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Cultivation and characterization of pterygium as an *ex vivo* study model for disease and therapy

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ABSTRACT

Purpose: Development of *ex vivo* model to study pathogenesis, inflammation and treatment modalities for pterygium.

Methods: Pterygium obtained from surgery was cultivated (3 months). Gravitational attachment method using viscoelastic facilitated adherence of graft and outgrowing cells. Medium contained serum as the only growth supplement with no use of scaffolds. Surface profiling of the multi-layered cells for hematopoietic- and mesenchymal stem cell markers was performed. Examination of cells by immunohistochemistry using pluripotency, oxidative stress, stemness, migration and proliferation, epithelial and secretory markers was performed. The effect of anti-proliferative agent Mitomycin C upon secretion of pro-inflammatory cytokines IL-6 and IL-8 was assessed.

Results: Cells showed high expression of migration- (CXCR4), secretory- (MUC1, MUC4) and oxidative damage- (8-OHdG) markers, and low expression of hypoxia- (HIF-1α) and proliferation- (Ki-67) markers. Moderate and low expression of the pluripotency markers (Vimentin and ΔNp63) was present, respectively, while the putative markers of stemness (Sox2, Oct4, ABCG-2) and epithelial cell markers- (CK19, CK8-18) were weak. The surface marker profile of the outgrowing cells revealed high expression of the hematopoietic marker CD47, mesenchymal markers CD90 and CD73, minor or less positivity for the hematopoietic marker CD34, mesenchymal marker CD105, progenitor marker CD117 and attachment protein markers while low levels of IL-6 and IL-8 secretion *ex vivo*, were inhibited upon Mitomycin C treatment.

Conclusion: Ex vivo tissue engineered pterygium consists of a mixture of cells of different lineage origin, suitable for use as a disease model for studying pathogenesis *ex vivo*, while opening possibilities for new treatment and prevention modalities.

1. Introduction

Pterygium is a common ocular surface disorder characterized by an active wing-shaped overgrowth of epithelial and fibrovascular tissue growing from the conjunctiva towards the limbus and onto the cornea, thus giving cosmetic alterations to the ocular surface and in advanced cases impairing vision [1–4]. It consists of a head which falls onto the anterior cornea, a neck which covers the superficial limbus, and a body which overlies the sclera. The first sign of pterygium is formation of a cap which appears as halo in front of the head and goes deep into the epithelium without respecting the limbal epithelium stem cell border [5]. It is not fully known whether the stem cell deficiency alone or other factors drive the centripetal migration of the pterygium cells onto the cornea, or how sclera support may play a role in the process.

The pathogenesis of pterygium remains uncertain, although it is thought to be mainly caused by ultraviolet (UV) radiation. A wide range of alternative pathogenic factors have been proposed, including viral infections, epigenetic aberrations, epithelial-mesenchymal transition, immunologic and anti-apoptotic mechanisms, angiogenic and lymphangiogenic stimulation, deregulation of extracellular matrix (ECM) modulators and growth factors, inflammation cascades, recruitment of bone-marrow-derived stem- and progenitor cells, and modifications in the cholesterol metabolism. Most of these factors are rather related to the development and maintenance of the disease than to its origin [6].

In the present study, cells were adherently cultivated and grown out of the pterygium using gravitational force from viscoelastic material for more than three months or until they formed multi-layered structures. Surface marker profiling using hematopoietic- and mesenchymal stem

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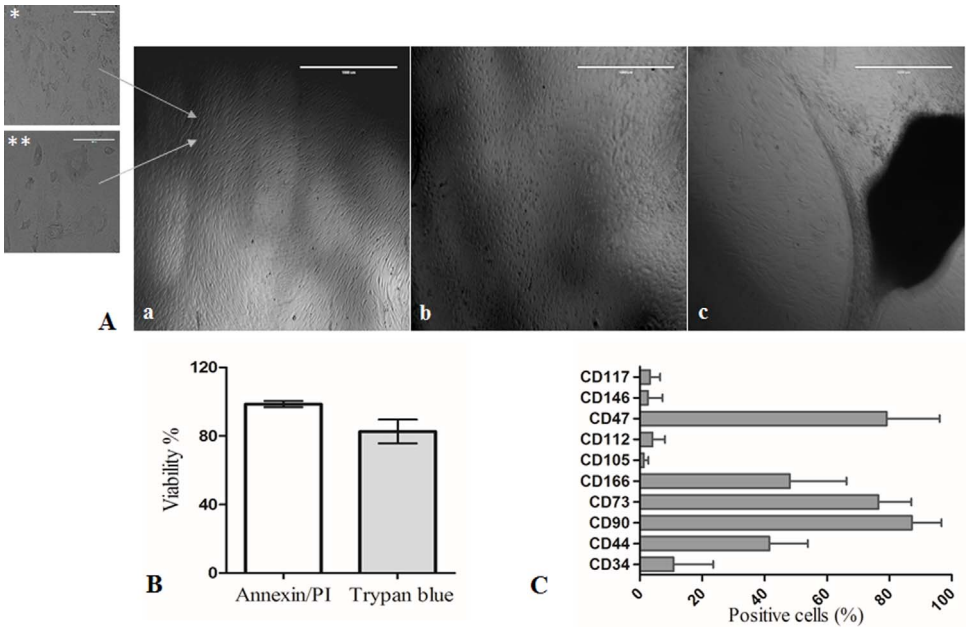

Fig. 1. Cell morphology, viability and phenotyping of the cultivated pterygium. Fibroblastic cells (Aa), pterygium epithelial cells (Ab) and graft with outgrowing cells forming 3D layered structure (Ac) (Magnification: 10×, (*) 20× and (**) 40×). Cellular viability of the 3D outgrowing cells from the pterygium using Annexin V-FITC/PI assay and Trypan blue exclusion test (B). Cell surface markers phenotyping of the 3D outgrowing cells by FACS analysis; a plot of the percent positive cells versus different markers is shown as mean ± SD (C).

Table 1
Immunophenotyping of the multi-layered outgrowing pterygium cells. Expression of different surface markers shown as percent (%).

