Comparison of culture media indicates a role for autologous serum in enhancing phenotypic preservation of rabbit limbal stem cells in explant culture

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Abstract In this study, we aimed to compare the effects of six different cell culture media and autologous serum (AS) on the phenotypic characteristics of rabbit limbal epithelial stem cells (LESC) cultivated on porous polyethylene terephthalate (PET) membranes. Limbal explants from rabbit corneas were grown on PET membrane inserts in five different media: DMEM-F12 with fetal bovine serum (FBS) (DMEM-F12-FBS), with pluripotin (DMEM-F12-pluripotin) and with autologous serum (DMEM-F12-AS), Epilife, Keratinocyte Serum Free Medium (KSFM) and Defined-Keratinocyte Serum Free Medium. The effects of different media were evaluated by total cell yield from explants, measuring the expression of proteins by immunofluorescence and gene expression by Real Time PCR. In all five media tested, most of the limbal epithelial cells (LEC) which proliferated from explants were positive for cytokeratin (CK) 14 (85–90%), indicating that all five media

support the growth of LESC from explants. The expression of differentiation markers; CK 3 and 12 was highest in DMEM-F12-FBS (56%), was lower in Epilife and KSFM (26 and 19%, respectively), with the lowest values (13%) obtained in DMEM-F12-AS. Gene expression of limbal cultures on PET membrane inserts was compared to fresh limbal tissue. In DMEM-F12-FBS, DMEM-F12-pluripotin, and DMEM-F12-AS, expression of potential LESC markers CXCR4 and polycomb complex protein BMI-1 were similar to limbal tissue. DMEM-F12 with 10% AS maintained a higher percentage of potential stem cell marker genes and lower expression of genes involved in differentiation compared to Epilife or KSFM. Our study shows that rabbit LEC can be cultivated on PET inserts using DMEM-F12 with autologous serum without a requirement for amniotic membrane or feeder cells.

Keywords Limbal epithelial stem cells · Limbal explant culture · Gene expression · Protein expression · Autologous serum

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Introduction

The cornea is a regenerating stratified squamous epithelium. The homeostasis of corneal cells is required for a clear cornea and good vision, and this

is mediated by stem cells located in the basal layers of the corneoscleral limbus (Schermer et al. 1986; Tseng 1989). The current theory of corneal epithelial renewal is based on the presence of a group of unipotent stem cells (SC) termed limbal epithelial stem cells (LESC) residing in the basal limbus. Their concomitant migration, transformation (through an intermediary state termed transient amplifying cell; TAC) and final homing in the cornea is critical to the formation of terminally differentiated corneal epithelial cells (Yoon 2014). Several proteins have been identified as possible markers of LESCs; these are, cytokeratin (CK) 14,15,19 (Schlötzer-Schrehardt and Kruse 2005), p6, (Ghoubay-Benallaoua et al. 2011) CXCR4, polycomb complex protein BMI-1 and intermediate filament protein; vimentin. These cells are negative for CK3/12 (Schermer et al. 1986) and connexin 43 (Yoon et al. 2014). Due to the expression of the drug efflux transporter ABCG2, LESCs can be characterized and purified by the efflux of various molecules such as Hoechst 33342 and JC-1 by flow cytometry. They are designated as side population (SP) cells (de Paiva et al. 2005; Selver et al. 2011). LESCs are envisioned to be present in a unique microenvironment termed the limbal niche. Its anatomical location is the Palisades of Vogt and comprises cellular and extracellular components (Dziasko and Daniels 2016). The limbal niche is similar to other stem cell niches that are spatially distributed in various tissues (O'Brien and Bilder 2013). LESCs function is affected by their proximity to cells in the niche compartment, the interaction of these cell groups involves SDF-1/CXCR4 signaling pathways (Xie et al. 2011).

