GENOMIC AND PROTEOMIC CHARACTERIZATION OF PHYSIOLOGICAL AND PATHOLOGICAL ANGIOGENESIS IN THE HUMAN EYE

Ph.D. THESIS

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LIST OF ABBREVIATIONS

ABCG2	ATP-binding cassette sub-family G member 2 (CD338)
ACAN	Aggrecan/cartilage-specific proteoglycan core protein (CSPCP)/chondroitin sulfate proteoglycan 1
AKT1	RAC-alpha serine/threonine-protein kinase
AKT3	RAC-gamma serine/threonine-protein kinase
ALCAM	Activated leukocyte cell adhesion molecule (CD166)
AREG	Amphiregulin
bFGF	Basic fibroblast growth factor
BMI	Body mass index
BM-MSC	Bone marrow-derived mesenchymal stem cell
BMP2	Bone morphogenetic protein 2
BMP6	Bone morphogenetic protein 6
BMP7	Bone morphogenetic protein 7
BSA	Bovine serum albumin
CAMs	Cell adhesion molecules
CAV1	Caveolin-1
CCL5	Chemokine (C-C motif) ligand 5/RANTES
CCNA1	Cyclin-A1
CDH1	Cadherin/ CAM 120/80 or epithelial cadherin
CECs	Corneal epithelial cells
CLDN7	Claudin-7
CNTF	Ciliary neurotrophic factor
COL2A	Collagen, type II, alpha
CT-1	Cardiotrophin 1
CTGF	Connective tissue growth factor
CXCL10	C-X-C motif chemokine 10/interferon gamma-induced protein 10
CXCL12	Chemokine (C-X-C motif) ligand 12/ stromal cell-derived factor-1
CXCR1	Interleukin 8 receptor, alpha
CXCR3	Chemokine receptor CXCR3
CXCR4	Chemokine receptor CXCR4
DCN	Decorin
DKK1	Dickkopf-related protein 1

DMEM	Dulbecco-modified Eagle's medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
ECM	Extracellular matrix
EDA	Ectodysplasin-A
EDN1	Endothelin 1/ preproendothelin-1
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EG-VEGF	Endocrine-gland-derived vascular endothelial growth factor
ELISA	Enzyme-linked immunosorbent assay
EREG	Epiregulin
ERK1/2	Extracellular signal-regulated kinases
ET-1	Endothelin 1
F3	Platelet tissue factor, factor III, thromboplastin
FACS	Fluorescence-Activated Cell Sorting
FAM3B	Protein FAM3B
FC	Fold change
FCS	Fetal calf serum
FGF1	Fibroblast growth factor 1/Heparin-binding growth factor 1
FGF7	Fibroblast growth factor 7/Keratinocyte growth factor
FITC	Fluorescein-5-isothiocyanate
flt-3L	Fms-related tyrosine kinase 3 ligand
FOXA1	Forkhead box protein A1/hepatocyte nuclear factor 3-alpha
FOXA2	Forkhead box protein A2/hepatocyte nuclear factor 3-beta
FOXD3	Forkhead box D3
FOXG1	Forkhead box protein G1
FOXN1	Forkhead box protein N1
FOXO4	Forkhead box protein O4
fvERMs	Fibrovascular epiretinal membranes
G-CSF	Franulocyte colony-stimulating factor
GDF15	Growth differentiation factor 15
GDF9	Growth differentiation factor 9
GJA1	Gap junction alpha-1 protein/connexin 43
GJB1	Gap junction beta-1 protein/connexin 32

GM-CSF	Granulocyte-macrophage colony-stimulatory factor
gp130	Glycoprotein 130
HCAM	Homing Cell Adhesion Molecule (CD44)
HEPES	4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen DR
HLA-G	Human leukocyte antigen G
HRAS	Transforming protein p21
HSC	Hematopoietic stem cell
HSPA9	Stress-70 protein, mitochondrial
ICAM1	Intercellular Adhesion Molecule (CD54)
IFNE	Interferon epsilon
IGF2	Insulin-like growth factor 2
IL-	Interleukin
IL-11	Interleukin 11/adipogenesis inhibitory factor
IL17	Interleukin 17
IL17F	Interleukin 17 F
IL18	Interleukin-18/interferon-gamma inducing factor
IL1A	Interleukin-1 alpha
IL1B	Interleukin 1 beta
IL1R1	Interleukin 1 receptor, type I
IL36RN	Interleukin 36 receptor antagonist
IL-6	Interleukin 6
IL6R	Interleukin 6 receptor (CD126)
IL-8	Interleukin 8
INF	Interferon
INHBA	Inhibin, beta A
IPA	Ingenuity Pathway Analysis
IPKB	Ingenuity Pathways Knowledge Base
ISL1	Insulin gene enhancer protein
JAK	Janus kinase
KAT2B	P300/CBP-associated factor (PCAF/K(lysine) acetyltransferase 2B
KITLG	Stem Cell Factor
KLF4	Kruppel-like factor 4

LAMA1	Laminin subunit alpha-1
LAMA3	Laminin subunit alpha-3
LAMC1	Laminin subunit gamma-1
LEF1	Lymphoid enhancer-binding factor 1
LESCD	LESC deficiency
LESCs	Limbal epithelial stem cells
LFA-1	Lymphocyte function-associated antigen 1 (CD11a)
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharides
MAP2K	Mitogen-activated protein kinase kinase
MAP2K1	Dual specificity mitogen-activated protein kinase kinase 1
MAPK	Mitogen-activated protein kinases
MAPK1	Mitogen-activated protein kinase 1
MAPK14	Mitogen-activated protein kinase 14/p38-α
MAPK3	Mitogen-activated protein kinase 3
MCAM	Melanoma cell adhesion molecule (CD146)
MCP-1	Monocyte chemotactic protein-1
MMP1	Matrix metalloproteinase-1/interstitial collagenase
MMP14	Matrix metalloproteinase-14
MMP3	Stromelysin-1/matrix metalloproteinase-3
MMP9	Matrix metallopeptidase 9
MMPs	Matrix metalloproteases
MSC	Mesenchymal stem cell
MSX1	Msh homeobox 1
MYOD1	Myogenic differentiation 1
NCAM1	Neural cell adhesion molecule (CD56)
NFKB1	Nuclear factor kappa-B p105 subunit
NFKBIA	IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha)
NFκB	NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells
NOV	Nephroblastoma overexpressed
NT5E	Ecto-5'-nucleotidase (CD73)
OSGIN1	Oxidative stress induced growth inhibitor 1
OSMR	Oncostatin M receptor

PAI-1	Plasminogen activator inhibitor 1
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGFRβ	Platelet-derived growth factor receptor beta
PDR	Proliferative diabetic retinopathy
PE	Phycoerythrin
PEDF	Pigment epithelium-derived factor
PFA	Paraformaldehyde
PGE	Prostaglandin E
PKC	Protein kinase C
PLG	Plasmin
PMEL	Premelanosome protein
PRKCA	Protein kinase C alpha
PRKCB	Protein kinase C beta type
PRR	Pattern recognition receptors
PTX3	Pentraxin-related protein 3
RAF1	RAF proto-oncogene serine/threonine-protein kinase
RAR	Retinoic acid receptor
RMA	Robust microarray analysis
RNA	Ribonucleic acid
ROCK2	Rho-associated protein kinase
SERPINE1	Plasminogen activator inhibitor-1 /serine protease inhibitor E1
SOCS1	Suppressor of cytokine signaling 1
SOCS3	Suppressor of cytokine signaling 3
SOX3	SRY-related HMG-box 3
SOX6	Transcription factor SOX-6
SOX9	SRY (sex determining region Y)-box 9
SPP1	Secreted phosphoprotein 1
SRC	Proto-oncogene tyrosine-protein kinase
STAT	Signal transducer and activator of transcription
STAT3	Signal transducer and activator of transcription 3
TACs	Transient amplifying cells
TGFB	Transforming growth factor, beta
TGFB1	Transforming growth factor beta 1

TGFB2	Transforming growth factor-beta 2
TGFBI	Transforming growth factor, beta-induced
TGFBR1	Transforming growth factor, beta receptor I/activin A receptor type II-like kinase
TGFBR2	Transforming growth factor, beta receptor II
TIMP-1	Tissue inhibitor of metalloproteinase 1
TNFα	Tumor necrosis factor alpha
TSP-1	Thrombospondin 1
VCAM-1	Vascular cell adhesion protein 1 (CD106)
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A
VEGFR2	Vascular endothelial growth factor receptor 2
VIM	Vimentin
vWF	Von Willebrand factor
WNT1	Proto-oncogene protein Wnt-1
WNT5A	Protein Wnt-5a

1. MAGYAR NYELVŰ ÖSSZEFOGLALÓ

Az emberi szem nagy tárházat kínál szövetregeneráció és sejtterápia szempontjából. Különösen az, hogy a cornea szövet limbális őssejteket (LESC) tartalmaz. Génexpresszión alapuló microarray segítségével azonosított molekuláris markerek és upstream regulátorok, azok fehérje szinten történő megerősítése az LESC-k könnyebb azonosítását, a sejtekhez kötődő jelátviteli útvonalak pontosabb megismerését teszi lehetővé, amely a szembetegségek hatékonyabb sejtterápián alapuló gyógyításához is vezethet. Annak ellenére, hogy a szemben számos őssejt tulajdonságokkal rendelkező sejttípus található, betegségek vagy a megváltozott környezet által felborított egyensúly gyulladásos és fibrovaszkuláris folyamatok kialakulásához vezethet, csakúgy, mint proliferatív diabeteszes retinopathia (PDR) esetén. A PDR-ben található fibrovaszkuláris epiretinális membránok létrehozásában, illetve kialakításában részt vevő sejtek sejtfelszíni markerek alapján nem kellően leírtak és nem ismert ezen sejtek immunválaszban és angiogenezisben betöltött szerepe sem.

Az LESC-k gén expressziós transzkripciós profilját hasonlítottuk differenciált cornea epithel sejtek (CEC)-khez. A két sejttípus között 1830 gén expressziójában találtunk több mint kétszeres emelkedést illetve csökkenést. Számos a sejtmozgásban (381), sejtosztódásban (567), fejlődésben (552), sejthalálban és túlélésben (520) és a sejt-sejt közötti jelátvitelben (290) szereppel bíró molekulát azonosítottunk, amelyek a LESC-k legfontosabb biológiai funkciójában is részt vesznek. Számos a sejtfelszínen is kifejeződő molekulát áramlási citometriás mérésekkel is igazolni tudtunk. A teljes transzkripciós profilból kiválogatva a géneket speciális csoportokba rendezve analizáltuk, az őssejt jelleg fenntartásában, differenciációban, sejtadhézióban, angiogenezisben betöltött szerepük alapján, a citokineket és növekedési hormonokat kódoló génekkel egyetemben. Az eredmények azt mutatják, hogy a LESC-k nem csak az epithel irányú differenciálódásban és szövet regenerációban, hanem az angiogenezis és az extracelluláris matrix kialakításának szabályozásában is kulcs szerepet játszanak. Az őssejt jelleg fenntartásában, a differenciálódásban és az angiogenezisben is fontosnak találtunk néhány gyulladást keltő citokint. Ezek közül az interleukin-6 és az interleukin-8 szerepelt a legtöbbet az említett biológiai folyamatokhoz kapcsolódó útvonalakban; a két citokin jelenlétét a LESC tenyészetek felülúszójában is igazolni tudtuk.

Az fvERM–ből kinövő, osztódó sejtek *ex vivo* körülmények között egy sejtrétegű tenyészetet hoztak létre. Ezek a sejtek negatívak voltak néhány *endotheliális* sejt markerre (CD31, VEGFR2), részben pozitív hematopoietikus - (CD34, CD47) és a mesenchymalis őssejt markerekre (CD73, CD90/Thy-1, PDGFRβ), azonban nem fejeztek ki CD105 a membránjukban. Az eddig nem közölt CD146/MCAM és CD166/ALCAM markerek alacsony illetve magas expressziós szintet mutattak a sejttenyészetben. TNFα kezelés hatására 11 angiogenezisben szerepet játszó *factor* szekrécióját (DPPIV/CD26, EG-VEGF/PK1, az ET -1, az IGFBP-2 és 3, IL-8/CXCL8, MCP-1/CCL2, MMP-9, PTX3/TSG-14, szerpin E1/PAI-1, szerpin F1/PEDF, TIMP-1 és a TSP -1) mutattuk ki. A membránokból kinövő sejtek mechano-stimuláció hatására intracelluláris Ca^{2+} növekedéssel válaszoltak, amely viabilitásuk mellett felveti a mechanizmus esetleges szerepét a fvERM-hez kapcsolódó húzóerő által kiváltott *retina leválásnak.*

Összefoglalva elmondhatjuk, hogy a LESC-k fontos szerepet játszanak az epithel irányú differenciálódásban, a szövetregenerációban, angiogenezisben és az ECM felépítésében. A PDR-ben szenvedő betegek fvERM membránjaiban lévő sejtek kevert fenotípust mutattak, nem lehetett egyértelműen a hematopoietikus, mesenchymális őssejt vagy progenitor sejt eredetet bizonyítani. A sejtek nagy osztódási potenciállal rendelkeztek, TNFα hatására gyulladásos és angiogén molekulákat termeltek. Működőképességük és viabilitásuk a mechano-stimulációra végbemenő calcium változásokban is tükröződött, amely a PDR-ben is végbemenő, fvERM általi húzóerő *ex vivo* kísérletes modellje lehet.

2. INTRODUCTION

The eye is the most important sensory organ of our body. It can be divided into two large parts: the anterior segment covered mainly by the cornea, and the posterior segment wrapped up by the sclera. The cornea and the sclera are connected by a ring called the limbus (**Figure 1**). The protective and refractive function of the outermost surface of the eye is provided by the highly transparent and strong tissue - the cornea. During organogenesis this is the last part of the eye which is formed from the neural crest[1].

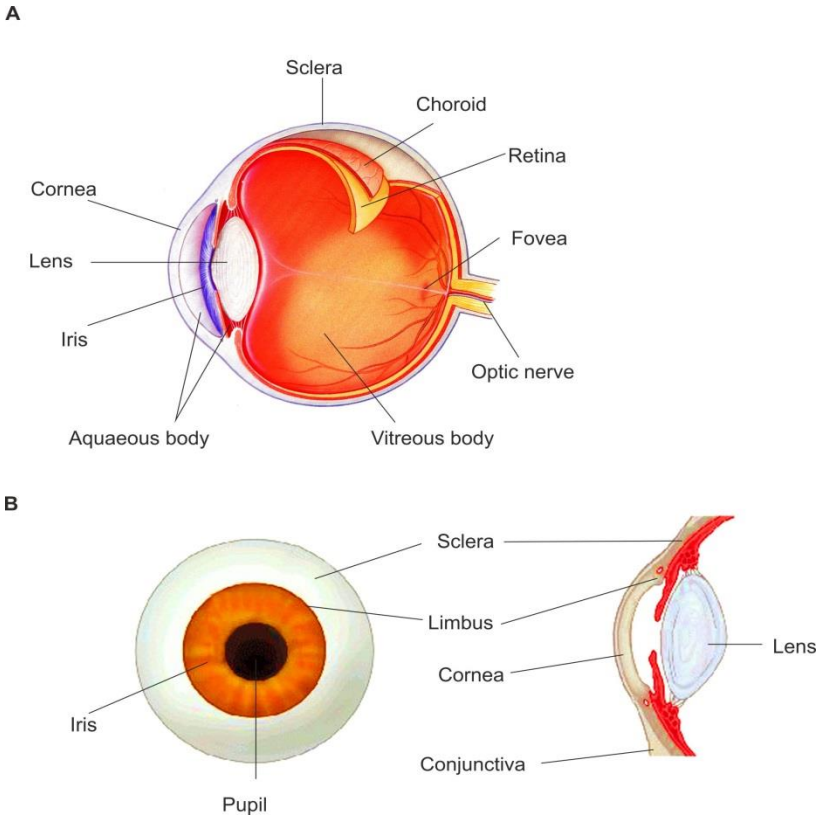

Figure 1. Structure of the human eye

A. Simplified schematic layers of the eye. The anterior segment is covered by the highly transparent and impermeable cornea, **B.** The limbus connects the cornea and the sclera together. (Modified pictures from http://healthfavo.com/human-eye-diagram.html, Health, Medicine and Anatomy Reference Pictures)

The cornea has a rather complex structure compared to other body surface tissues. A distinct acellular Bowman's layer and acellular collagenous basement (Descemet's) membrane separate the single inner cell layer of the cornea – the cornea endothelial cells, from the stroma, which is the biggest middle thick layer of the tissue. The cornea endothelial cells play critical role in the maintenance of correct hydration of the corneal stroma. The latter contains collagen rich extracellular matrix with embedded, resting flattened stromal keratocytes and fibroblast-like cells. The surface of the cornea consists of stratified squamous epithelium acting as protective, impermeable barrier of the eye (**Figure 2**). Due to persistent physical, microbial, chemical and biological damage, the central corneal epithelial cells (CECs) need to be continuously replaced from the limbal epithelial stem cells (LESCs).

Animal studies have shown that CECs can differentiate from approximately 100 progenitor cells, which means there is extremely low frequency of LESCs in the limbal tissue[2]. Stem cells and progenitors cells usually need special niches to maintain their multipotency and proliferation potential. In humans, only six limbal epithelial crypts have been identified as niches of the LESCs - they located at the basal cell layer of the limbal epithelium at the corneo-scleral junction[3-8]. These data have further been strengthened by findings from animals as well[7]. The limbal epithelial crypts provide a concentrated and safe place for harboring LESCs, and also, a rich vascular supply with growth factors and metabolites for their sustained persistence[9-14] (**Figure 2**). The stem cell niches are important because they block cell division in both the corneal and conjunctival epithelium, therefore act as border guards delineating the two distinct tissues. As mentioned before, the CECs differentiate from LESCs which can undergo asymmetric division and give rise to transient amplifying cells (TACs). TACs proliferate in the basal layer of the limbus before they differentiate and then migrate centripetally and superficially while becoming more and more differentiated and post-mitotic[15,16]. During the differentiation from TACs into mature CECs, the cells lose their ability to proliferate[12,17]. The function of LESCs is not only in providing a source for epithelial cells' differentiation, but also, they play a key role in the maintenance of a balanced immunological state in the cornea and corneal tissue regeneration as well[18].

