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Short review of the Ph.D. Thesis

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

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Table of contents

Abstrakt **4**

Abstract **5**

Abbreviations **5**

1.Introduction **8**

1.1. Cornea 8

1.2. Limbal epithelial stem cells 9

1.3. Limbal stem cells deficiency 10

1.4. Conjunctiva 11

1.5. Amniotic membrane 11

2. Hypotheses and aims of work **12**

3. Material and Methods **14**

4. Results **15**

5. Discussion **18**

Part 1: Preparation of the cell cultures 18

Part 2: Preparation of HAM 20

6.Conclusion **23**

7. References **24**

8. List of publications related to the Thesis **30**

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

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
DMEM	Dulbecco's Modified Eagle Medium
CFE	colony forming efficiency
COM	complex medium
GCs	goblet cells
hAECs	human amniotic epithelial cells
HAM	human amniotic membrane
hAMSCs	human amniotic mesenchymal stromal cells
HLA	human leucocyte antigen
hPLM	human platelet lysate medium
hSM	human serum medium
IGF-I	insulin-like growth factor-I
IL	interleukin
K	keratin
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
PCR	polymerase chain reaction
SEM	scanning electron microscopy
SOX2	sex determining region Y-box 2
TACs	transient amplifying cells
TP63 (p63)	tumor protein p63
ΔNp63	ΔN isoform of TP63 gene

1.Introduction

1.1. Cornea

Cornea is a transparent avascular tissue that consists of the outermost epithelium with its basement membrane, Bowman´s layer, stroma, Descemet´s membrane and the inner endothelium and it is formed by three main cell types: epithelial cells, keratocytes and endothelial cells.

1.1.1. 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, wing and basal cells, with the lifespan of 7 to 10 days (Hanna et al., 1961). 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.

1.1.2. Bowman´s layer

Bowman´s layer is an acellular condensate of collagen and proteoglycans between corneal epithelium and stroma. Biological function of Bowman´s layer is still unclear. The layer does not regenerate after an injury and as consequence of damage it can results in a scar.

1.1.3. Stroma

The corneal stroma consists of extracellular matrix, keratocytes and nerve fibres. 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). Keratocytes 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.

1.1.4. Descemet´s membrane

Descemet´s membrane is the basement membrane secreted by endothelial cells. In case of Descemet´s membrane rupture, the membrane does not regenerate, and the aqueous humour penetrates to the corneal stroma (stromal oedema).

1.1.5. Endothelium

The corneal endothelium is a single layer of cells located on the posterior side of the cornea with the cell density 3500 cells/mm^2 (Watsky et al., 1989). 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.

1.2. 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). Corneal epithelial stem cells, called limbal epithelial stem cells (LESCs), reside in the basal layer of the limbus, the transition zone between cornea and conjunctiva (Cotsarelis et al., 1989; Schermer et al., 1986). LESCs are located in the region with stromal invagination known as Palisades of Vogt (Goldberg and Bron, 1982). 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., 2007).

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).

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). Many other genes have been considered as LESCs markers based on their expression in the basal epithelia, like ATP-binding cassette transporter group 2 protein (*ABCG2*) (Chen et al., 2004), integrin α9 (Stepp et al., 1995), N-cadherin (Hayashi et al., 2007), keratin 19 (*K19*) (Kasper et al., 1988), vimentin (Schlotzer-Schrehardt and Kruse, 2005), or ATP-binding cassette, sub-family B, member 5 (*ABCB5*) (Ksander et al., 2014). On the other hand, limbal basal cells lack differentiation markers K3, 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 balance between cell proliferation, differentiation, migration and apoptosis is necessary and is driven by the various cytokines and growth factors. Insulin-like growth factor-I (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 (Trosan et al., 2012).

1.3. Limbal stem cells deficiency

The functional or anatomical loss of limbus results in limbal stem cells deficiency (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 disorder can affect one (unilateral) or both (bilateral) eyes with partially or totally damaged limbus. The most successful treatment is transplantation of limbal tissue or LESCs from same patient with unilateral disease (autologous transplantation) (Pellegrini et al., 1997, Rama et al., 2010). 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).