Various diseases of the ocular surface lead to Limbal stem cell deficiency (LSCD), loss of limbal function, interruption of the replenishment of corneal epithelial cells, which ultimately results in vision loss. Current treatment approaches of LSCD are; (a) Auto or allotransplantation of limbal tissue explants or (b) *in vitro* expansion of cells from limbal tissue, to be transplanted to the ocular surface (Pellegrini et al. 2014). Clinically, the most widely used treatment for LSCD is autologous limbal stem cell transplantation (Pellegrini et al. 2007). With a reported success rate of 76%, culturing and transplantation of LECs is emerging as a promising treatment approach (Baylis et al. 2011).

One major limitation of limbal explant transplantation is that fresh limbal epithelial tissue has been

reported to contain less than 1% LESCs in human/rabbit (Budak et al. 2005). *In vitro* expanded cells establish a mixed population including LESCs, TACs, and differentiated cells. The most widely used technique for restoring corneal function in limbal deficiency is to transplant LESCs expanded *ex vivo* on AM (Zhao and Ma 2015). By *in vitro* expansion on human amniotic membrane (AM), SP cells were reported to be increased to 12.3 and 25.8% for human and rabbit samples, respectively (Selver et al. 2011). Although culturing explants on AM is currently the predominant method of *ex vivo* propagation of LESCs, drawbacks of using AM exist including; availability issues, the possibility of contamination and transfer of infections from the mother. Therefore, other supporting materials have been recently investigated (Joe and Yeung 2014).

Mesenchymal stem cells (MSC) are present in different tissues and human MSC derived from various sources are currently in use for the treatment of a wide variety of diseases (Sharma et al. 2014). Culture media for MSC growth and expansion contain fetal bovine serum (FBS), growth factors, and hormones with xenogenic origin; pointing to issues of safety if cultivated cells are to be used in a clinical setting. Therefore, various approaches to cultivate MSC in defined serum-free media are being developed (Jung et al. 2012). Both autologous and allogeneic sera have been investigated as an alternative to FBS for stem cells (Kinzebach and Bieback 2013). As media for LESCs culture are based on Green medium containing DMEM/F12, L-glutamine, FBS, cholera toxin, insulin, hydrocortisone, and antibiotics (Rheinwald and Green 1975), to which different groups have made various small molecule additions, the same safety concerns apply to these cells. Although most clinical trials for LSCD have used variations of six main cell culture protocols, a systematic analysis of the inclusion of all media components has not been performed (Tseng et al. 2010). To circumvent xenogeneic contamination from FBS, pooled human serum has been utilized in LSCD. Transplantation of human LESCs propagated on AM in an autologous serum-based medium has been reported to be successful in the treatment of LSCD (Kolli et al. 2010). There is no consensus on an ideal LESCs culture medium. Therefore, there is a need to define media showing optimal cell growth and with minimal xenogeneic components.

The objective of this work was to provide information on the feasibility of culturing rabbit limbal tissue in cholera toxin free medium with minimal xenogeneic medium additives, by using autologous rabbit serum (AS) as a substitute for FBS and using PET membrane inserts as a physical support.

This paper compares the effectiveness of six different culture media [DMEM-F12 with the addition of FBS, autologous rabbit serum (AS) or pluripotin, KSFM, Epilife, Keratinocyte Serum Free Medium (KSFM) and Defined-Keratinocyte Serum Free Medium (D-KSFM)] on the growth and phenotype of LESC grown *ex vivo* from rabbit limbal tissue on PET membrane inserts.

Materials and methods

Harvesting of limbal tissue from rabbits

The animal study was approved by the Dokuz Eylul University, Laboratory Animals Local Ethical Committee (protocol number 36/2014). New Zealand white rabbits weighing 2–3 kg and 1–1.5 years of age were used in this study. Following general anesthesia with xylazine hydrochloride (5 mg/kg) together with ketamine hydrochloride (50 mg/kg), approximately 1 × 2 mm in size limbal explant biopsy was taken from the rabbits.

The limbal tissues were placed in optisol GS (Bausch+Lomb, Rochester, NY, USA) and transported to the laboratory.

