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Kultivace limbálních a mezenchymálních buněk na různých typech nosičů pro využití v oftalmologii

The culture of limbal and mesenchymal cells on various feeders for their use in ophthalmology

Ph.D. Thesis

Supervisor: doc. Mgr. Kateřina Jirsová, Ph.D.

Prague, 2019

DECLARATION

I, Peter Trošan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. I also confirm that the work presented hasn't been used to achieve another or the same university degree. On the other hand I would like to emphasized, that I am co-author of two herein mentioned papers (paper 4 and 5), which are going to be used in PhD. Thesis of my colleague from our laboratory, Ingrida Šmeringaiová, MSc.

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Abstrakt

Deficience limbálních kmenových buněk (LSCD) je onemocnění charakterizované poruchou, nedostatkem či absencí limbálních kmenových buněk zodpovídajících za homeostázu a obnovu rohovkového epitelu. Důsledkem onemocnění je zánět na povrchu oka, neovaskularizace a ztráta transparence rohovky, což může vézt až ke ztrátě vidění. Prakticky jedinou formou léčby je transplantace limbální tkáně nebo kultivovaných limbálních epitelových buněk (LECs) na poškozený povrch oka. Lidská amniová membrána (HAM) je přitom využívaná jako nosič pro kultivaci LECs a uplatňuje se i v léčbě povrchu oka včetně LSCD. Předkládaná dizertační práce se zabývá buněčnou terapií LSCD, zejména inovací kultivačních podmínek pro limbální epitelové buňky a přípravou vhodného nosiče pro přenos buněk na poškozenou rohovku. V průběhu studia jsem použil široké spektrum metod, např. kultivaci různých typů buněk (LECs, mezenchymálních kmenových, amniových epitelových, spojivkových epitelových, pohárkových a 3T3 buněk), imunohistochemii a imunocytochemii, mikroskopii, proliferační testy a testy tvorby kolonií, reverzní transkripční a kvantitativní real-time PCR a statistickou analýzu.

Během studia jsem se podílel na přípravě protokolu pro zlepšení kvality kultivace limbálních epitelových buněk bez použití xenogenních médií. Tuto metodu plánujeme využít pro přípravu buněk pro transplantace v léčbě LSCD, jelikož tento typ léčby v České republice není dosud zaveden. Dále jsme z explantátu korneosklerálního terče připravili kulturu spojivkových epitelových a pohárkových buněk pro využití v léčbě poškozené spojivky. Pomocí inzulínu-podobnému růstového faktoru-1 jsme dokázali zvýšit diferenciaci myších mezenchymálních kmenových buněk na buňky podobné rohovkovým epitelovým buňkám s možným využitím při bilaterální léčbě LSCD. Pro standardizaci přípravy HAM jsme zavedli přípravu laboratorního dekontaminačního roztoku jako možnou náhradu za komerčně vyráběný produkt a tuto metodu začali používat při přípravě amniové membrány pro oční transplantace a léčbu dlouhodobě se nehojících ran. Dále jsme vyvinuli deepitelizaci HAM, která vede ke vzniku HAM použitelné pro kultivaci buněk, ale i vitálního epitelu, který může být dále využit metodami tkáňového inženýrství.

Klíčová slova: rohovka, limbální kmenové buňky, spojivka, mezenchymální kmenové buňky, diferenciace, amniová membrána

Abstract

Limbal stem cell deficiency (LSCD) is a disease characterized by the deficiency of stem cells in the limbus, which are responsible for the homeostasis and renewal of the corneal epithelium. This disorder results in corneal neovascularization, chronical inflammation and opacification, which may lead to loss of vision. The most successful treatment is the transplantation of limbal tissue or cultured limbal epithelial cells (LECs) onto the damaged ocular surface. The human amniotic membrane (HAM) is used as the feeder of the LECs culture, as well as for the LSCD treatment. HAM is also widely used in clinical practice, particularly for the treatment of chronic wounds.

This dissertation is particularly concerned on cell therapy for LSCD, on preparation of cells suitable for grafting onto the ocular surface, on the improvement of the LECs culture conditions, and on the preparation of appropriate carrier for the transfer of cells onto the damaged cornea. During my work I have used a wide spectrum of methods, e.g. cell cultures (LECs, mesenchymal stem, amniotic epithelial, conjunctival epithelial, goblet and 3T3 cells), immunohisto- and immunocytochemistry, microscopy, proliferation and colony forming assays, reverse transcription and quantitative real-time PCRs and statistical analysis.

For the improvement of the safety of LECs culture we prepared a protocol with the use of xeno-free media. This method could be used in transplantations for LSCD therapy, as this treatment is still missing in the Czech Republic. Moreover, we prepared the culture of conjunctival epithelial and goblet cells from corneoscleral rims for the treatment of conjunctival defects. We also demonstrated enhanced differentiation of murine mesenchymal stem cells into corneal-like cells by using of insulin-like growth factor-I with the corneal extract, as a possible future treatment of bilateral LSCD.

We established a new laboratory decontamination solution for the HAM decontamination as a possible substitution of a commercially available reagent. This procedure is already in use for the preparation of HAM for ocular transplantations and for the treatment of nonhealing skin wounds. We also prepared undamaged de-epithelialized HAM and viable amniotic epithelial cells by removing of the epithelial layer from the HAM after trypsin/EDTA treatment. The denuded HAM can be used for cell culture and epithelial cells stay viable for other methods of tissue engineering.

Key words: cornea, limbal stem cells, conjunctiva, mesenchymal stem cells, differentiation, amniotic membrane

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Abbreviations

ABCB5	ATP-binding cassette, sub-family B, member 5
ABCG2	ATP-binding cassette transporter group 2
AECs	apoptotic epithelial cells
AMCs	apoptotic mesenchymal cells
BM	basement membrane
CFE	colony forming efficiency
CLET	cultivated limbal epithelial cell transplantation
COM	complex medium
DECs	dead epithelial cells
DMEM	Dulbecco's Modified Eagle Medium
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FBS	fetal bovine serum
FGF-β	fibroblast growth factor-β
GCs	goblet cells
hAECs	human amniotic epithelial cells
HAM	human amniotic membrane
hAMSCs	human amniotic mesenchymal stromal cells
HGF	hepatocyte growth factor
HLA	human leucocyte antigen
hPLM	human platelet lysate medium
hSM	human serum medium
IFN-γ	interferon- γ
IGF-I	insulin-like growth factor-I
IL	interleukin
K	keratin
KGF	keratocyte growth factor
KLF4	Kruppel-like factor 4
LDS	laboratory decontamination solution
LECs	limbal epithelial cells
LESCs	limbal epithelial stem cells

LSCD	limbal stem cell deficiency
MSCs	mesenchymal stem cells
MUC	mucin
NANOG	nanog homeobox
OCT-4	octamer-4
P	passage
PCR	polymerase chain reaction
SEM	scanning electron microscopy
SLET	simple limbal epithelial transplantation
SOX2	sex determining region Y-box 2
TACs	transient amplifying cells
Tap63	transactivated isoform of *TP63* gene
TGF	transforming growth factor
TP63 (p63)	tumor protein p63
ΔNp63	ΔN isoform of *TP63* gene

1. Foreword

Approximately 1.3 billion people live with some form of vision impairment, more than 36 million of them are blind by the data of the World Health Organization (Bourne et al., 2017). The corneal diseases are the 4th most common cause of blindness worldwide. The corneal damage can be caused mainly by immune or inherited disorders, mechanical injury, chemical or thermal burn. If the corneal damage is more extensive and involves the limbus, the defect can lead to limbal stem cell deficiency (LSCD). In such cases, the cornea cannot heal properly because of the absence of limbal epithelial stem cells (LESCs). Transplantations of limbal tissue or LESCs are the only effective ways to treat this disease. LESCs can be isolated from the healthy eye, expanded *in vitro* and applied on damaged area. The various factors as the isolation of cells, the composition of convenient culture media and culture substrates, criteria for the quality control or surgical procedures directly influences the quality of the LESCs transplantation and must be further explored and improved. Therefore, the main aim of my study was to test various conditions for culture of LESCs and compare the efficiency and maintenance of stemness or differentiation of cells.

The damage of the cornea is often connected with the conjunctival damage. Conjunctiva and tear film participate on maintaining homeostasis of the ocular surface. For the successful conjunctival reconstruction, it is important to expand both conjunctival cell types, epithelial and goblet cells (GCs). The preparation of conjunctival epithelium with both cell types from human corneoscleral rims was the second aim of my dissertation.

The transplantation of LESCs has its limitation in a low number of LESCs in the limbus and in the harmful immunological rejection if the cells from genetically unrelated donor are transplanted. Therefore, other sources of autologous stem cells, e.g. mesenchymal stem cells (MSCs), are explored to treat LSCD. MSCs have the powerful immunomodulatory effects and are able to differentiate into various cell types. The differentiation of MSCs into corneal cells and their future use for transplantation purposes was another aim of the dissertation. Mouse experimental model was chosen by reason of easy obtainability of MSCs and standardized laboratory model of damaged cornea.

The appropriate carrier is necessary for the transfer of the LESCs on the ocular surface. The human amniotic membrane (HAM), obtained from placenta after Caesarean delivery, is the most frequently used scaffold in ophthalmology. One of the important steps in HAM preparation is its decontamination. As only a limited number of certified tissue

decontamination solutions are commercially available, we wanted to compare their effectivity with laboratory decontamination solution (LDS).

The wound healing effect of HAM is the main reason for its use in ophthalmic surgery. It is broadly discussed whether intact or denuded (without the epithelial cells) HAM is more suitable for LESCs culture. Moreover, human amniotic epithelial cells (hAECs) have multipotent differentiation ability and express the stemness markers, characteristic for the embryonic stem cells. Both, the denuded HAM and hAECs, can be therefore used separately for various purposes and research. Several protocols were published, but they were focused only on gaining of cells or HAM. The requirement of method for obtaining both aims in one step leads us to the study of comparison of various enzymatic technics for de-epithelialization of HAM.

2. Introduction

2.1. Cornea

The eye is a primary sense and very complex organ. It comprises from three major tissues of different embryonic origins: the lens and the cornea are derived from the surface ectoderm and the retina from anterior neural plate (Graw, 2010). The cornea is a transparent avascular tissue that is exposed to the outer environment and protects the eye against infections and mechanical damage. The anterior corneal surface is covered by the tear film and the posterior surface is in contact with the aqueous humor. The parameters of the adult human cornea are 11-12mm horizontally and 9-11mm vertically. It is approximately 515µm thick at the central part (Rapuano et al., 1993) and it constitutes two-thirds of the refractive power of the eye (40-44D).

Cornea consists of five layers: the outermost epithelium with basement membrane, Bowman´s layer, stroma, Descemet´s membrane and the inner endothelium (Figure 1) and it is formed by three main cell types: epithelial cells, keratocytes and endothelial cells.

2.1.1. Epithelium

The corneal epithelium is a physical barrier to the outside environment. It is composed of five to six nonkeratinized, stratified squamous cell layers and it is approximately 50µm thick (Reinstein et al., 2008). The epithelium consists of superficial cells, wing cells and basal cells, with the lifespan of 7 to 10 days (Hanna et al., 1961). The epithelium with the tear film contributes to the maintenance of the smooth corneal surface.

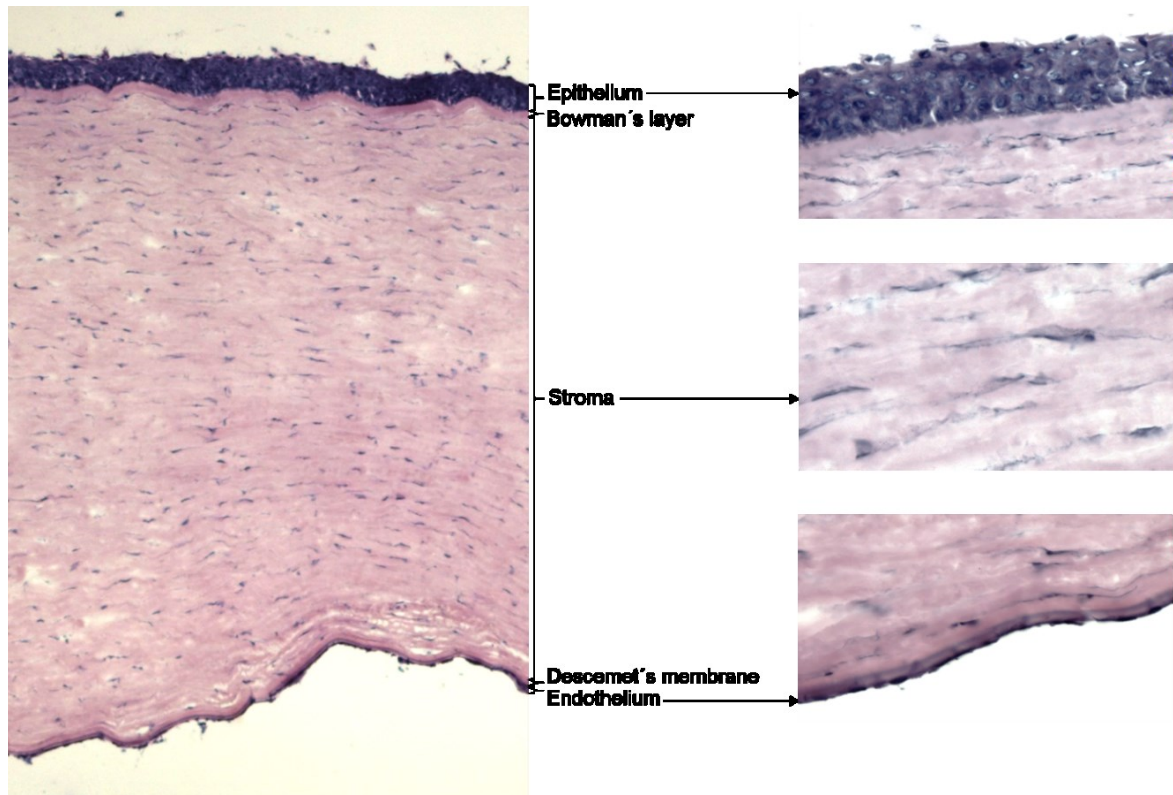

Figure 1: Anatomy and histology of the human cornea stained with hematoxylin and eosin (laboratory archive).

The most superficial cells of corneal epithelium form 2-3 layers of terminally differentiated cells (Figure 2). They are flat and exhibit polygonal morphology with the diameter of 40 to 60µm and thickness of 2 to 6µm. Their surface is covered with microvilli to increase cell surface area and for closer association with the tear film (Pfister, 1973). The superficial cells are joined by desmosomes and tight junction complexes, which prohibit tears from entering the intracellular spaces. Between superficial and basal cells, 2-3 layers of wing cells are located. They are less flat than underlying superficial cells. Basal layer is formed by a single layer of cuboidal or columnar cells approximately 20µm tall. Only stem cells, transient amplifying cells (TACs) and basal cells possess mitotic activity in corneal epithelium (Wiley et al., 1991). Wing and superficial cells are differentiated from basal cells and migrate to the anterior of the cornea, squamous cells then age and slough off into the tear film. Basal cells are strongly attached to the basement membrane by hemidesmosomal system to preserve the epithelium from separating from other corneal layers. Basal cells secrete type VII collagen and via connection of hemidesmosomes to the anchoring fibrils of type VII collagen, which penetrates through the basement membrane to the stroma, the adhesion of basal cells to the basement membrane and stroma is maintained

(Bentz et al., 1983; Gipson et al., 1987). The integrin subunits α5, α6 and β4 are localized specifically to the basal membrane of the basal cells (Stepp et al., 1993).

The epithelial basement membrane is 40 to 60nm thick and major components are type IV collagen and laminin, both produced by basal cells. Their functions are to anchor epithelial cells to the stroma and to provide scaffolding during embryonic development, migration, differentiation, and maintenance of the differentiated epithelial phenotype (Torricelli et al., 2013).

2.1.2. Bowman´s layer

Bowman´s layer is an acellular condensate of collagen and proteoglycans between corneal epithelium and stroma. In humans, it is about 12µm thick and it becomes thinner with aging. Bowman´s layer is well developed in higher mammals; lower mammals have a thinner layer (Hayashi et al., 2002). The exception is a rabbit which does not have Bowman´s layer in the adult (Cintron et al., 1983). Major components of Bowman´s layer are collagens types I, III and V (Jacobsen et al., 1984; Marshall et al., 1991a, b; Zimmermann et al., 1988). However, biological function of Bowman´s layer is still unclear. One of the speculations is that it acts as a corneal ligament to maintain corneal structure. On the other hand, many mammals do not have Bowman´s layer and still exhibit a well-organized epithelial structure. Other speculation suggests that this layer acts as a barrier to inhibit extension of viruses that can infect the corneal epithelium. Bowman´s layer is a visible indicator of ongoing negative chemotactic effects of the epithelium on the keratocytes. The layer does not regenerate after an injury and as consequence of damage it can results in a scar.