Multi-layered outgrowing pterygium cells		
HSC markers	CD34	10.8 ± 12.7%
	CD47	79.1 ± 17%
Progenitor marker	CD117/c-kit	3.3. ± 3.2%
MSC markers	CD90	87.2 ± 9.5%
	CD73	76.4 ± 11%
	CD105	1.3 ± 1.4%
ECM attachment proteins markers	CD146/MCAM	2.6 ± 4.7%
	CD166/ALCAM	48.1 ± 18.1%
	CD44/H-CAM	41.5 ± 12.3%
	CD112/Nectin-2	4.1 ± 4%

cell (HSC and MSC) markers to determine the possible phenotype and origin of these cells was also used. Furthermore, full characterization of markers for pluripotency and stemness: Vimentin (Vim), tumour/transformation-related protein 63 (p63/TP63), Sex Determining Region Y-related HMG-box (Sox2), Octamer-binding transcription factor 4 (Oct4), and ATP-binding cassette sub-family G member 2 (ABCG-2), oxidative stress: 8-hydroxy-2' −deoxyguanosine (8-OHdG) and hypoxia-inducible factor 1 alpha (HIF-1α), migration and proliferation:C-X-C chemokine receptor type 4 (CXCR4) and (Ki-67/MKI67), epithelial: Cytokeratin (CK19 and 8–18) and secretory markers: Mucin (MUC1 and MUC4) was done.

Inflammatory processes in the pterygium can cause reactive wound formation, which may induce dysregulated and inappropriate tissue remodelling, fibrotic proliferation, enhanced vascularization and deposition of ECM, leading to formation of hypertrophic scarring and recurrence. Inflammation can induce the angiogenic pathways, which can result in neovascularization and contribute to further pterygium growth. Mitomycin C (MMC), an anti-mitotic agent often used in recurrent pterygium ablation surgery can induce apoptosis of keratocytes and myofibroblasts [6]. Herein, the effect MMC has upon the secretion of pro-inflammatory cytokines, interleukin 6 (IL-6) and IL-8 was checked after repeated pterygium graft cultivation, thus resembling closely its application *in vivo* for recurrent cases.

2. Materials and methods

2.1. Pterygium harvesting and cultivation

All tissue collection complied with the Guidelines of the Helsinki Declaration and was obtained from surgery following a patient signed consent. The tissue harvesting was approved by the Local Committee for Medical Research Ethics at University of Oslo. The removed graft was then cultivated in 24-well cell culture plates using Dulbecco-modified Eagle's medium (DMEM) with 4.5 g glucose/L supplemented, 10% fetal bovine serum (FBS) and 200 mM/mL L-glutamine (Sigma-Aldrich, Germany), as well as 1% antibiotic/antimycotic solution (PAA, Pasching, Austria). Before cultivation, viscoelastic (ProVisc, Alcon, Fort Worth, TX, USA) [7] was added on top of the explant to allow flattening and adherence of the tissue onto the surface of the well. Medium was changed every other day and the tissues were cultivated for more than three months or until they formed multi-layered cells which could be lifted easily from the cell culture plate by Colibri forceps before fixation and further analysis.

2.2. Cell viability assay

Cell viability was determined using Annexin V-Fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) assay (MBL International, Woburn, MA, USA) and trypan blue exclusion test (Sigma Aldrich, MO, USA). For both assays, the cells were collected by trypsinization in culturing media, then centrifuged and resuspended in Binding Buffer for the Annexin V-FITC/PI assay. Furthermore, they were stained with Annexin V-FITC, PI, and Annexin V-FITC/PI and analysed by Flowing Software 2.5 (PerttuTerho, Turku Centre for Biotechnology, University of Turku, Finland). 50 μL cell suspension was mixed with equal parts of trypan blue solution and cells were counted in a Hemocytometer (Burker chamber).

2.3. Immunophenotyping of cells

The immunophenotype of the long-term multi-layered cultures of pterygium containing outgrowing cells was determined by flow cytometry. FITC-, R-phycoerythrin (PE)- and allophycocyanin (APC)- con-

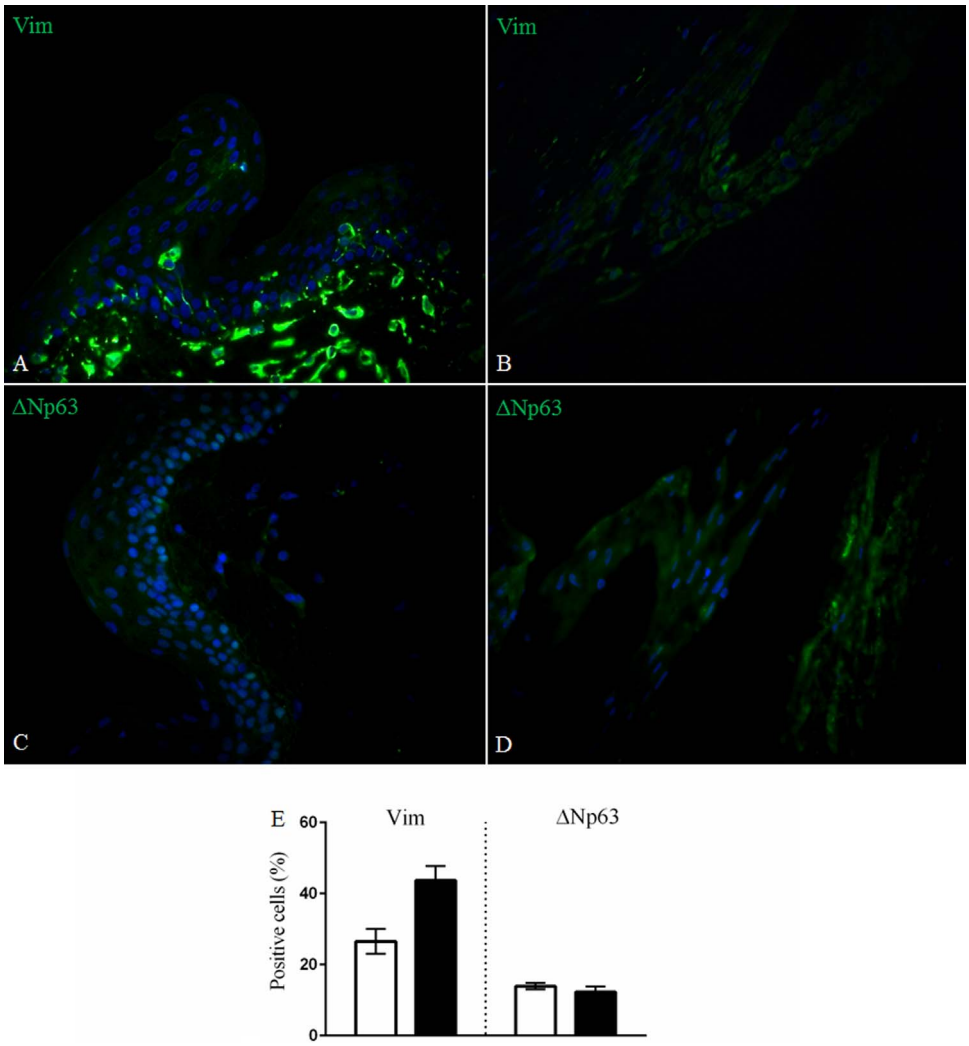

Fig. 2. Pterygium tissue and 3D outgrowing cells stained for pluripotency markers. Pterygium tissue-containing cultures (A, C), and outgrowing cells forming 3D structures (B, D) were stained for Vim and ΔNp63, respectively (Magnification: 40×). White bar represents data from pterygium tissue, while the black bar shows results from independently grown 3D outgrowing cells. Data are expressed as mean ± SD.