Preparation and cultivation of limbal explants

Limbal tissue was manipulated in a laminar flow hood under aseptic conditions. The explants were directly placed on high pore density, polyethylene terephthalate six-well culture inserts (PET membrane insert, 0.4 µm pore size, BD Falcon, 353090, Franklin Lakes, NJ, USA), with the epithelial surface facing upward. The explants were subsequently cultured with one of six culture media. Initially, the explants were covered with 1–2 drops of medium and kept for 2 days to allow the explants to adhere to the insert. Following this, 2 mL of medium were added on top and below the explants. The cultures were maintained in a 37 °C humidified incubator with 5% CO₂. No feeder cells

were used to support cell growth. The medium was changed every 2 days for 14 days.

Culture media

Six different culture media were used for the cultivation of limbal explants:

1. DMEM/F12-FBS: Dulbecco's Modified Eagle's medium and Ham's F12 medium (1:1, DMEM/F12; Sigma, D6421, St. Louis, MO, USA), supplemented with 16% (v/v) fetal bovine serum (FBS; Sigma), 2.5 mM L-glutamine (Sigma).
2. DMEM/F12-Pluripotin: DMEM/F12, supplemented with 200 nM pluripotin (Calbiochem, Billerica, MA, USA), 16% FBS, 2.5 mM L-glutamine.
3. DMEM/F12-AS: DMEM/F12, supplemented with 10% (v/v) autologous rabbit serum, 2.5 mM L-glutamine.
4. Epilife Medium (Gibco, MEPI500CA, Carlsbad, CA, USA), supplemented with 5% FBS, 0.2 ng/mL epidermal growth factor (EGF; Sigma), 1% human corneal growth supplement (HCGS; Gibco), 0.2% bovine pituitary extract (Sigma), 5 µg/mL human insulin (Biochrom, Billerica, MA, USA), 0.18 µg/mL hydrocortisone (Sigma), 5 µg/mL transferrin (Calbiochem).
5. KSFM: Keratinocyte Serum Free Medium (KSFM; Gibco, 10725018), supplemented with 10% FBS, 0.2 ng/mL EGF, 30 ng/mL bovine pituitary extract.
6. D-KSFM: Defined-Keratinocyte Serum Free Medium (D-KSFM; Gibco).

All media contained 1% penicillin streptomycin (5000 U/mL penicillin and 5000 µg/mL streptomycin) (Gibco).

Isolation of cells from PET membrane inserts

Cells were isolated from PET membrane inserts using dispase. The explants were removed and the cells remaining on the inserts were incubated with 2 mg/mL dispase II (Roche, Basel, Switzerland) in DMEM-F12 solution at 37 °C for 20 min., while shaking at 80 rpm. After dispase II digestion, epithelial sheets detached from PET inserts were incubated with 0.25% trypsin-EDTA (Sigma) solution at 37 °C for ten min., with shaking at 80 rpm. This second incubation

enabled the isolation of single cells. They were counted with a hemocytometer for the determination of the number of cells proliferating from each explant, or used for protein expression analysis by immunocytochemistry.

Isolation of cornea and conjunctiva cells

Cornea and Conjunctiva tissues were separately incubated in 4 mg/mL dispase II/DMEM-F12 solution at + 4 °C overnight with shaking at 20 rpm. Following digestion, tissues were scraped gently with a cell scraper and harvested cells were incubated with 0.25% Trypsin-EDTA solution at 37 °C for ten min., with shaking at 80 rpm.

Immunocytochemistry

The isolated conjunctival or corneal cells and cells grown in culture were placed in compartments of a Cytospin (Hettich, Tuttlingen, Germany) and centrifuged at 2000 rpm for 8 min to prepare slides (2500 cells per slide). These were dried for 15 min at room temperature and fixed in cold methanol.