2.1.3. Stroma

The corneal stroma consists of extracellular matrix, keratocytes and nerve fibres. It comprises 80-85% of the corneal thickness. The biochemical properties of the stroma are responsible for the transparency, stability in shape and physical strength of the cornea. The transparency of the stroma is due to a precise organisation of the stromal fibres and extracellular matrix (Boote et al., 2003; Maurice, 1970). The collagen fibres are self-assembled into parallel bundles – fibrils, and these fibrils are packed in parallel layers – lamellae. The fibrils of each lamella are parallel with one another, but at right angles to those of adjacent lamellae (Maurice, 1970). Human corneal stroma contains 200 to 250 flattened lamellae (Maurice, 1970) up to 0,2mm broad and 2µm thick (Komai and Ushiki,

1991; Polack, 1961). The peripheral stroma is thicker than the central stroma and the packing density is higher in the anterior part than in the posterior lamellae (Bergmanson et al., 2005).

The extracellular matrix of the corneal stroma consists of collagens, proteoglycans and glycosaminoglycans. The collagen fibrils are made of a heterodimeric complex of type I and type V collagens (Fini and Stramer, 2005). Proteoglycans are made of a small protein core to which one or more glycosaminoglycan chains are attached. The glycosaminoglycans constitute of chondroitin sulphate/dermatan sulphate and keratan sulphate, which can absorb and retain large amounts of water and regulate hydration.

Keratocytes, quiescent mesenchymal cells, are a major cell type of the stroma. They are involved in maintaining the extracellular matrix environment and stromal homeostasis by synthesizing collagens, glycosaminoglycans and matrix metalloproteinases. The keratocytes are not terminally differentiated in the central stroma. They remain in a G0 stage and consequently they can proliferate in case of corneal wound healing (Cvekl and Tamm, 2004).

2.1.4. Descemet´s membrane

Descemet´s membrane is the basement membrane secreted by endothelial cells. Development of the membrane begins in utero at the 8-week stage. It is composed by type IV and type VIII collagens (Nakayasu et al., 1986; Tamura et al., 1991) and laminin (Fitch et al., 1990). Its thickness ranges from 3μm at birth to 8-10μm in adults (Johnson et al., 1982). In case of Descemet´s membrane rupture, the membrane does not regenerate, and the aqueous humour penetrates to the corneal stroma (stromal oedema).

2.1.5. Endothelium

The corneal endothelium is a single layer of cells located on the posterior side of the cornea. The cells are polygonal in shape and about 75% of them are hexagonal. The endothelial monolayer is 10μm thick at birth with the cell density 3500 cells/mm^2 (Watsky et al., 1989). Corneal endothelial cells have no mitotic activity in humans, monkeys and cats, but they proliferate in rabbits. The cells are arrested in the G1 cell stage (Zieske, 2004). In humans, the cell density gradually decreases throughout life approximately 0.6% per year (Bourne et al., 1997). With the decreased number of cells, remaining endothelial cells enlarge themselves and cover the denuded Descemet´s membrane. There is a risk of corneal oedema if the cell density decreases below 500 cells/mm^2.

The endothelial cells are metabolically and secretory active. Their major function is regulation of the water content in the corneal stroma through ion transport systems. The maintaining of the dehydrated state of the cornea is required for the optical transparency. Na+ K+-ATPase pumps are present on the lateral side of the endothelial cells (Stiemke et al., 1991), the basal surface contains numerous hemidesmosomes for adhesion to Descemet´s membrane.

2.2. Limbal epithelial stem cells

The central corneal epithelium undergoes continuous renewal throughout life but has very limited capacity to renew by itself (Ebato et al., 1988). Conjunctival epithelium was considered as the possible source of corneal epithelial cells for many years (Friedenwald, 1951; Shapiro et al., 1981; Thoft and Friend, 1977). As the first, Davanger and Evensen speculated that epithelial cells renewal depended on the limbus (Davanger and Evensen, 1971). Next experiments confirmed that corneal epithelial stem cells, called limbal epithelial stem cells, reside in the basal layer of the limbus, the transition zone between cornea and conjunctiva (Cotsarelis et al., 1989; Schermer et al., 1986). The limbus provides them a unique environment with rich and complex morphology, dense innervations and vascularization (Lawrenson and Ruskell, 1991; Meyer, 1989; Papas, 2003; Van Buskirk, 1989). LESCs are located in the region with stromal invagination known as Palisades of Vogt (Goldberg and Bron, 1982). The structure of the palisades is unique and can vary over time with age or as a response to surgery or disorders (Fatima et al., 2008; Hatch and Dana, 2009; Zheng and Xu, 2008). The palisades contain vascularized limbal epithelial crypts, that provides the characteristics that correspond to the stem cell niche (Dua et al., 2005; Shortt et al., 2007a). The cells are protected in the crypts from UV rays by melanocytes. Moreover, external protection is ensured by eyelids. The limbus also acts as a barrier which prevents overgrowth of conjunctival cells and growth of blood vessels to the corneal epithelium (Dua and Azuara-Blanco, 2000). It is widest in superior and inferior cornea, where the most of LESCs has been found (Goldberg and Bron, 1982).

The LESCs are slow cycling during homeostasis, but in case of injury they become highly proliferative (Cotsarelis et al., 1989; Lehrer et al., 1998). They have an unlimited proliferative capacity and divide asymmetrically to self-renew and to produce TACs (Castro-Munozledo and Gomez-Flores, 2011). TACs migrate centripetally through the basal epithelium and undergo a limited number of divisions on the central cornea. After that TACs migrate superficially and give rise to post-mitotic suprabasal wing cells that

subsequently differentiate into terminally differentiated superficial squamous cells, which form the corneal epithelial layer (Tseng, 1989). The superficial cells are lost from the surface by exfoliation (squamification). The corneal epithelial mass is constant under the normal circumstances and therefore the rate of cellular entry due to centripetal motion and mitosis into any epithelial volume must equal the cell loss from the same volume (Sharma and Coles, 1989). Thoft and Friend proposed the XYZ hypothesis of corneal epithelial maintenance. This theory proposes that the anterior migration of basal cells (X) and centripetal migration of cells from limbus (Y) is equal to epithelial cell loss from the corneal surface (Z) (Figure 2) (Thoft and Friend, 1983).

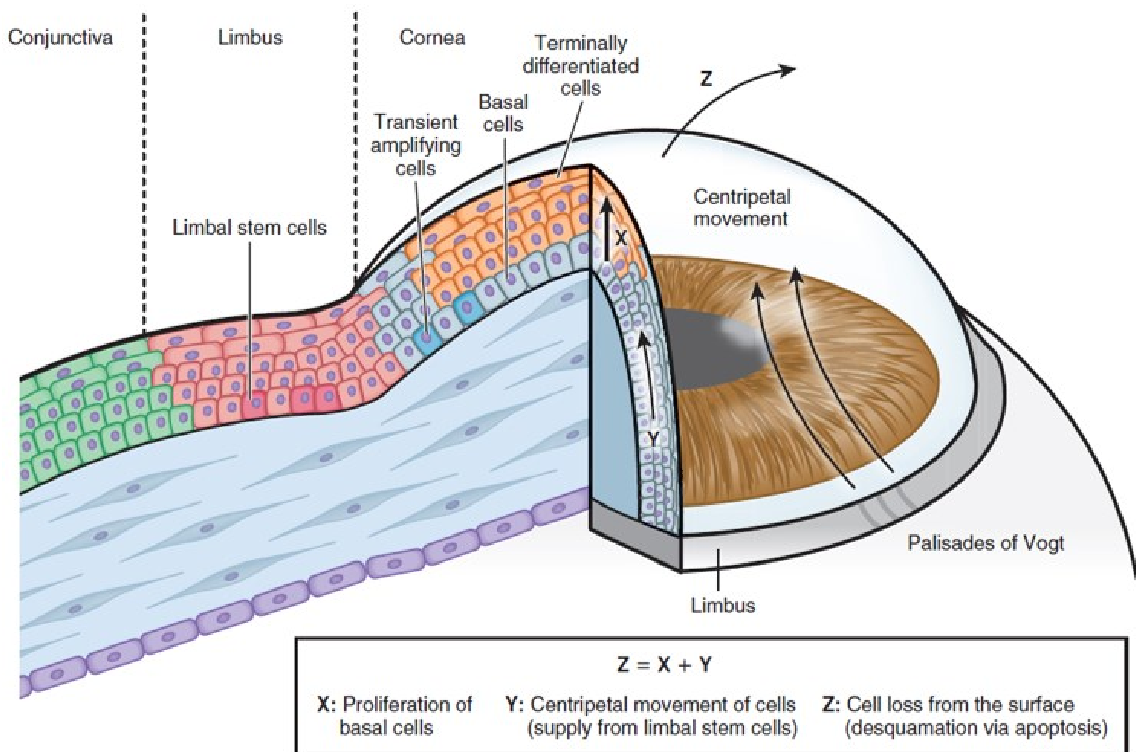

Figure 2: Lineage of corneal epithelial cells (Cornea, 4th edition, 2017, Elsevier Inc.)

Cotsarelis et al. postulated that only up to 10% of limbal basal cells are stem cells (Cotsarelis et al., 1989). The characteristics of LESCs include small size, high nuclear to cytoplasmic ratio, clonogenicity and absence of differentiation markers expression coupled with positive expression of progenitor markers (Barrandon and Green, 1987; Kurpakus et al., 1990; Pellegrini et al., 2001; Romano et al., 2003; Schermer et al., 1986). A single specific marker for identifying LESCs has not yet been discovered, therefore the expression of panel of putative stem cells markers is used. The positive expression of tumor protein p63 gene (*TP63*, also known as *p63*) is considered as the main LESCs

marker, especially one particular isoform Δ*Np63α* (Pellegrini et al., 2001). The *TP63* gene generates 6 isoforms: the transactivated isoforms (TAp63) and the ΔN isoforms (ΔNp63). Both transcripts have three splice variants α, β and μ based on different C termini (Yang and McKeon, 2000). In normal healthy corneal epithelium all isoforms of *TP63* gene are missing. The variant ΔNp63α was located in the resting stem cells and TACs in the basal epithelium and is responsible for the maintenance of the proliferative potential of LESCs and their ability to migrate to the cornea. The β and μ isoforms of *ΔNp63* gene play role in epithelial differentiation during corneal regeneration (Di Iorio et al., 2005).

Many types of stem cells exhibit a side population (SP) phenotype. These cells can pump out a DNA binding dye (Hoechst 33342) through the ATP-binding cassette transporter group 2 protein (ABCG2). The ABCG2 protein was immunolocalised in the cell membrane and cytoplasm of limbal basal cells (Chen et al., 2004). Only 1% of limbal epithelial cells expressed gene *ABCG2* and these cells were able to proliferate and differentiate from clones in culture (Budak et al., 2005). ABCG2 has therefore been proposed as a putative marker of LESCs.

Many other genes have been considered as LESCs markers based on their expression in the basal epithelia. The protein integrin α9 has been localised in the epithelium (Stepp et al., 1995), but experiments with wounded murine corneas indicated its association with TACs (Stepp and Zhu, 1997). N-cadherin, which is expressed in a subpopulation of limbal basal cells, is important in interactions between LESCs and cells in the niche (Hayashi et al., 2007). Epithelial cells contain various types of intermediate filaments. Keratin 19 (K19) was localised in the limbal basal epithelia and therefore suggested as marker for LESCs (Kasper et al., 1988; Lauweryns et al., 1993). Later, it was demonstrated that K19 was also expressed in the corneal and conjunctival epithelia (Chen et al., 2004; Ramirez-Miranda et al., 2011). Vimentin is localised in limbal cells as well, but is more specific for transitional cells between the cornea and limbus (Schlotzer-Schrehardt and Kruse, 2005). ATP-binding cassette, sub-family B, member 5 (ABCB5) was identified as novel marker for LESCs that is required for the maintenance of stemness and for corneal development and repair (Ksander et al., 2014).

Barbaro et al. reported that coexpression of CCAAT enhancer binding protein delta (C/EBPΔ), polycomb complex protein BMI-1, and ΔNp63α identified mitotically quiescent limbal stem cells, which generate holoclones in culture (Barbaro et al., 2007). On the other hand, limbal basal cells lack differentiation markers keratin 3 (K3), keratin 12 (K12) and

gap junction-mediated connexin 43 which are specific for the corneal epithelium (Chaloin-Dufau et al., 1990; Matic et al., 1997; Schermer et al., 1986).

The proliferative capacity and differentiation potential of human corneal epithelium can be evaluated by clonal analysis with colony forming cell assay. Isolated limbal cells and TACs generate holoclones and paraclones in culture under appropriate conditions (Barrandon and Green, 1987; Pellegrini et al., 1999; Rochat et al., 1994). Human holoclone-forming cells are located only in limbus (Majo et al., 2008; Pellegrini et al., 1999), and they are required to restore massive corneal epithelial defects permanently (De Luca et al., 2006; Green, 2008). Meroclones are formed by intermediate type of cells and give rise to paraclones. Paraclones have the properties of TACs and have limited number of division (Barrandon and Green, 1987; Pellegrini et al., 1999). The transcript variant *ΔNp63α* of LESCs marker gene *TP63* is highly expressed in holoclones, poor in meroclones and no expression is measured in paraclones derived from the limbus (Di Iorio et al., 2005). The successful rate of LESCs transplantation is associated with the percentage of p63-bright holoclone-forming stem cells in the culture. If more than 3% of p63 positive cells from total number of clonogenic cells are in the culture, the transplantation is successful in 78% of patients. On the contrary, only 11% of successful transplantation was documented when less than 3% of p63 positive cells were in the culture (Rama et al., 2010).

The balance between cell proliferation, differentiation, migration and apoptosis is necessary and is driven by the various cytokines and growth factors. Jia et al. demonstrated that genes for growth factors and cytokines are upregulated in murine corneal cells after the damage of the cornea (Jia et al., 2011), many of them already 3 to 6 hours after the injury (Figure 3) (Trosan et al., 2012). It was shown that epidermal growth factor (EGF) (Gospodarowicz et al., 1977; Savage and Cohen, 1973; Zieske et al., 2000), fibroblast growth factor-β (FGF-β) (Gospodarowicz et al., 1977; Rieck et al., 1992), hepatocyte growth factor (HGF) (Daniels et al., 2003; Wilson et al., 1993), keratinocyte growth factor (KGF) (Sotozono et al., 1995) and insulin-like growth factor-I (IGF-I) (Lee et al., 2006a; Yanai et al., 2006) are involved in proliferation and migration of corneal epithelial cells. HGF regulates apoptosis (Kakazu et al., 2004), while transforming growth factor-β1 (TGF-β1) and TGF-β2 can inhibit proliferation of corneal epithelial cells *in vitro* (Haber et al., 2003; Pancholi et al., 1998).

The differentiation potential of IGF-I on limbal stem cells in murine model has been reported. IGF-I, which is overexpressed in the corneal tissue after injury, migrates to

the limbus, enhances the expression of its receptor and induces differentiation of LESCs into corneal epithelial cells, without any effect on cell proliferation (Figure 3) (Trosan et al., 2012).

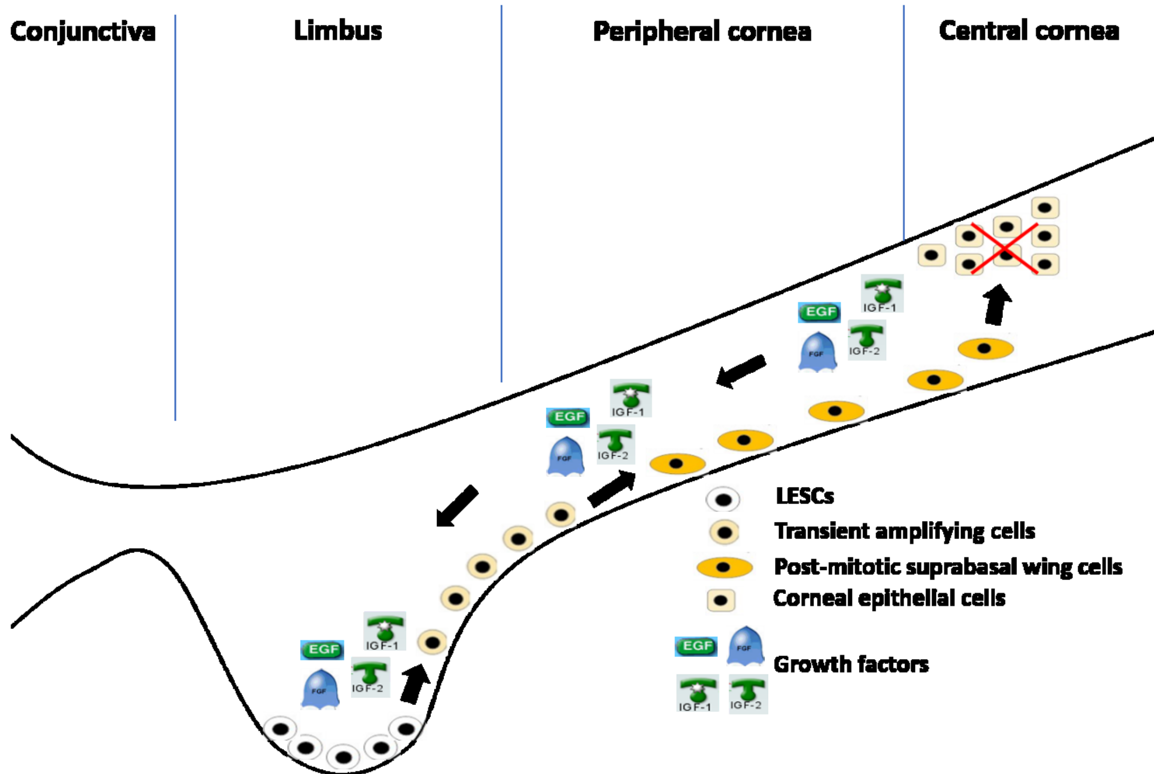

Figure 3: The differentiation of LESCs after damage of the cornea (author´s illustration).