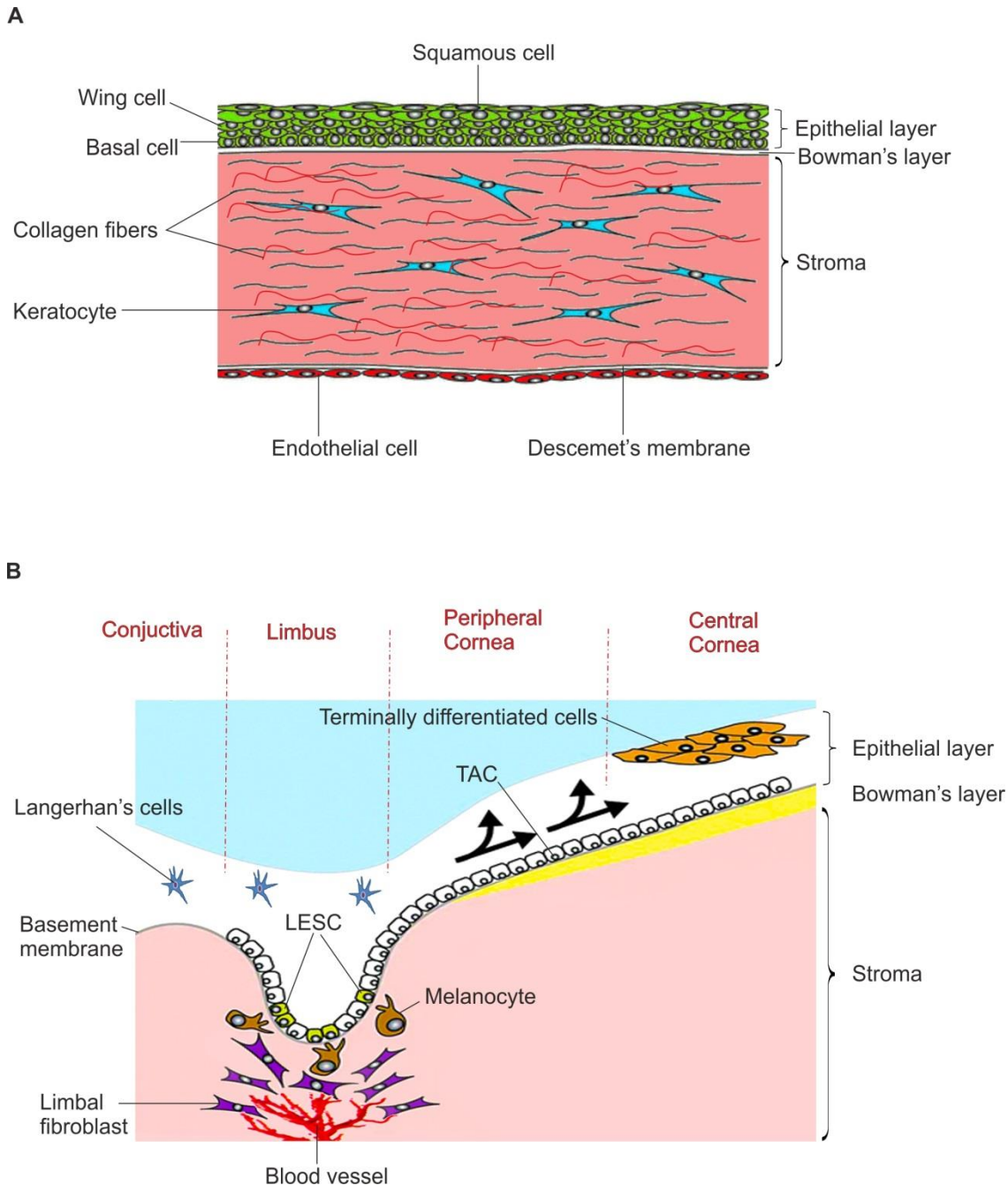

Figure 2. Structure of the cornea and the limbal stem cell niche

A. The five layers of the cornea. The top layers are the stratified squamous epithelium which is separated by the Bowman's layer from the thicker stroma. The latter is highly collagenous with resting keratocytes and fibroblasts as well. Descemets's membrane separates the stroma from the cornea endothelial monolayer. (Note: the newly discovered sixth, Dua's layer, is not mentioned here for simplicity)[19]. **B.** LESCs are located at the basal layer of the limbus. The niche made by limbal fibroblast-like cells and blood vessels provide the important metabolites and cytokines for the LESCs. These cells then differentiate into TACs which migrate laterally and horizontally, differentiating into non-proliferative, central corneal epithelial cells.

(Modified image based upon the work of Secker GA, Daniles JT: Limbal epithelial stem cells of the cornea[5], and Li W et al.: Niche regulation of corneal epithelial stem cells at the limbus[14]; downloaded from http://www.stembook.org/node/588)

Injuries - traumatic, chemical and iatrogenic, or diseases of the LESCs - inborn or acquired, can all lead to partial or total LESC deficiency (LESCD) or corneal neovascularization accompanied by inflammation. In LESCD, the failure of corneal re-epithelialization and the invasion of blood vessels cause a painful state which can lead to blindness. LESCD can be treated with autologous limbal graft transplantation from a healthy donor eye, if available, however, such treatment does not provide guarantee for the functionality of the graft itself[20,21]. Despite intense research in this field in the last couple of years, the real phenotype and biological behavior of LESCs is still not known in detail.

The appearance of new vessels in the cornea during LESCD can be based upon immunological and angiogenic processes interplaying in the tissue affected. The formation of new vessels from existing capillaries is a complex and strictly controlled multistep process[22,23]. The proliferation and migration of vessel wall-derived endothelial cells is usually limited, therefore, neovascularization is mainly depended on the circulating or tissue resident endothelial progenitor cells (EPCs), and the balance of pro- or anti- angiogenic factors released by the activated cells surrounding them[23]. Neovascularization plays a key role in wound healing and tissue regeneration, as well as various ischemic and inflammatory diseases[23]. Pathological neovascularization is a hallmark of many ocular diseases such as age-related macular degeneration (AMD), diabetic macular edema (DME), neovascular glaucoma, corneal neovascularization, pterygium and proliferative diabetic retinopathy (PDR)[23,24].

PDR is the most advanced stage of the diabetic retinopathy (DR) caused by diabetes mellitus. The disease destroys the micro- and macro- vasculature in the posterior segment of the eye, and is frequent in patients having the disease for 10 or more years[25]. Based upon the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR), the 10-year incidence of new retinopathy was 89% in the group diagnosed before age 30 years, 79% in the insulin-taking group of 30 years or older and 67% in the non-insulin-taking group[26]. The disease itself affects those individuals who are in their most productive years, therefore, it poses great socio-economic burden on the society[27]. The symptoms of the disease are visual field defects and blurred vision (**Figure 3A**), while funduscopy can reveal fibrovascular membranes and neovascularizations with cotton wool spots, flame- and dot-blot hemorrhages as well as hard exudates **(Figure 3B)**. The pathophysiology of PDR is based upon the neovascularization formation in the retina, followed by formation of fibrovascular

proliferations which can lead to tractional retinal detachment causing blindness[28] (**Figure 3C**).

The molecular background of DR remains still unclear. Several hypotheses regarding growth hormone involvement, sluggish platelet and erythrocyte circulation with consequent focal capillary occlusions and retinal ischemia, activation of aldose reductase pathway and consequent damage of intramural pericytes that altogether cause saccular outpunching of capillaries, ruptured microaneurysms, intra- and epi- retinal hemorrhages and exudation, have all been described as causes of DR[29,30]

The pro-inflammatory cytokines have been known stimulators of the production of angiogenic and vasoproliferative factors. Whenever pro-angiogenic factors are mixed with an ischemic environment, they can provoke neovascularizations in the retina, which can further lead to proliferations and penetration through the internal limiting membrane, the final product being fibrovascular epiretinal membranes (fvERMs) formation between the retina and the posterior hyaloid face. Strengthening this hypothesis are the high levels of pro-inflammatory cytokines such as interleukin 6 (IL-6), IL-8 and tumor necrosis factor alpha (TNFα) that have been measured in samples from the vitreous body of patients with PDR[31,32]. So far, the origin of the cells found in fvERMs has not been well understood. Although attempts to assess the presence of $CD34^+$ and $CD31^+$ vascular endothelial cells have been made using histological means in post-vitrectomy membranes[33-35], no such assessment has been made when the cells are cultivated *ex vivo* under adherent conditions.

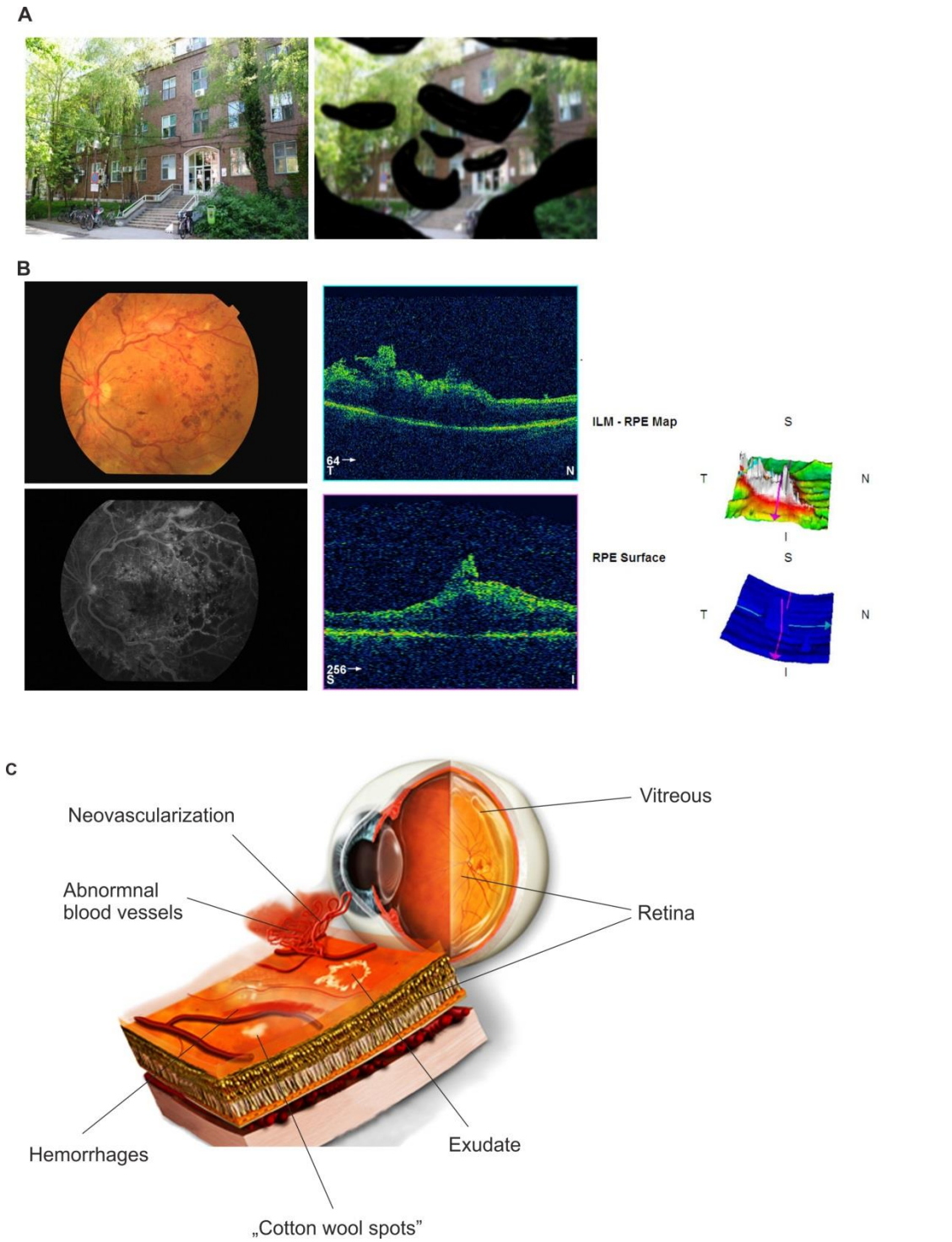

Figure 3. Clinical symptoms and pathomechanism of proliferative diabetic retinopathy (PDR)

A. Patients with PDR suffer from visual field defects (the difference of visual perception is shown between normal subject (left) and patient having DR (right), **B.** Funduscopic, fluorescein-angiography and OCT images of a patient suffering from PDR, recruited in the study. The same cotton wool spots, exudates, neovascularizations and hemorrhages are shown in **C.**

3. AIMS OF THE STUDY

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

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

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

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

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

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

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

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

4. MATERIALS AND METHODS

4.1. Tissue collection

All tissue collection complied with the Guidelines of the Helsinki Declaration (1964) and was approved by the National Medical Ethics Committee of Hungary (14415/2013/EKU – 183/2013 and DEOEC RKEB/IKEB 3094/2010) and the National Medical Ethics Committee of the Republic of Slovenia. Limbal tissue and CEC collection was done within 12 hours of biologic death from cadavers and followed the EU Member States' Directive 2004/23/EC on presumed consent practice for tissue collection. Biologic death was always confirmed by a clinical team at the particular Clinic of death (mostly Internal Medicine) which was then followed by delivery to the Pathology Mortar. All relevant data pertaining to the status of the patient before death were checked before tissue collection.

FvERMs were obtained from patients (Mean Age: 62.7±9.0 years) undergoing vitrectomy due to intravitreal hemorrhage in PDR (**Table 1** shows the data for each patient).

	Donor 1	**Donor 2**	**Donor 3**
Age (years)	54	72	62
Gender	male	female	male
Type of diabetes	1	2	2
Duration of diabetes (years)	29	33	4
Insulin therapy	yes	yes	yes
BMI	28.1	31.6	35.1
Arterial hypertension	yes	yes	yes
Hyperlipidemia	yes	yes	yes

Table 1. Data of patients with proliferative diabetic retinopathy (PDR)

4.1.1. Isolation of LESCs and CECs

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

4.1.2. Isolation of fvERM outgrowing cells and hRPEs

Immediately after the isolation, fvERMs were transported and cultivated *ex vivo* under adherent conditions in L-glutamin containing DMEM:F12 (Sigma-Aldrich, Ljubljana, Slovenia) supplemented with 10% fetal calf serum (FCS), antibiotics and antimycotics (PAA Laboratories GmbH, Pasching, Austria) and kept until reaching confluence. For control experiments, hRPE cells were used. The cells were isolated from cadavers and cultivated *ex vivo* (protocol modified from Thumann et al.[36]). The number of viable cells was determined with trypan blue exclusion assay after detachment with trypsin for further experiments.

4.2. Surface protein expression analysis by flow cytometry

The phenotype of the isolated cells was determined by multicolor flow cytometry using FITC-, PE- and APC- conjugated antibodies against selected surface proteins (details are shown in **Table 2).** In the case of LESCs and CECs, the antibodies against MSC-related markers, such as CD29/Integrin β1, CD44/HCAM, CD45, CD54/ICAM1, CD73, CD90/Thy-1, CD117/c-kit and CD146/MCAM were used in a concentration specified in the manufacturers' protocol (all antibodies were obtained from Biolegend, San Diego, CA, USA). For the fvERM outgrowing cells and hRPEs, antibodies against CD11a/LFA-1, CD14, CD18/Integrin β2, CD29/Integrin β1, CD34, CD44/HCAM, CD49a, CD49d, CD51/Integrin αV, CD54, CD73, CD90/Thy-1, CD338/ABCG2, CD106/VCAM-1, CD166/ALCAM, PDGFRβ, HLA-G (Biolegend); CD105 (BD Bioscience, San Jose, CA, USA), CD47, CXCR4, CD146/MCAM, HLA-DR, VEGFR2, CD45, CD117 and CD31 (obtained from R&D Systems, Minneapolis, MN, USA) were used with the matching isotype controls as well. Cells were washed with their growth medium and twice with Fluorescence-Activated Cell Sorting (FACS) buffer after harvesting with 0.025% trypsin-EDTA. Samples were labeled for 30 minutes on ice, then washed again with FACS buffer and fixed in 1% paraformaldehyde (PFA)/Phosphate buffered saline (PBS) and analyzed within 1 day. Measurement of the samples was carried out with FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) and the data were analyzed using FlowJo (TreeStar, Ashland, OR, USA) software. The results were expressed as means of positive cells (%)$\pm$SD in the case of LESCs and CECs and means of positive cells (%)$\pm$SEM in the case of hRPE and fvERM, respectively. Hierarchical clustering was performed by the R software[37].

Antibody	Corp.	Cat. No.	Clone	Concentration
CD11a (LFA-1)	BioLegend	350604	TS2/4	25 μg/mL
CD14	BioLegend	325606	HCD14	200 μg/mL
CD18 (Integrin β2)	BioLegend	302106	TS1/18	400 μg/mL
CD29 (Integrin β1)	BioLegend	303004	TS2/16	100 μg/mL
CD31 (PECAM)	R & D Systems	FAB3567P	9G11	25 μg/mL
CD34	BioLegend	343504	581	25 μg/mL
CD44 (H-CAM, Hermes)	BioLegend	338804	BJ18	50 μg/mL
CD45	R & D Systems	FAB1430A	2D1	10 μg/mL
CD47	R & D Systems	FAB4670A	472603	10 μg/mL
CD49a (Integrin α1)	BioLegend	328304	TS2/7	50 μg/mL
CD49d (Integrin α4)	BioLegend	304304	9F10	12.5 μg/mL
CD51 (Integrin αV)	BioLegend	327908	NKI-M9	100 μg/mL
CD54 (ICAM-1)	BioLegend	353108	HA58	100 μg/mL
CD73	BioLegend	344004	AD2	100 μg/mL
CD90 (Thy-1)	BioLegend	328108	5E10	50 μg/mL
CD105	BD Biosciences	561443	266	200 μg/mL
CD106 (VCAM-1)	BioLegend	305806	STA	6.25 μg/mL
CD117 (c-kit)	R & D Systems	FAB332P	47233	50 μg/mL
CD140b (PDGFRβ)	BioLegend	323606	18A2	400 μg/mL
CD146 (MCAM)	R & D Systems	FAB932A	128018	10 μg/mL
CD166 (ALCAM)	BioLegend	343904	3A6	50 μg/mL
CD184 (CXCR4)	R & D Systems	FAB173A	44717	25 μg/mL
CD338 (ABCG2)	BioLegend	332020	5D3	25 μg/mL
HLA-DR	R & D Systems	FAB4869F	L203	25 μg/mL
HLA-G	BioLegend	335910	87G	100 μg/mL
VEGFR2	R & D Systems	FAB357P	89106	50 μg/mL

Table 2. Details of the fluorochrome-conjugated antibodies used in FACS

4.3.1. Microarray and Data Analysis

Total RNA from each analyzed sample was extracted by miRNeasy Mini Kit (QIAGEN, QIAGEN Inc.,Valencia, CA, USA) and quantified with NanoDrop (Thermo Fisher Scientific, Wilmington, DE, USA). RNA integrity was confirmed with Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). For microarray-based gene expression profile analysis Affymetrix GeneChip Human Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA, USA) were used. The array contained 28,869 gene transcripts. For the whole genome gene expression analysis 150 ng of total RNA was subjected to Ambion WT Expression Kit (Ambion, Life Technologies, Carlsbad, CA, USA) and GeneChip WT Terminal Labeling Kit (Affymetrix) according to the manufacturers' protocols. After washing, the arrays were stained using the FS-450 fluidics station (Affymetrix) and signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA, USA). The scanned

images were processed using GeneChip Command Console Software (AGCC) (Affymetrix) and the CEL files were imported into Partek Genomics Suite software (Partek, Inc. MO, USA). Robust microarray analysis (RMA) was applied for normalization. Gene transcripts with a maximal signal values less than 32 across all arrays were removed to filter for low and non-expressed genes, reducing the number of gene transcripts to 23,190. Differentially expressed genes between groups were identified using one-way ANOVA analysis in Partek Genomics Suite Software. Clustering analysis was made using the same name module in a Partek Genomics Suite Software.

