama *et al.* discovered that 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 (Rama et al., 2010). Therefore, this group uses only transplants prepared from cultures containing more than 3% of the p63α-bright cells (Rama et al., 2010), while others have used the percentage of p63α-bright cells to evaluate the purity of their culture (Shahdadfar et al., 2012).

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 (Kim et al., 2002). 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 (de Paiva et al., 2005).

Regarding CK19 expression, there are controversial data in the literature - some groups describe CK19 as marker for conjunctival epithelium (Donisi et al., 2003; Elder et al., 1997; Sacchetti et al., 2005), while others use it as LESC marker (Sacchetti et al., 2005; Schlötzer-

Schrehardt and Kruse, 2005). Our gene array analysis revealed an upregulation of CK19 in the *ex vivo* cultured LESCs compared to differentiated corneal epithelium (Table 9). Beside the stem cell marker positivity, our LESCs showed proliferative potential (higher MKI67 expression/Ki-67^{+}) 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 (Dua et al., 2005; Figueira et al., 2007; Merjava et al., 2011; Merjava et al., 2009), which together with the low expression of terminally differentiated cornea epithelial markers (CK3 and CK12 (Chaloin-Dufau et al., 1990; Schermer et al., 1986)) 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 ITG α9, 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 (Wolosin and Wang, 1995), 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 (Liu et al., 2011); 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 (Ko et al., 2010) and Fibronectin 1 (FN1) (FC: 74.9), which is involved in cell adhesion and migration processes during wound healing (Nishida et al., 1983), 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/Itg α1, CD49b/Itg α2, CD49f/Itg α6 and CD29/Itg β1 have been previously reported as basal limbal and basal corneal cell markers (Notara et al., 2010; Stepp et al., 1993) - 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 (Burillon et al., 2012; Nakajima et al., 2013; Nishida et al., 2004b) and transdifferentiated bone marrow-derived stem cells (Cai et al., 2010; Katikireddy et al., 2013; Yao and Bai, 2013) are a possible substitute for corneal epithelial cells in bilateral LESCD (Nakajima et al., 2013; Yao and Bai, 2013). Transdifferentiation of hair follicle stem cells to substitute corneal epithelial cells (Blazejewska et al., 2009; Meyer-Blazejewska et al., 2011) 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 (Eberwein and Reinhard, 2012).

6.2. Progenitor Cells of the Posterior Segment

Stem cell therapy holds great promise for retinal diseases (Tibbetts et al., 2012). 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 (Cramer and MacLaren, 2013; Ramsden et al., 2013).

Tropepe *et al.* and Coles *et al.* were the first to propose that human CBE contains NPCs which are able to make new neural cell types (Coles et al., 2004; Tropepe et al., 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 (Imitola et al., 2004; Olstorn et al., 2007; Park et al., 2002), 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 (Bhatia et al., 2011; Bhatia et al., 2009; Cicero et al., 2009; Gualdoni et al., 2010; Moe et al., 2009).

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 (Fischer and Reh, 2001; O'Malley and Allen, 1967). In agreement with previous findings (Bhatia et al., 2009), we could not detect any cell division or Ki67 positivity in uninjured human retina *in situ* (negative data are not shown).

In response to PVR formation, proliferation was observed in either the $GFAP^-$ or the $GFAP^+$ cells at the proximal Pla and RP (Figure 10C). These findings partially correspond to the results of Ducournau *et al.* although this group described a few $Rhodopsin^+$ cells next to the $GFAP^-$ pigmented cells in eyes with extensive PVR formation (Ducournau et al., 2012). 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 (Coles et al., 2004; Mayer et al., 2003; Xu et al., 2007), although the method is not specific for stem cells as some other cell types like epithelial cells can also form spheres (Cicero et al., 2009). There is also evidence that small spheres are not derived from single cells but develop as aggregation of cells (Pastrana et al., 2011). 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 (Pastrana et al., 2011). It also means that the population of cells within the sphere is heterogeneous in terms of their differentiation stage and commitment (Arsenijevic et al., 2001). 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 (Ahmad et al., 2000b; Coles et al., 2004; Mayer et al., 2003). Coles *et al.* found that spheres formed only from the CB and iris, such spheres or cells could not be isolated from the neuroretina or RPE; Mayer *et al.* could isolate spheres from the neuroretina and CB regardless of sex, age or post-mortem time (Mayer et al., 2003). Xu *et al.* characterized spheres derived from CB which consist of proliferating cells expressing immature neuronal and glial markers (Liu et al., 2007; Xu et al., 2007). 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, a combination 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.

Moe *et al.* demonstrated that CBE spheres contain proliferating epithelial-like cells with decreased expression of NPC markers compared to CNS neurospheres, which is in agreement with Cicero *et al.* who showed that CBE-derived spheres are pigmented and display more epithelial characteristics (Cicero et al., 2009; Moe et al., 2009). Gualdoni *et al.* showed that cells derived from CBE express significant amount of NPC markers but fail to differentiate to photoreceptors (Gualdoni et al., 2010). Bathia *et al.* separated pigmented and non-pigmented epithelial cells of CBE and found that only non-pigmented CBE proliferate to form spheres in culture, expressing high level of epithelial markers and limited level of NPC markers (Bhatia et al., 2009).

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 (Lengner et al., 2008). 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 (Table 11). The presence of Pax6 and Sox2 could further be confirmed at a protein level by immunofluorescent staining (Figure 12G and 12H insert).