2.3. Limbal stem cells deficiency

LESCs are responsible for the homeostasis and renewal of the corneal epithelium. The functional or anatomical loss of limbus results in LSCD. This complex corneal disorder can be caused by chemical or thermal burn, radiation, genetic or autoimmune disorders (Stevens-Johnson syndrome, aniridia, ectodermal dysplasia), multiple surgeries, contact lens use, infection or drug use (Dua et al., 2000). LSCD results in corneal neovascularization, chronical inflammation and opacification. The laboratory diagnosis of LSCD is based on the detection of GCs in the corneal epithelium consequence of invasion of the conjunctival epithelium into the cornea (Dudakova et al., 2018; He and Yiu, 2014). The disorder can affect one (unilateral) or both (bilateral) eyes with partially (Chen and Tseng, 1990) or totally damaged limbus (Huang and Tseng, 1991). The most successful treatment is transplantation of limbal tissue or LESCs from same patient with unilateral disease (autologous transplantation). The successful vision improvement and corneal stability was firstly reported after transplantation of conjunctival limbal autograft by

Kenyon and Tseng (Kenyon and Tseng, 1989). As an advantage of this technique it is no risk of immune rejection and no need of immunosuppression, as a big disadvantage it is requirement of large limbal graft with the risk of causing LSCD in the healthy eye (Chen and Tseng, 1990). The cultivated limbal epithelial cell transplantation (CLET) was firstly reported by Pellegrini on two patients (Pellegrini et al., 1997). A small autologous limbal biopsy was taken out from the healthy eye, expanded on the fibrin or HAM and then transplanted onto the affected eye (Kolli et al., 2010; Rama et al., 2010). Long-term analysis with 10 years monitoring showed the restoration and renewal of corneal epithelium in 76.6% of eyes of 112 patients (Rama et al., 2010). The success of transplantation is associated with the percentage of p63-bright holoclone-forming stem cells in culture (mentioned above). The cost and availability of the ex vivo culture system are the limitations of this technique. On the other hand, the CLET is a successful approach with the minimal risk for the donor eye and the possibility to repeat the application. Moreover, the European Medicine Agency and the European Commission approved and registered autologous limbal stem cell expanded cultures (Holoclar) for the clinical use. It is the very first product containing stem cells approved for the therapy.

A new technique, simple limbal epithelial transplantation (SLET), is based on 2x2mm biopsy from the healthy eye, which is divided into 10 to 15 small pieces and transplanted using HAM as scaffold with fibrin glue on the affected eye. The expensive laboratory cell culture from the explants is not required. The successful rate of SLET is 76% with the follow-up of 1.5 years (Basu et al., 2016). Main complications linked with this therapy are focal recurrence of LSCD and progressive conjunctivalization.

In case of bilateral ocular damage, the transplantation of living-related conjunctival allograft, cadaveric keratolimbal allografts, or their combination is applied (Haagdorens et al., 2016). The main risk for failure is rejection of the allografts and therefore the long-term success is limited (Daya and Ilari, 2001). The systemic and long-term immunosuppression is also required.

Because use of allograft transplant is problematic, other types of cells suitable for LSCD treatment are also being investigated. Autologous cultured oral mucosal epithelial cell transplantation has been already clinically tested (Liu et al., 2011; Nakamura et al., 2011). Positive visual improvement in 95% of eyes after 6 months decreased to rate of 53% after 30 months (Nakamura et al., 2011). The use of MSCs in the treatment of damaged ocular surface was tested mostly in animal models, where the therapeutic potential of MSCs was comparable with the LESCs (Holan et al., 2015). Human

embryonic stem cells can be differentiated into corneal-like cells by using of limbal fibroblast-conditioned medium (Ahmad et al., 2007). Moreover, they can be transferred to partially wounded human cornea *in vitro* to form corneal epithelial-like cells (Hanson et al., 2013b). Other alternative sources of cells were studied and used to improve the treatment of LSCD in animal models, such as hair follicle stem cells (Meyer-Blazejewska et al., 2011), umbilical cord stem cells (Reza et al., 2011), or human undifferentiated immature dental pulp stem cells (Gomes et al., 2010).

The HAM and fibrin glue are the most successfully used substrates and scaffolds for cultured LESCs and for transfer of cells onto ocular surface, respectively (Chen et al., 2007; Pellegrini et al., 1997; Sangwan et al., 2011). HAM itself degraded after 4 weeks after application and ocular surface stayed stable with no immune response. Fibrin gels degraded 3 days after transplantation (Talbot et al., 2006). Other natural scaffolds, like collagen, silks, keratin, and chitosan, are also studied. Collagen, biocompatible easily available carrier with low immunogenicity, has been used for culture of stratified epithelial cells and applied onto ocular surface (Dravida et al., 2008; Fagerholm et al., 2014). Synthetic materials have some advantages compared to natural, such as nonbiologic nature, no immunogenicity, no transmission of diseases and better control during manufacture. Hydrophilic siloxane hydrogel contact lenses have been used for treatment of LSCD (Di Girolamo et al., 2009). Several polymer carriers, such as polymers made of poly (lactide-co-glycolide) (PLGA) (Deshpande et al., 2013; Ortega et al., 2014) and nanofiber scaffolds prepared from poly(L-lactic) acid (PLA) (Cejka et al., 2016; Holan et al., 2015), have been reported for culture and transferred of LESCs onto damaged ocular surface.

Mouse fibroblasts (3T3 cells) are quite often used as a feeder layer to culture isolated limbal epithelial cells (Pellegrini et al., 1997; Shortt et al., 2007b). Theoretically, the use of xenomaterials can lead to potential undetectable viral transmission, tumorigenesis or immune rejection, but no advance reaction consequent to the use of 3T3 cells has been observed. Nevertheless, the research is focusing on xenobiotic-free culture systems, like autologous human serum (Shandadfar et al., 2012) or human cord blood serum (Chakraborty et al., 2012).

2.4. Conjunctiva

Conjunctiva is a mucous membrane; it extends between the mucocutaneous junction at the margin of the eyelids and corneoscleral limbus. It is subdivided into three zones, palpebral, forniceal and bulbar (Singh et al., 2015). Conjunctiva provides coverage

of the ocular surface, which function as a mechanical barrier against pathogens. Similar to other mucosal surfaces, the ocular surface is persistently exposed to allergens and commensal bacteria that pose a risk of inflammation and infection. With the production of aqueous and mucous components of tear film, conjunctiva is, therefore, part of the mucosal immune defence system (Gipson, 2016; Kessing, 1968; Siebelmann et al., 2013). Conjunctiva actively secretes fluid from its stroma to the surface, and it can absorbs electrolytes, water and other components from the tear film (Candia, 2004).

Conjunctiva is composed of non-keratinizing stratified epithelium and highly vascularised stroma. The epithelium consists of 6-9 layers of epithelial cells, GCs, Langerhans cells and melanocytes. The attachment of epithelial cells to the basement membrane is mediated by few hemidesmosomes. The superficial epithelial cells express human leucocyte antigen (HLA) class I and class II antigens (Fujihara et al., 1997). Conjunctival epithelium is positive for K6, K7, K8, K14, K18 and K19, but K3 and K12, that are typical for cornea, missing (Dhamodaran et al., 2014; Krenzer and Freddo, 1997; Merjava et al., 2011).

The GCs are dispersed among conjunctival epithelia and attached to neighbouring epithelial cells via tight junctions. They account up to 10% of the conjunctival basal cells (Pellegrini et al., 1999). The highest concentration is in the palpebral conjunctiva and it decreases to the limbus part (Kessing, 1968; Ralph, 1975). The density of GCs is affected by age, environmental factors and ocular diseases, such as ocular pemphigoid, keratoconjunctivis sicca, Stevens-Johnson syndrome or chemical injuries (Kessing, 1968; Nelson and Wright, 1984; Waheed and Basu, 1970).

The lubrication of the ocular surface and stabilization of the ocular film is supported by the mucins, high-molecular weight glycoproteins. Human GCs secrete the gel forming mucin 5AC (MUC5AC), soluble MUC2, and membrane associated MUC16. Transmembrane mucins MUC1, MUC4 and MUC16 are expressed by corneal and conjunctival epithelial cells as well (Gipson, 2004, 2016).

Conjunctival stem cells, in contrast to LESCs, are not located in certain regions, but are dispersed throughout the conjunctival tissue. The highest cell density is in the nasal part of the lower fornix and the medial canthus (Stewart et al., 2015; Wei et al., 1995). Usually, colony-forming efficiency and the expression of stem cell markers (*ABCG2, ΔNp63, Hsp70*) are used for their identification (Stewart et al., 2015). Conjunctival stem cells are bipotential and give rise to both epithelial cells and GCs (Pellegrini et al., 1999).

Moreover, GCs can be generated from limbal epithelial cells influenced by the conjunctival environment (Li et al., 2010).

2.5. Amniotic membrane

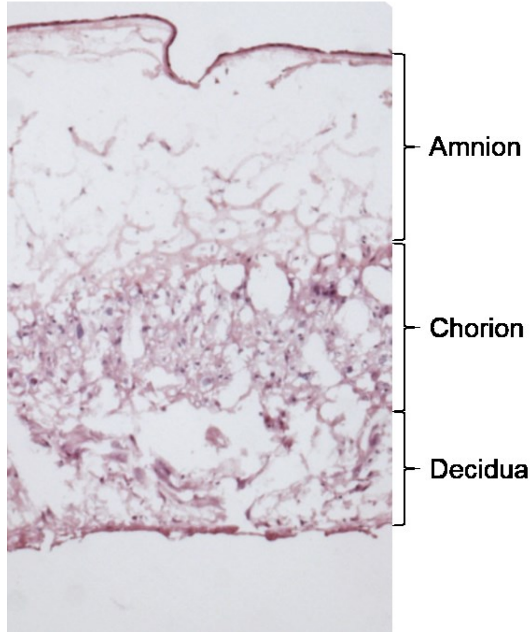

Figure 4: Histology of the placenta section stained with hematoxylin and eosin (laboratory archive).

HAM is the most frequently used carrier for culture of LESCs. It is the innermost layer of the placenta (Figure 4), and it is composed of a monolayer of epithelial cells, a thick basement membrane and an avascular stroma (Figure 5) (Niknejad et al., 2008). HAM is 0,02-0,05mm thick and semi-transparent, it has no nerves, muscles or lymph vessels, and it is supplied with nutrients and oxygen from chorionic fluid, amniotic fluid or foetal blood vessels. HAM possesses numerous metabolic functions, e. g. transport of water and soluble compounds or production of bioactive factors (Mamede et al., 2012). HAM promotes epithelialisation and wound healing mediated by numerous growth factors and cytokines (EGF, HGF and KGF) (Koizumi et al., 2000). The anti-fibrotic effect of HAM is mediated by down-regulation of TGF-β, what leads to tissue reconstruction, not to scar formation (Lee et al., 2000; Tseng et al., 1999). The reduction of inflammation is based on suppression of expression of pro-inflammatory cytokines, interleukin-1α (IL-1α), IL-2, IL-8, IL-10, interferon-γ (IFN-γ), tumor necrosis factor-β, and platelet derived growth factor (Solomon et al., 2001). The inhibitors of matrix metalloproteases and anti-angiogenic compounds like trombospondin-1 and endostatin were also found in the HAM

(Hao et al., 2000; Kim et al., 2000). The antimicrobial effect of HAM is due to its close adherence to the wound surface, and it can be used as a physical barrier against bacterial infiltration (Talmi et al., 1991). Moreover, HAM produces cystatin E, the analogue of cysteine proteinase inhibitor, which exhibits antiviral properties (Ni et al., 1997). It was believed that HAM has no immunogenicity, later was observed that small amounts of HLA class Ia (HLA-A, B, C, DR) and Ib (HLA-G, E) antigens are expressed by cells in HAM (Adinolfi et al., 1982; Kubo et al., 2001). It was also demonstrated that the levels of HLA-A, B, C antigens are lower than as found on other placental and somatic cells (Rylova et al., 2015). The presence of HLA antigens can lead to inflammatory reaction if fresh HAM is used for transplantation (Akle et al., 1981). Cryopreservation of HAM decreases the possibility of immunologic rejection due to nonviable epithelial cells after freezing (Adinolfi et al., 1982; Hammer et al., 1997). By contrast, HLA class II antigens are not expressed by amnion epithelial cells (Adinolfi et al., 1982; Hammer et al., 1997).

Figure 5: The HAM sections stained with hematoxylin and eosin (left) and with collagen type IV antibody (green) (right). The nuclei were stained with propidium iodide (red). Nitrocellulose membrane was used as support for HAM (left) (author´s archive).

Two cell types are located in the HAM, hAECs and human amniotic mesenchymal stromal cells (hAMSCs). The hAECs, derived from embryonic ectoderm, have many microvilli at their apical surface, large irregular nucleus with large nucleolus and many intracytoplasmic organelles and pinocytic vesicles (Pollard et al., 1976). The niche of hAECs is located between two different microenvironments, amniotic fluid and basement membrane (BM), therefore both microenvironments have influence on hAECs with amniotic fluid soluble compounds and insoluble compounds of BM (collagens, fibronectin, nidogen and laminins) (Lambshead et al., 2013; Niknejad et al., 2012). The hAECs have highly multipotent differentiation ability and could be differentiated into all three germ layers (Miki et al., 2005). It was reported that hAECs express pluripotent stem cells-specific transcription factors, such as octamer-4 (*OCT-4*), nanog homeobox (*NANOG*), sex determining region Y-box 2 (*SOX2*), or surface markers associated with embryonic stem

cells, such as Kruppel-like factor 4 (*KLF4*), zinc finger protein 42 (*REX-1*), stage specific embryonic antigen 3 and 4 (*SSEA-3* and *-4*), *TRA-1-60* and *TRA-1-81* (Miki, 2018; Simat et al., 2008). It was also shown that both amniotic cell types expressed *OCT-4, NANOG*, and *KLF4* more that bone marrow-derived MSCs (Koike et al., 2014). The function of epithelial cells is regulated by BM, which is composed of collagen type III, IV (α1, α2, α5 and α6 chains), VII, XV, XVI, XVII and XVIII, fibronectin, heparan sulphate, nidogen 1 and 2, fibulin-2, fibrillin-2, perlecan, agrin and laminins (Niknejad et al., 2008). The hAMSCs, derived from embryonic mesoderm, are important as a source of extracellular matrix components (Insausti et al., 2014).

The ability to differentiate, low immunogenicity and anti-inflammatory effect indicate the potential of hAECs for treatment of various diseases and disorders, e.g. type I diabetes (Chen et al., 2007) or cardiovascular regeneration (Fang et al., 2012). The hAECs can be used as a feeder for culture of various stem cells, including human LESCs (Chen et al., 2007), or human and murine embryonic stem cells (Avila-Gonzalez et al., 2015; Lai et al., 2009). The conditioned medium of hAECs has immunosuppressive activity, inhibits the chemotactic activity of neutrophils and macrophages as well as reduces the proliferation of T and B cells after mitogenic stimulation (Cargnoni et al., 2014; Li et al., 2005). Intact or denuded (de-epithelialized) HAM can be used for the culture and transfer of the LESCs on the damaged ocular surface. For the growth of enzymatically dispersed LSCs is more suitable denuded HAM (de Melo et al., 2007; Shortt et al., 2009; Zhang et al., 2013), while intact HAM mostly supports the growth of limbal explants (Kolli et al., 2008; Li et al., 2007; Sudha et al., 2008). Denuded amniotic BM supports growth, stratification and differentiation of LESCs, but not their stemness (Dietrich-Ntoukas et al., 2012; Koizumi et al., 2007).

Several approaches exist to denude HAM, such as most frequently used trypsin/ethylenediaminetetraacetic acid (EDTA) (Madhira et al., 2008; Sangwan et al., 2003), then sodium dodecyl sulphate (SDS) (Wilshaw et al., 2006), Tris/EDTA followed by incubation with SDS (Roy et al., 2016), Tris/EDTA/aprotinin (Wilshaw et al., 2008), EDTA (Koizumi et al., 2007), thermolysin (Siu et al., 2008), dispase (de Melo et al., 2007), NaOH (Saghizadeh et al., 2013) or ammonium hydroxide (Noguchi et al., 1995).