4.3.2. Pathway analysis

To identify the relationships between selected genes, the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA) was used. Excel datasheets containing gene IDs with the assigned gene expression values were uploaded into the program. The Ingenuity Pathways Knowledge Base (IPKB) provided all known functions and interactions which were published in the literature. ANOVA was used to calculate a p-value to determine the probability that each biologic function or canonical pathway assigned to the data set was due to chance alone. For the representation of the relationships between the genes, the 'Pathway Designer' tool of the IPA software was used.

4.4. Transmission electron microscopy

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

4.5.1. Treatment and supernatant collection of fvERM outgrowing cells

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

4.5.2. Supernatant collection from LESCs

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

4.6.1. Measurement of secreted cytokine concentrations by ELISA

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

4.6.2. Measurement of secreted angiogenesis related cytokines by Angiogenesis Protein array

The Proteome Profiler Human Angiogenesis Array Kit (R&D Systems) provides a platform for detection of 55 different angiogenesis related cytokine in one raw. Measurements were carried out according to the manufacturer’s protocol. Briefly, the membranes were blocked with the manufacturers blocking solution, while 300 µl pre-prepared supernatant used in the analysis was added to 200 µl Array Buffer 4 and 1ml Array Buffer 5, then the mixture was incubated with 15 µL reconstituted Detection Antibody Cocktail for additional 1hr. After the incubation, the mixture solution – containing the sample and the detection antibodies, were added to the membranes and incubated overnight at 4°C. After four washing steps, the membranes were incubated for 30 minutes with streptavidin-HRP solution, then the antibody-antigen reaction was

visualized with a chemiluminescent reaction. The membranes were exposed to X-ray films and developed with KODAK Medical X-Ray Processor 101. Data analysis was performed according to the manufacturer's recommendation with ImageJ software.

4.7. Calcium dynamics in the fvERM outgrowing cells

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

4.8. Statistical analysis

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

5. RESULTS

5.1. Gene expression profiling of LESCs and CECs

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

	Ingenuity Canonical Pathways	**-Log(B-H-P-value)**	**Ratio**
Top Canonical Pathways	*Hepatic Fibrosis/Hepatic Stellate Cell Activation*	8.36E-05	32/142 (0.225)
	Inhibition of Angiogenesis by TSP1	2.22E-04	12/34 (0.353)
	RAR Activation	5.01E-04	35/179 (0.196)
	Antigen Presentation Pathway	1.25E-03	11/40 (0.275)
	Axonal Guidance Signaling	1.31E-03	65/432 (0.15)
Top Tox (toxicological) Pathways	*Hepatic Fibrosis*	4.25E-06	27/93 (0.29)
	Glutathione Depletion - CYP Induction and Reactive Metabolites	6.6E-05	7/12 (0.583)
	Liver Proliferation	1.5E-04	39/189 (0.206)
	Persistent Renal Ischemia-Reperfusion Injury (Mouse)	4.41E-04	10/30 (0.333)
	Increases Renal Proliferation	4.54E-04	24/101 (0.238)

Table 3. The most significantly affected canonical pathways found in LESCs
IPA was used to calculate the canonical pathways from the gene expression profile of LESCs.

With further deep-analysis, more biological functions and diseases from the significantly changed expression levels of groups of genes were determined. 733 molecules were found to be involved in cancer development, 344 in reproductive system diseases, 282 in dermatological diseases, 402 in gastrointestinal diseases and 257 in endocrine system related disorders. Within the cellular functions' group of genes, 567 were involved in cellular growth and proliferation, 552 in cellular development, 520 in cell death, 381 in cellular movement and 290 in cell-to-cell signaling. In system development, the IPA software found 371 genes involved in organogenesis and 350 in tissue development. High number of genes belonged to the cardiovascular development (271) and tumor morphogenesis (140) group. Only a small number of molecules related to visual system development and function (98) could be detected (**Table 4**). Furthermore, only 42 genes could be identified as related to clinical chemistry and hematology, 142 genes to be involved in cardiotoxicity, 205 in hepatotoxicity and 195 in nephrotoxicity, respectively **(Table 4)**.

5.2. Customized gene array data – upstream regulators in LESCs

Groups from the significantly and differentially expressed upstream regulators in LESCs were generated in particular, those which were extensively related to physiological maintenance of the cells. 257 genes collected and divided into subgroups of interest such as stemness and proliferation, differentiation, cytokines and growth factors, angiogenesis and cell adhesion. Although there were significant inter-donor differences, the heatmap and the functional clustering of the genes demonstrated a clear distinction between the LESCs and the control differentiated CECs. The genes that were mostly affected were involved in ion-, nucleotide- or protein binding, protein secretion as well as receptor or enzyme activities (**Figure 4**). Most of the upregulated genes were involved in ECM re-organization or ECM-to-cell connection such as *FN1* (Fibronectin 1 with 74.934 fold change), *MMP9* (type IV collagenase with 14.243 FC), *MMP1* (interstitial collagenase, 13.875 FC), *ICAM1* (Intercellular adhesion molecule 1, 13.681 FC) and *ITGA5* (fibronectin receptor with 13.455 FC respectively). Cytokines and growth hormones were also included in the selection (**Table 5**).

Function	Name	p value	Molecules
Diseases and Disorders	*Cancer*	5.96E-27-1.23E-03	733
	Reproductive System Disease	1.61E-16-1.19E-03	344
	Dermatological Diseases and Conditions	3.42E-16-1.15E-03	282
	Gastrointestinal Disease	4.31E-13-8.26E-04	402
	Endocrine System Disorders	2.65E-10-7.46E-04	257
Molecular and Cellular Functions	*Cellular Movement*	5.90E-18-1.43E-03	381
	Cellular Growth and Proliferation	1.31E-10-1.12E-03	567
	Cellular Development	3.26E-09-1.07E-03	552
	Cell-To-Cell Signaling and Interaction	8.23E-09-1.48E-03	290
	Cell Death and Survival	1.04E-08-1.48E-03	520
Physiological System Development and Function	*Cardiovascular System Development and Function*	9.52E-10-1.23E-03	271
	Tumor Morphology	6.48E-09-9.47E-04	140
	Organismal Development	9.59E-09-1.48E-03	371
	Visual System Development and Function	1.34E-07-1.48E-03	98
	Tissue Development	2.59E-07-1.48E-03	350
Clinical Chemistry and Hematology	*Decreased Levels of Albumin*	1.63E-03-3.94E-01	6
	Increased Levels of Alkaline Phosphatase	2.79E-03-1.18E-01	16
	Increased Levels of Creatinine	8.01E-03-8.01E-03	8
	Increased Levels of Potassium	1.48E-02-5.41E-01	7
	Increased Levels of Albumin	1.09E-01-2.21E-01	5
Cardiotoxicity	*Cardiac Stenosis*	6.92E-04-3.13E-01	15
	Congenital Heart Anomaly	3.64E-03-5.28E-01	23
	Cardiac Arteriopathy	4.20E-03-6.33E-01	42
	Pulmonary Hypertension	5.95E-03-1.18E-01	11
	Cardiac Hypertrophy	8.04E-03-1.00E00	51
Hepatotoxicity	*Liver Proliferation*	2.37E-04-3.13E-01	39
	Liver Cholestasis	6.93E-04-5.84E-01	22
	Liver Cirrhosis	7.33E-04-2.21E-01	31
	Liver Damage	8.26E-04-2.21E-01	33
	Liver Hyperplasia/Hyperproliferation	8.39E-03-5.03E-01	80
Nephrotoxicity	*Renal Proliferation*	6.16E-06-2.21E-01	38
	Renal Damage	7.17E-04-5.03E-01	37
	Renal Tubule Injury	7.17E-04-2.21E-01	24
	Renal Necrosis/Cell Death	1.84E-03-1.00E00	52
	Renal Inflammation	8.70E-03-1.00E00	34

Table 4. Top biological and toxicological functions found in LESCs
Biological functions based upon the most affected pathways in LESCs as determined by the IPA software.

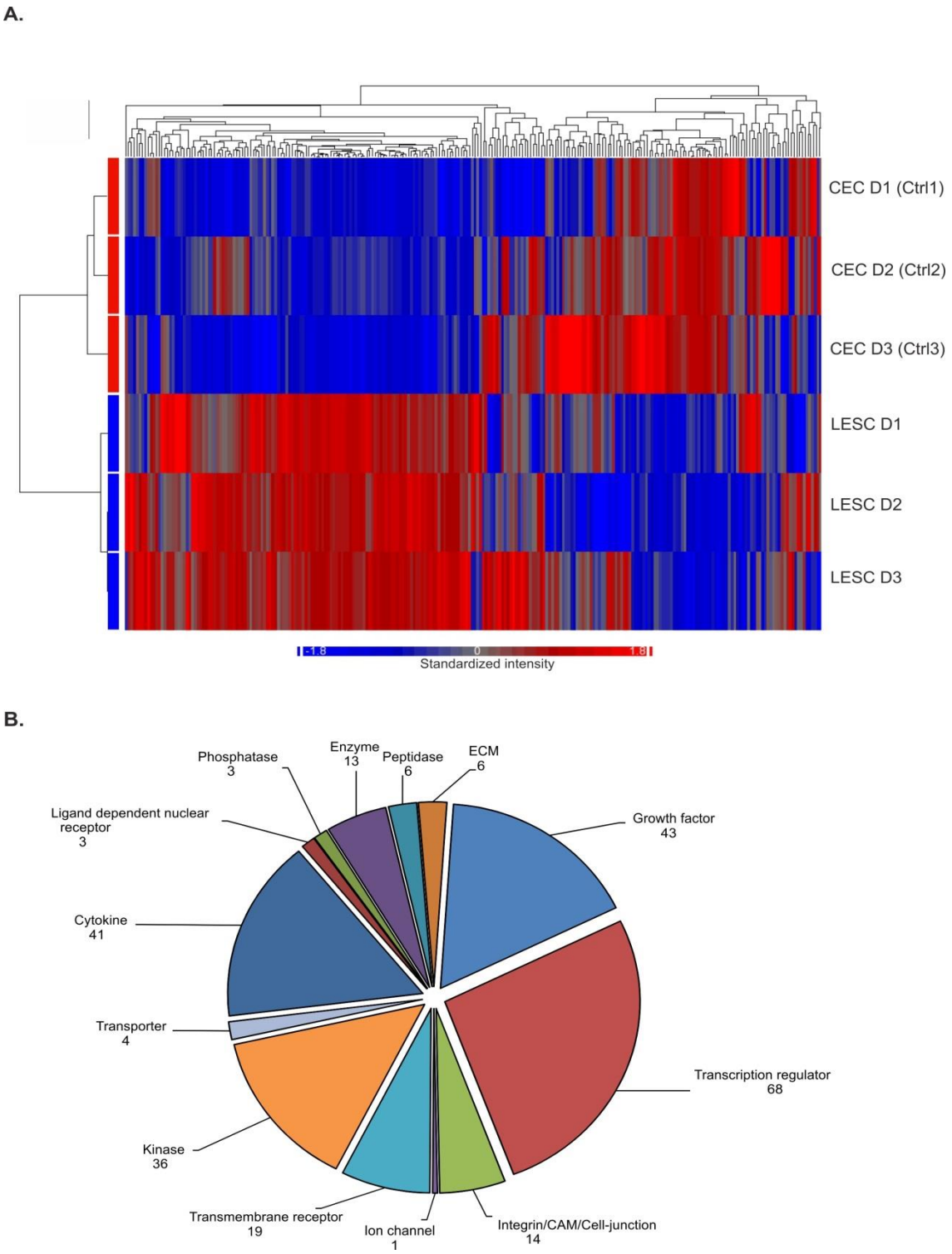

Figure 4. Heatmap of the differentially expressed genes in LESCs compared to CECs

A. Heatmap of the transcripts and functional clustering of 257 genes expressed in a significantly different way in LESCs compared to CECs, and related to stemness, epithelial differentiation, tissue organization and angiogenesis. Red and blue colors indicate high and low expression, respectively. The cluster analysis and dendrogram show the difference between the two cell types, **B.** Distribution of the 257 significantly differentially expressed genes by molecule type as defined by the IPA software.

Symbol	Entrez gene name	FC	z-score	p-value	Molecule
Fold Change up-regulated					
FN1	Fibronectin 1	74.934	2.979	8.37E-05	enzyme
CCNA1	Cyclin A1	27.199		3.42E-02	other
IL1B	Interleukin 1, beta	24.948	4.924	8.09E-15	cytokine
INHBA	Inhibin, beta A	21.815	1.352	2.27E-04	growth factor
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	18.854	-0.927	1.40E-02	other
GDF15	Growth differentiation factor 15	16.924	1.999	2.63E-02	growth factor
PTHLH	Parathyroid hormone-like hormone	16.2	1.972	8.72E-03	other
OSMR	Oncostatin M receptor	15.366	1.982	1.83E-02	TM receptor
CXCL10	Chemokine (C-X-C motif) ligand 10	15.171	0.911	2.31E-02	cytokine
MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa type IV collagenase)	14.243	0.689	1.72E-02	peptidase
IL1R1	interleukin 1 receptor, type I	13.972	2.603	5.76E-03	TM receptor
MMP1	Matrix metallopeptidase 1 (interstitial collagenase)	13.875	1.188	4.01E-03	peptidase
ICAM1	Intercellular adhesion molecule 1	13.681	2.961	1.36E-03	TM receptor
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	13.455	2.411	1.46E-02	other
SH3KBP1	SH3-domain kinase binding protein 1	12.752		4.98E-02	other
AKT3	V-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	11.843		4.76E-02	kinase
LOXL2	Lysyl oxidase-like 2	11.734	1.992	1.88E-03	enzyme
CEACAM5	Carcinoembryonic antigen-related cell adhesion molecule 5	10.588		1.23E-02	other
SLPI	Secretory leukocyte peptidase inhibitor	8.53	-2.433	1.06E-02	other
PDZK1IP1	PDZK1 interacting protein 1	8.485		1.23E-02	other

Table 5. Top 20 up-regulated genes in LESCs
Top 20 up-regulated genes determined by the IPA software. The genes shown code for cytokines, enzymes and receptors, and are involved in cell-migration, ECM re-organization and immune responses.
(FC: Fold change, p-value: p- value of overlap, TM receptor: transmembrane receptor)

Symbol	Entrez gene name	FC	z-score	p-value	Molecule
Fold Change down-regulated					
CRTAC1	Cartilage acidic protein 1	-72.277			
LPA	Lipoprotein, Lp(a)	-11,623		4.98E-02	Other
ETV1	Ets variant gene 1	-7,444	1.969	1.83E-02	transcription regulator
EDNRB	Endothelin receptor type B	-7,25		3.38E-02	G-protein coupled receptor
BMP7	Bone morphogenetic protein 7	-6,436	0.733	1.17E-04	growth factor
NREP	Neuronal regeneration related protein	-5,823	-0.248	3.81E-03	Other
CFTR	Cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	-5,766	-1.993	1.49E-01	ion channel
DCN	Decorin	-5,066	0.172	6.04E-07	Other
RORA	RAR-related orphan receptor alpha	-4,781	-0.439	2.61E-03	ligand-dependent nuclear receptor
LEF1	Lymphoid enhancer binding factor 1	-4,441	-0.306	2.01E-02	transcription regulator
BDKRB1	Bradykinin receptor B1	-4,1	-2.000	6.89E-04	G-protein coupled receptor
GJA1	Gap junction protein, alpha 1	-3,94	-1.480	5.54E-04	transporter
FAM3B	Family with sequence similarity 3, member B	-3,9		3.20E-08	cytokine
P2RX7	Purinergic receptor P2X, ligand-gated ion channel, 7	-3,885		1.15E-02	ion channel
KAT2B	K(lysine) acetyltransferase 2B	-3,829	1.963	8.27E-02	transcription regulator
ODC1	Ornithine decarboxylase, structural 1	-3,63		4.98E-02	Enzyme
EPHX2	Epoxide hydrolase 2, cytoplasm	-3,469		3.42E-02	Enzyme
MAT1A	Methionine adenosyltransferase I, alpha	-3,386	-0.215	1.82E-02	Enzyme
CTSL2	Cathepsin L2	-3,385		4.98E-02	peptidase
DUSP1	Dual specificity phosphatase 1	-3,358	-1.881	6.12E-02	phosphatase
NOV	Nephroblastoma overexpressed	-3,149	0.555	1.94E-02	growth factor

Table 6. Top 20 Down-regulated genes in LESCs
Top 20 down-regulated genes determined by the IPA software. The genes shown code for cytokines, enzymes and receptors, and are involved in cell-migration, ECM re-organization and immune responses.
(FC: Fold change, p-value: p- value of overlap, TM receptor: transmembrane receptor)

5.3. Customized gene networks – upstream regulators in LESCs

5.3.1. Stemness and proliferation

122 upstream regulators were found to be related to stemness and proliferation in LESCs, in particular, to be related to MSCs. The heatmap of the significantly expressed genes within this group, besides the cluster analysis, showed a clear difference between the LESCs and the CECs **(Figure 5A)**. These differential genes coded for transcriptional factors, surface molecules, cytokines and growth factors, all playing a key role in the maintenance of multipotency and proliferation capacity of stem cells **(Figure 5B)**. Up- and down- regulation was also found in 66 and 56 genes, respectively, and within the custom selected gene cluster. The 10 most highly expressed upstream regulators were: *CCNA1* (27.199 fold), *IL1B* (24.948*), GDF15* (16.924), *ICAM1* (13.681), *TGFB* (16.745), *SOX9* (4.859 fold), *VIM* (4.368), *NT5E* (4.009), *TGFBR2* (3.772) and *BMP6* (3.494), while the 10 most down-regulated regulators were: *BMP7* (-6.436 fold), *LEF1* (-4.441), *GJA1* (-3.94), *KAT2B* (-3.829), *KLF4* (-3.041), *EGF* (-2.563), *FOXN1* (-2.11), *SOX6* (-1.984), *GDF9* (-1.865) and *HSPA9* (-1.838). The expression pattern of the selected genes confirmed that the LESCs have a higher proliferation potential and stemness-related gene expression than differentiated CECs. The SRY related HMG-box family members *SOX9* and *SOX6*, both involved in chondrogenesis and proliferation, were down-regulated in the LESCs.