jugated antibodies were used to measure the expression of CD34, CD44, CD90/Thy-1, CD73, CD105 and CD166/ALCAM (all from Biolegend, San Diego, CA, USA); CD47, CD117/c-kit, CD146/MCAM, CD112 (all from R & D Systems, Minneapolis, MN, USA) (for further details refer to S1Table). Samples were measured by FACS Calibur flow cytometer (BD Biosciences Immunocytometry Systems) and data were analysed by Flowing Software 2.5 (PerttuTerho, Turku Centre for Biotechnology, University of Turku, Finland).

2.4. Immunofluorescent staining

Pterygium obtained right after surgery and that cultivated as long-term multi-layered cultures were collected and fixed in 4% paraformaldehyde at room temperature, later dehydrated in ascending alcohol series and embedded in paraffin; 3–4 μm thick tissue sections were prepared using a rotary microtome, then mounted onto histological slides. After heat-induced antigen retrieval and blocking, immunofluorescent labelling was performed. The samples were characterized for markers of pluripotency and stemness (Vim, ΔNp63, Sox2, Oct4, ABCG-2,), oxidative stress (8-OHdG, HIF-1α), migration and proliferation-(CXCR4 and Ki-67), epithelial- (CK19 and 8–18) and secretory markers (MUC1 and MUC4) (S2 Table summarizes the primary antibodies and dilutions used for immunofluorescent staining). Nuclear staining was performed using 4′, 6-diamidino-2-phenylindole (DAPI). Fluorescent images were taken by a ZEISS Axio Observer.Z1 (ZEISS, Oberkochen, Germany) microscope. The quantification of positive cells was carried out using standard ImageJ software by three independent individuals. The number of positive cells on the full field of view was taken into account with the help of nuclear (DAPI) staining. Multiple pictures were taken of each sample and the results averaged out as mean ± standard deviation (SD).

2.5. Secretion of inflammatory cytokines by ELISA

High glucose-containing DMEM medium with 5% FBS was applied to the multi-layered outgrowing cells and the supernatants were collected after 24 h. In addition, treatment of the cells with 0.5 mg/mL MMC (Kyowa, Takeda Belgium-Brussels) was applied for 5 min and exchanged by fresh medium for another 15 min prior to the supernatant collection, thus resembling the clinical use of MMC in surgery. Limbal epithelial stem cell (LESC) cultures cultivated in high glucose-containing medium with 10% FBS were also used as a control. The secreted cytokines, IL-6 and IL-8, were analysed by a commercial ELISA kit (R & D, Germany) according to the manufacturer's protocol. Three independent experiments were performed on outgrowing cells from four different pterygium donors.