The best established method for the isolation of viable hAECs is the trypsin/EDTA treatment (Evron et al., 2011; Miki et al., 2007; Pratama et al., 2011) or dispase application (Diaz-Prado et al., 2010; Rutigliano et al., 2013).

Each of the mentioned techniques has different effects on biological and physical properties of HAM and hAECs. Some of the treatments may damage the integrity of the HAM or BM, the viability of hAECs and hAMSCs can be affected, or the activity of growth factors decreased. EDTA itself does not remove the epithelium completely, and the application of dispase can lead to BM damage. Moreover, each of these studies was focused on either de-epithelialization of HAM or on obtaining viable hAECs only.

3. Hypotheses and aims of work

LESCs culture (Hypothesis 1):

LESCs are commonly used for the treatment of LSCD. The unilateral form of disease is the most successfully treated by autologous transplantation of limbal tissue or cultured LESCs. The cells are usually cultured with the use of xenogeneic components (FBS, 3T3 cells, etc.), what can potentially result in transmission of infection diseases or immune reaction. Various carriers, such as HAM, fibrin glue or nanofiber scaffolds are used for the transfer of cells on the damaged ocular surface. We supposed that the replacement of animal components will increase the safety of the grafted cells. Moreover, we hypothesized that the combination of culture of human LESCs in xenobiotic-free culture media on plastic or fibrin glue (which is used as a medical treatment) may result in improvement of this culture technique and it has a direct positive effect on higher rate of successful transplantation. We supposed that suggested conditions would lead to comparable stemness and proliferative activity of cultured LESCs as typically used complex medium (COM) containing xenogeneic material.

Aims:

- To culture LESCs in standard complex medium and xenobiotic-free culture systems (supplemented with human serum or platelet lysate) on plastic or fibrin glue carrier.
- To compare the growth kinetics, cell proliferation, differentiation, maintenance of stemness, apoptosis and contamination by fibroblast-like cells between the groups.

Preparation of conjunctival epithelial and goblet cells culture (Hypothesis 2):

Conjunctival stem cells, which give rise to epithelial and GCs, are distributed throughout conjunctival tissue, and it is suggested that both can be generated from limbal epithelium. It was reported that IL-13 has positive effect on GCs numbers and secretion of MUC5AC. We considered the possibility to culture the conjunctival epithelium containing

GCs from corneoscleral rims. Moreover, we hypothesized that culture and differentiation of these cells could be enhanced by the effect of IL-13.

Aims:

- To culture the conjunctival epithelium with the presence of GCs from corneoscleral rims.
- To evaluate the effect of human recombinant IL-13 on GCs numbers, mucin expression and stemness of cultured conjunctival cells.

Differentiation of MSCs (Hypothesis 3):

Allograft transplantation suitable for bilateral corneal damages is less effective than autograft. Therefore, other sources of autologous stem cells are being investigated and tested, as a potential replacement for the LESCs. In this respect, MSCs have proven to be a promising stem cell type due to their immunomodulatory and regenerative potential. Insulin-like growth factor-I (IGF-I) was identified as the main factor responsible for the differentiation of LESCs into corneal epithelial cells in mice. We hypothesized that the culture of murine MSCs with IGF-I and corneal extract may result in differentiation into corneal epithelial cells and it could increase the therapeutic effect of MSCs.

Aim:

- To characterize the differentiation potential of bone marrow-derived MSCs into corneal-like cells as a possible source of cells suitable for autologous transplantation in bilateral corneal damage.

Decontamination of HAM (Hypothesis 4):

HAM is used for its strong wound healing effect in clinical practice, particularly in ophthalmic surgery. Various protocols and solutions are used for the decontamination of placenta and HAM after tissue retrieval by caesarean section. Process of decontamination may be affected by several variables such as temperature, length of treatment, pH or concentration of the disinfectant. Only a limited number of certified tissue decontamination solutions is commercially available. We supposed that expensive commercial decontamination product could be substituted with similar antimicrobial efficiency and toxicity by our own decontamination solution.

Aims:

- To prepare LDS based on the composition of commercially available product.

- To compare the antimicrobial efficiency, toxicity and stability (pH, osmolarity) of commercial (BASE•128) and LDS decontamination solutions on HAM model.

De-epithelialization of HAM (Hypothesis 5):

HAM can be used preclinically as a feeder layer for cell culture, or clinically for stimulation of wound healing treatment as a source of cells for cell therapy. Deepithelialized HAM is more suitable for culture of LESCs compared to intact. Moreover, amniotic epithelial cells, debris after de-epithelialization, have the ability to differentiate, low immunogenicity, anti-inflammatory effect and express the stemness genes. Therefore, these cells have potential for treatment of various disorders. Various de-epithelialization approaches have been published, but with the aim to obtain deepithelialized HAM or with the aim to receive viable cells only. We hypothesized that by using a gentle enzymatic method would be possible to achieve both products, unimpaired deepithelialized HAM and viable amniotic epithelial cells.

Aim:

- To find effective and safe enzymatic HAM de-epithelialization method leading to harvesting of both denuded undamaged HAM and viable human amniotic epithelial cells.

4. Material and Methods

To achieve the aims of our work, a wide spectrum of methods was used. Individual detailed protocols are described in the appended publications. The methods mentioned below were managed by the author of this Doctoral Thesis.

LESCs culture (Paper 1):

- Preparation and culture of LESCs from limbal explants cut from corneoscleral rims.
- Culture and preparation of 3T3 feeder layers.
- Determination of the clonal growth ability of the cultured cells with colony forming efficiency assay.
- RNA extraction, reverse transcription and the detection of gene expression of *TP63, ABCB5, BMI1, K15* and *K3* genes by quantitative real time polymerase chain reaction (PCR) analysis.
- Statistical analysis.

Preparation of conjunctival epithelial and goblet cells culture (Paper 2):

- Preparation of the limbal explants and cell culture.
- Culture and preparation of 3T3 feeder layers.
- Determination of the clonal growth ability of the cultured cells with colony forming efficiency assay.
- RNA extraction, reverse transcription and detection of gene expression of *TP63, MUC5AC, MUC4, K3, K7*, and *K12* genes by quantitative real time PCR analysis.

Differentiation of MSCs (Paper 3):

- Isolation, culture and purification of murine MSCs from the bone marrow of BALB/c mice.
- Preparation of the corneal extract by harvesting and cutting the corneas into small pieces.
- Differentiation of MSCs into corneal-like cells by culture cells with corneal extract and recombinant IGF-I.
- RNA extraction, reverse transcription and detection of gene expression of *K12, keratocan* and *lumican* genes by quantitative real time PCR analysis.
- Determination of the metabolic activity of living cells by Wst-1 assay.
- Indirect fluorescent immunohistochemistry of K12 and immunostaining with phalloidin on differentiated and control cells.
- Stimulation of spleen cells with Concanavalin A and subsequent culture of lymphocytes with untreated or differentiated MSCs to compare their immunosuppressive properties.
- Statistical analysis.

Decontamination of HAM (Paper 4, 5):

- Processing of the placenta with the preparation of the HAM.
- Trypan blue staining and the light microscopy of the HAM samples.
- Processing of the placenta with the peeling off and caring of the HAM.

De-epithelialization of HAM (Paper 6):

- Processing of the placenta with the preparation of the HAM.
- HAM de-epithelialization and isolation and culture of the hAECs.

- Hematoxylin and eosin staining of the HAM with the examination by light microscopy.
- DNA isolation and its concentration measurement.
- Immunostaining of the HAM with anti-collagen type IV α2 chain and anti-laminin α5 chain antibody.
- Preparation of the samples for scanning electron microscopy (SEM).
- Determination of metabolic cell activity by Wst-1 assay.
- RNA extraction, reverse transcription and detection of gene expression of *SOX2, OCT-4, OCT-4A* and *NANOG* genes by reverse transcription PCR analysis.
- Statistical analysis.

5. Results

The most important results of the work performed are summarized below. For more detailed results, please see the appended publications.

LESCs culture

Limbal epithelial cells (LECs) cultures were expanded in two completely xeno-free culture media (human serum medium - hSM and human platelet lysate medium - hPLM) and compared with the COM used as a gold standard. LECs grown on both plastic and fibrin and in all experimental groups displayed no difference in morphology, they were cuboidal with high nucleocytoplasmic ratio (Figure 6A). All explants had similar percentage of successful cultures, about 70% (Figure 6B).

LECs cultured in COM and hSM on fibrin expanded earlier and reached confluence more rapidly than cells cultured in the same media on plastic. LECs cultured in COM achieved confluency significantly earlier than cells cultured in hSM and hPLM on both surfaces (Figure 6C).

The contamination by fibroblast-like cells was recognized under all tested conditions (Figure 6D), but more often observed in the explants grown on fibrin. However, the statistical significance was confirmed only for cultures in COM (Figure 6E).

The TP63 gene expression of p63α isotype was not statistically different among all groups, except for the cells cultured in hSM on plastic compared to that grown on fibrin. Bmi-1 expression was higher in LECs cultured in COM on fibrin compared to culture in both xenogeneic media on fibrin, and it was higher in cultures grown in COM than in

hPLM on plastic. No statistically significant difference was observed in expression of K15 among all groups (Figure 8B).

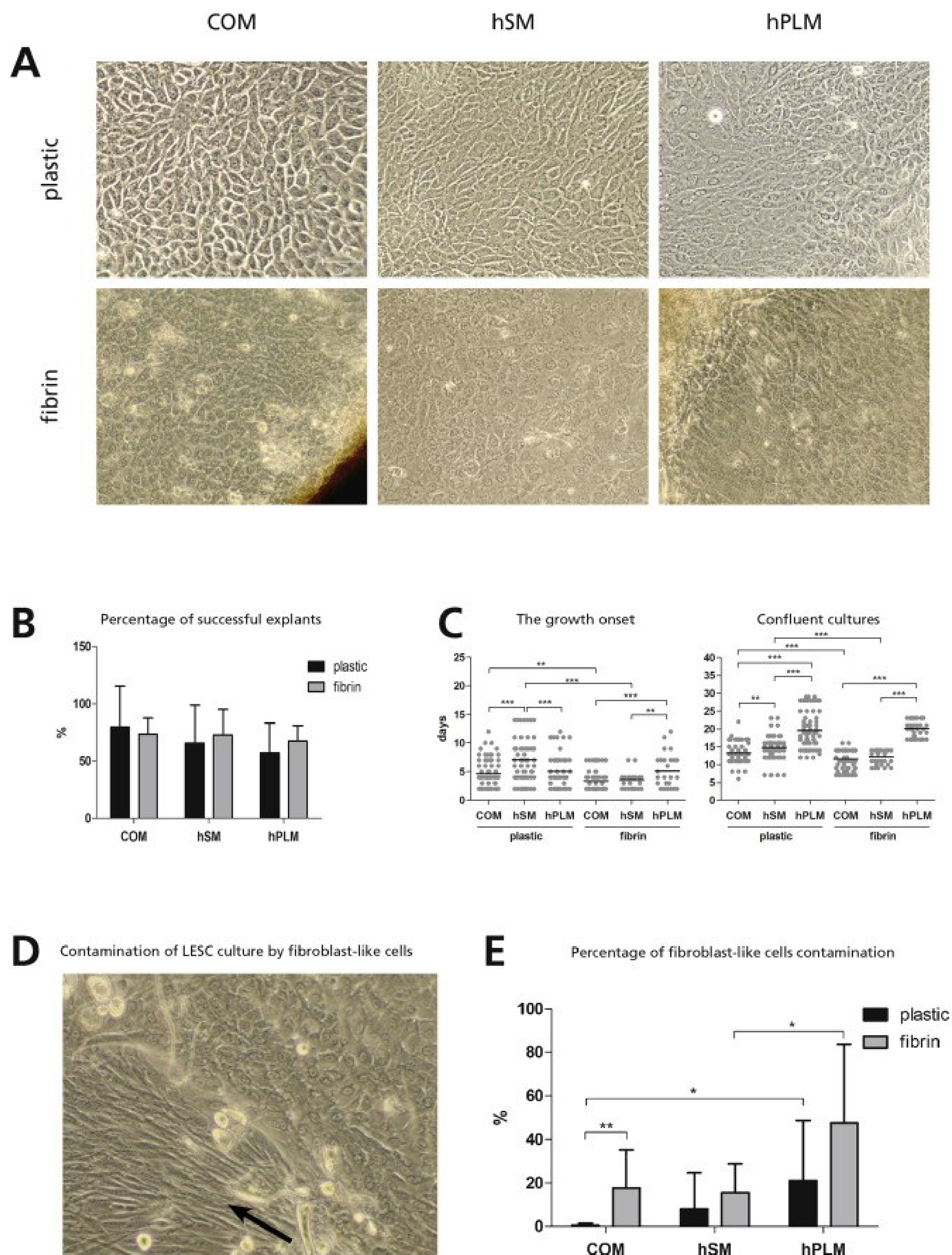

Figure 6: LECs growth and morphology. Cell morphology in cultures cultured in COM, hSM and hPLM on plastic or fibrin (A). The percentage of successfully grown explants in relation to the number of plated explants (B). The start of outgrowth of LECs cultures from day 0 (plating) until full confluence (harvest) (C). Fibroblast-like cell contamination (D) and the percentage of LECs cultures with contamination in relation to successfully grown explants (E).

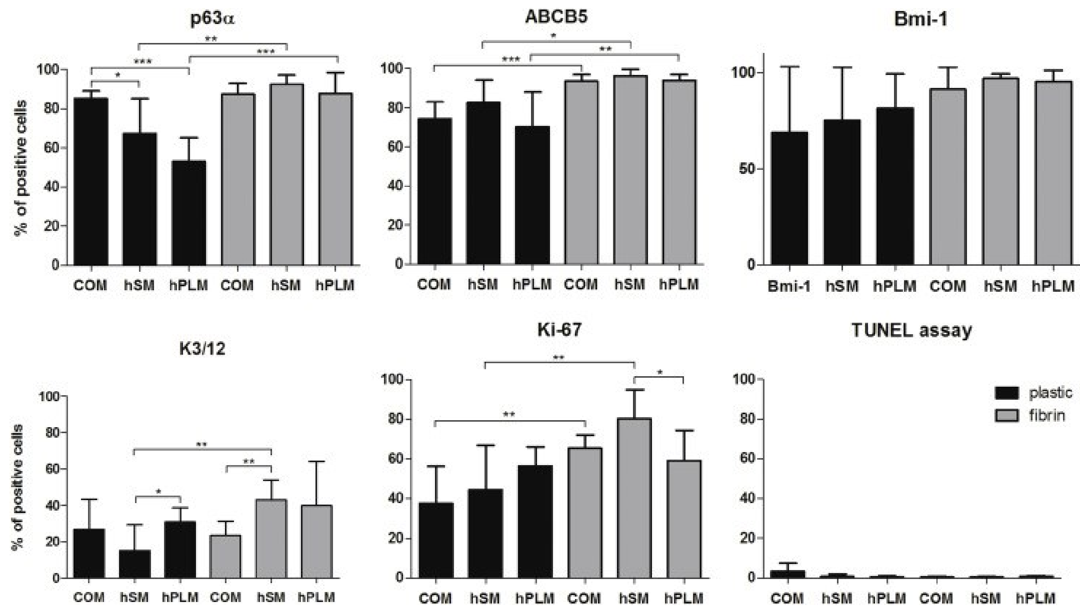

Figure 7: Percentage of cells stained for putative LESCs markers p63α, ABCB5, and Bmi-1; differentiation corneal epithelial cell marker K3/12; proliferation cell marker Ki-67, and percentage of apoptotic LECs cultured either on plastic or on fibrin substrate in COM, hSM or hPLM.

The number of differentiated K3/12-positive cells varied between 15–43% in all cultures cultured on both substrates in all media. A significantly lower percentage of K3/12-positive cells was found only in cultures grown on plastic in hSM compared to hPLM. More K3/12-positive cells were detected in LECs cultured in hSM compared to COM on fibrin (Figure 7). The gene expression of *K3* was higher in cells grown on plastic than on fibrin with the significant difference for cultures in COM and hSM. The LECs cultured in hPLM had significantly higher *K3* expression than in COM and hSM on fibrin (Figure 8B).

The cultures grown on plastic had slightly lower cell proliferation compared to that cultured on fibrin. The only significant differences were observed between cells cultured in COM and hSM media (Figure 7).

No significant difference in the presence of apoptotic cells was observed in cultures grown in all conditions by TUNEL assay (Figure 7). Slightly more apoptotic cells (up to 3%) were detected in cultures in COM on plastic, compared to other cultures in all media and both substrates (up to 1%).