5.3.2. Differentiation

LESCs play a key role in the re-epithelialization of the cornea, so 42 genes were analyzed that are related to terminal and epithelial differentiation. Based on the transcription level of the selected genes, LESCs segregated well from the differentiated CECs (**Figure 6A**). This group of upstream regulators consisted of growth factors, cytokines, adhesion molecules, transcription regulators and enzymes (**Figure 6B**). Transcriptional regulators such as *FOXA1* and *PMEL* were up-regulated in contrast to *FOXG1* (-1.165), *FOXD3* (-1.1), *MYOD1* and *OSGIN1* (-1.109) which were among the downregulated genes (**Figure 6C**). The pericellular matrix proteoglycan decorin coding gene *DCN* (-5.066) was found to be down-regulated in LESCs. Within the collection of cytokines and growth factors which play a role in epithelial differentiation, *IL1B* (24.948), *INHBA* (21.815), *IL1A* (7.853), *TGFB1* (6.745), *EREG* (3.836), *BMP6* (3.494) and *DKK1* (2.88) were up-regulated, while *BMP7* (-6.436 fold), *FGF1* (-2.96), *FGF7* (-1.473), *IL18* (-1.152) and *IGF2* (-1.126) were down-regulated (**Figure 6D**).

5.3.3. Cytokines and growth factors

Cytokines and growth factors have an important function in differentiation, maturation of cells, cell-cell communication, as well as controlling immunogenicity. Their expression level was different in LESCs compared to CECs (**Figure 7A**). The most up-regulated gene in this group was *IL1B* (24.948 FC), followed by *CXCL10* (15.171), *IL1A* (7.853), *IL8* (5.849), *EDN1* (5.504), *IFNE* (4.601), *IL6* (2.57), *SPP1* (2.077) and *CCL5* (1.973). These genes code for pro-inflammatory cytokines, while other cytokine family genes which have similar pro-inflammatory properties, such as *IL17* (-1.129), IL-1 superfamily members *IL18* (-1.152) and *IL36RN* (-1.059), were down-regulated. Human *EDA* (-1.113) which belongs to the TNF family was within the most down-regulated genes, while the top down-regulated gene was *FAM3B* (-3.900) (**Figure 7B**). Growth factors are important in the maintenance of stemness, pluripotency of stem- or progenitor cells, playing a key role in differentiation, tissue and organ development. The detected genes in this group were related to epidermal growth factor (EGF) and TGF beta (TGFβ) superfamily. In particular, *INHBA* (21.815 fold), *GDF15* (16.924), *TGFB1* (6.745) and *BMP6* (3.494) – all members of the TGFβ family, were among the most up-regulated genes. Other genes such as epiregulin - *EREG* (3.836), amphiregulin - *AREG* (4.047) and connective tissue growth factor *CTGF* (6.513) were in the top up-regulated genes as well, while other TGFβ superfamily members such as *BMP7* (-6.436) and *GDF9* (-1.865), acidic fibroblast growth factor, *FGF1* (-2.96) and *FGF7* (-1.473), were all down-regulated. Similarly, NOV-like CTGF- member of the CCN protein family: nephroblastoma overexpressed/*NOV* (-3.149) and the EGF (-2.563) gene expression responsible for regulation of cell division and proliferation were both down-regulated (**Figure 7C**).

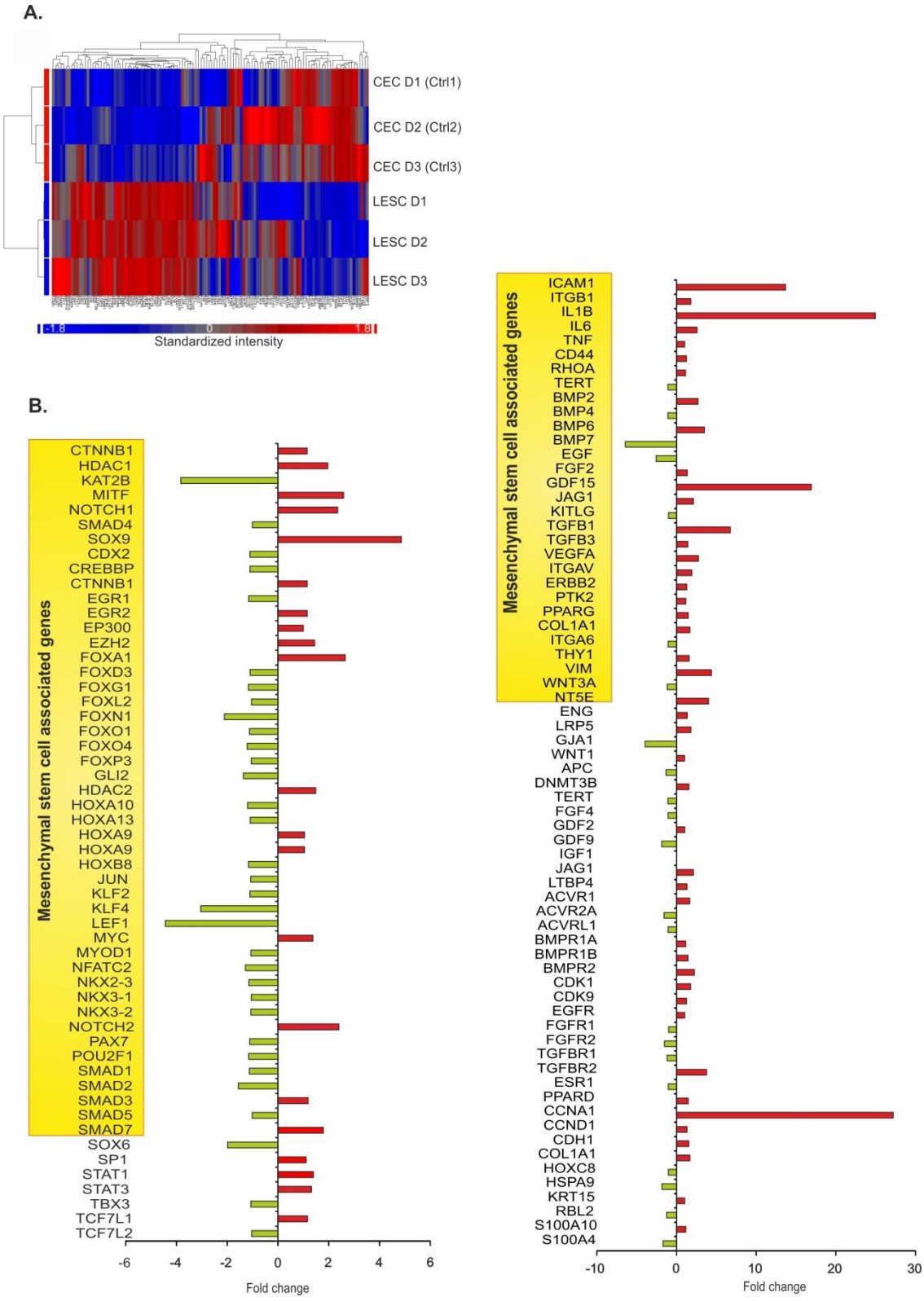

Figure 5. Upstream regulators as determinants for stemness and multipotency

A. Genes involved in the maintenance of stemness, cell cycle and multipotency-related transcriptional factors, and **B.** growth factors, cytokines and corresponding receptors. Genes characteristic for MSCs are grouped and highlighted as well.

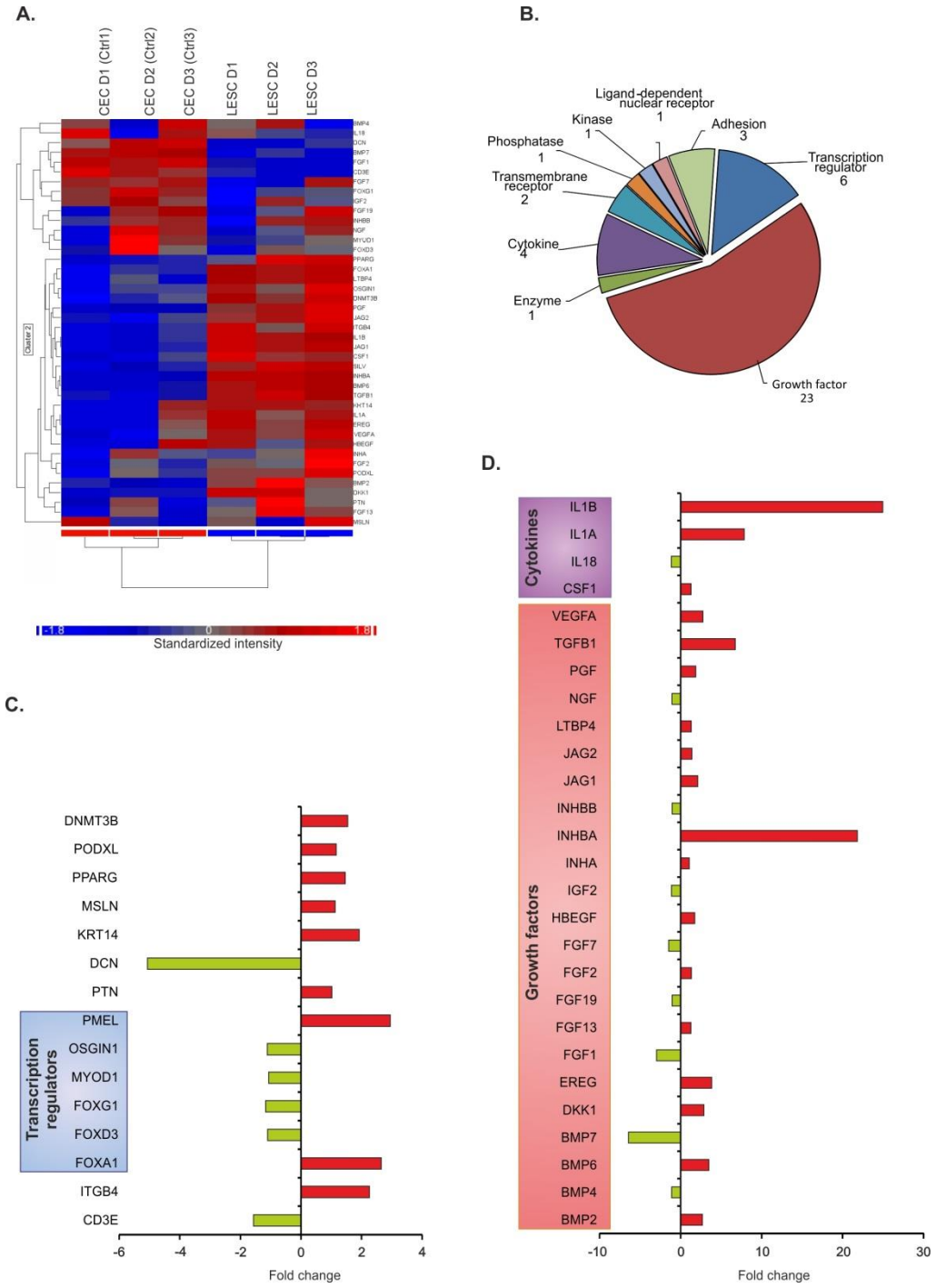

Figure 6. Expression of 42 epithelial- and terminal differentiation-related genes

A. Expression of transcriptional factors, transmembrane receptors, enzymes and adhesion molecules involved in epithelial- or terminal differentiation. **B**. Subgroups of cytokines- and growth factor-coding genes involved in epithelial differentiation of stem cells, **C**. Distribution of the selected 42 upstream regulators by molecule type, such as, **C.** transcriptional regulators and **D.** growth factors and cytokines.

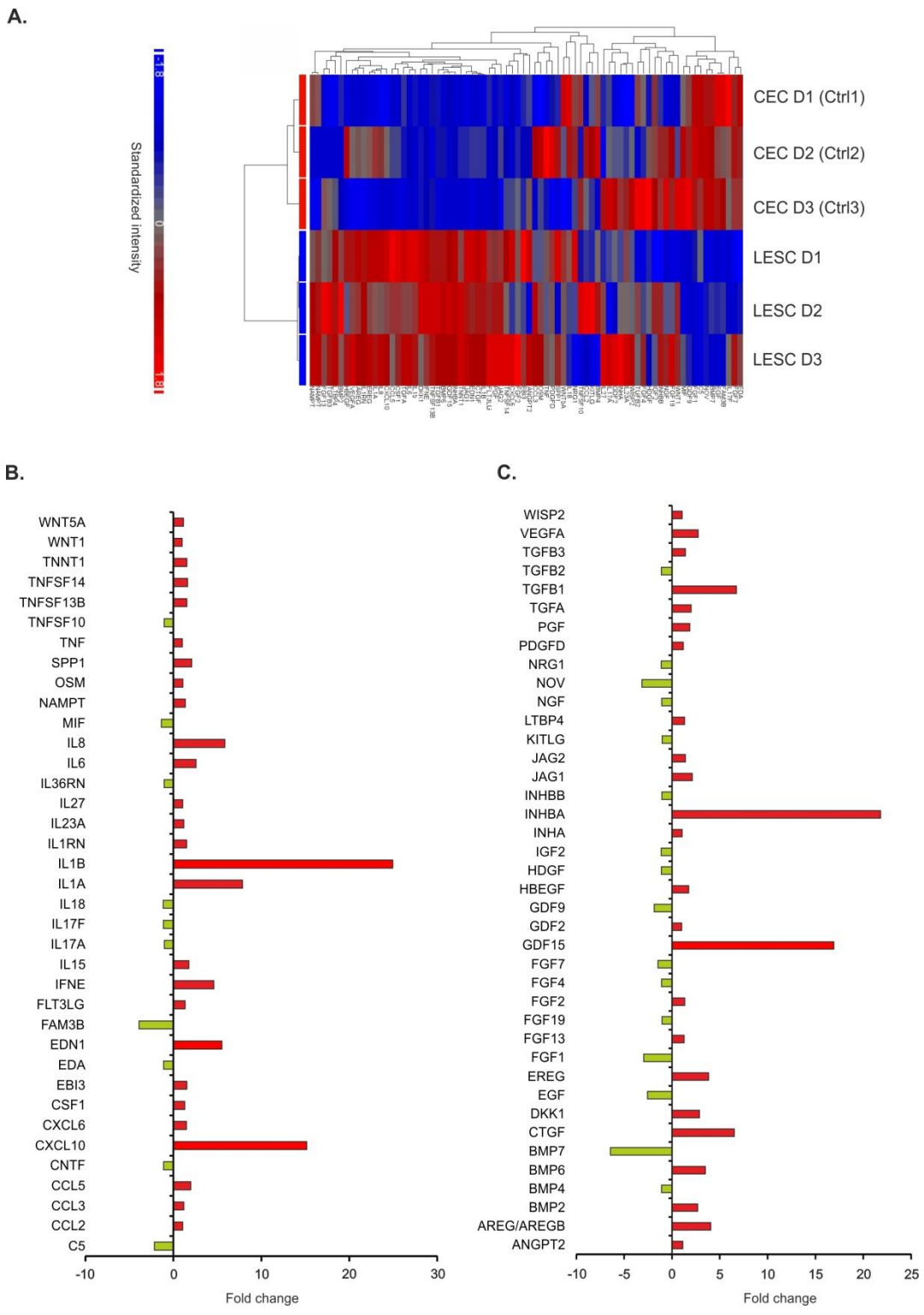

Figure 7. Transcriptional pattern of the cytokines- and growth factors- coding genes

A. In comparison to CECs, the LESCs expressed 37 cytokines- and 40 growth factors-coding genes in a significantly different manner, **B**. Selection of significantly and differentially expressed genes of cytokines, **C.** expression of differentiation and growth factors genes.

5.3.4. Cell adhesion

To further distinct the multipotent LESCs within the heterogeneous population of epithelial cells, surface markers including ECM-cell and cell-cell adhesion, as well as cell migration genes were used as putative markers. The upstream regulators of 54 genes coding for molecules involved in cell adhesion were analyzed **(Figure 8A,E)**. In the first subgroup, highly expressed transcriptional factors and transmembrane receptors were collected. This group contained *MAP2K* (12.088), *AKT3* (11.843), *TGFBI* (6.745), *CTGF* (6.513), *SPP1* (2.077) and *SRC* (1.931) as up-, and *AKT1* (-1.026), *ROCK2* (-1.076), *PRKCA* (-1.154), *HRAS* (-1.5), *PRKCB* (-1.583) and *NOV* (-3.149) as down-regulated genes **(Figure 8C)**. The next subgroup, included genes coding for integrins, cell adhesion molecules (CAMs), proteolytic enzymes and matrix metalloproteases (MMPs) – all involved in the ECM breakdown and tissue healing and remodeling (**Figure 8D**). Within this cluster, *MMP9* (14.243), *MMP1* (13.875), and *MMP14* (1.836) were found to be the most up-regulated genes, while the other family member *MMP3* was down-regulated (-1.105). Laminins, which encode important proteins of the basal membrane, were all up-regulated - *LAMA3* (3.289), *LAMC1* (1.724) and *LAMA1* (1.428), in the LESCs. Integrins, CAMs and tight junctions, which are very important in cell-cell adhesion and tissue organization were up-regulated - *ICAM-1* (13.681), *CAV1* (1.608) and *CLDN7* (3.056), while *GJA1* (-3.94) was down-regulated within this custom-made group. In particular, *CDH1* (1.536), an important component of the desmosomal junction formation and stratified epithelium transformation was up-regulated; the desmosome formation between the LESCs grown on lens capsule could be demonstrated using transmission electron microscopy (**Figure 8B**). Altogether, the expression of 11 integrin-coding genes was different between the LESCs and the differentiated CECs - 8 out of them were up-, while 3 were down-regulated.