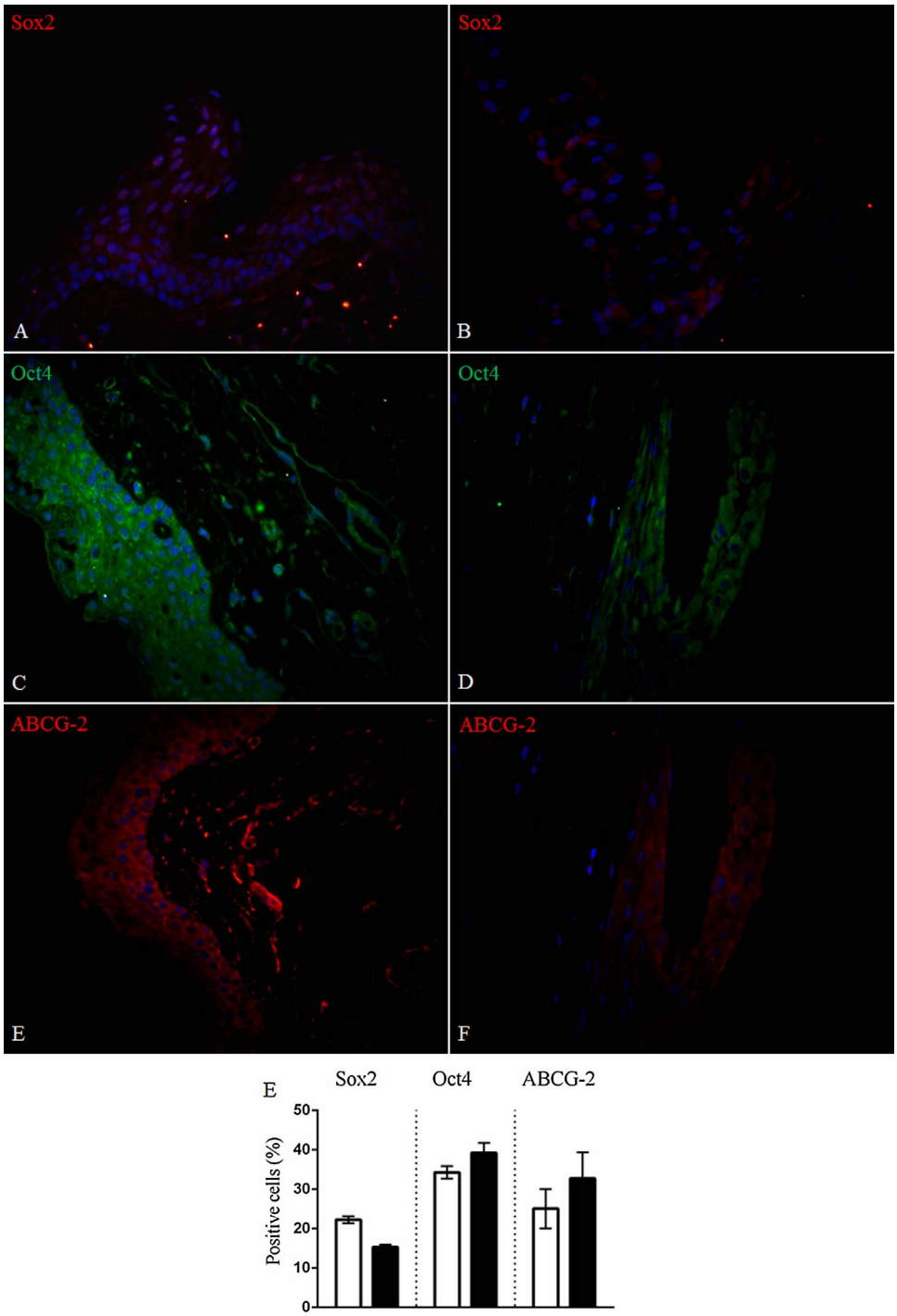

Fig. 3. Pterygium tissue and 3D outgrowing cells stained for markers of stemness. Pterygium tissue-containing cultures (A, C and E), and outgrowing cells forming 3D structures (B, D and F) were stained for Sox2, Oct4 and ABCG-2, respectively (Magnification: 40×). White bar represents data from pterygium tissue, while the black bar shows results from independently grown 3D outgrowing cells. Data are expressed as mean ± SD.

2.6. Statistical analysis

Each experiment was performed at least three times and each sample was tested in triplicates. Data are expressed as mean ± standard deviation (SD). Statistically significant differences were determined by student-*t*-tests, a p-value ≤ 0.05 was regarded significant.

3. Results

Pterygium morphology depends on two types of cells: fibroblastic which are long-shaped (Fig. 1Aa), and squamous epithelial cells tightly packed together (Fig. 1Ab). After long term cultivation, pterygium formed spontaneous multi-layered structure that was easily tangible and removable from the plate for further analysis (Fig. 1Ac). The viability of the outgrowing cells tested by the Annexin V-FITC/PI assay and Trypan blue method was (98.6 ± 1.7% and 82.6 ± 6.9%), respectively (Fig. 1B). The expression of hematopoietic cell surface markers CD34 and CD47 was (10.8 ± 12.7%and 79.1 ± 17%), respectively. CD117/c-kit, a progenitor cell marker was low (3.3 ± 3.2%), while a very high expression of the MSC markers CD90 (87.2 ± 9.5%) and CD73 (76.4 ± 11%) were measured, in contrast to the MSC marker CD105 which was (1.3 ± 1.4%). The

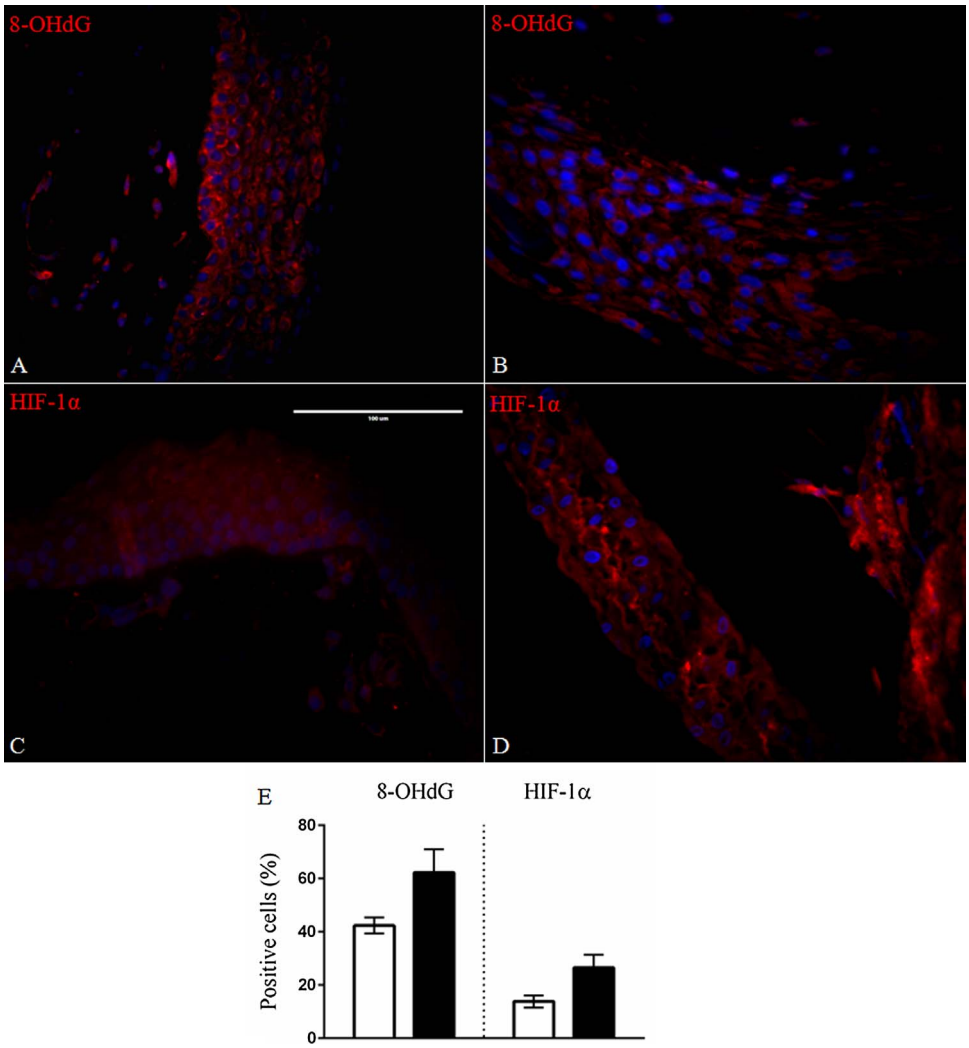

Fig. 4. Pterygium tissue and 3D outgrowing cells stained for oxidative stress markers. Pterygium tissue-containing cultures (A, C), and outgrowing cells forming 3D structures (B, D) were stained for 8-OHdG and HIF-1α, respectively (Magnification: 40×). White bar represents data from pterygium tissue, while the black bar shows results from independently grown 3D outgrowing cells. Data are expressed as mean ± SD.

expression of ECM attachment proteins, which is important for the maintenance of the cellular growth were tested next: CD146/MCAM (2.6 ± 4.7%), CD166/ALCAM (48.1 ± 18.1%), CD112/Nectin-2 (4.1 ± 4%) and CD44/homing-associated cell adhesion molecule (H-CAM) (41.5 ± 12.3%) (Fig. 1C). The expression of surface markers (%) is summarised in Table 1.