The colony forming efficiency assay (CFE) showed no statistically significant differences among all experimental groups (Figure 8A). Cells cultured in COM had similar CFE values on plastic (3.97%) and on fibrin (3.21%), as well as LECs cultured in hSM (2.42% vs. 4.13%) and hPLM (2.92% vs. 4.48%).

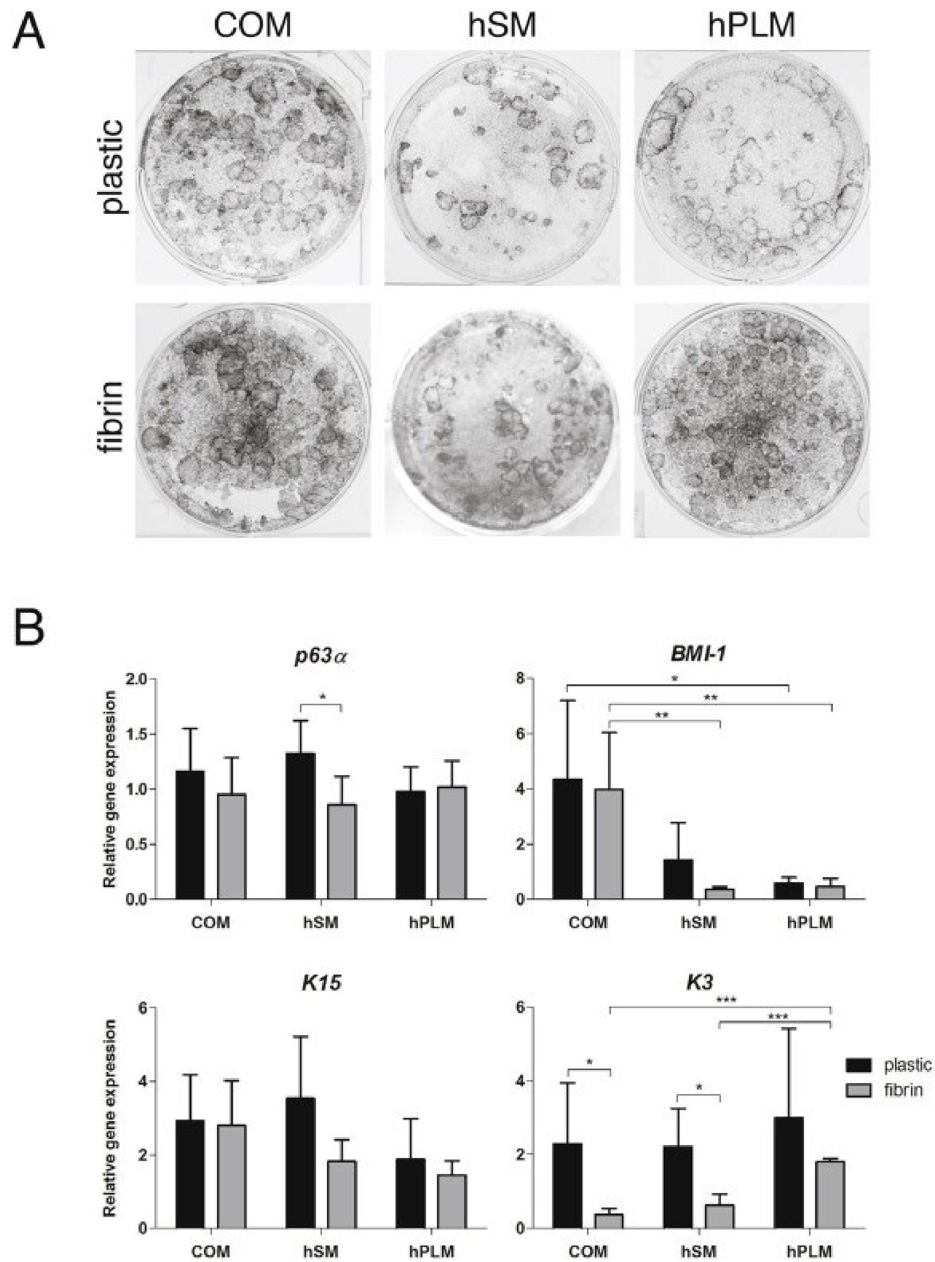

Figure 8: Colony forming efficiency assays of LECs cultured in COM, hSM and hPLM on plastic or fibrin (A). The Expression of *p63α* isotype of *TP63, BMI1, K15* and *K3* genes (B) determined by qPCR.

Preparation of conjunctival epithelial and goblet cells culture

Cell cultures became confluent after 9 days of culture (P0). After first passage, cells reached the confluence on day 9-12 (P1), after second passage, cells were harvested on day 12-14 without reaching confluence (P2). The cell viability after passaging was comparable among the passages and groups (IL-13- or IL-13+ cell cultures), about 90%, only P0 culture with the IL-13 had viability of 83%. Unattached cells were rinsed and evaluated one day after P1, named P1d1. Their viability in both groups were significantly lower (about 41%) than in P0-P2 cultures (Figure 9A).

Strong positivity for K7 showed conjunctival epithelial cells and GCs in all groups and passages. The only difference was in the P2 IL-13- group, where the K7 positivity was 80% compared to 99% in other cultures (Figure 9B).

In P0, the morphology of epithelial cells in both groups was cobblestone in shape with a high nuclear-to-cytoplasmic ratio, while the morphology of GCs was round to oval-shaped (Figure 9Ca, Ce). A mixture of cuboidal and flattened epithelial cells with superficially located GCs appeared at P1 (Figure 9Cb, Cf). In P2, the differentiated flattened epithelial cells with low nuclear-to-cytoplasmic ratio and with superficially located GCs appeared in both groups (Figure 9Cc, Cg). Unattached oval-shaped cells were detected in P1d1 (Figure 9Cd, Ch).

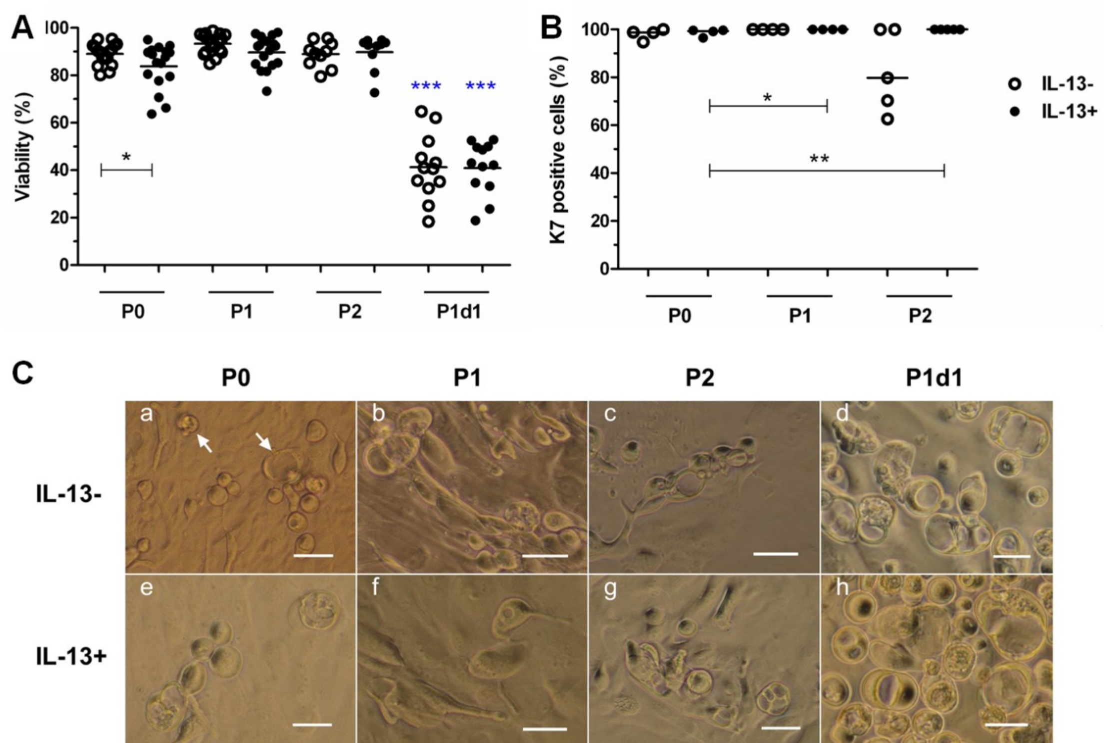

Figure 9: The viability, immunofluorescence staining of K7, and morphology of IL-13- or IL-13+ cell cultures. (A) Percentages of cell viability at the end of culture of P0, P1, and P2 cultures and unattached GC-enriched subpopulation harvested on P1d1. The line indicates the median. The asterisks represent statistically significant difference (*P < 0.05, ***P ≤ 0.001). Blue asterisks indicate significant decrease of viability in P1d1 IL-13- or P1d1 IL-13+ group vs. P0, P1, and P2 IL-13- or IL-13+ groups, respectively. (B) P0, P1, P2, and P1d1 cells were analysed by immunofluorescence staining for K7 protein. All data of positive percentages are presented in the vertical scatter plot graph with line indicating median. *P < 0.05, **P ≤ 0.01. (C) Cell morphology at the end of culture of P0, P1, and P2 cultures and unattached GC-enriched subpopulation (P1d1) observed under inverted phase contrast microscope. White arrows show examples of GCs. Scale bars: 50 μm.

Detection of gene expression showed strong expression of *K7* gene in IL-13- and IL-13+ conditions and in all passages (Figure 10). The median values of *K7* expression were higher in all passages of IL-13+ cells compared to IL-13- cells. In the IL-13+ cultures, *K7* expression was significantly higher at the end of P2 compared to P0. The gene expression of the *K3* and *K12* genes were expressed at much lower levels than the expression of *K7* gene (Figure 10).

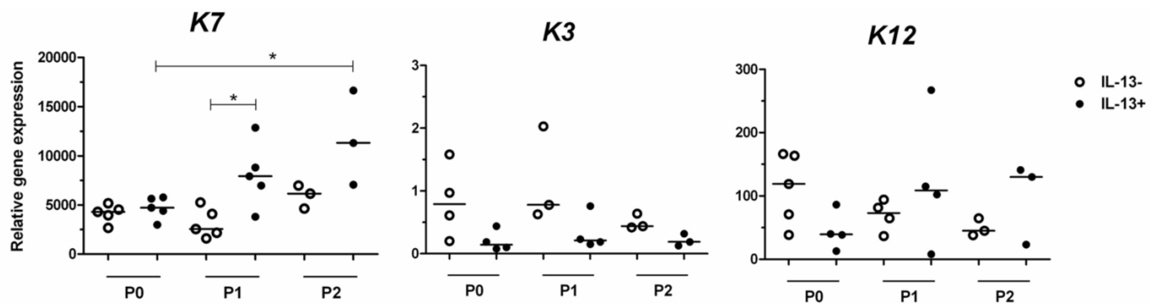

Figure 10: Relative gene expression of *K7, K3*, and *K12* genes in IL-13- or IL-13+ cell cultures determined by qPCR. Cells were analysed at the end of culture, P0 cells originating from limbal explants, P1 and P2 cells are from culture after passages. All data are presented in a vertical scatter plot graph with line indicating median. *P < 0.05.

The presence of single and grouped GCs in all cultures under both IL-13 conditions was verified by AB/PAS staining (Figure 11C). The absolute number of GCs in the P1 IL-13- group was significantly higher than that in the P1 IL-13+ group. Among the IL-13- cultures, significantly higher number of GCs was present at P1; among the IL-13+ group at P2 (Figure 11A). P1d1 GC-enriched population showed slightly higher AB/PAS-positivity in IL-13- group (27.6%) compared to IL-13+ group (24.4%) with no significant difference between groups (Figure 11B).

MUC5AC immunostaining confirmed the presence of single and grouped GCs in all tested groups (Figure 12B). Same results were confirmed by qPCR but with no statistical significance (Figure 12A). MUC4 was expressed in all groups, among IL-13+ cultures in P1 and P2 expression was higher than in IL-13- cultures but with no statistical significance (Figure 12A).

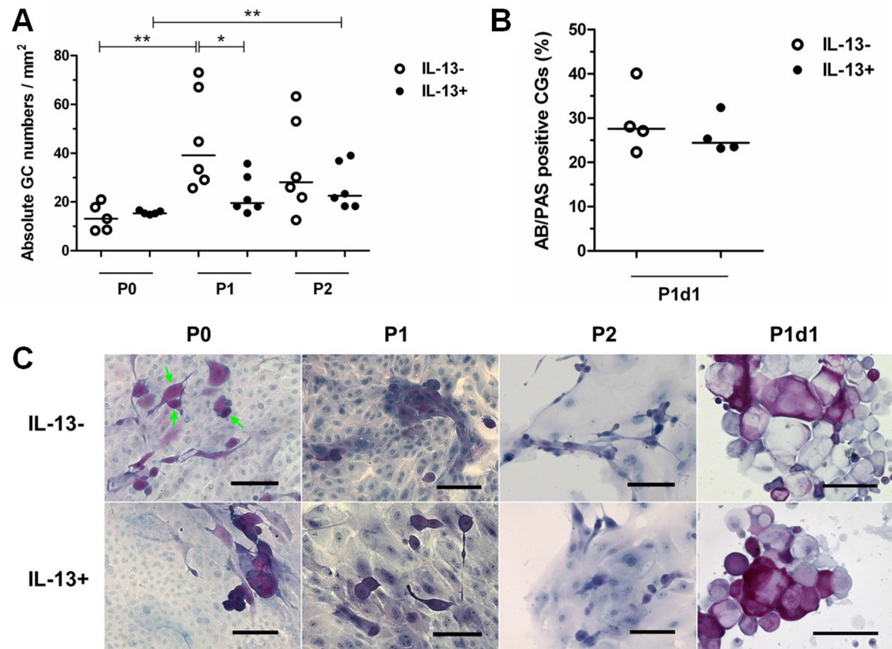

Figure 11: AB/PAS staining of GCs in IL-13- or IL-13+ cell cultures of P0, P1 and P2 cells and P1d1 subpopulation. (A) Distribution of absolute numbers of AB/PAS-positive GCs in individual groups presented in a vertical scatter plot graph with line indicating median. (B) Distribution of percentages of AB/PAS-positive GCs in individual groups of P1d1 subpopulation presented in a vertical scatter plot graph with line indicating median. *P < 0.05, **P ≤ 0.01. (C) Green arrows indicate examples of GCs. Scale bars: 100 µm.

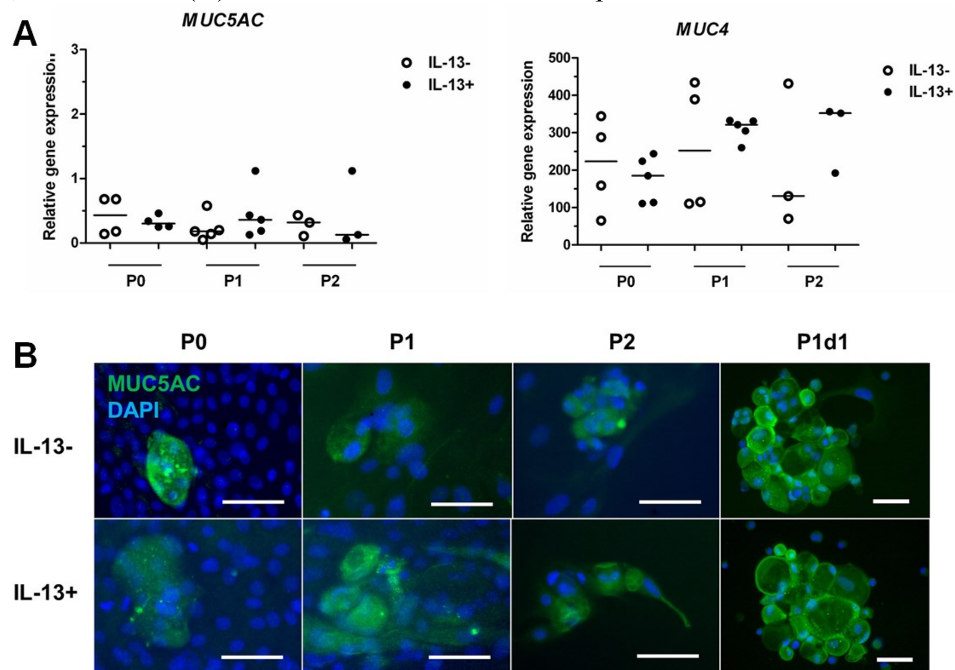

Figure 12: Relative gene expression of *MUC5AC* and *MUC4* genes and immunofluorescence staining of MUC5AC in IL-13- or IL-13+ cell cultures of P0, P1 and P2 cells and P1d1 subpopulation (all fixed at the end of culture). (A) The relative gene expression of *MUC5AC* and *MUC4* was analysed by qPCR. All data are presented in vertical scatter plot graphs with line indicating median. (B) Cells were analysed by immunofluorescence staining for MUC5AC (green). Nuclei were counterstained with DAPI (blue). Scale bars: 50 µm.