5.3.5. Angiogenesis

The dataset was filtered for molecules which may have a role in pathological angiogenesis in the cornea. The heatmap of the detected 48 genes shows the differential expression profile of LESCs and CECs (**Figure 9A**). The molecule types coded by the angiogenesis-related genes could be divided into transcriptional factors, enzymes and cytokines including angiogenic growth factors as well (**Figure 9B**). The fibronectin (*FN1)* gene, which is important in new vessel sprout formation, had the highest up-regulation (74.934), followed by *SERPINE1* (18.854) and *MMP9* (14.243). The

coagulation factor III (thromboplastin) gene *F3* (7.054) was also highly expressed in the LESCs. The most down-regulated genes were *PLG* (-2.521), *TIMP1* (-1.658), *FOXO4* (-1.213), *TGFBR1* (-1.179) (**Figure 9C**). Certain cytokines and growth factors which are also important in angiogenesis were up-regulated in the LESCs: *ILB1* (24.948), C-X-C motif chemokine 10, *CXCL10* (15.171), *TGFB1* (6.745) and *VEGFA* (2.742) (**Figure 9D**). In addition, IL-6 and IL-8, two very potent angiogenic cytokines, were also up-regulated in these cells: *IL-6* (2.57) and *IL-8* (5.849), similar to *EDN1* (5.504), *EREG* (3.836) and *BMP2* (2.686). Only four of the angiogenic cytokines were down-regulated in the LESCs: acidic FGF - *FGF1* (-2.96), *IL17F* (-1.129), *TGFB2* (-1.106) and *KITLG* (-1.015).

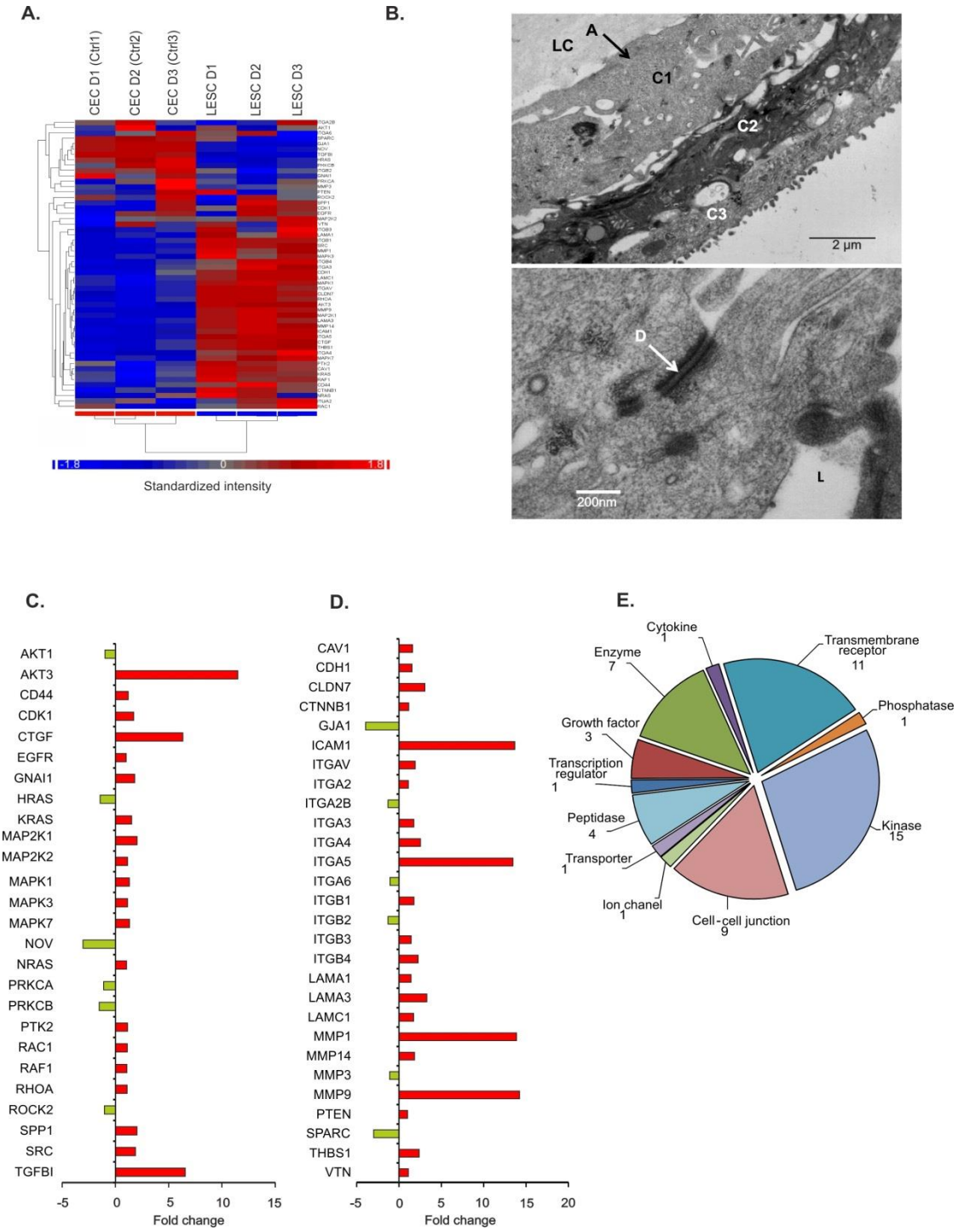

Figure 8. Differential changes in selected genes related to cell-cell junction, cell-to-cell and cell-to-extracellular matrix (ECM) adhesion.

A. Collection of significantly different expressions of transcriptional factors, kinases and transmembrane receptors related to cell-cell connection and adhesion, **C** and **D**. CAMs, integrins and ECM receptors determining the tissue origin of the LESCs, **E**. Molecule types of the selected 54 upstream regulator in the two groups of cells, **B**. TEM pictures from LESCs grown on lens capsule (LC) and showing cell-cell junctions among the cells (LC = Lens capsule, A = attachment between LC and cells; C1', C2 and C3 = three cell-layers, L = translucent space, D = Desmosomes).

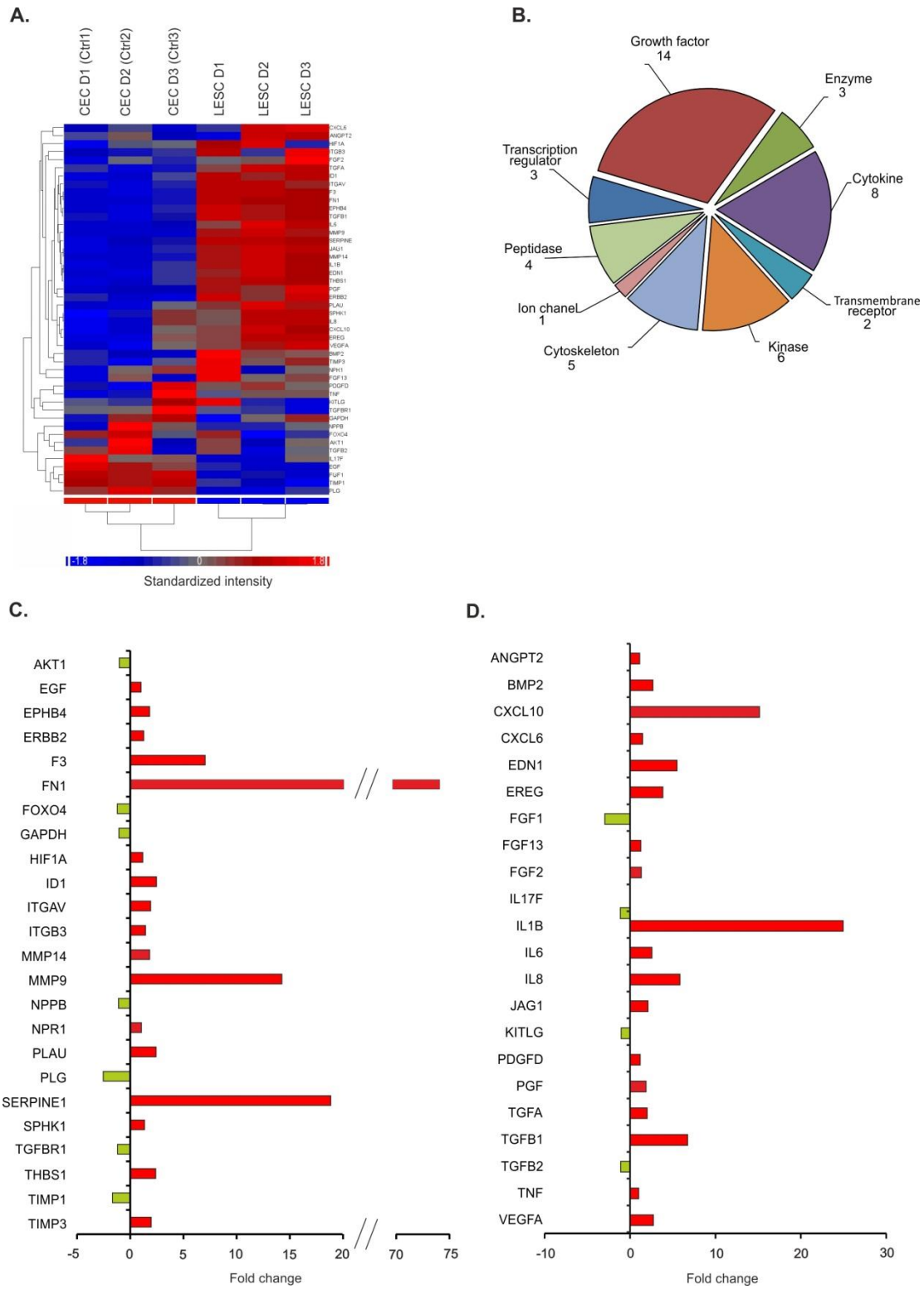

Figure 9. Expression of angiogenesis-related genes in LESCs

A. MMPs, proteolytic enzymes and ECM proteins play a key role in the angiogenic processes, **B**. Cytokines and growth factors are important players during angiogenesis and endothelial cell activation as well as EPC/stem cell differentiation, **C** and **D**. Most of the angiogenic molecules belong to the shown molecule types.

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

IL-6 and IL-8 are important cytokines not only in the inflammatory processes, but play a key role in maintaining stemness, differentiation and immunosuppression of stem cells, as well as epithelial differentiation and angiogenesis[38-41]. Their gene expressions were up-regulated in LESCs: *IL-6* (2.570) and *IL-8* (5.849), respectively. Using the IPA software analysis, the IL-6 signaling pathways were further confirmed of being present in the LESCs compared to CECs, together with some other well-known pathways described below (**Figure 10A**).

Secretion of IL-6 depends on many stimulatory signals; hallmark pathway is the response of activated cells to pro-inflammatory stimuli, such as IL-1β- and TNFα-mediated activation. This signaling is further mediated by the NFκB and JNK (JUN, C-Fos) transcriptional factors and can lead to IL-6 and IL-8 release in parallel to collagen type I production (COL1A1), which is the major component of connective tissue. The second network affected is the autocrine or IL-6-mediated-IL-6-secretion through RAF1, MAP2K and ERK1/2. This process needs to be initiated by the IL-6 receptor (IL6R), however, the JAK-STAT pathway (STAT3) can also induce release of angiogenic factors such as VEGF and activation of SOX3. As shown before in our dataset, *IL1B* was highly up-regulated with a 24.948 fold change hand-in-hand with its receptor *IL1R1* (13.972) and *IL1A* (7.853). Although a subunit of the receptor for IL-6 coding gene was down-regulated - *IL6R* (-2.640), a member of the type I cytokine receptor family - oncostatin M receptor (*OSMR*), was found to be highly up-regulated (15.366) in the LESCs. In our dataset, *SOCS3* was up-regulated (1.397), while *SOCS1* was down-regulated (-1.120). Four MAPKs were slightly up-regulated in the LESCs: *MAP2K1* (2.088), *MAPK1* (1.339), *MAPK14* (1.011) and *MAPK3* (1.163), while the members of the NFκB pathway were down-regulated: *NFKB1* (-1.178) and *NFKBIA* (-1.193). CXCL10 in presence of high amount of IL-6 has been shown to induce migration of trophoblasts through activation of the CXCR3 receptor[42]. Interestingly, *CXCL10* was among the highest up-regulated genes (15.171) in the LESCs compared to the CECs (**Figure 10B**).

The pathways in which IL-8 participates are in general more complex than for other cytokines, due to its multipotent effect on the different cell types. IL-8 can act as chemotactic factor for neutrophils, can activate immune cells[43], stem cells and has been also described as potent pro-angiogenic cytokine, especially in the eye (**Figure 11A**)[44, 45]. The molecular background of such angiogenic processes has not been well described, but IL-8 seems to be an important activator of circulating EPCs with myeloid

origin. IL-8 can bind to G protein-coupled serpentine receptors such as CXCR1 and CXCR2 and beside immunological activation, it can induce rearrangement of cytoskeletal proteins, increase the expression of VCAM and ICAM1, and the migration as well as vessel formation of endothelial cells and stem cell-like EPCs, in parallel with increase in vascular permeability (**Figure 11B**).

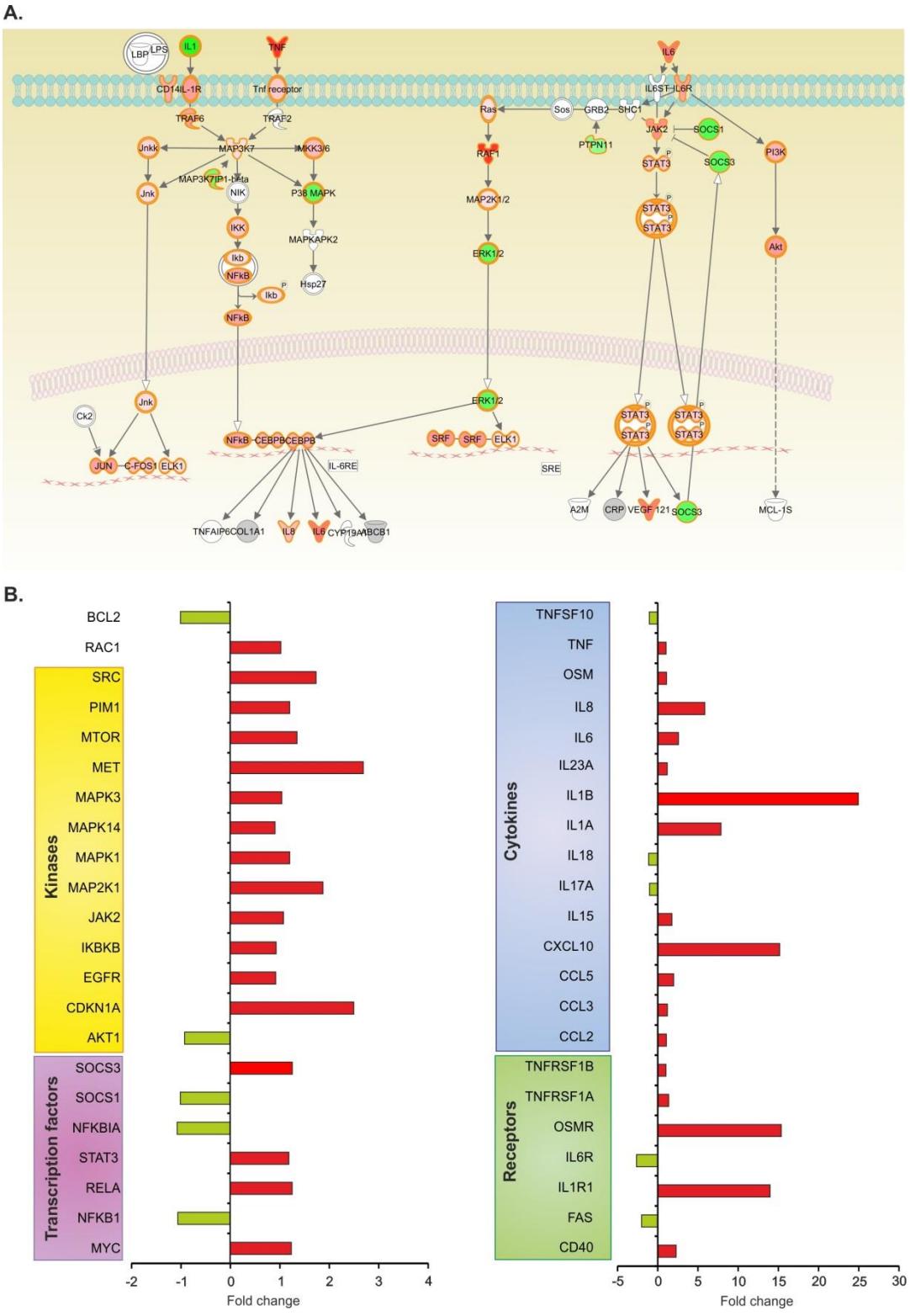

Figure 10. Networks related to IL-6 signaling pathway as detected by the IPA software.

The colored genes appear in the studied dataset: red color means up-, while green color means down-regulated. The grey colored genes did not fit the cut off level. **A** and **B**. 44 upstream regulators of the IL-6 signaling pathway in LESCs when grouped upon biological functions of a molecule type.