Immunofluorescent staining of a wide range of markers involved in the different pathways were examined in the pterygium grafts obtained directly from surgery and compared to those from long-term cultivated 3D outgrowths from the pterygia. The expression of pluripotency markers (Vim and ΔNp63) was (26.5 ± 3.5% and 13.9 ± 0.9%), respectively, in the pterygium graft tissue only, and (43.7 ± 4% and 12.3 ± 1.5%), respectively, in the multi-layered outgrowing cells (Fig. 2), while other stemness markers (Sox2, Oct4 and ABCG-2) showed positivity in (22.2 ± 0.9%, 34.2 ± 1.6% and 25 ± 5%) of the cells in the pterygium graft itself, respectively, and (15.3 ± 0.6%, 39.2 ± 2.5% and 32.7 ± 6.6), respectively, within the multi-layered outgrowing cells (Fig. 3). Oxidative stress markers (8-OHdG and HIF-1α) were determined in the pterygium graft tissue (42.4 ± 3% and 13.8 ± 2.3%), respectively and in the multi-layered outgrowing cells (62.3 ± 8.6% and 26.6 ± 4.8%), respectively (Fig. 4). The expression of migration and proliferation markers – CXCR4 and Ki-67, was (27.2 ± 4.5% and 5.1 ± 0.4%), respectively, in the pterygium graft tissue, and (67.3 ± 4.7% and 1.2 ± 0.3%), respectively, in the multi-layered outgrowing cells (Fig. 5). The epithelial cell markers expression (CK19 and CK8-18) was (0% and 24.3 ± 6.5%) for the pterygium graft tissue, and (11.9 ± 3.7% and 0%), respectively, for the multi-layered outgrowing cells (Fig. 6), while that for the secretory markers (MUC1 and MUC4) was (13.1 ± 4.3% and32.2 ± 3.3%), respectively, for the pterygium graft tissue, and (63.7 ± 5.7% and 55.3 ± 5%), respectively, for the multi-layered outgrowing cells (Fig. 7). Expression of all markers (%) is summarised in Table 2.

The multi-layered outgrowing cells from the pterygium biopsies secreted moderate and low levels of the pro-inflammatory cytokine IL-6and IL-8, respectively, which levels were significantly decreased upon treatment with the anti-proliferative agent MMC, in contrast to the level secreted by LESCs (control) for IL-6 ($p < 0.05$). The IL-8 secretion upon MMC treatment showed no significant change, although it was lower than that secreted by LESCs (Fig. 8A and B). Both, the inflammatory cytokines' release and the treatment by MMC have clinical relevance in case of recurrent pterygia.

4. Discussion

Being a benign process of uncontrolled cell proliferation, pterygium is a common ocular surface disorder which can vary from locally invasive, mild dysplasia to carcinoma *in situ*, characterized by proliferation, inflammation, fibrosis, angiogenesis and ECM breakdown

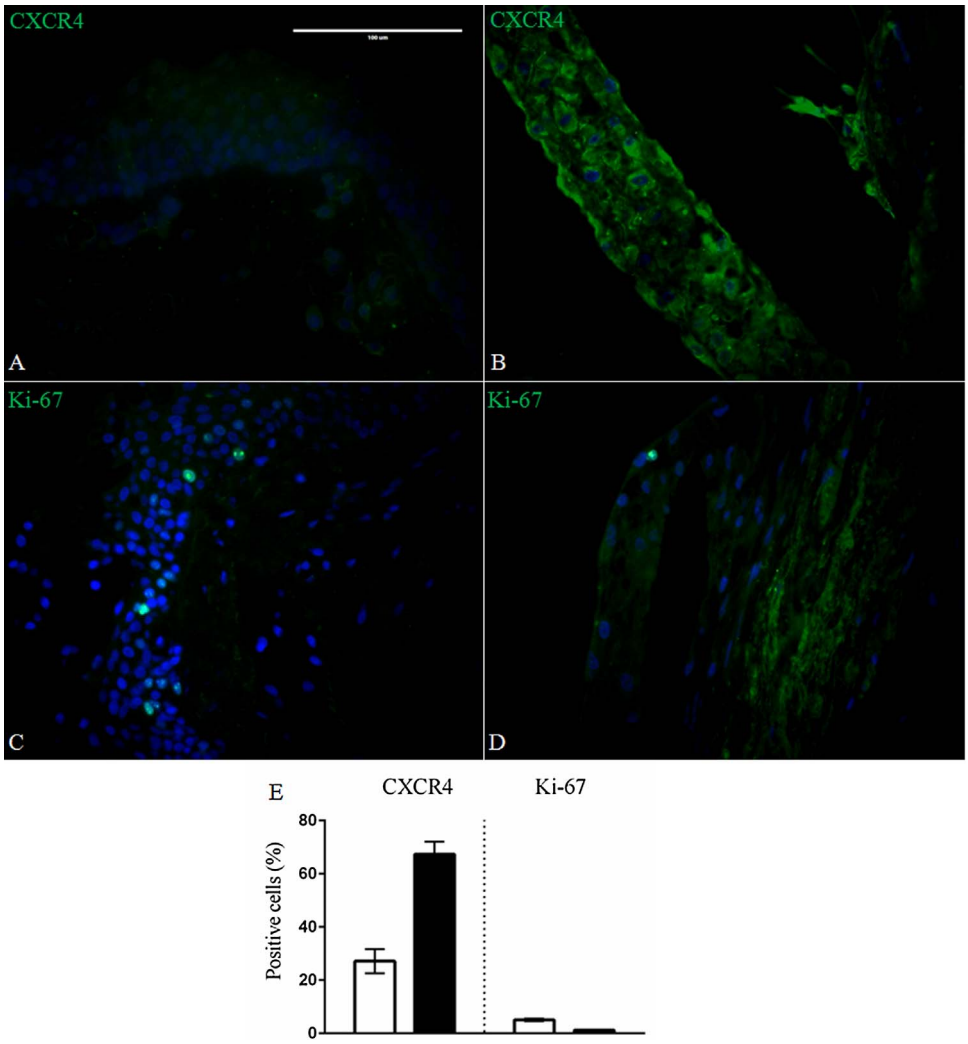

Fig. 5. Pterygium tissue and 3D outgrowing cells stained for migration and proliferation markers. Pterygium tissue-containing cultures (A, C), and outgrowing cells forming 3D structures (B, D) were stained for CXCR4 and Ki-67, respectively (Magnification: 40×). White bar represents data from pterygium tissue, while the black bar shows results from independently grown 3D outgrowing cells. Data are expressed as mean ± SD.