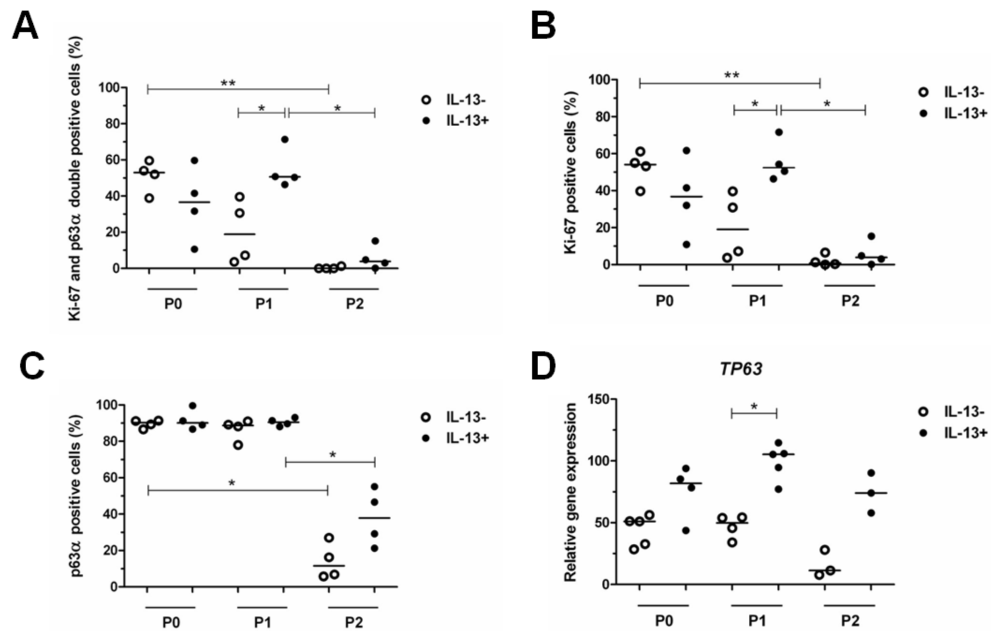

Figure 13: Immunofluorescence staining and double staining of Ki-67 and p63α and the relative *TP63* gene expression in IL-13- or IL-13+ cell cultures. Distribution of percentages in P0, P1, and P2 groups for Ki-67 and p63α double staining (A), and Ki-67 (B) and p63α (C) immunostaining. (D) qPCR analysis of relative *TP63* gene expression. All data are presented in vertical scatter plot graphs with line indicating median. *P < 0.05, **P ≤ 0.01.

Ki-67 and p63α immunostaining demonstrated a high percentage of positivity, particularly in the P0 and P1 cultures, and a low percentage of positivity in the P2 cultures (Figure 13A). Between the P0, P1, and P2 IL-13- and IL-13+ groups, P1 IL-13+ cells had significantly higher Ki-67 and p63α double positivity compared to P1 IL-13- cells (Figure 13A). A similar pattern of antigen expression was seen for Ki-67 staining versus Ki-67 and p63α double staining; indicating almost 100% Ki-67 co-localization with p63α in P0, P1, and P2 cells (Figure 13B). Significant decrease of p63α positivity was demonstrated in P2 cell cultures (Figure 13C). The addition of IL-13 into the P2 culture increased the p63α positivity without significance. *TP63* gene expression was present in all evaluated cultures under the IL-13- and IL-13+ conditions (Figure 13D). All IL-13+ cell cultures had higher median of *TP63* expression than IL-13- cultures. The statistical significance was proved only in the P1 cell cultures.

Statistical analysis of the CFE data demonstrated significantly higher growth potential of the P0 IL-13+ group compared to the P0 IL-13- group. Similarly, the P1 IL-13+ group had significantly higher growth potential than the P1 IL-13- group (Figure 14B). The growth potential decreased in both IL-13 groups in P2 and P1d1.

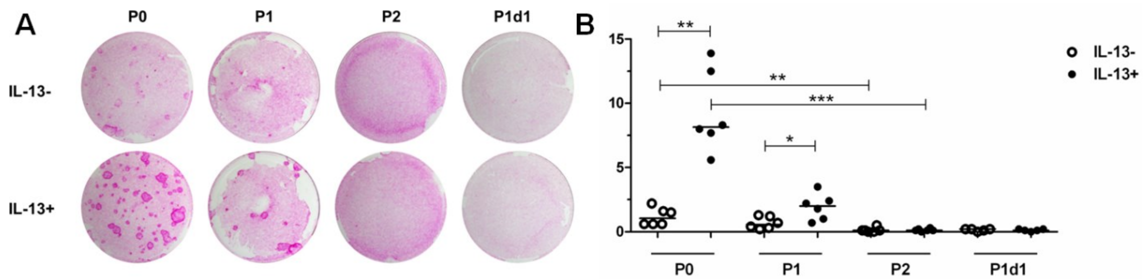

Figure 14: Comparison of total CFE. At the end of culture, P0,P1 and P2 cells, and the unattached GC-enriched subpopulation (P1d1, harvested on day 1 after passage of primary cells) were cultured with growth-arrested 3T3 mouse fibroblasts to compare their growth ability under IL-13- and IL-13+ conditions. All total CFE data are presented in vertical scatter plot graphs with line indicating median. $*P < 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. (A) Colonies grown in CFE assay and stained with 2% rhodamine B (day 12). (B) Distribution of total CFE percentages of the P0, P1, P2, and P1d1 groups.

Differentiation of MSCs

The MSCs were cultured alone (untreated) or with the corneal extract or with the corneal extract and recombinant IGF-I (20ng/mL) for 3, 7 or 10 days. Analysis by qPCR showed that the expression of *K12*, keratocan and lumican were already upregulated after a 3-day culture and gradually increased during the 10-day incubation of MSCs with the corneal extract. Adding IGF-I to the culture medium significantly increased differentiation (Figure 15).

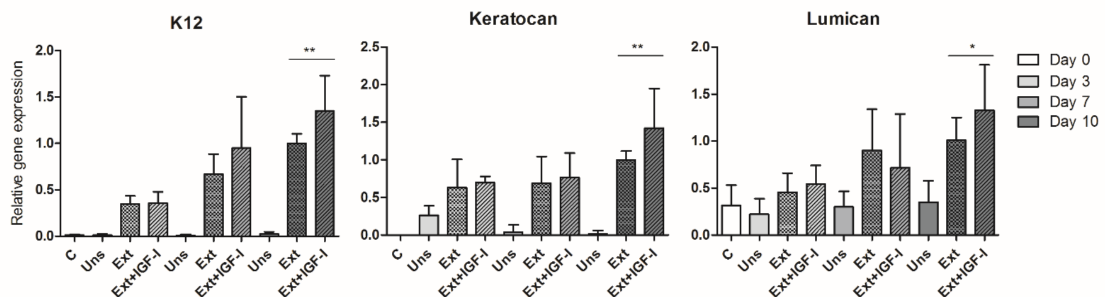

Figure 15: The expression of genes for *K12*, keratocan and lumican in untreated and differentiated MSCs determined by qPCR. The cells were cultured for 3, 7 or 10 days untreated (Uns), with the extract from the corneas (Ext) and in the presence of the extract and IGF-I (Ext + IGF-I). Each bar represents mean ± SD from 4 - 5 determinations. The asterisks represent statistically significant ($*P < 0.05$, $**P < 0.01$) difference in the gene expression between MSCs treated only with the extract or with the extract and IGF-I. Freshly purified MSCs are marked as a control (C).

The expression of cell surface markers CD45, CD11b, CD73, CD44 and CD105 was comparable in the control and differentiated MSCs (Figure 16A). The morphology showed that both types of cells had a typical fibroblast-like shape and adhered to plastic

and glass surfaces (Figure 16B). Results from the WST-1 assay showed that proliferative activity was even higher in differentiated cells than in untreated MSCs (Figure 16C). Both control and differentiated MSCs inhibited the production of IL-2 and IFN-γ in spleen cells stimulated with Concanavalin A (Figure 16D).

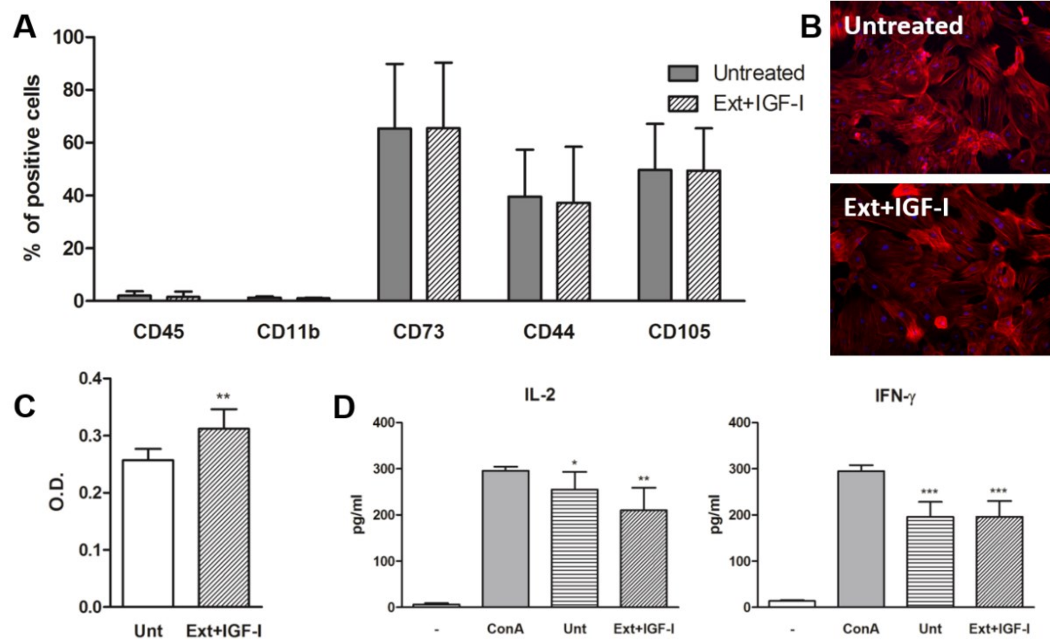

Figure 16: The comparison of the expression of cell surface markers (A), morphology (B), metabolic activity (C) and immunosuppressive properties (D) of untreated (Unt) and differentiated MSCs after 10 days of treatment (Ext + IGF-I). Flow cytometry analysis of CD45, CD11b, CD73, CD44 and CD105 markers expressed in untreated or differentiated MSCs is demonstrated (A). The cells for the immunofluorescence analysis were stained with phalloidin for F-actin (red filaments) and the nuclei with DAPI (blue). Scale bars represent 25µm (B). The metabolic activity was observed by adding WST-1 reagent to the cell culture to form formazan and absorbance was measured using a Sunrise Remote ELISA Reader at a wave-length of 450nm (C). Spleen cells were cultured unstimulated or were stimulated with Con A in the presence of untreated or differentiated MSCs. The production of IL-2 and IFN-γ was determined in the supernatants after a 24h (IL-2) or 48h (IFN-γ) incubation period by ELISA (D). Each bar represents mean ± SD from 3 determinations.

HAM decontamination

HAMs were decontaminated in BASE•128 or LDS or incubated in DMEM medium, this last one for control (Co). The tissues were stained with Trypan blue dye for determination of viability of epithelial cells. For comparison, fresh HAMs before decontamination were stained as well. The visually confirmed increase in the percentage of dead epithelial cells (DECs) was observed when longer decontamination periods (C2, C3) were used (Figure 17). Compared to Co, decontamination by both BASE•128 and LDS at all conditions increased the average percentage of DECs; however, the only statistically significant increase was in LDS at C3.

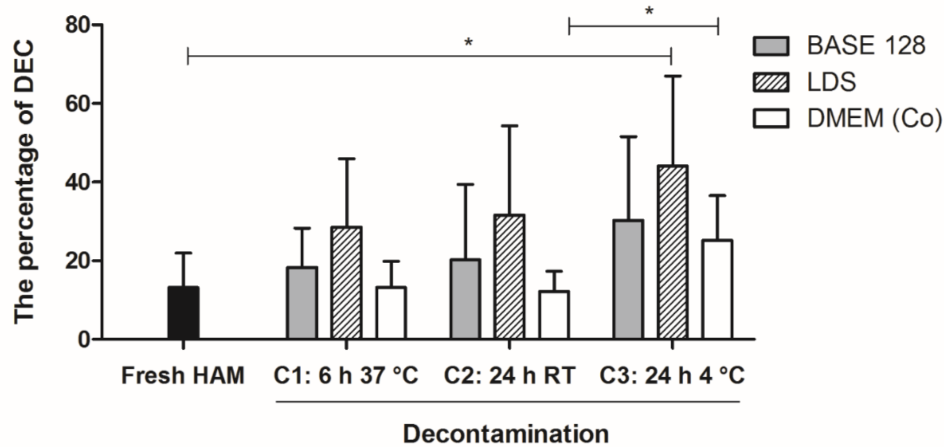

Figure 17: The mean percentage of the dead epithelial cells in fresh and decontaminated HAM. BASE•128, LDS or DMEM medium as a control solution (Co) were used. The HAM specimens were incubated for 6h at 37°C (C1), for 24h at room temperature (RT) (C2), or for 24h at 4°C (C3). The asterisks represent statistically significant difference (*P < 0.05).

The percentage of apoptotic epithelial cells (AECs) and apoptotic mesenchymal cells (AMCs) were determined by TUNEL assay. The samples were cryopreserved after decontamination, then thawed and used for the analysis. In the fresh HAM samples the percentage of AECs was less than 1% and the percentage of AMCs was 42.0%. In all HAMs after decontamination and in all conditions the number of AECs remained low, about 1–2% (Figure 18A). The percentage of AMCs increased significantly up to 87.9% (C1, Co), compared to fresh HAM (Figure 18B). Changes in the mean percentage of AEC among groups were not statistically significant.

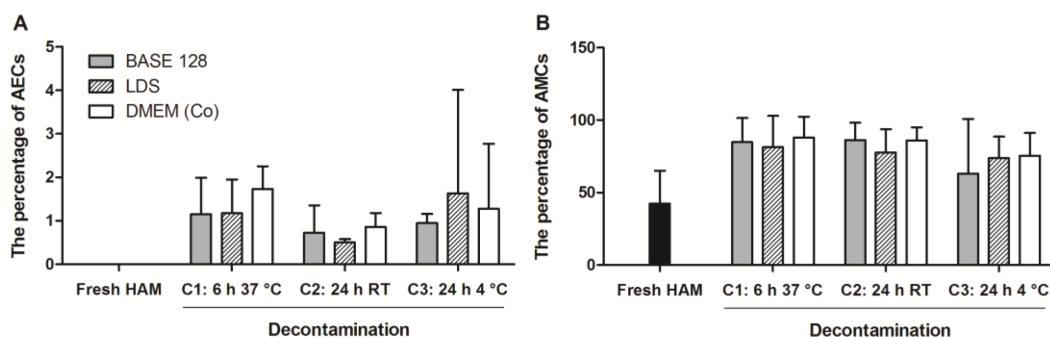

Figure 18: The mean percentage of the apoptotic epithelial cells (AECs; A) and apoptotic mesenchymal cells (AMCs; B) after HAM decontamination determined by TUNEL assay. BASE•128, LDS or DMEM medium as a control solution (Co) were used. The HAM specimens were incubated for 6h at 37°C (C1), for 24h at room temperature (RT) (C2), or for 24h at 4°C (C3).

The BASE•128 and LDS were compared in their antimicrobial efficiency and stability immediately after thawing aliquots frozen for 1, 3 or 6 months. The difference in the inhibition of growth was mostly not statistically significant. Both solutions were the most effective at elimination of *Proteus mirabilis* and the lowest antimicrobial activity was measured against *Staphylococcus aureus.* The BASE 128 had lower antimicrobial efficiency against *Escherichia coli* and *Enterococcus faecalis* than LDS. The antimicrobial activity of both solutions against *S. aureus* slightly decreased with cold storage (Table 1).

Organism	IZ (mm), mean ± SD							
	BASE•128				LDS			
	T0	T1	T2	T3	T0	T1	T2	T3
P. mirabilis	49.8 ± 0.4	49.0 ± 0	49.0 ± 0	49.2 ± 0.8	49.8 ± 0.4	49.3 ± 0.5	50.0 ± 0 (0.001)***	49.0 ± 1.1
P. aeruginosa	39.6 ± 2.9	39.4 ± 3.1	39.7 ± 2.5	39.1 ± 3.8	40.3 ± 2.9	40.0 ± 2.5	39.5 ± 2.0	38.3 ± 3.3
S. aureus	30.5 ± 0.5 (0.015)*	29.0 ± 0	27.3 ± 0.5	28.2 ± 0.1	29.2 ± 0.8	28.7 ± 0.5	27.2 ± 0.4	27.3 ± 0.5
E. coli	35.3 ± 0.5	36.7 ± 0.5	36.0 ± 0	35.3 ± 0.5	37.8 ± 0.4 (0.003)**	37.8 ± 0.4 (0.007)*	37.3 ± 0.8 (0.009)*	36.5 ± 0.5 (0.013)*
E. faecalis	32.3 ± 0.5	32.7 ± 0.5	31.5 ± 0.5	31.0 ± 0.6	37.0 ± 0 (0.002)**	36.0 ± 0.6 (0.004)**	36.7 ± 0.5 (0.004)**	36.5 ± 0.5 (0.004)**

Table 1: The effect of cold storage of BASE•128 and LDS, on their antimicrobial activity against five bacterial strains. The efficiency of both solutions was tested in fresh (T0) or aliquots frozen for 1 (T1), 3 (T2) or 6 months (T3). The effect is expressed as a mean ± standard deviation (SD) of a diameter (mm) of the inhibitory zone (IZ), assessed by agar-well diffusion method *(*P ≤ 0.05; **P ≤ 0.005; ***P ≤ 0.001*).