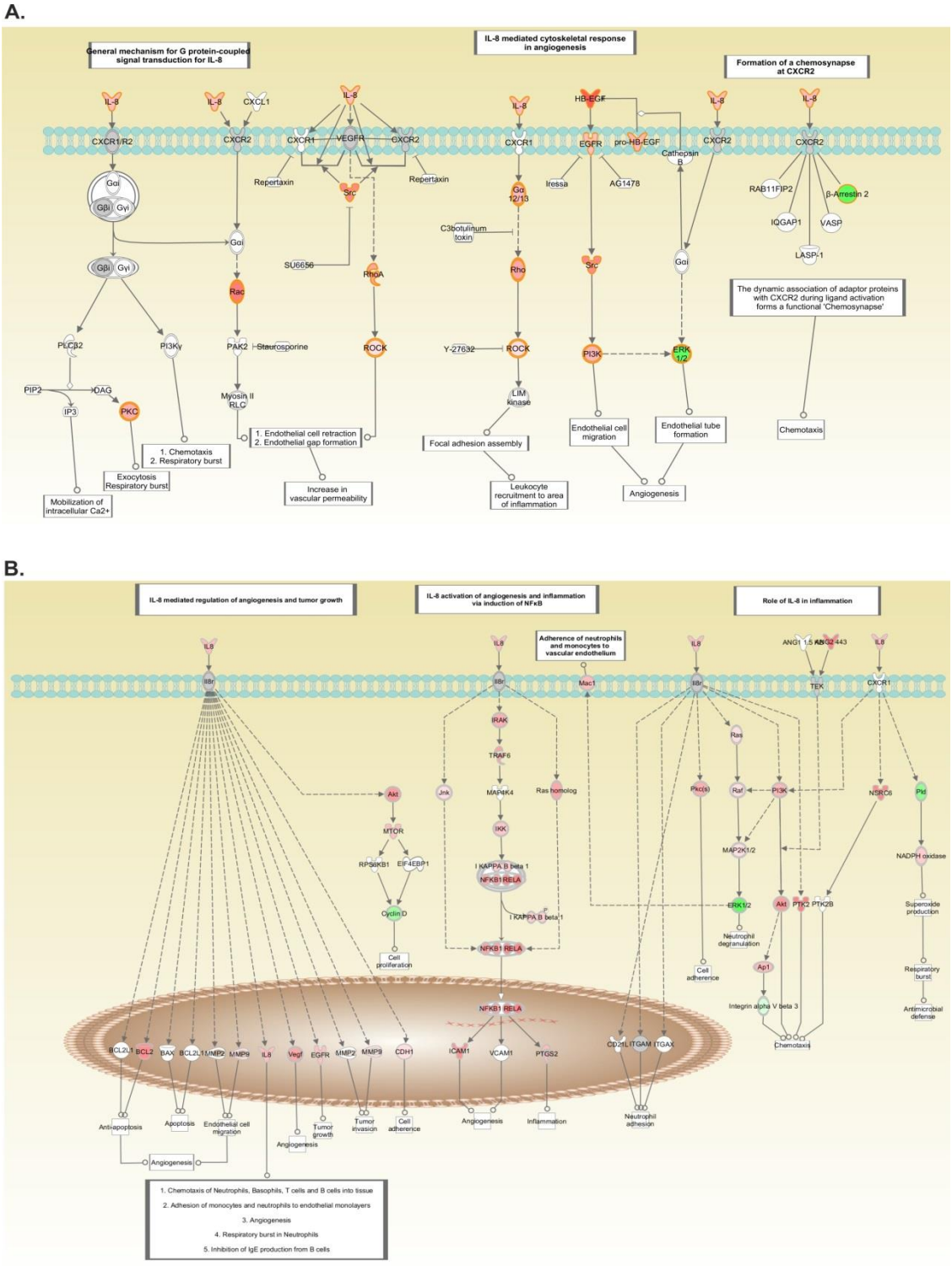

Figure 11. Networks of IL-8 mediated signaling pathways generated by the IPA software

A. IL-8 plays a key role in innate immunity, **B.** and as pro-angiogenic cytokine. The colored genes appear in the studied dataset: red color means up-, while green color means down-regulated. The grey colored genes did not fit the cut off level.

5.5. IL-6 and IL-8 secretion by LESCs

The level of IL-6 and IL-8 cytokines in the LESC culture supernatants were measured by ELISA and were continuously high and correlated well with the gene expression data. At day 9, the level of IL-6 was 5885.24±685.6 pg/mL (Data are Mean±SEM), and at day 13 it was 6147.14±530.21 pg/mL, with no significant difference at both time points (p=0.778) (**Figure 12**). The same pattern could be observed in the IL-8 levels, 6665.00±312.41 pg/mL could be detected at day 9 and 7429.52±352.43 pg/mL at day 13, without significant difference between the two (p=0.14).

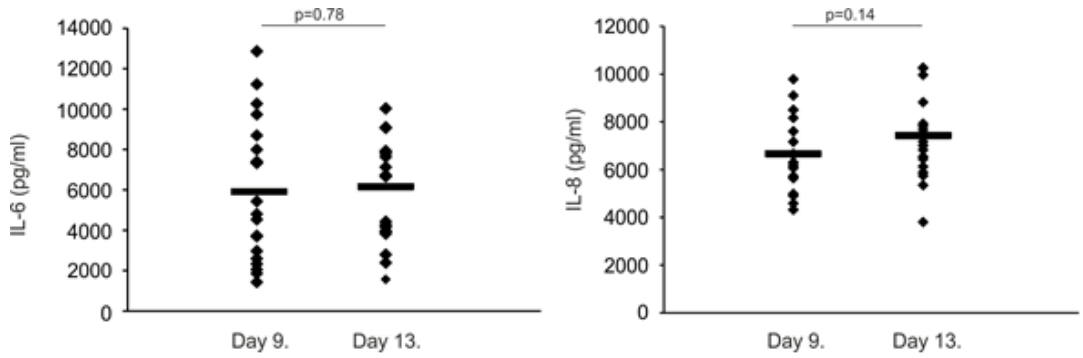

Figure 12. Secreted IL-6 and IL-8 levels by LESCs

The levels of secreted IL-6 and IL-8 as measured by ELISA in the supernatants of long term LESC cultures (N=21, p values were determined by Student's t-test).

5.6. Phenotype analysis of LESCs and CECs

Genes coding MSC-related surface markers were measured at the protein level as well. Interestingly, no difference was found in the positivity for CD90 (8.75±19.56% in LESCs and 1.77±3.54% in CECs, respectively; p=0.4748, data shown are Mean±SD) and CD73 (89.86±6.15% in LECSs and 76.93±17.43% in CECs, respectively; p=0.2374). In the LESC cell cultures, more cells expressed the stem cell factor receptor CD117/c-kit (19.87±24.92%) compared to the CECs (0±0), however, this difference was not statistically significant (p=0.1491) due to a high inter-donor variance (**Figure 13A).** CAMs and integrins showed more difference between the two cell types. At a protein level, CD146/MCAM was found not to be expressed on the surface of CECs (0±0%) compared to LESCs (82.40±14.60%, p=0.0002) and higher number of positive cells for ICAM1 in CECs (56.19±12.46%) than in LESCs (4.37± 7.63%) (p=0.0001) could be observed. No difference could be found between LESCs and CECs in the

expression of CD29/Integrin β1 (97.01±1.87% and 78.28±15.84%, respectively), and CD44/HCAM (16.55±23.21% and 19.83±21.55%, respectively). The percentage of CD47 positive cells, which plays a role in cell viability and immune regulation, was significantly higher in LESCs (98.98±0.57%) compared to CECs (25.9±27.44%) (p=0.0039), showing higher viability and inhibition of phagocytosis in the LESCs (**Figure 13A)**. The percentage of the positive cells for the selected surface proteins within the cell cultures showed different pattern in LESCs compared to CECs when using a cluster analysis (**Figure 13B**).

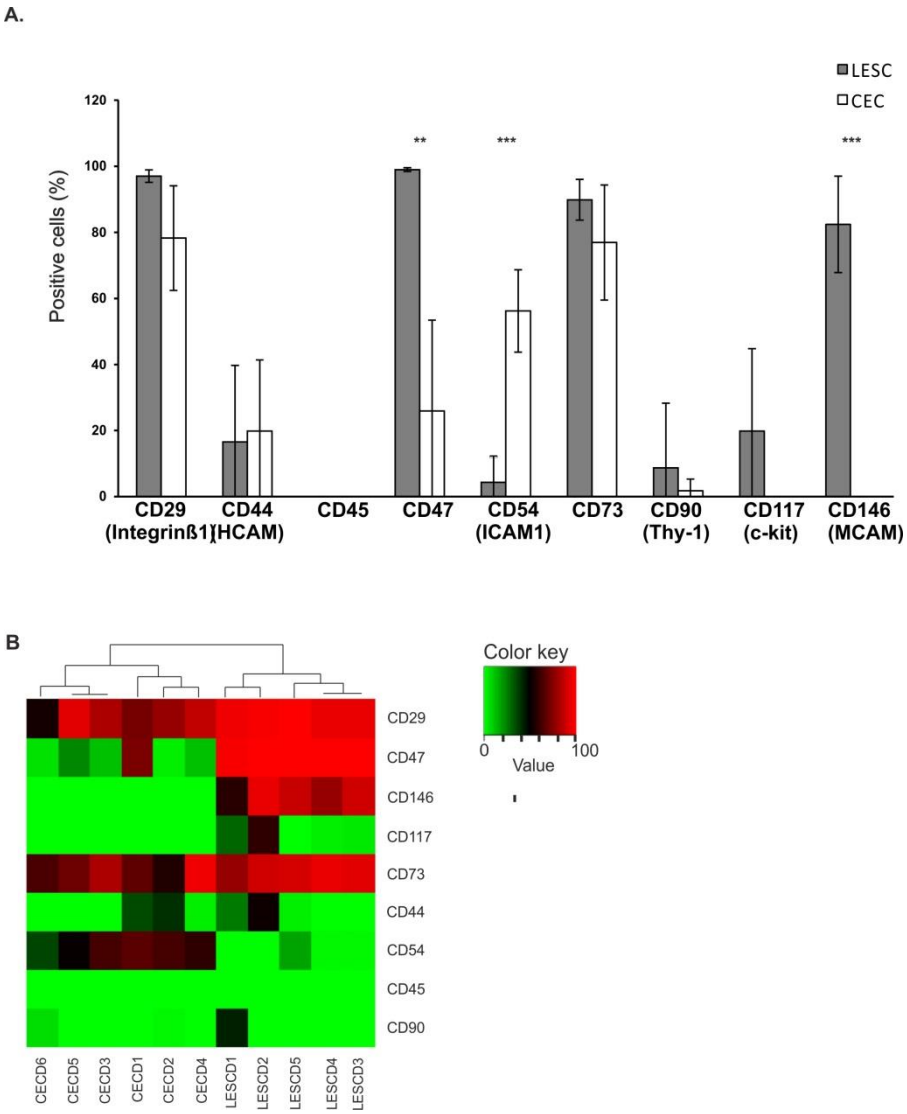

Figure 13. Surface protein level analysis on LESCs and CECs by FACS.

A. Positivity of the LECSs and CECs for CD73, CD90/Thy-1, CD117/c-kit, CD146/MCAM, CD29/Integrin β1, CD44/H-CAM, CD47 was determined by flow cytometry. CD45 was used as a negative control in these cells (Data shown are Mean±SD; p<0.05 *, p<0.01 **, p<0.001 ***; N=6 for the CECs, and N=5 for the LESCs), **B.** Cluster analysis showed a clear difference between the LESCs and the CECs.

5.7. Immunophenotyping of the fvERM outgrowing cells

The fvERM outgrowing cells assumed an elongated, fibroblastoid-like morphology when cultivated under adherent conditions *ex vivo* (**Figure 14A**). The surface marker expression profile of the cultivated fvERM cells was compared to that of primary hRPE cells (**Figure 14B** (cluster analysis) and **Table 7**). The *ex vivo* cultured fvERM cells showed no purely common hematopoietic or monocyte phenotype. Similarly, these cells expressed no CD45, CD11a (LFA-1) and HLA-G, like the primary hRPE cells (an exception being the very low CD11a expression in one of the hRPE donors). Higher percentage of the primary hRPE cells were positive for CD14 (66.60±11.26%) compared to the fvERM cells (1.81±1.06%, p=0.005), while inversely, higher CD47 expression was observed on the fvERM (97.95±0.44%) compared to the primary hRPE cells (88.04±5.48%) – the latter showing that the outgrowing fvERM cells were indeed viable cells. Both cell types had a low surface expression of HLA-DR (0.08±0.08% in fvERM cells vs. 1.00±1.00% in hRPE), while the percentage of CD117/c-kit (0.94±0.76% and 19.80±16.53%), CXCR4 (0.41±0.25% and 7.28±5.22%) and CD338/ABCG2 (0.80±0.08% and 17.63±15.09%) cells was, in general, lower in the fvERM compared to the primary hRPE, respectively. Only the expression of CD34 was more abundant in the fvERM cultures (21.81±15.78%) compared to the primary hRPE (2.34±1.17%), however, this difference was not statistically significant. Similarly, the expression of CD73, CD105 and PDGFRβ was not significantly different between the fvERM cells and the primary hRPEs, while a significant difference in the CD90 expression was measured between the two cell types (68.19±0.46% in fvERM cells vs. 91.16±6.66% in primary hRPE, p= 0.03).

Among the cell adhesion molecules (CAMs) and integrins - all being important for maintaining the fate of the cells in their environment, significantly lower expression of CD18/Integrin β2 (p=0.01) and CD51/Integrin αV (p= 0.004) was found on fvERM cells compared to primary hRPE cells. The expression of CD29/integrin β1, CD49a/integrin α1, CD49d/ integrin α4, CD54/Intercellular adhesion molecule 1 (ICAM-1), CD106/V-CAM 1, CD146/MCAM and CD166/ALCAM on fvERM cells were similar to those detected on primary hRPEs.

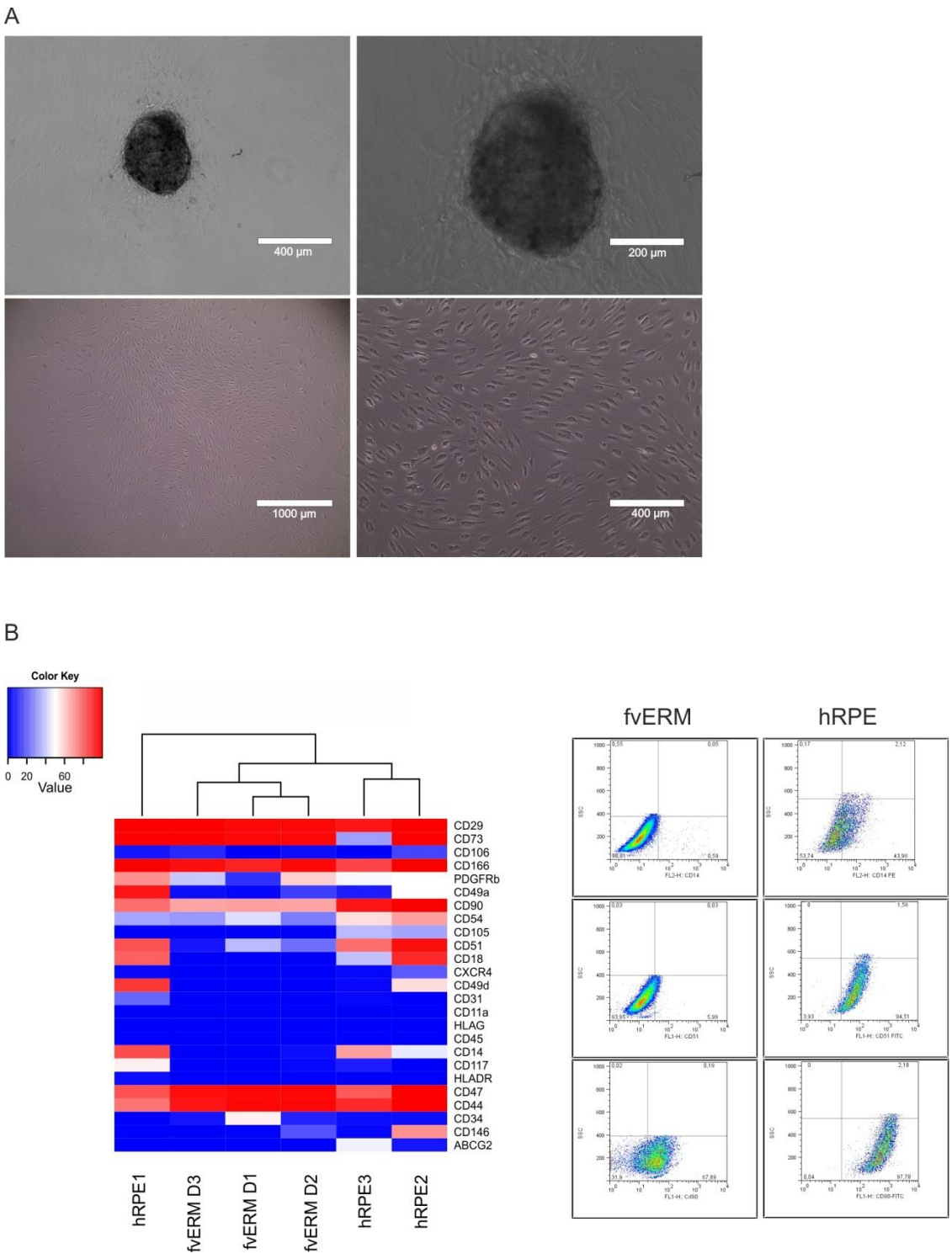

Figure 14. Morphology and immunophenotype of the fvERM outgrowing cells

A. Morphology and **B.** hierarchical clustering based upon the expressed surface markers on fvERM outgrowing cells, in comparison to primary hRPE cells. (fvERM D1, 2 and 3 are fvERM Donor 1, 2 and 3 respectively; Data shown are representative of 3 independent experiments on 3 different donor fvERM samples)

		fvERM cells	Primary hRPE cells	
Hematopoietic Monocyte markers	CD11a (LFA-1)	0.00 ± 0.00	0.00 ± 0.00	
	CD14	1.81 ± 1.06	66.60 ± 11.26	**
	CD34	21.81 ± 15.78	2.34 ± 1.17	
	CD45	0.00 ± 0.00	0.00 ± 0.00	
	CD47	97.95 ± 0.44	88.04 ± 5.48	
	CD117/c-kit	0.94 ± 0.76	19.80 ± 16.53	
	CXCR4	0.41 ± 0.25	7.28 ± 5.22	
	HLA-DR	0.08 ± 0.08	1.00 ± 1.00	
	HLA-G	0.00 ± 0.00	0.00 ± 0.00	
	CD338 (ABCG2)	0.80 ± 0.08	17.63 ± 15.09	
MSC Fibroblast markers	CD73	98.37 ± 0.32	76.55 ± 22.76	
	CD90/Thy-1	68.19 ± 0.46	91.16 ± 6.66	*
	CD105/Endoglin	0.29 ± 0.29	23.10 ± 11.60	
	PDGF Rβ	36.46 ± 14.11	56.59 ± 7.66	
CAMs Integrins	CD18 (Integrin β2)	0.25 ± 0.17	69.86 ± 16.38	*
	CD29/Integrin β1	98.34 ± 0.48	98.38 ± 1.40	
	CD31/PECAM	0.00 ± 0.00	7.60 ± 6.52	
	CD44/H-CAM	96.78 ± 1.06	89.03 ± 6.71	
	CD49a/Integrin α1	6.06 ± 3.53	50.36 ± 25.67	
	CD49b/ Integrin α2	0.11 ± 0.07	49.30 ± 25.56	
	CD51 Integrin αV	21.07 ± 9.14	85.98 ± 5.54	**
	CD54/ICAM-1	32.65 ± 5.45	52.47 ± 10.83	
	CD106/V-CAM 1	4.17 ± 2.60	6.43 ± 3.85	
	CD146/MCAM	5.91 ± 5.69	24.58 ± 23.52	
	CD166/ALCAM	95.70 ± 1.64	95.28 ± 4.47	

Table 7. Surface markers expression on fvERM outgrowing cells and primary hRPE cells

The expression of different groups of surface markers was compared between fvERM outgrowing- and primary hRPE cells. The two cell types showed differential expression of CD14, CD18/Integrin β2, CD51/Integrin αV and CD90/Thy-1. (Data shown represent percentage of positive cells within the total cell culture and are representative of 3 independent experiments on 3 different donor fvERM samples, Mean±SD; $p<0.05$ *, $p<0.01$ **)