[8–10]. The pathogenesis of pterygia is partly unknown, but might be due to an alteration of the normal stationary limbal epithelial basal cells giving rise to a zone of motile daughter cells − pterygium cells, which leave the limbal region and migrate as a group centripetally along the corneal basement membrane dissolving the Bowman's layer. Matrix metalloproteinases (MMP) take a role in the process of squamous metaplasia and goblet cells' hyperplasia [11–14].

Pterygium as a structure consists of a mixture of epithelial and fibroblastic cells, proliferation of which closely determines its recurrence and severity [6]. The stratified outgrowing cell model for pterygium expansion *ex vivo* shown here has the advantage of giving immediate adherence of the graft to the cell culture plate (*e.g.* using viscoelastic material), therefore, providing both cell types an equal chance to expand, without the use of any growth factors. The outgrowing cells expressed CK8-18 −a typical epithelial cell marker also expressed by other human corneal cells such as epithelial and endothelial cells [15,16], as well as the LESC marker CK19 [17,18].

Cellular homeostasis or the balance between proliferation and apoptosis can be disrupted in pterygia [19]. The disorder shares many similarities with cancer, in terms of cell proliferation, invasion and recurrence after resection. The proliferation marker Ki-67 has been found to be overexpressed in the epithelial cell layer of pterygium rather than in the stromal fibroblasts, in particular, the head and the body parts compared to the healthy conjunctiva. Accordingly, corneal invasion by the pterygium might be associated with proliferation of the epithelium, although it is clear that fibroblasts play important role in epithelial–mesenchymal interactions [6,20–22]. Ki-67 has also been expressed in conjunctival and eyelid tumours [23], the positivity of which was low in the pterygium graft containing cultures, which was additionally five times less expressed in the multi-layered outgrowing tissue. The SDF-1/CXCR4 signalling pathway has been described in developmental processes and adult angiogenesis to have a role in vascular endothelial cell migration and proliferation [24]. LESCs themselves possess migratory capacity or positivity for CXCR4 [17], while this marker is expressed by vascular endothelial cells in proliferative diabetic retinopathy membranes as well [25]. The pterygium graft-containing cultures showed 2.5 times less positivity for CXCR4 compared to multi-layered outgrowing cell cultures. Vimentin, a crucial cytoskeletal mediator for the repair of cellular function during wound-healing from lens epithelial cells [26] and in LESCs cultivated in long-term cultures [18], was found highly expressed by the pterygium-graft containing and the stratified outgrowing cells. Similarly, ΔNp63 was expressed at a steady and similar extent in both cell cultures, the protein being known for its role in activation or inhibition of apoptosis in a cell- and tissue-type specific manner. In fact, ΔNp63α is the most present isoform in the central corneal epithelium which seems to be lost during apoptotic cell death [27]. Furthermore, ΔNp63 is important in the regulation of LESC proliferation, and its expression in the limbal

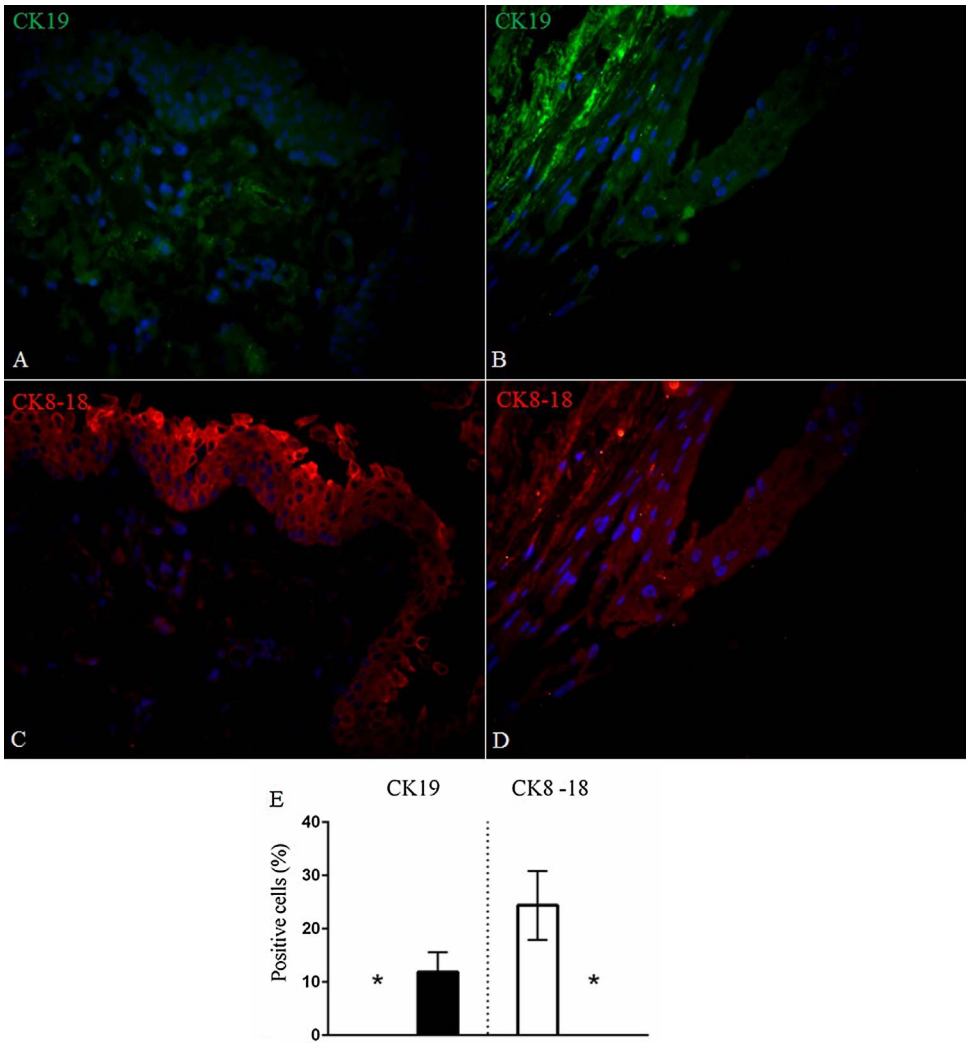

Fig. 6. Pterygium tissue and 3D outgrowing cells stained for epithelialcell markers. Pterygium tissue-containing cultures (A, C), and outgrowing cells forming 3D structures (B, D) were stained for CK19 and CK8-18, respectively (Magnification: 40×). White bar represents data from pterygium tissue, while the black bar shows results from independently grown 3D outgrowing cells. Data are expressed as mean ± SD.

epithelium plays a role in the homeostasis of corneo-limbal epithelium in rabbit limbal epithelial cells [28].