Because use of allograft transplant is problematic, other types of cells suitable for LSCD treatment are also being investigated. The use of mesenchymal stem cells (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).

The human amniotic membrane (HAM) and fibrin are the most successfully used substrates and scaffolds for cultivated LESCs and for transfer of cells onto ocular surface, respectively (Pellegrini et al., 1997; Sangwan et al., 2011).

1.4. Conjunctiva

Conjunctiva is a mucous membrane which provides coverage of the ocular surface. Conjunctiva is composed of non-keratinizing stratified epithelium and highly vascularised stroma. The epithelium consists of 6-9 layers of epithelial cells, goblet cells (GCs), Langerhans cells and melanocytes. The lubrication of the ocular surface and stabilization of the ocular film is supported by the mucins, high-molecular weight glycoproteins (Gipson, 2016).

Conjunctival stem cells are dispersed throughout the conjunctival tissue. They are bipotential and give rise to both epithelial cells and GCs (Pellegrini et al., 1999).

1.5. Amniotic membrane

HAM is the most frequently used carrier for cultivation of LESCs and one of very effective products used for the healing of chronic wounds. It is the innermost layer of the placenta, and it is composed of a monolayer of epithelial cells, a thick basement

membrane and an avascular stroma (Niknejad et al., 2008). HAM promotes epithelialisation and wound healing mediated by numerous growth factors and cytokines (Koizumi et al., 2000). It was believed that HAM has no immunogenicity, later was observed that small amounts of human leucocyte antigens (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).

Two cell types, human amniotic epithelial cells (hAECs) and human amniotic mesenchymal stromal cells (hAMSCs) are located in HAM. The hAECs have highly multipotent differentiation ability and express pluripotent stem cells-specific transcription factors (Miki et al., 2005; Miki, 2018; Simat et al., 2008).

2. Hypotheses and aims of work

LESCs culture (Hypothesis 1):

The LESCs, used for the treatment of LSCD, are usually cultivated with the use of xenogeneic components, what can potentially result in transmission of infection diseases or immune reaction. We supposed that the replacement of animal components will increase the safety of the grafted cells. Moreover, we hypothesized that the culture of LESCs on fibrin glue may results in improvement of this culture technique.

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):

We considered the possibility to cultivate the conjunctival epithelium containing GCs from corneoscleral rims. Moreover, we

hypothesized that cultivation and differentiation of these cells could be enhanced by the effect of interleukin-13 (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. We hypothesized that the cultivation of murine MSCs with IGF-I and corneal extract may results 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):

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 laboratory decontamination solution (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):

Deepithelialized HAM is more suitable for cultivation of LESCs compared to intact. Moreover, amniotic epithelial cells have the ability to differentiate, low immunogenicity, anti-inflammatory effect and express the stemness genes. We hypothesized that by using a gentle enzymatic method would be possible to achieve both products in one step, 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.

3. 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:

- Preparation and culture of LESCs from limbal explants.
- Culture and preparation of 3T3 feeder layers.
- Isolation, culture and purification of murine MSCs from the bone marrow of BALB/c mice.
- Differentiation of MSCs into corneal-like cells.
- Processing of the placenta with the preparation of the HAM.
- HAM de-epithelialization and culture of the hAECs.
- Determination of the clonal growth ability of the cultured cells with colony forming efficiency assay.
- Determination of the metabolic activity of living cells by Wst-1 assay.
- RNA extraction, reverse transcription and the detection of gene expression by quantitative real time polymerase chain reaction (PCR) analysis.
- Immunohistochemistry and immunocytochemistry.
- Statistical analysis.

4. Results

The most important results of the work performed are summarized below. For more detailed results, please see the 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 complex medium (COM) used as a gold standard.

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

The number of differentiated K3/12-positive cells varied between 15–43% in all cultures cultivated on both substrates in all media. The cultures grown on plastic had slightly lower cell proliferation compared to that cultured on fibrin. The only significant differences were observed between cells cultivated in COM and hSM media.

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

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).