5.8. Detection of angiogenic factors secreted by the fvERM outgrowing cells

Altogether 55 angiogenesis-related markers were screened from the supernatants of the fvERM outgrowing cells under presence or absence of TNFα treatment. Unstimulated fvERM cells expressed high amount of serine protease inhibitor E1 (Serpin E1), also known as endothelial plasminogen activator inhibitor 1 (PAI-1; at pixel density 28891±1096.02) and tissue inhibitor of metalloproteinase 1 (TIMP-1; at pixel density 45238.5±1170.26) as shown in **Figure 15**. After 24 hours of pro-inflammatory stimulation by TNFα, both factors increased further in the supernatants of the treated compared to the control untreated cells (Serpine E1 with pixel density 87418.5±243.24 and TIMP-1 with 113313±9050.26, respectively). More importantly, 11 secreted angiogenesis-related factors could be detected in the cell culture media of fvERM cells treated by TNFα that were otherwise absent in the untreated controls. Besides Serpine E1, the anti-angiogenic and tumorigenic pigment epithelium-derived factor (PEDF, also known as Serpin F1) was induced and secreted (at pixel density 20601.5±1045.10). Endothelin 1 (ET-1; at pixel density 11427±2065.46), a molecule that has been implicated in the development and progression of vascular disorders and usually secreted by endothelial cells upon stimulation by pro-inflammatory cytokines or hypoxia, could also be detected upon TNFα treatment (**Figure 15**). In addition, TNFα stimulation caused expression of 14 TNF-inducible proteins, among them being the pentraxin-related protein 3 (PTX3), which is a marker for rapid primary local activation of innate immunity and inflammation (at pixel density 51756+2533.56). Monocyte chemotactic protein-1 (MCP-1 or CCL2; at pixel density 15799.5+5861.92) and IL-8 (also referred as CXCL8; at pixel density 94931+9130.87) were both released by the TNFα treated, but not the untreated fvERM outgrowing cells – these molecules play an important role as monocyte chemo-attractant proteins. High pixel density of thrombospondin 1 (TSP-1; 28239±2942.27) could also be measured in the proteome profiler array of the TNFα stimulated fvERM outgrowing cells, referring to its many angiogenic and anti-angiogenic functions that depend upon its binding factor alternatives. Endocrine-gland-derived vascular endothelial growth factor (EG-VEGF)/prokineticin (PK), which is a new members of the angiogenic cytokine family, was also secreted by the TNFα stimulated cells, although in low amounts (at pixel density 3698+627.20), and it could not be detected in the control cell culture supernatants. Dipeptidyl peptidase-4 **(**DPPIV, also known as CD26), which plays a key role in the glucose metabolism, underwent induction upon TNFα treatment (at pixel density 15854.5+2201.93). In addition, two members of the insulin-like growth factor-

binding proteins (IGFBPs) appeared upon the TNFα pro-inflammatory stimulus: IGFBP-2 (at pixel density 15965.5±222.03) and IGFBP-3 (at pixel density 41872.5±2607.81). Matrix metallopeptidase 9 (MMP-9), which has many biological functions, among them being facilitation of angiogenesis upon inflammatory stimulation, also increased upon TNFα treatment (at pixel density 8989.5+134.35), while it was absent in the supernatants from the control fvERM outgrowing cells.

A

	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	23-24
A	(+)CTRL		Activin A	ADAMTS-1	ANG	Ang-1	Ang-2	Angiostatin Plasminogen	AR	Artemin		(+)CTRL
B	CF III TF	CXCL16	DPPIV CD26	EGF	EG-VEGF PK1	Endoglin CD105	Endostatin Collagen XVIII	ET-1	FGF acidic FGF-1	FGF basic FGF-2	FGF-4	FGF-7 KGF
C	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IL-1ß IL-1F2	IL-8 CXCL8	TGF-ß1	Leptin	MCP-1 CCL2
D	MIP-1γ CL3	MMP-8	MMP-9	NRG1-ß1 HRG1-ß1	Pentraxin 3 TSG-14	PD-ECGF	PDGF-AA	PDGF-AB PDGF-BB	Persephin	PF4 CXCL4	PlGF	Prolactin
E	Serpin B5 Maspin	Serpin E1 AI-1	Serpin F1 PEDF	TIMP-1	TIMP-4	TSP-1	TSP-2	uPA	Vasohibin	VEGF	VEGF-C	
F	(+)CTRL											(-)CTRL

B

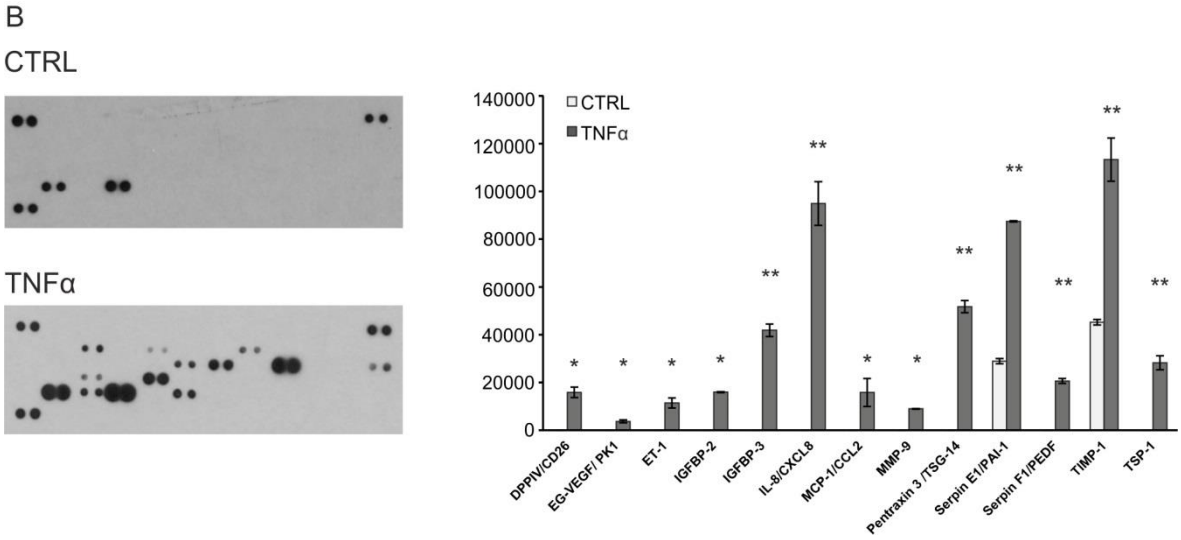

Figure 15. Angiogenic factors secreted by the fvERM outgrowing cells.

The fvERM cells were treated with 100ng/mL TNFα for 24 hours. Supernatants were collected and the cytokine levels were determined by a Human Angiogenesis Array Proteome Profiler. **A.** Map of the 55 angiogenesis-related proteins detected on the membranes. **B**. Panel of the secreted proteins of control and TNFα treated cells. (Mean±SD; $p<0.05$ *, $p<0.01$ **)

5.9. Surface marker expression of fvERM outgrowing cells upon TNFα treatment

In correlation to the FACS surface immunophenotype of the fvERM outgrowing cells, TNFα treatment increased the percentage of CD54/ICAM-1 positive cells within the cell cultures and the amount of the surface protein as well (**Figure 16A,B**), but had no influence on the VEGFR2 or CXCR4 expression, indicating that these cells do not participate directly in the angiogenesis process through endothelial differentiation.

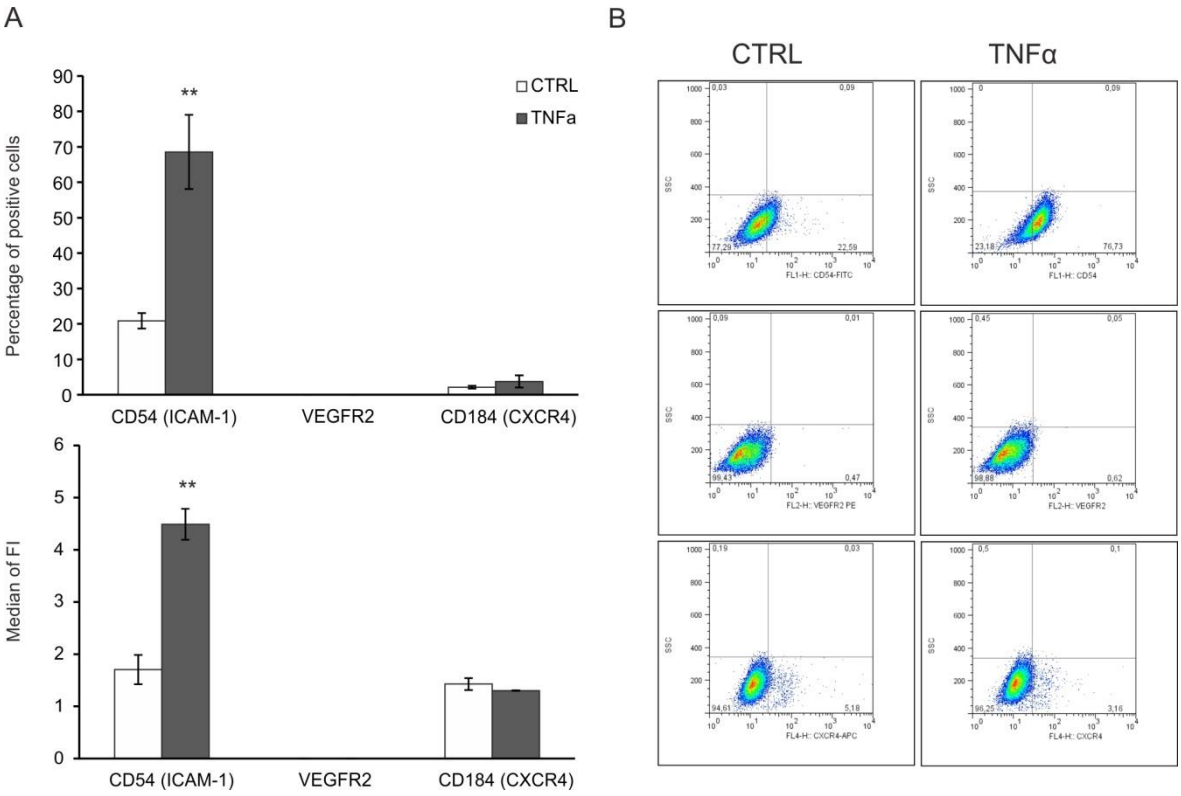

Figure 16. Surface marker-analysis of the TNFα treated fvERM outgrowing cells

A. Percentage of positive cells for the selected markers and their expression levels of the surface proteins as measured by flow cytometry. Not just the number of positive cells, but the protein level on the surface of the fvERM outgrowing cells increased in case of CD54 in response to recombinant TNFα treatment. **B.** Color-dotplots of untreated and treated cells. (Data shown are Mean±SD of 3 independent experiments on 3 different donor fvERM samples, $p<0.01$ **)

5.10. Functionality and viability of the fvERM outgrowing cells

The dynamics of $[Ca2^{+}]_i$ upon mechanical stimulation reflects well upon the functionality and viability of the outgrowing fvERM outgrowing cells, and such mechanical tractional forces can be common in fvERMs in late stages of PDR[46]. Mechano-stimulation was induced by a glass micropipette applied to a single cell (**Figure 17A**), which caused and intracellular calcium propagation that could be followed from the cell body to the periphery (**Figure 17B**). The fvERM outgrowing cells responded to mechano-stimulation by increasing their $[Ca2^{+}]_i$ in a monophasic manner (**Figure 17C,D**). The parts of interest are shown by colored arrows superimposed onto the morphology image. The colors correspond to the traces showing the time courses of the 360⁄380 ratio, proportional to $[Ca2^{+}]_i$ for the selected areas. The resting levels, the increase in 360/380 ratio upon stimulation and the amplitudes of the ratio of the responses corresponding to the resting levels and the changes in $[Ca2^{+}]_i$ can all visualized in **Figure 17**.

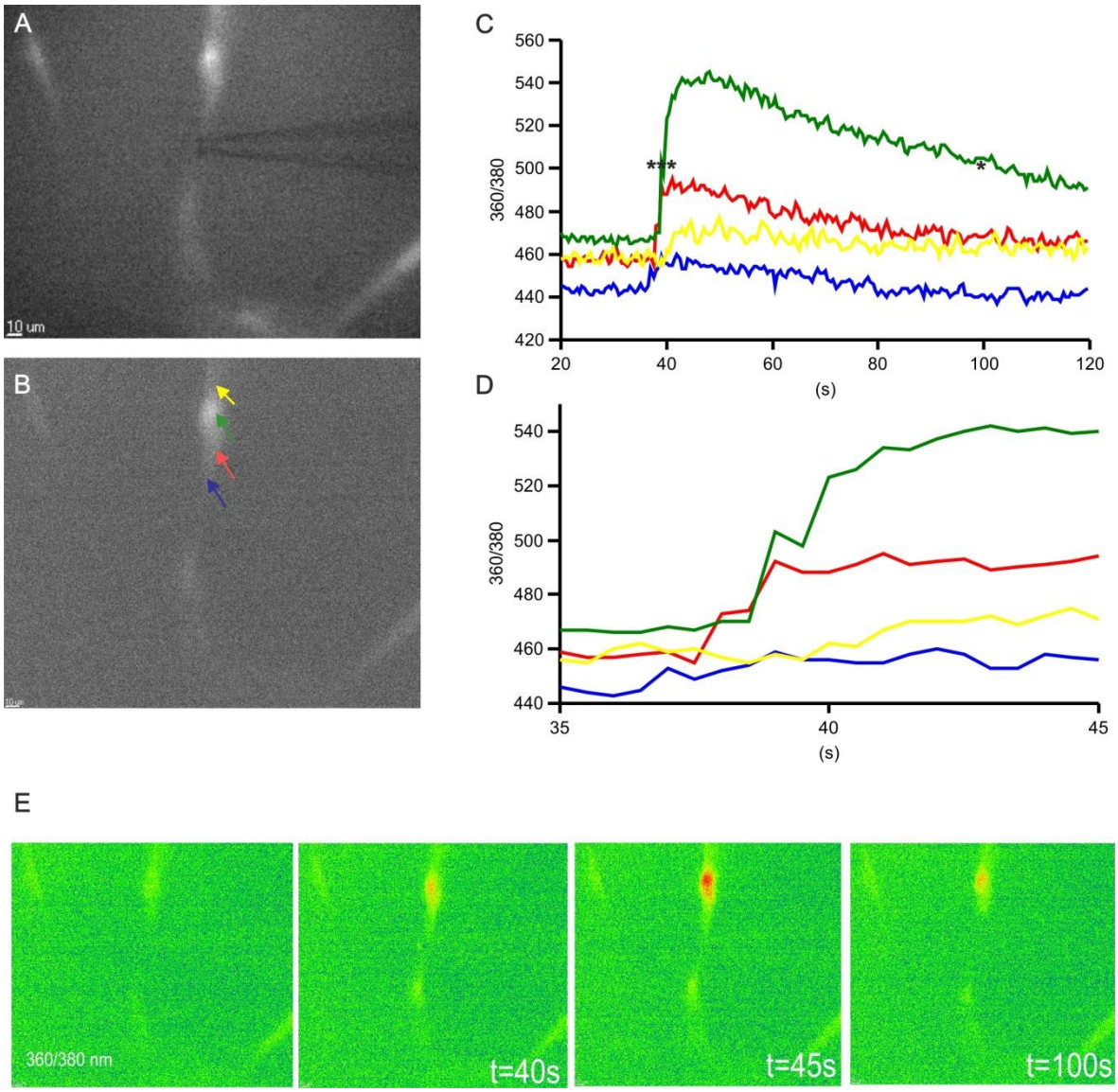

Figure 17. Intracellular propagation of calcium signal upon mechanical stimulation in fvERM outgrowing cells from PDR

A. A glass pipette was used for mechanical stimulation of cells. **B.** Regions of interest (ROIs) are presented in different colors to which the traces on panel **C** and **D** correspond. **C**. The intracellular calcium concentration changes in time are marked with colored traces before and after the mechanical stimulation, the different colors corresponding to the regions of the cell marked accordingly. **D.** Enlarged region of the traces in **C** showing that calcium increase starts in the cell region marked in blue and then propagates with a time delay to the cell regions marked in red-green and then yellow. **E.** The intracellular calcium concentration distribution shown as color-coded images, corresponding to the four selected instants marked in **C** with asterisk.

6. DISCUSSION

The importance of the LESCs in corneal biology and regeneration is well known, the absence or removal of which can cause defective corneal regeneration[47,48]. In humans, several diseases are known with acquired or genetic loss of LESCs, such as Steven Johnson syndrome, chronic limbitis, ocular pemphigoid or chronic inflammation-caused LESCD. Inflammatory processes with uncontrolled neovascularization very likely change the environment in such a way, that the small niches of stem cells become non-functional[4,13,49]. This process leads to decreased epithelial differentiation and transparency of the cornea, resulting in a serious and painful disease with subsequent loss of vision[50]. The treatment of LESCD is based upon the replacement of stem or progenitor cells in the limbus, with the more appropriate *ex vivo* culturing and proof-of-functionality of the LESCs becoming widely accepted today[51]. Other cells such as embryonic stem cells, bone-marrow and Wharton jelly-derived stem cells have been attempted for LESCD treatment in animal models with relatively good outcomes as well[52,53]. Unlike the new directives, most of the cell therapies used so far culture the limbal epithelial cells on 3T3 mouse feeder fibroblasts supplemented with FCS-containing medium[54], therefore, causing risk for murine (animal) viral transmission, prions and contamination with other unknown factors[55,56]. Although the overall success rate of the cell therapies has been reported to be 76%[56] with replacement of corneal epithelial cells, the recovery of stemness is still unknown[52]. According to the new directives and directions for quality, safety and efficacy of advanced-therapy medicinal products (ATMPs), animal material-free methods would be more plausible and accurate way to go[57,58], however, the right quantity and phenotype of the cells needed for it has not been determined yet. The latter is crucial in stem cell-based therapies, since the purity of the product (the percentage of stem cells within the cell culture) determines the outcome[21,56]. As mentioned earlier, the LESCs continuously replace the corneal epithelium, but during the epithelial expansion and differentiation they lose their multipotency[59]; therefore, it is important to distinguish between niche cells, LESCs, TAMs and CECs within the cell culture used for therapy. One of the key features in cell therapy products is the amount of LESCs or cells which are capable of keeping their stemness after being transplanted.