If physical parameters were compared, the LDS had higher pH values after storage than BASE•128 (Table 2). The higher difference was measured between the solutions after 6 months. The osmolarity values of the LDS were measured between 277.50 units (T3) and 392.00 units (T0), while the values of BASE•128 were mostly below range of the osmolarity measurement instrument used in the study (Table 2).

Parameter	Mean ± SD							
	BASE•128				LDS			
	T0	T1	T2	T3	T0	T1	T2	T3
pH	7.46	7.61 ± 0.03	7.52 ± 0.09	7.58 ± 0.07	7.36 ± 0.07	7.61 ± 0.07	7.60 ± 0.03	7.72 ± 0.19
Osmol (mOsm/l)	BR	288.50 ± 19.90	BR	BR	392.00	285.00 ± 4.24	285.00 ± 8.49	277.50 ± 2.12

BR below range of TearLAB® Osmometer

Table 2: The comparison of the pH and osmolarity of the BASE 128 and LDS solutions, stored for different time-periods, i.e. 0 (T0), 1 (T1), 3 (T2) or 6 months (T3).

De-epithelialization of HAM

All three enzymatic methods (TrypLE Express, trypsin/EDTA, and thermolysin) used for de-epithelialization of HAM had comparable efficiency, only few epithelial cells occasionally rested on denuded HAM. The thermolysin application led to loss of mesenchymal spindle-shaped cell morphology of hAMSCs, showing rather round cell shape (Figure 19D).

Collagen type IV and laminin α5 chain showed clear positivity in BM of all control tissues and after TrypLE Express and trypsin/EDTA treatment (Figure 19E, F, G, I, J, K). In half of the specimens, the positive signal of collagen type IV and laminin α5 was spread throughout the whole amniotic stroma after thermolysin application. In these samples the positive line representing BM was not apparent (Figure 19H, L).

The results from SEM showed that BM is well preserved after trypsin/EDTA treatment, only some residues of extracellular matrix are detectable (Figure 19O, S, W). When TrypLE Express was used for de-epithelialization, the BM stayed mostly intact, but partial damage was observed (Figure 19N, R, V). The damaged BM and numerous lesions were observed after thermolysin treatment. The collagen network of compact layer was clearly visible under BM, suggesting aggressive proteolysis (Figure 19P, T, X).

The viability of obtained hAECs immediately after de-epithelialization reached approximately 6% after TrypLE Express, and about 60% when trypsin/EDTA was used. After thermolysin treatment, only dead cells and cellular fragments were observed (Figure 20A). The hAECs harvested after trypsin/EDTA treatment were successfully cultured and their morphology changed from cuboidal shape at the beginning to more mesenchymal-like shape cells in the 4th and 5th passage (Figure 20B). The higher proliferation activity was observed in later passages. The metabolic activity was slightly, but not significantly, increased when hAECs were co-cultured with EGF for 24 hours (Figure 20C). We detected the expression of two stem cell markers in cultured hAECs, SOX2 was present up to 2nd passage and NANOG up to 4th passage. The expression of OCT-4 was present in all passages. Unfortunately, no band was observed when primers for transcription variant specific for stem cells (OCT-4A) were used (Figure 20D).

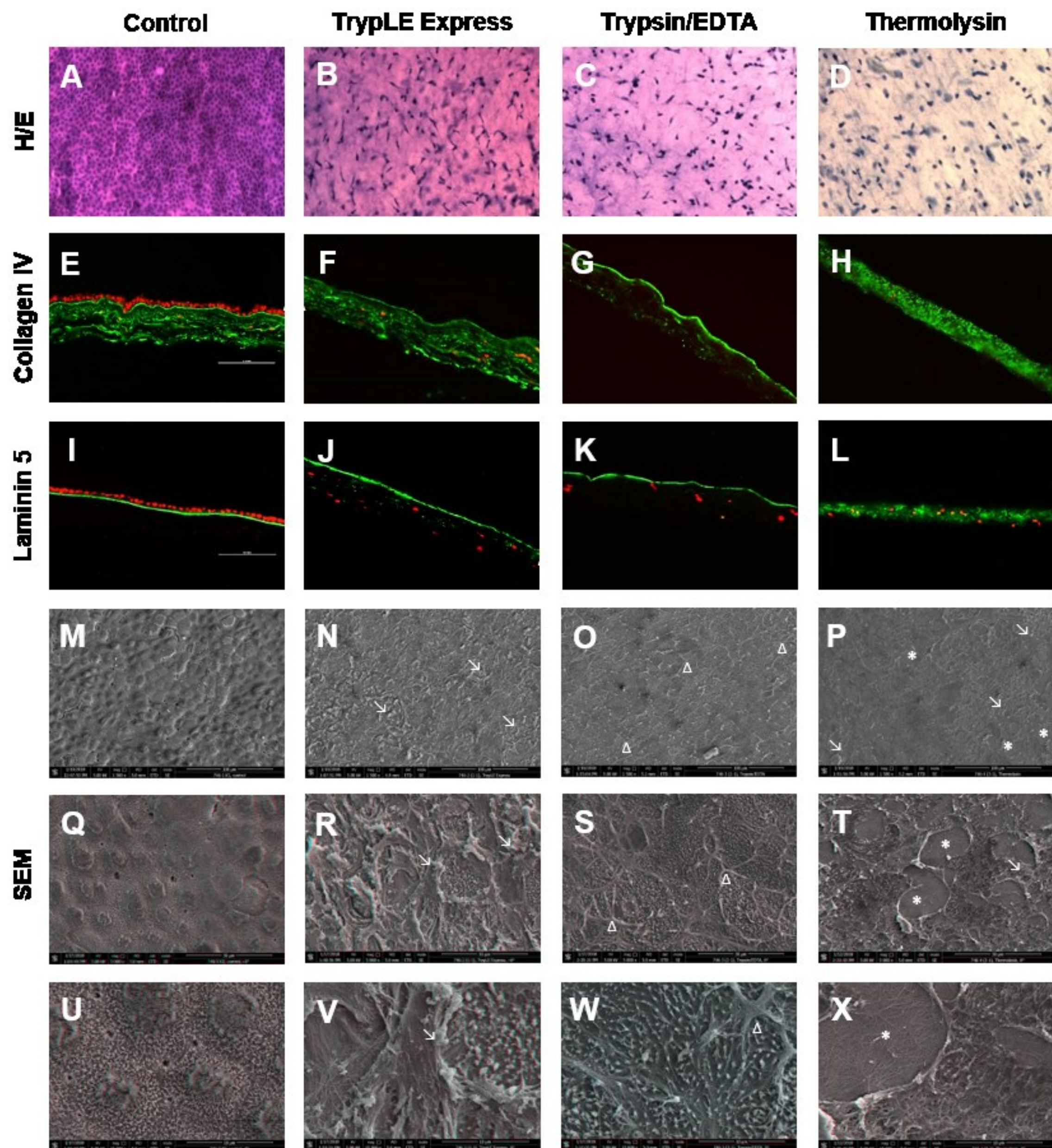

Figure 19: Comparison of the intact (Control) and denuded HAMs by using of light, florescent and SEM. The specimens were stained with hematoxylin/eosin (H/E; A-D), collagen type IV α2 chain (E-H) or laminin α5 (I-L). Scale bars represent 100 µm. Scanning electron micrographs (M-P) and stereo anaglyphs (Q-X) were prepared by SEM. Areas of damaged BM are marked by arrows, ruptured gaps by *, the residues of ECM by Δ. Red-green or red-cyan glasses required for proper view of stereo anaglyphs.

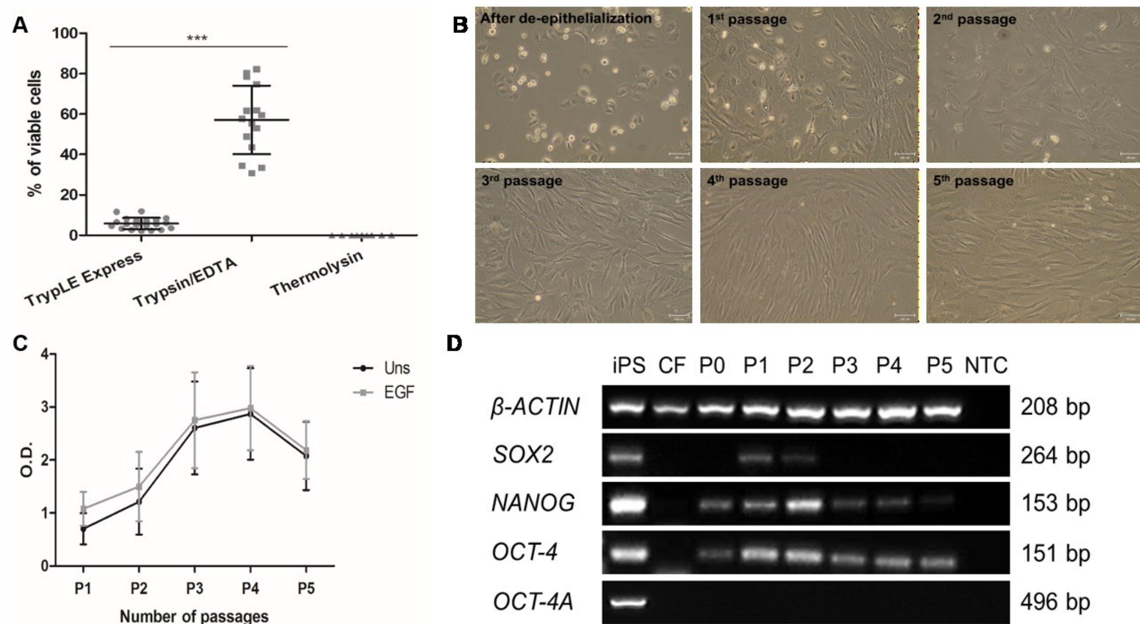

Figure 20: The viability, morphology, growth and expression pattern of hAECs. Comparison of the hAECs viability after TrypLE Express, trypsin/EDTA and thermolysin treatment (A). Cells were stained with Trypan blue and counted via haemocytometer. Each bar represents mean ± SD from 15 determinations (***P < 0.001). The morphology of cultured hAECs after trypsin/EDTA treatment in complete DMEM medium was analysed by light microscopy after de-epithelialization and before each passage (B). Scale bars represent 100 µm. The metabolic activity of unstimulated (Uns) and stimulated hAECs with EGF (EGF) were measured after each passage by WST-1 assay (C). Each bar represents mean ± SD from 3 determinations. The expression pattern of hAECs after de-epithelialization and at each passage was analysed by RT-PCR (D). The induced pluripotent cells (iPS) were used as a positive and corneal fibroblast as negative control (CF). Sample without cDNA (NTC) was used as non-template control. One representative experiment of 3 (with identical results) is shown.

6. Discussion

The maintenance of healthy and transparent cornea is important for the protection of the inner parts of the eye and for penetrating light into the eye. The cornea and limbus can be damaged by chemical, mechanical or thermal injury, or by various disorders. This injury can lead to LSCD, followed by corneal conjunctivalization, neovascularization, epithelial defects and chronic inflammation connected with loss of corneal clarity and vision (Sangwan, 2001). Despite great success of LSCD treatment with transplanted limbal tissue or cultured LESCs, there are still some limitations and complications. Current trend is to improve and to standardize the culture methods, to replace xenogeneic components in culture media, to increase the number of LESCs in cell culture or to reduce the cost. Another challenge is to find the best carrier for the cell culture and for transferring of ex vivo cultured LESCs onto damaged ocular surface.

The outputs of my dissertation can be divided into two parts. Preparation of the cell cultures suitable for the treatment of LSCD is the first part. In the second section I focused on the decontamination and de-epithelialization of the HAM, the most suitable carrier for the transfer of LESCs onto damaged corneal epithelium and one of very effective products used for the healing of chronic wounds.

Part 1: Preparation of the cell cultures

To improve the safeness of the LESCs culture, we prepared the xeno-free culture protocol. LECs cultures, *ex vivo* expanded in two completely xeno-free culture media (human serum medium - hSM and human platelet lysate medium - hPLM), were compared with the complex medium used as a gold standard (Brejchova et al., 2018). Fibrin tissue glue was used as a substrate for LECs culture as it promotes cell growth, maintains LESCs stemness, and enables easy transfer to the patient's eye (Forni et al., 2013a).

In all our experimental conditions, 57% (hPLM, plastic) to 80% (COM, plastic) of explants gave rise to confluent cultures. Comparable results were reached during explant expansion in FBS supplemented complex medium on amniotic membrane (Kethiri et al., 2017) or on plastic (Ghoubay-Benallaoua et al., 2011). Our cultures reached full confluency approximately after three weeks. We were the first ones to publish the comparison of the cell growth kinetics of complex and xeno-free media. The shortest time needed for reaching the confluence was achieved in both hSM and COM on fibrin. This may reflect the fact that fibrin promotes better cell attachment and growth (Reinertsen et

al., 2014). The cell growth in hPLM on fibrin was significantly delayed. This fact can be negatively connected to the higher fibroblast-like cell contamination in hPLM cultures. Resulting from our experience, longer LECs culture leads to increased and subsequently overgrowth of the fibroblast-like cells in the culture wells. The contamination was mostly lower on plastic than on fibrin substrate.

We observed that more than 50% of cells were stained for stem cell markers p63α, Bmi-1, K15 and ABCB5 in all conditions. Similar results were achieved for p63- and ABCB5-staining in LECs cultures grown in COM on plastic with significantly more p63α-positive cells in COM compared to xenofree media (Lopez-Paniagua et al., 2013; Loureiro et al., 2013). In contrast to that, we did not find any significant difference in *TP63* gene expression of *p63α* isotype in cells grown in all three media. The higher percentages of p63α- and ABCB5-positive cells were detected on fibrin compared to plastic in all tested media. These results confirm the suggestion that fibrin scaffold maintains LESCs stemness (Forni et al., 2013b; Han et al., 2002).

It was reported that about 60-80% of K3- and K12-positive cells were observed in culture with COM on plastic, what indicates the unwanted differentiation of cells into corneal phenotype (Lopez-Paniagua et al., 2016; Loureiro et al., 2013). Unlike these studies, we detected less than 43% of K3/12-positive cells in all conditions. Moreover, the cells cultured in hSM on plastic had expressed even lowest positivity (15%). The higher *K3* gene expression was found in the xeno-free media compared to COM on fibrin. Similarly, LECs culture in hPLM led to increased cell differentiation compared to hSM. This can indicate that hPLM supports better cell differentiation into corneal epithelial phenotype.

The positivity for Ki-67, cell cycle marker indicating dividing cells, was detected in comparable number in all three media on plastic. However, the cells grown in COM and hSM on fibrin exhibited significantly higher proliferative activity, which is in accordance with previous study (Forni et al., 2013b). Only a low rate of apoptosis was observed in our cultures, with no significant difference among the studied conditions. It means that our method is safe and LESCs are well protected.

The outcome of this study is a xeno-free culture protocol for improving the safety of LECs transplantation in patients with LSCD. Preparation of LECs culture with the hSM media and fibrin showed same or better results compared to COM. The use of hSM led to adequate growth rate and stemness maintenance. Second xeno-free media, hPLM, was less efficient as higher number of contamination and differentiated cells were noticed.

The impairment of ocular surface often invades both the cornea and conjunctiva. Conjunctival disorders or injuries can cause scarring of the conjunctiva what consequently can lead to anatomical or functional defect. For its successful cell therapy, it is important to expand both conjunctival cell types, conjunctival epithelial and GCs. It is difficult to maintain the growth of GCs during culture of conjunctival cells *in vitro*.