UV-B radiation is considered to be the main factor responsible for pterygium formation due to its capacity to cause oxidative stress, which plays an important role in its recurrence as well. Oxidative stress can induce growth factor production, angiogenesis, chronic inflammation and collagenolysis. When sunlight triggers pterygium inception, the proliferating fibroblasts generate reactive oxygen species that further sustain inflammation [5]. UV light can activate epithelial cells close to the limbus to produce cytokines such as IL-6 and IL-8, and enhance inflammation, proliferation, angiogenesis and anti-apoptosis. IL-8 is secreted from activated monocytes, fibroblasts, endothelial and epithelial cells, while IL-6 is synthesized by fibroblasts, endothelial cells and keratinocytes in response to cytokines as well as TNF-α and IL-1. In pterygia, IL-6 and IL-8 are believed to promote angiogenesis within the epithelium, or to stimulate mitosis in these cells and in fibroblasts together with VEGF, fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF). An abundant immunopositivity for IL-6 and IL-8 has indeed been found in pterygium epithelial cells [4,29], while in the 3D pterygium outgrowing cells, only IL-6 appeared to be highly expressed. Likely, the expression of these cytokines is closely related to the factors associated with the niche where pterygia sit *in vivo*, where they are closely related to the neighbouring limbus and its abundant vasculature underlying it. A balanced antioxidant defence in patients following primary pterygium surgery is also important [30]. 8-OHdG is a ubiquitous marker of oxidative stress with a high mutagenic potential. It is a sensitive and stable biomarker for DNA damage estimation and has been expressed more in the epithelium of the head of primary pterygium compared to the healthy conjunctiva. While pterygium is considered to be a non-metastatic lesion with limited local invasion, the presence of 8-OHdG could confirm a visible genetic instability which is in contrast to its benign clinical course [6,31].

HIF-1α plays an important role in the modulation of cell metabolism and survival pathways. A coordinated activation and increase of HIF-1α and heat shock proteins in pterygium, may represent an adaptive process for the survival of cells undergoing stressful conditions [32].

Pterygium epithelial cells seem to keep their potential to divide and thus support the view of this disease being caused by altered limbal stem cells, also being confirmed by the presence of telomerase in pterygium epithelium [11]. Although there have been reports on the presence of stem cell markers within the eye, to the best of the authors' knowledge, there has been no evidence for their presence in pterygium. Sox2 has been described as being expressed in many epithelial cell types, including conjunctival epithelial cells. In the conjunctival ocular surface epithelial cells, but not ocular stromal cells, the pluripotent stem cell marker Oct4 has been found expressed [33]. ABCG-2 has a role in the maintenance of retinal stem cells under the regulation of

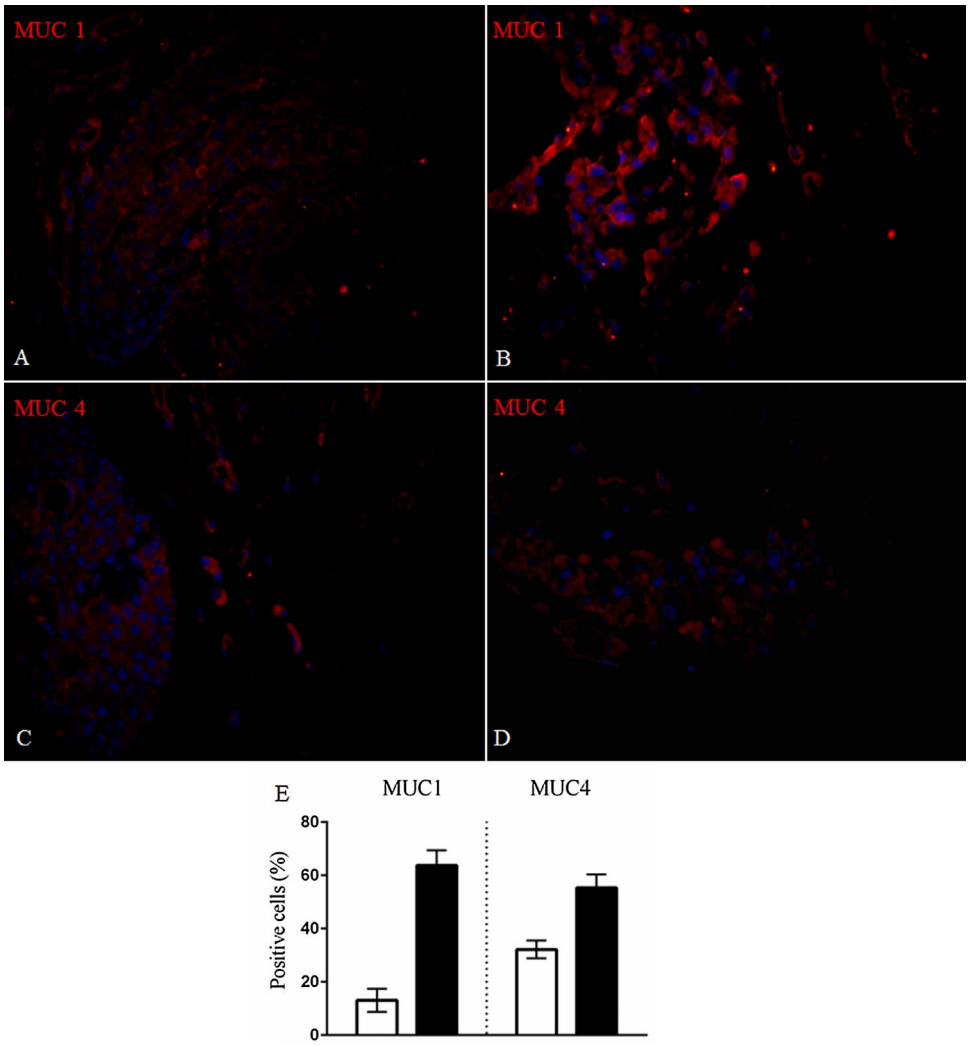

Fig. 7. Pterygium tissue and 3D outgrowing cells stained for secretory markers. Pterygium tissue-containing cultures (A, C), and outgrowing cells forming 3D structures (B, D) were stained for MUC1 and MUC4, respectively (Magnification: 40×). White bar represents data from pterygium tissue, while the black bar shows results from independently grown 3D outgrowing cells. Data are expressed as mean ± SD.