In the maintenance of stemness, the SRY-related HMG-box family member SOX is important. In our LESC cultures, SOX9 expression was up-regulated, while SOX6 was down-regulated, indicating no chondrogenic differentiation but high proliferative

capacity of the LESCs. Similarly, Wnt2, Wnt6, Wnt11 and Wnt16b have been reported to be typically expressed in the limbal region and to be important for the LESCs proliferation[60]. We could confirm that the expression of *WNT1* and *WNT5A* was up-, while *WNT3A* was down- regulated in our LESCs, along the wider lines of the results mentioned above. The limbal epithelial crypt cells express S100A4 and A9 proteins[61]; our LESCs, the *S100A4* was down-regulated, indicating they are not crypt cells. We could not confirm a difference between the LESCs and differentiated CECs in the other known or putative LESC markers[62], such as *CXCL12*, *COL2A*, *ISL1*, *FOXA2*, *NCAM1*, *ACAN*, *GJB1* and *MSX1*. In our dataset, only *FOXA1* was up-regulated and *GJA1* was down-regulated, the latter also being known as negative marker for LESCs[63] (**Figure 5**). At protein level, the LESCs were different from the CECs in their higher positivity for CD146/MCAM, CD47 and CD117/c-kit, showing a pattern typical for stem cells and higher multipotency. The phenotype analysis demonstrated that the classical MSC markers, such as CD90/Thy-1 and CD73 are not good enough to differentiate between LESCs and CECs. The CAMs and integrins are important for attachment to ECM proteins, and their expression pattern is typical for the tissue of origin. Indeed, the integrin expression is able to define the cell phenotype and seems to be useful in classifying MSCs from various tissues besides the well-known MSC markers we have reported before[57]. The results of our gene array data analysis strengthen the fact that the LESCs cultured in medium containing human serum as the only growth supplement can keep their integrin and CAM pattern that relates them to their limbal tissue phenotype (**Figure 13**).

Not only the attachment, but the ECM breakdown is important in the initial steps of tissue healing and remodeling[64,65]. MMPs play a key role in this process, which were up-regulated in the LESCs, and their pattern implicates a preferred degradation of collagens to rebuild the ECM[64]. Along the upregulated MMP genes, the limbal basal membrane's laminin and vitronectin[66, 67] and their genes were also up-regulated in the LESCs (**Figure 8**). Wound healing can often lead into angiogenesis, which can have a very important and controllable pathological role in the limbus[41,65,68,69]. Fibronectin plays a key role in the *de novo* formation of new vessels, sprouting and differentiation of vessel wall resident and endothelial progenitor/stem cells into endothelial cells[70-72]. Beside the most upregulated fibronectin gene, several possible members of angiogenesis were also detected (**Figure 9**).

The two highest up-regulated gene products found in our LESCs seem to have an opposite effect on the angiogenesis pathway: IL-1β can induce-, while CXCL10 can inhibit the formation of new vessels[73-77]. Furthermore, CXCL10 has been found to be expressed by human limbal epithelial progenitor cells[78], while its absence decreased the level of IL-6 in mice corneas[79]. During wound healing, *TGFB1* is highly expressed and it can induce *VEGF* expression which was also up-regulated in the LESCs, capable of provoking angiogenesis in a damaged tissue[80,81]. Similar to the VEGF, ET-1 has many direct and indirect angiogenic effects - it stimulates endothelial cells and fibroblasts to produce pro-angiogenic proteases[82,83] (**Figures 7 and 9**). At gene and at protein level as well, we observed IL-6 and IL-8 secretion by the LESCs in culture (**Figure 12**). IL-6 and IL-8 play a role in inflammation, angiogenesis and MSC differentiation[84] (**Figures 10 and 11**). Our results indicate that both pro- and anti-angiogenic genes are expressed at the same time or in a balanced fashion in LESCs, maintaining an avascular state in the normal cornea. Loss of this control can be initiated by either a decreased production of anti-angiogenic molecules or increased production of pro-angiogenic and inflammatory factors. Although transplantation of LESCs has been known to suppress corneal inflammation and angiogenesis, the molecular mechanism how LESCs participate in the processes has not yet been fully understood[11,52,68,85,86]. Limbal niche cells have been found to have a differentiating ability towards angiogenic progenitors and inhibition of vascular endothelial differentiation of LESCs[85].

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

Neovascularization is a key mediator of corneal diseases, but it is also an important player in PDR. Many cell types have been shown to have a direct or indirect function in angiogenesis. In the retina, the EPCs have been described as key members of retinal neovascularization formation[87-89] as well as the pathology of DR[90-94]. Nevertheless, the real phenotype of the cells found in fvERMs and capable of growing out of the membranes is not clear; in particular, it is not known what type of cells initiate angiogenesis in PDR. The EPCs express hematopoietic stem cell- and monocyte- surface markers such as CD31, CD34, CD45, CD14 and VEGFR2. Our *ex*

vivo cultured fvERM and primary hRPE cells did not show an EPC - or retinal endothelial cell phenotype[95], although they expressed CD14, CD31 and CD34 to certain levels. Interestingly, the surface marker expression pattern of the primary hRPE cells appeared to be the closest to an EPC phenotype[96], although no CD45 positivity could be detected on their surface, meaning no haematopoietic origin of these cells[97]. The CD117/c-kit marker is characteristic of retinal progenitor cells[98-100] and angiogenic cells[89,101], which to a certain extent was the case with our primary hRPE, but it was absent from the fvERM outgrowing cells (**Figure 14**).

CD73, CD90 and CD105 are known as putative MSC (fibroblast-like) markers[57]; while CD73 and CD90 were present on both cell types, CD105 did not reach a sufficient expression level to qualify the cells as MSCs. CD73 has been described as a retinal photoreceptor progenitor marker in mice[102-104] and found on human RPE cells as well[105]. ABCG2, often referred as a stem cells- and retinal progenitor cells marker, was expressed at a low level on the fvERM outgrowing cells (<1%), while it was much higher on the primary hRPE cells (17.63$\pm$15.09%)[106]. The immune status of the cells is also very important - no HLA-G expression of this immunosuppressive marker could be detected on both cell types, while HLA-DR expression was very low on these cells[107-110] (**Figure 14**).

CAMs and integrins play a key role in the cell-cell and cell-ECM contacts during immune response. In PDR, the cellular and soluble forms of ICAM-1 have been frequently shown in epiretinal membranes, serum or vitreous[111-114] and diabetic fibrovascular membranes[115] of patients. Our fvERM outgrowing cells increased the ICAM-1 expression upon TNFα pro-inflammatory stimulus, similar to the primary hRPE cells[97,116-119], meaning that these cells function as activated epithelial cells for leukocyte adhesion[120,121]. CD11a (LFA-1) interacts to ICAM-1 and plays a key role in lymphocyte co-stimulation. No CD11a (LFA-1) positivity was observed in both cell types under basal conditions. Previously, CD29/ integrin β1[122], CD51/integrin αV and CD44/HCAM have been described to be present on the surface of hRPE cells[120,123,124], but we detected more positivity for integrin α1 and integrin α2 in the primary hRPE cell cultures compared to the studies published to date[123]. The integrin pattern of our cells differed in their CD18/integrin β2 and CD51/integrin αV expression. The fvERM outgrowing cells expressed low levels of the α-subunit-containing integrins, besides a reported expression of integrin α4 in DR[123,125]. Interestingly, the presence of integrin β2 subunit has been considered an important factor in RPE-T cell interactions[126] (**Figure 14**).

The CD146 expression on fvERM outgrowing- or primary hRPE cells was described the first time by our group. The cells expressed low levels of CD146 of this generally considered MSC marker[57], but also a novel endothelial biomarker, which plays an essential role in the angiogenesis by interacting directly with VEGFR2 found on endothelial cells[96]. In addition, CD146 has been accepted as a marker of a new EPC subset as well[97]. Although no data exists about positivity of fvERM outgrowing- and primary hRPE cells for CD166, cancer-, stem- and retinal endothelial- cells have been shown to express it[94].

The PDGF receptor and its subunits have already been detected on RPE cells[127,128] and investigated in ERMs of patients with proliferative vitreoretinopathy (PVR)[129]. The PDGFRβ subunit has been found to be less frequent than the PDGFRα subunit[129,130]. The PDGF produced by hRPE cells can act as autocrine growth factor[131] and influence wound healing and migration of these cells towards wounds; the interaction of the ligand with its receptor seems to be exaggerated during wound repair and, therefore, ERM formation. Indeed, cells isolated from ERMs removed during vitrectomy for PVR show expression of PDGF receptor and are usually identified as RPE cells[95] (**Figure 14**).

The presence of TNFα changed the CXCR4 expression in fvERM outgrowing cells, similar to how it acts upon HUVEC or Langerhans cells[132]. IL-1β or TNF-α can increase the CXCR4 mRNA expression in RPE cells[132], which indicates that fvERM outgrowing cells are not the migrating cells in DR in response to increased local SDF1α[94], nor that cell migration occurs via the well-known SDF1α-CXCR4 axis (**Figure 16**).

VEGFR2 plays a crucial role in endothelial differentiation of progenitors mediated by pro-angiogenic or inflammatory signals. We investigated the possible expression of VEGFR2 on fvERM outgrowing cells upon stimulation with TNFα and found no expression of this receptor under such treatment (**Figure 16**). Despite this finding, cells that are not directly involved in the angiogenesis can secrete pro- or anti-angiogenic factors during cell differentiation to either support- or inhibit- vessel formation in their microenvironment, respectively. In PDR the presence of TNFα in the vitreous is an important marker of the disease[113,133].

In vitro, the fvERM cells secreted MCP-1/CCL-2 and IL-8/CXCL-8 upon TNFα treatment - phenomenon which has been observed in ARPE -19 cells and primary hRPE cells also. Besides TNFα, IL-1β and co-culturing with T lymphocytes have all been shown to induce IL-6, IL-8 and MCP-1 secretion in ARPE-19 cells[134-136]; in addition,

TLR3 ligands have been shown to have the same effect on hRPE cells[119]. MCP-1 is chemokine for monocytes and can induce apoptosis in hRPE cells[137]. Furthermore, MCP-1 has been detected in the aqueous humor of patients with diabetic retinopathy[138] and its level was higher in a rodent model of this disease[139]. MMPs play a key role in the first stage of cell migration, connective tissue remodeling and degradation of basal lamina and surrounding extracellular matrix (ECM) during neovascularization[140]. MMP-9 appeared, while TIMP-1 levels increased upon TNFα treatment of fvERM outgrowing cells. Although the MMP-9 level has been previously determined in PDR[141-143], nothing is known about its level in healthy retina[142]. TIMP-1 and TIMP-2 have both been measured in the vitreous of patients with PDR[144] - increased levels of TIMP-1 have been associated with the disease[142,144-146]. The fvERM outgrowing cells expressed and secreted a basal level of TIMP-1, probably serving as a counter-coup to angiogenesis via inhibiting MMPs[147] (**Figure 15**).

PTX3 has many immunological activities in innate immunity, mainly in the inhibition of the uptake of apoptotic cells by antigen presenting cells (APCs)[148,149]. TNFα could induce PTX3 in ARPE-19 cells[150], but no such data have been shown in primary hRPE and fvERM outgrowing cells. EG-VEGF/PK-1 is a novel selective angiogenic mitogen with a special pro-survival effect in different cell types[151], including endothelial cells, dendritic cells (DCs), monocytes and neutrophils; no reports have been made about its presence in primary hRPE and fvERM outgrowing cells. Interestingly, PK1 and PK2 have been shown to induce monocyte differentiation and activation, suggesting an immunomodulatory function at local sites of inflammation[152,153] (**Figure 15**).

Hypoxic conditions stimulate Serpin E1/PAI-1 by hRPE[154]. Serpin E1/PAI-1 is a principal inhibitor of tissue plasminogen activator (tPA) and urokinase. PAI-1 could facilitate retinal angiogenesis in a model of oxygen-induced retinopathy[155]. Serpin F1/PEDF plays a very important anti-angiogenic and anti-oxidant role in the eye[156]. It has been found in the vitreous[157] and serum[158] of patients with PDR (**Figure 15**).

IGFBPs have been detected in the vitreous of patients suffering from neovascular ocular diseases[159]. In particular, IGFBP-2 and IGFBP-3 appear to be potent inhibitors of IGF-I and IGF-II activity in Muller cells[160] and inhibitors of cell growth of retinal endothelial cells[161]. Overexpression of ET-1 can lead to retinal edema, degeneration and neuronal death in animal models[162,163]. Dipeptidyl peptidase-4 (DPP4) is an important player in glucose metabolism, but it has also been described for its role in immune regulation and apoptosis. Thrombospondin-1 (TSP-1) is a natural inhibitor

of angiogenesis. High levels of TSP-1 have been detected in drusen found in early stage age-related macular degeneration[164]. The existence of TSP-1 in the vitreous has been controversial and sometimes reported as present[165] or absent[166] in PDR patients (**Figure 15**).

So far, the origin of the cells found in the fvERMs has not been well understood. There is also lack of knowledge regarding the fvERMs cells specific features, including the Ca^{2+}-signaling pathways. The knowledge of Ca^{2+} dynamics is important toward understanding cell biology. Mechanical force modulates a wide array of cell physiological processes. Cells from diverse tissues detect mechanical load signals by similar mechanisms, but respond differently. The diversity of responses reflects the genotype of the cell and the mechanical demands of the resident tissue. It has been shown that hyperglycemia alters the tight control of $[Ca2^{+}]_i$ dynamics in retinal cells and may lead to the development of DR[167]. Calcium signaling upon mechanical stimulation has been previously demonstrated in human corneal endothelial-[168], RPE-cells[169], rat retinal astrocytes and Müller cells[170]. Mechanosensory function of Müller cells in the retina upon application of tractional force to the living retina showed that Müller cells respond to retinal stretch both with fast changes, as evidenced by transient $[Ca2^{+}]_i$ increases, and with slower changes in protein expression[171]. A model for Ca^{2+} waves in networks of glial cells has been studied[172] which confirms the importance of Ca^{2+} signaling measurements. It has also been shown that calcium signaling mediates mechanically induced human MSC proliferation[173]. The calcium signaling has been also recognized as a regulator of hematopoiesis[174] - the application of shear stress to hematopoietic progenitor cells has been shown to stimulate hematopoiesis[175]. Store-operated Ca^{2+} entry has been shown to be expressed in human endothelial progenitor cells[176]. $[Ca^{2+}]_i$ stores are essential for injury-induced Ca^{2+} signaling and re-endothelialization[177] (**Figure 17**).

An unwanted consequence of the appearance of fibrovascular membranes in PDR is the tractional force these membranes can impose upon the retina, which can eventually lead to retinal detachment. Mechanical stimulation of the cells in the membranes or the retina can result in $[Ca^{2+}]_i$ changes that can propagate through the cells as intercellular waves. Generation of such calcium waves occurs by the release of calcium from internal stores. The waves do not evoke changes in the cell membrane potential, but may constitute a pathway for extraneuronal signaling[178]. Along that line, isolated retinal ganglion cells can respond directly to mechanical deformation with pannexin-mediated ATP release and auto-stimulation of the $P2X_7$ receptors[179].

Furthermore, stimulation of RPE cells with mechanical stress can upregulate MMP-2 and fibronectin (FN) expression through activation of the p38 pathway[180].

Overall, we could isolate and cultivate cells from fvERMs obtained from patients with PDR and show their proliferative potential, phenotype, pro-angiogenic activity and response to mechano-stimulation. Our results suggest that these cells play a role in the pathomechanism of abnormal neovascularization as being activated by pro-inflammatory stimuli.

7. SUMMARY

The human eye as an organ possesses great potential for regeneration and cell therapy, in particular, its corneal surface which contains LESCs. Identifying molecular markers and upstream regulators in the LESCs using genome-wide microarray transcriptional profiling, as well as verification of those at protein expression level can provide a better identification and more specific understanding of the signaling molecules associated with these cells, therefore, better application in ocular surface disease therapy. Although the eye is composed of many cell types with stem cell-like properties, altered cellular environments or disease states can tip the balance towards pro-inflammatory and fibrovascular processes as is the case with PDR. The surface marker phenotype of the cells obtained from human fvERMs in PDR has not been fully characterized nor the role these cells play in immunity and angiogenesis.

Genome-wide microarray transcriptional profiling compared the LESCs to differentiated human CECs. More than 2 fold increase and decrease in expression could be found in 1830 genes between the two cell types. A number of molecules functioning in cellular movement (381), proliferation (567), development (552), death and survival (520), and cell-to-cell signaling (290) were detected having top biological functions in LESCs and several of these were confirmed by flow cytometry surface protein analysis. Custom-selected gene groups related to stemness, differentiation, cell adhesion, cytokines and growth factors as well as angiogenesis could be analyzed. The results show that LESCs play a key role not only in epithelial differentiation and tissue repair, but also in controlling angiogenesis and extracellular matrix integrity. Some pro-inflammatory cytokines were found to be important in stemness-, differentiation- and angiogenesis-related biological functions: IL-6 and IL-8 participated in most of these biological pathways as validated by their secretion from LESC cultures.

The cultivated fvERMs formed proliferating cell monolayers when cultivated *ex vivo*. These cells were negative for some endothelial cell markers (CD31, VEGFR2), partially positive for hematopoietic- (CD34, CD47) and mesenchymal stem cell markers (CD73, CD90/Thy-1, PDGFRβ) and negative for CD105. The CD146/MCAM and CD166/ALCAM, which have not been previously reported in cells from fvERMs, showed low and high expression levels, respectively. Secretion of 11 angiogenesis-related factors (DPPIV/CD26, EG-VEGF/PK1, ET-1, IGFBP- 2 and 3, IL-8/CXCL8, MCP-1/CCL2, MMP-9, PTX3/TSG-14, Serpin E1/PAI-1, Serpin F1/PEDF, TIMP-1

and TSP-1) were detected upon activation of fvERM cells by TNFα. Mechano-stimulation of the outgrowing cells induced intracellular calcium propagation representing their functional viability and the role it can have in fvERM-related tractional retinal detachment.

Overall, new biological functions or signaling-related pathways were identified in human LESCs which play a crucial role in cellular adhesion and migration, epithelial differentiation and tissue repair, as well as angiogenesis and extracellular matrix integrity. A method for isolating and cultivating cells coming from fvERMs from PDR was established and characterization of their origin as mixed or not pure hematopoietic, mesenchymal or epithelial was achieved. The fvERM outgrowing cells had a clear proliferative potential, were capable of releasing pro-inflammatory and angiogenic molecules upon stimulation with TNFα and showed functionality and viability reflected through calcium dynamics upon mechano-stimulation, which can serve as a study model for tractional forces present in fvERMs in PDR *ex vivo*.

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10. APPENDIX

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