The most common NPC marker is Nestin, which is an intermediate filament protein and can be upregulated in pathological conditions like gliosis (Gilyarov, 2008). During neuro-and gliogenesis, Nestin is replaced by tissue-specific intermediate filaments like neurofilaments or GFAP (Gilyarov, 2008; Lin et al., 2009). 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 (Challen and Little, 2006; Wan et al., 2010) 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 a 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 (Ding et al., 2010; Watanabe et al., 2004). Froen *et al.* showed Nestin and Claudin positivity, but not double positivity of cells in spheres derived from CBE (Froen et al., 2011), which suggest that the spheres are a mixture of two different cell types: epithelial and neural progenitor-like cells. In contrast to that, Cicero *et al.* showed homogeneous population of epithelial cells in the spheres of CBE (Cicero et al., 2009).

We show that sphere-like structures isolated from the vitreous of patients with PVR show high GFAP positivity as well (Figure 12F insert, Figure 13D). PCR could also reveal that GFAP is expressed significantly higher in PVR spheres than in CBE spheres (Table 11). No RPE65 (RPE marker) (Figure 12F) 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 (Bhatia et al., 2011; Bhatia et al., 2009; Das et al., 2006; Lawrence et al., 2007). Furthermore, it has been recently shown that Müller glia isolated from the human RP during vitrectomies can be an efficient source for producing photoreceptors (Giannelli et al., 2011) 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.

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. Összefoglalás

Az őssejt-therápia ígéretesnek mutatkozik az olyan súlyos látásromlást okozó károsodások ill. betegségek kezelésében, mint a vegyi sérülések, égések, időskori macula degeneráció vagy retinitis pigmentosa. Az embrionális őssejtek kapcsán felmerülő etikai kérdések és az indukált pluripotens sejtek esetleges tumor-képző tulajdonságától való félelem miatt a felnőtt szöveti őssejtek használhatósága sokkal valószerűbb.

Munkánk során sikeresen izoláltunk, tenyészettünk és karakterizáltunk a szaruhártya epithel regenerációjáért felelős limbális epitheliális őssejteket (LESC), valamint a vékony és áttetsző tulajdonsággal bíró lencsetokról (LC) bizonyítottuk, hogy az LESC-transzplantáció számára alkalmas biológiai hordozó.

Legfőbb eredményeinknek tartjuk az állati eredetű anyagok teljes kivonását az LESC-tenyésztési procedúrából. A tenyésztés során a humán szérum, mint egyetlen tápanyag-kiegészítőforrást használtuk. Emellett az LESC jellemzésére szolgáló panelt korábban nem vizsgált sejt adhéziós molekulákkal (CD44/HCAM, CD146/MCAM, CD166/ALCAM) és sejtfelszíni glikoproteinekkel bővítettük ki.

A limbális graftokból kiinduló sejtek a LC felszínén 2 hét alatt többrétegű hámot hoztak létre. A kinövő sejtek felszínükön őssejt (ABCG2, CK19, TP63/p63, VIM/Vim), a cornea epitheliális irányba való elköteleződés (CK8/CK18, alacsony CK3/CK12) valamint a nagy proliferációs kapacitásra (MKI67/Ki67) utaló markereket expresszálják, így létrehozva egy őssejtekből és tranziensen amplifikálódó sejtek keverékéből álló populációt, annak minden előnyével.

Irodalmi adatok alapján feltételezzük, hogy a sugártest epithelsejt rétege (CBE) olyan neuronális pluripotens sejteket (NPC) tartalmaz, amelyek érzékelve a retina károsodását, pl. retina leválásnál és következményesen kialakuló proliferatív vitreoretinopáthia (PVR) esete, célzott migrációval és funkcionális differenciációval reagálnak.

Kísérleteink során azonban nem sikerült NPC-t detektálnunk az emberi szemben, a pars plana ill. reitna periférián található ún. perifériás cystákon kívül. Mi több, $GFAP^+$ sejteket is csak a retina perifériás részén detektáltunk. Eredményeink alátámasztják a humán $GFAP^-$ sejtek proliferációját PVR-ben, de azok őssejt mivoltára vagy fotoreceptorrá való differenciálódásának képességére nem találtunk bizonyítékot. Csupán néhány $Rhodopsin^+$ sejt csoportosulást figyeltünk meg a PVR okozta jelentős fotoreceptor károsodástól perifériásabban.

Szemben a humán eredményekkel, egerekben PVR indukálást követően a CB kifejezett hyperplasiáját és emelkedett Nestin pozitivitást tapasztaltunk.

PVR-es retina leválás miatti vitrectomia során az operatőrök gyakran észlelnek, a retina perifériához közeli vitreusban gömbszerű struktúrákat. Azért, hogy kiderítsük, ezeknek a szféráknak az eredetét és jellemzőit, a vitreusból nyert mintákat tenyésztés előtt és után is megfestettük. Az immunfluoreszcens festés és transzmissziós elektron mikroszkópia alapján a szférák pigmentált és nem pigmentált sejtekből, valamint Nestin és GFAP pozitív centrális és β-III tubulin^{+} külső részből állnak, továbbá retina pigment epithelsejtes kontaminációmentesek.

Munkánk során a PVR-re adott gliális és nem-gliális válasz megerősítést kapott, de a gliális sejtek vitreusba való migrációja továbbra sem bizonyított.

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10. 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.
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   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.
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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.
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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.
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IF:2.281

5. Petrovski, G., **Albert, R.**, Kaarniranta, K., Moe, M.C., Fésüs, L., Berta, A., Das, D.K.: Autophagy in the eye: A double-edged sword.
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11. Keywords

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12. Tárgyszavak

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