Table 2
Expression of a wide range of markers expressed in pterygium grafts obtained directly from surgery and compared to long-term cultivated 3D outgrowths from pterygium shown as percent (%).

		Pterygium graft	Multi-layered outgrowing pterygium cells
Pluripotency markers	Vim	26.5 ± 3.5%	43.7 ± 4%
	ΔNp63	13.9 ± 0.9%	12.3 ± 1.5%
Stemness markers	Sox2	22.2 ± 0.9%	15.3 ± 0.6%
	Oct4	34.2 ± 1.6%	39.2 ± 2.5%
	ABCG-2	25 ± 5%	32.7 ± 6.6%
Oxidative stress and hypoxia markers	OHdG	42.4 ± 3%	62.3 ± 8.6%
	HIF-1α	13.8 ± 2.3%	26.6 ± 4.8%
Migration and proliferation markers	CXCR4	27.2 ± 4.5%	67.3 ± 4.7%
	Ki-67	5.1 ± 0.4%	1.2 ± 0.3%
Epithelial cell markers	CK19	0%	11.9 ± 3.7%
	CK8-18	24.3 ± 6.5%	0%
Secretory markers	MUC1	13.1 ± 4.3%	63.7 ± 5.7%
	MUC4	32.2 ± 3.3%	55.3 ± 5%

Notch signalling. The expression of ABCG-2 in canine limbal epithelial cells has been considered a useful marker for presence of putative corneal epithelial stem cells and proliferation [34,35]. The *ex vivo* 3D tissue engineered pterygium outgrowths and the graft-containing cultures expressed all of these stem cell markers (Sox2, Oct4 and ABCG-2).

It is believed that the multifunctional molecules MUC1 and MUC4 are involved in the barrier function, protection and lubrication of the ocular surface, signalling and that they are osmosensors. These proteins can be expressed by both corneal and conjunctival epithelia [36,37], while their expression in the pterygium graft-containing cultures and the stratified outgrowing cells was clearly high, probably making the *ex vivo* model for pterygium being close to a goblet cell hyperplasia as well.

MMC has often been used to reduce recurrence of pterygium in connection with conjunctival autografts, amniotic membrane transplantation and radiation or chemotherapeutical agents' treatment [38,39]. MMC suppresses the proliferation of fibroblasts by inhibiting DNA synthesis, hence it can also affect normal tissues and cause complications such as scleral necrosis, corneal perforation, corneal oedema, secondary glaucoma, corneal calcification and cataracts, thus it must be used with caution [1]. In this study, MMC reduced the level of secreted cytokines (in particular IL-6), which provides a combined anti-inflammatory and anti-proliferative therapy for pterygium at a

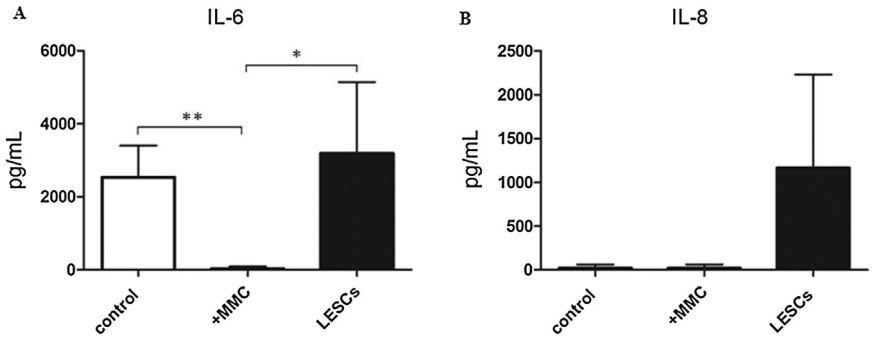

Fig. 8. Cytokine secretion by 3D outgrowing cells from pterygium tissue grown *ex vivo*. Treatment by Mitomycin C (MMC) and limbal epithelial stem cells (LESCs) is shown for comparison. The significant data for secretion of IL-6 is presented with asterisk (*), while IL-8 showed no significant expression.

cellular and molecular level.

The pterygium outgrowing cells were still viable after having undergone more than three months cultivation time, which was further confirmed by the high level expression of CD47. The surface marker profile of *ex vivo* cultivated outgrowing cells showed that CD44, a protein expressed actively on dividing cells [40], is moderately expressed in the stratified outgrowing cells. The expression of ECM attachment proteins, which is vital for the maintenance of cellular growth within the pterygium, was low (CD146/MCAM, CD112/Nectin-2) and moderate (CD166/ALCAM, CD44/H-CAM). Those markers could also be found in LESCs, while the expression of CD73 and CD105 in 3D outgrowing cells from pterygium was in general lower than that found in LESCs [17]. Expression of CD105 has been described previously to have a role in neovascular endothelial cells formation in the subepithelial area of primary pterygia, while a correlation between the expression of VEGF in the stroma and CD105-MVD in primary pterygia has also been proposed. Overexpression of VEGF and CD105-MVD can contribute to pterygium progression by increasing angiogenesis and growth [41]. Expression of the hematopoietic cell surface markers CD34 and 'don't eat me' signal CD47 in the 3D outgrowing cell from pterygium cells was low and high, respectively. The population of 3D outgrowing cells from pterygium contained a low level of the early progenitor cell marker CD117/c-kit.

In conclusion, it has been demonstrated that human pterygium explants can give rise to 3D outgrowing cells in a medium containing FBS as the only growth supplement. The cells were viable, could proliferate and migrate out of the grafts and form a stratified structure very much like the one *in vivo*. The expanding cells carried proteins related to an undifferentiated state, but also a commitment towards epithelial lineage. Stemness markers suggest a flexible cell phenotype in the long-term cultures. Manipulating the cell sheets was similar to handling primary human tissues, presuming a possibility for use in tissue engineering and drug discovery.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.clae.2017.04.002

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