We have successfully prepared conjunctival epithelium, composed of epithelial and GCs, using limbal explants from human corneoscleral rims (Stadnikova et al., 2019). The number of GCs in our culture was about 13-15 GCs/mm^2, what was more than 0.5-0.6 GCs/mm^2 reported by Ang (Ang et al., 2005), and lie between the GC levels obtained by Pellegrini et al. in non-confluent and confluent cultures (Pellegrini et al., 1999). In our study we used IL-13 with the aim to accelerate GCs proliferation and mucin synthesis. One of our ideas was to separate GCs and use them for treatment of conjunctival damage. The positive effect of IL-13 on murine GC numbers (Contreras-Ruiz et al., 2013; Tukler Henriksson et al., 2015) and human MUC5AC secretion has been published (Garcia-Posadas et al., 2017). However, we did not find any difference in number of GCs between the IL-13+ and IL-13- groups. We were able to prepare subpopulation of cells with GC phenotype. The subpopulation of unattached cells P1d1, harvested one day after passage of primary cells, had typical GC morphology. AB/PAS staining revealed about 28% and 24% of positive cells in IL-13- and IL-13+ group, respectively. On the other hand, P1d1 cells did not exhibit clonogenic ability, which is consistent with the proposal that human conjunctival GCs are terminally differentiated (Pellegrini et al., 1999). Because there is no clonogenic activity, this subpopulation of unattached cells cannot be used for therapeutical purposes as it was thought.

The positivity for K7, conjunctival cell marker, was detected in epithelial and GCs as well (Jirsova et al., 2011). In our study, the IL-13+ cell cultures had significantly higher K7 expression and more histologically stained GCs in P2 cells compared to P0 cells. This finding is consistent with the increasing differentiation into conjunctival phenotype observed throughout P0, P1, and P2.

The expression of genes for K3 and K12, markers of corneal epithelial cells, was detected. We found that IL-13 does not alter their expression significantly. Moreover, the expressions of *K3* and *K12* genes were lower than the expression level of *K7* gene. Therefore, we conclude that our cultures differentiate more to the conjunctival than to the corneal phenotype. This finding is favorable, as we want to use these cells for the treatment of conjunctival damage.

We detected a high percentage of p63α positive cells (about 90%) in P0 and P1 culture with no significant difference between IL-13 groups. Results from qPCR detection showed higher expression of *TP63* gene in the IL-13+ cultures, in P1 with significant difference, than in IL-13- cultures. It confirms our hypothesis that IL-13 maintains the stemness of the cultures.

The clonogenic ability of P0 IL-13- cultures was 1%, what is similar to human conjunctival tissue (Garcia-Posadas et al., 2017; Stewart et al., 2015) and limbal explants (Lopez-Paniagua et al., 2016). The inhibition or stimulation of the colony formation by IL-13 was reported on the haematopoietic progenitor cells (Jacobsen et al., 1994; Xi et al., 1995). In our P0 IL-13+ cultures, the clonogenic ability increased to 8%. The same tendency was visible in *TP63* expression in IL-13+ cultures and in K7 staining in P2 IL-13+ cells. Thus, IL-13 possesses double effect, the maintenance of stemness and the preservation of differentiation. LESCs are divided via asymmetric cell division (Gomez-Flores et al., 2011). Therefore, the more stem cells present in the cell culture, the more differentiated cells will be generated (IL-13+ cultures). Vice versa, the decreased number of stem cells leads to decreased number of differentiated cells, because terminally differentiated cells do not proliferate (IL-13- cultures). This is confirmed by the increased number of Ki-67 positive proliferating cells in P1 IL-13+ cultures.

In conclusion, we prepared an engineered epithelium with the presence of stem cells, cells with conjunctival phenotype and a relatively high density of GCs. Moreover, we demonstrated that IL-13 maintains the stemness of the cultures. For the first time, we raise the possibility of using corneoscleral rims as an alternative source for engineering a conjunctival epithelium that could be used for further research on GCs and for treating patients with ocular surface disorders.

Despite progressive development of regenerative medicine and initial success, there is still problem with the long-term treatment of damaged ocular surface in allogeneic limbal or LESCs transplantations. As it was mentioned earlier, other autologous cell types are tested for usage in treatment of LSCD. Embryonic stem cells were used to differentiate into corneal epithelial-like cells (Ahmad et al., 2007), or corneal keratocyte phenotype (Chan et al., 2013), and transplanted on wounded human cornea *in vitro* (Hanson et al., 2013a). Induced pluripotent stem cells generated from various sources were used for differentiation into corneal epithelial-like cells (Hayashi et al., 2012; Yu et al., 2013), or for corneal repair (Chien et al., 2012). MSCs with their ability to differentiate and immunomodulatory properties have great potential to be used in stem cell therapy. The co-

culture of MSCs with corneal cells in limbal or corneal cell-conditioned medium leads to differentiation into keratocytes (Du et al., 2010; Park et al., 2012) or cells with markers and characteristics of corneal epithelial cells (Gu et al., 2009; Nieto-Miguel et al., 2013; Rohaina et al., 2014).

Previously, we found that IGF-I supports the differentiation of LSCs into corneal-like cells (Trosan et al., 2012). We were curious whether mouse bone marrow-derived MSCs have the potential to differentiate into corneal epithelial cells using the extract from the cornea, and whether the differentiation process could be increased by the IGF-I.

We observed that already after a 3-day culture of the MSCs with the extract, the cells started to express corneal markers and their expression gradually increased, what is in accordance with the previously mentioned differentiation studies. Adding IGF-I into these cultures it increased significantly the expression of genes for *K12*, keratocan and lumican, while IGF-I alone had no effect on the differentiation. Differentiated MSCs did not change their morphology, as it was shown in other studies (Jiang et al., 2010; Rohaina et al., 2014). No difference in expression of mesenchymal cell surface markers were found between untreated and differentiated cells. Differentiated MSCs had slightly enhanced metabolic activity compared to untreated cells. This is in accordance with the studies which compared the proliferative and metabolic activity of differentiated cells (Huang et al., 2012; Kaplan et al., 2005). The immunosuppressive potential of MSCs was proved by inhibition of the production of IFN-γ and IL-2, with no difference between differentiated and unstimulated cells. In this study we showed that IGF-I supports differentiation of mouse MSCs into the cells expressing markers of corneal epithelial cells and keratocytes. The differentiation into keratocytes was not expected. On the other hand, the population of cells in the cornea is heterogenic. Therefore, the use of differentiated heterogenic MSCs might be more effective in bilateral LSCD treatment. The differentiated cells still possess the characteristics of MSCs and furthermore they suppress the production of proinflammatory cytokines by activation of T lymphocytes. All of these properties can be used in treatment and regeneration of damaged or diseased cornea.

Part 2: Preparation of HAM

Successful stem cell therapy is based on three keystones, the selection of cells, culture conditions and choice of material that is used as a carrier or scaffold for the transplantation. Various biological or synthetical materials have been tested already. Despite of developing new scaffolds, the HAM still remains as gold standard for feeder of

LECs culture. It is not only the carrier for cells or tissue for healing the skin and corneal defects, but it is also the source of cells with stem cell properties. HAM is obtained from the placenta after elective caesarean section and has to be decontaminated before its application. Various protocols and solutions for sterilization are known, but the toxicity of disinfectants on HAM cells is still not good examined.

Therefore, we compared the effect of the commercial solution BASE•128 and laboratory prepared LDS, with analogous composition of antibiotics-antimycotics, on HAM structure with the focus on the cell viability (Smeringaiova et al., 2017). In fresh HAM, we observed 4.8-28.1% of dead epithelial cells, what is similar to other studies (Hennerbichler et al., 2007; Laurent et al., 2014). The higher viability of epithelial cells was achieved using BASE•128 and with the decontamination protocol for 6h and 37°C, followed by 3h and 24h storage. The worst survival rate was detected in control tissue at 4°C. The temperature between 12 and 24°C was also evaluated as the most suitable for preservation of cell morphology by Jackson et al. (Jackson et al., 2016). The viability of stromal MSCs was determined by TUNEL assay, showing 42% apoptotic cells before and 63-88% after the decontamination. This relatively high increase of hAMSCs apoptotic cells compared to hAECs (2%) can be explained by the release of hAECs from the basement membrane, while hAMSCs remain in the stroma.

We found that only small differences in cell viability were detected between BASE•128 and LDS. Thus, we also compared the antimicrobial efficiency and stability of both solutions after 1-, 3- and 6-months storage (Smeringaiova et al., 2018). The standard and clinical bacterial strains were used for this study. Both decontaminations were most effective in the following descending order against *P. mirabilis*, *P. aeruginosa*, *E. coli* and *E. faecalis*. The efficacy against *P. aeruginosa* was comparable with only small differences for both solutions. BASE•128 maintains its antimicrobial efficacy for longer storage, so it is more stable than LDS. On the contrary, LDS was more efficient against *E. coli* and *E. faecalis*. The lowest antimicrobial activity was measured against *S. aureus* in both solutions. The values of pH increased in both solutions after cold storage and 6 months; in LDS the increase was more prominent.

We showed that BASE•128 and LDS are comparable in cell viability and antimicrobial properties. BASE•128 is more practical from user's aspect, but LDS can be easily prepared anytime in the laboratory and used when commercial solution is not available. This result has a practical impact already. The most efficient protocol for both

solutions is used for decontamination and preparation of HAMs for grafting particularly in ophthalmic surgery and for treatment of non-healing wounds in our current clinical study.

Intact or denuded HAM can be used in experimental or clinical praxis. Intact HAM is more suitable for covering damaged ocular surface or particular corneal grafting. De-epithelialized HAM is more appropriate for LESCs culture. Because of the advantages of denuded HAM and hAECs with stem cells characteristics, we decided to find the best approach for obtaining of both at the same time. TrypLE Express (recombinant fungal trypsin-like protease), trypsin/EDTA or thermolysin (zinc neutral, heat-stable metalloproteinase) were used in our study (Trosan et al., 2018). The staining of HAM and DNA concentration measurement confirmed that all three solutions were efficient for de-epithelialization of the HAM. The detection of collagen type IV and laminin α5, as components of BM, showed that application of TrypLE Express and trypsin/EDTA did not disturb the integrity of the BM. This was in accordance with previous result (Zhang et al., 2013). Almost 50% of the specimens after thermolysin treatment showed the dispersion of the collagen type IV and laminin α5 in HAM stroma. The changes in cell morphology was also visible after application of this solution. Moreover, scanning electron microscopy confirmed the disruption of the BM. The certain damage of BM, fragility and difficulty handling HAM after thermolysin treatment was reported (Saghizadeh et al., 2013; Siu et al., 2008). The higher effectivity of this procedure was achieved by replacement of mechanical scrapping for simple washing (Hopkinson et al., 2008). In our experiments we were unable to denude HAM properly. Obtained results indicate that natural activity of this enzyme, cleavage of collagen IV, led to damage of the BM. Moreover, the de-epithelialization with thermolysin resulted also in complete loss of hAECs viability, while 60% and 6% of living cells were observed after trypsin/EDTA and TrypLE Express treatment, respectively.

The hAECs obtained by trypsin/EDTA, as this method presented the most viable cells after de-epithelialization, were used for subsequent culture. The proliferation activity of cells increased up to the 4th passage and the cell morphology changed from cuboidal to more mesenchymal-like cell shape. The change of morphology of the cultured hAECs was also observed (Fatimah et al., 2010). All these changes could be caused by epithelial to mesenchymal transition by autocrine production of TGF-β during the culture of hAECs (Alcaraz et al., 2013).

The stem cell markers gene expression was detected in cultured hAECs after each passage. The expression of *NANOG* was observed in each passage; *SOX2* was present in the first two passages only. The detection of expression of *OCT-4* gene is more difficult, as

it is encoded by two spliced variants. The *OCT-4A* variant is expressed in embryonic stem cells, *OCT-4B* is detected in various nonpluripotent cells (Atlasi et al., 2008; Lee et al., 2006b). The primers fitted on both variants were used in many studies (Fatimah et al., 2012; Garcia-Castro et al., 2015). In contrast to Izumi et al. (Izumi et al., 2009), we were not able to detect the expression of *OCT-4A* spliced variant, specific for hAECs. This can be caused by lower efficiency of our reverse transcription PCR compared to quantitative real time PCR and commercial primers with probes. In summary of gene expression experiments we can conclude that the stemness of hAECs decreases with each passage.

Out of three tested de-epithelialization protocols, the trypsin/EDTA treatment is the most efficient approach. It leads to successful de-epithelialization of HAM with undamaged BM and at the same time to harvesting of epithelial cells with good viability. The proliferation of hAECs increases and maintenance of stem cell markers decreases during culture and passaging.

7. Conclusion

The stem cell-based therapy noted a great and promising improvement in recent years. Despite the breakthrough in treatment of LSCD, a standardized therapy should be a continuous process. Therefore, I was working on improving culture protocols for preparing cells and tissues for ocular transplantation. I consider the following outputs as main results of my work:

- Preparation of a protocol for LECs culture with xeno-free media. This can simplify the approval process by State Institute for Drug Control and subsequently it can be used in clinical practise, as this treatment is still missing in Czech Republic (Hypothesis 1).
- The design of a protocol for culture conjunctival epithelial and GCs from corneoscleral rims, that can find application in treatment of conjunctival damage (Hypothesis 2).
- The enhancement of differentiation of murine MSCs into corneal-like cells by using of IGF-I with the corneal extract (Hypothesis 3).
- Test and development of a laboratory decontamination solution for decontamination of HAM, that is already in use for preparation of HAM for ocular transplantations and treatment of nonhealing wounds (Hypothesis 4).
- Establishment of a protocol in which both undamaged de-epithelialized HAM and viable hAECs can be obtained in one step (Hypothesis 5).

8. Souhrn

V posledních letech zaznamenala buněčná terapie na mezinárodní úrovni veliký pokrok. I navzdory průlomu v léčbě LSCD, je třeba standardizovanou terapii neustále zlepšovat. Z tohoto důvodu jsem pracoval na inovaci protokolů pro kultivaci a přípravu buněk a tkání pro transplantace v očním lékařství. Za hlavní výstupy mé práce považuji:

- Přípravu protokolu pro kultivaci limbálních epitelových buněk bez použití xenogenních médií, která povede ke zjednodušení schvalovacího procesu Státním ústavem pro kontrolu léčiv a následné využití metody v klinické praxi. Tento typ léčby v České republice dosud chybí. (Hypotéza 1).
- Návrh protokolu pro kultivaci spojivkových epitelových a pohárkových buněk z explantátu korneosklerálního terče. Metoda může najít uplatnění v léčbě poškození spojivky (Hypotéza 2).

- Pomocí použití IGF-I s rohovkovým extraktem jsme dosáhli zvýšení efektivity diferenciace myších MSCs do buněk podobných rohovkovým buňkám (Hypotéza 3).
- Přípravu a testování laboratorního dekontaminačního roztoku pro dekontaminaci amniové membrány, který se následně začal využívat při přípravě amniové membrány pro oční transplantace a léčbu dlouhodobě se nehojících ran (Hypotéza 4).
- Přípravu protokolu pro získání nepoškozené de-epitelizované amniové membrány a životaschopných buněk epitelu v jednom kroku (Hypotéza 5).

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10. List of publications related to the Thesis

1. Brejchova K, **Trosan P**, Studeny P, Skalicka P, Utheim TP, Bednar J, Jirsova K: Characterization and comparison of human limbal explant cultures grown under defined and xeno-free conditions. Exp Eye Res. 2018 Jun 18. Doi: 10.1016/j.exer.2018.06.019. PMID: 29928900. *IF:3.332.*

2. Stadnikova A, **Trosan P**, Skalicka P, Utheim T P, Jirsova K: Interleukin-13 maintains the stemness of conjunctival epithelial cell cultures prepared from human limbal explants. PLoS One. 2019 Feb 11;14(2): e0211861. Doi: 10.1371/journal.pone.0211861. PMID: 30742646. *IF:2.766.*

3. **Trosan P**, Javorkova E, Zajicova A, Hajkova M, Hermankova B, Kossl J, Krulova M, Holan V: The supportive role of insulin-like growth factor-I in the differentiation of murine mesenchymal stem cells into corneal-like cells. Stem Cells Dev. 2016 Jun 1;25(11):874-81. Doi: 10.1089/scd.2016.0030. PMID: 27050039. *IF: 3.777.*

4. Smeringaiova I, **Trosan P**, Mrstinova MB, Matecha J, Burkert J, Bednar J, Jirsova K: Comparison of impact of two decontamination solutions on the viability of the cells in human amnion. Cell Tissue Bank. 2017 Sep;18(3):413-423. Doi: 10.1007/s10561-017-9636-3. PMID: 28677080. *IF: 1.331.*

5. Smeringaiova I, Nyc O, **Trosan P**, Spatenka J, Burkert J, Bednar J, Jirsova K: Antimicrobial efficiency and stability of two decontamination solutions. Cell Tissue Bank. 2018 Jul 30. Doi: 10.1007/s10561-018-9707-0. PMID: 30062597. *IF: 1.331.*

6. **Trosan P**, Smeringaiova I, Brejchova K, Bednar J, Benada O, Kofronova O, Jirsova K: The enzymatic de-epithelialization technique determines denuded amniotic membrane integrity and viability of harvested epithelial cells. PLoS One. 2018 Mar 27;13(3): e0194820. Doi: 10.1371/journal.pone.0194820. PMID: 29584778. *IF: 2.766.*