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. Detection of gene expression showed strong expression of *K7* gene in IL-13- and IL-13+ conditions and in all passages. The median values of *K7* expression were higher in all passages of IL-13+ cells compared to IL-13- cells. The gene expression of the *K3* and *K12* genes were expressed at much lower levels than the expression of K7 gene.

The absolute number of GCs in the P1 IL-13-group was significantly higher than that in the P1 IL-13+ group. MUC5AC immunostaining confirmed the presence of single and grouped GCs in all tested groups. Same results were confirmed by qPCR but with no statistical significance.

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. Significant decrease of p63α positivity was demonstrated in P2 cell cultures. The addition of IL-13 into the P2 culture increased the p63α positivity without significance. All IL-13+ cell cultures had higher median of *TP63* expression than IL-13- 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.

Differentiation of MSCs

The MSCs were cultured alone or with the corneal extract or with the corneal extract and recombinant IGF-I 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.

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

from the WST-1 assay showed that proliferative activity was even higher in differentiated cells than in untreated MSCs. Both control and differentiated MSCs inhibited the production of IL-2 and interferon-γ in spleen cells stimulated with Concanavalin A.

HAM decontamination

HAMs were decontaminated in BASE•128 or LDS or incubated in DMEM medium, this last one for control. The visually confirmed increase in the percentage of dead epithelial cells was observed when longer decontamination period (24h) was used.

In the fresh HAM samples the percentage of apoptotic epithelial cells (AECs) was less than 1% and the percentage of apoptotic mesenchymal cells (AMCs) was 42.0%. In all HAMs after decontamination and in all conditions the number of AECs remained low, about 1–2%. The percentage of AMCs increased significantly up to 87.9%, compared to fresh HAM.

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.

If physical parameters were compared, the LDS had higher pH values after storage than BASE•128. The higher difference was measured between the solutions after 6 months.

De-epithelialization of HAM

All three enzymatic methods (TrypLE Express, trypsin/EDTA, and thermolysin) used for de-epithelialization of HAM had comparable efficiency. The thermolysin application led to loss of mesenchymal spindle-shaped cell morphology of hAMSCs, showing rather round cell shape.

Collagen type IV and laminin α5 chain showed clear positivity in BM of all control tissues and after TrypLE Express and trypsin/EDTA treatment. 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. The results from scanning electron microscopy (SEM) showed that BM is well preserved after trypsin/EDTA. When TrypLE Express was used for de-epithelialization, the basement membrane (BM) stayed mostly intact, but partial damage was observed. The damaged BM and numerous lesions were observed after thermolysin treatment.

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. 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. The higher proliferation activity was observed in later passages. 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.

5. Discussion

The outputs of my dissertation can be divided into two parts; the preparation of the cell cultures suitable for the treatment of LSCD and the decontamination and de-epithelialization of the HAM.

Part 1: Preparation of the cell cultures

To improve the safeness of the LESCs culture, we prepared the xeno-free culture protocol (Brejchova et al., 2018). In all our experimental conditions, 57% (hPLM, plastic) to 80% (COM, plastic) of explants gave rise to confluent cultures. 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.

We observed that more than 50% of cells were stained for stem cell markers p63α, Bmi-1, K15 and ABCB5 in all conditions. 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). 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).

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. For successful conjunctival cell therapy, it is important to expand both conjunctival cell types, conjunctival epithelial and GCs.

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). In our study we used IL-13 with the aim to accelerate GCs proliferation and mucin synthesis. However, we did not find any difference in number of GCs between the IL-13+ and IL-13- groups.

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). 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.

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. MSCs with their ability to differentiate and immunomodulatory properties have great potential to be used in stem cell therapy.

We observed that already after a 3-day culture of the MSCs with the corneal extract, the cells started to express corneal markers and their expression gradually increased. 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.

Part 2: Preparation of HAM

The HAM is used 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. Therefore, we compared the effect of the commercial solution BASE•128 and laboratory prepared LDS 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).

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). 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 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. 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.

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, trypsin/EDTA or thermolysin

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. Moreover, SEM 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). 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 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 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. 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.

6. 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).

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8. 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.*

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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.*