The cells were blocked with 1% bovine serum albumin (BSA, Sigma) in PBS (Thermo Scientific HyClone, Waltham, MA USA) and incubated 30 min., at room temperature (RT). After permeabilization, the cells were incubated with primary antibodies: anti-CK14 (1:100 Millipore, Darmstadt, Germany), anti-CK3/12 (1:100, Abcam, Cambridge, UK), anti-vimentin (1:100, Abcam) for one h at RT. After washing with PBS, the cells were incubated for one h with FITC- conjugated goat polyclonal secondary antibody (1:100, Abcam). Nuclear staining was performed with 4′-6′-diamino-2 phenylindole (DAPI, Invitrogen, Waltham, MA, USA). The stained cells were viewed under a fluorescent microscope (Olympus BX61, Tokyo, Japan).

Total RNA isolation from limbal tissue and cells, RNA quality control

Freshly isolated limbal tissues were incubated in 4 mg/mL dispase II/DMEM-F12 solution at + 4 °C overnight with shaking at 20 rpm. Following digestion, tissues were scraped gently with a cell scraper. For cells cultivated from explants, the limbal explant was initially removed. From this point on, the same procedure was used for limbal tissue and cells growing

from explants. Total RNA was isolated using a kit (AurumTM Total RNA Mini Kit; BIO-RAD, Hercules, CA, USA), according to the manufacturer's protocol. 1 mL lysis buffer was added directly to the digested tissues or PET inserts with cells.

DNase I digestion eliminated residual genomic DNA in the RNA samples (BIO-RAD, supplied with AurumTM Total RNA Mini Kit). The RNA was quantified by measuring the absorbance at 260 nm, and its purity was evaluated from the 260/280 ratio of absorbance (1.80–2.00) with Nanodrop (Thermo Scientific). The total RNA integrity number (RIN values > 5.0 regarded as acceptable) was evaluated using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). One µg total RNA was reverse transcribed with cDNA synthesis kit (iScriptTM cDNA Synthesis Kit; BIO-RAD) according to the manufacturer's protocol.

Reverse transcription and gene expression analysis by quantitative real-time PCR

For gene expression CXCR4, P63, ABCG2, Bmi-1 genes were selected for possible LESC markers and Connexin 43, CK3, CK12 genes were chosen as differentiation markers. For rabbit genes for which no PCR primers were reported in the literature, primers were designed using National Center for Biotechnology Information's (NCBI) databases. Primers for the genes used in this study are listed in Table 1; published rabbit primers are from Liu et al. (2010). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed by using a LightCycler 480 instrument (Roche Diagnostics, Nonnenwald, Penzberg, Germany). Products of PCR amplification were detected through intercalation of the SYBR green dye from Luminaris Color HiGreen qPCR Master Mix kit (Thermo-Scientific). The amplification cycles were as follows: 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, 52 °C for 30 s and 72 °C for 30 s. Each sample was tested in duplicate. To confirm their amplification specificity, the PCR products were subjected to a melting curve analysis.

Gene expression was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Seol et al. 2011) and (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ) (Mamo et al. 2008).

Table 1 Forward and reverse primer sequences used for real-time PCR of rabbit genes and their product size

Gene name (reference)	Sequence	PCR product size (bp)
	Forward (5'–3'); F Reverse (5'–3'); R	
GAPDH (Seol et al. 2011)	F: GGGTGGTGGACCTCATGGT R: CGGTGGTTTGAGGGCTCTTA	58
YWHAZ (Mamo et al. 2008)	F: GGTCTGGCCCTTAACCTCTCTGTGTTCTA R: GCGTGCTGTCTTTGTATGATTCTTCACTT	142
HPRT1 (Seol et al. 2011)	F: GCAGACCTTGCTTTTCCTTGGT R: GCAGGCTTGCGACCTTGAC	63
CXCR4 (D)	F: GGCCCTAGCCTTTTCCACT R: AAGAATGTCCACCCGCTTT	152
P63 (Liu et al. 2010)	F: GAAAACAATGCCAGACTCAATTT R: TCTGCGCGTGGTCTGTGTTAT	127
ABCG2 (D)	F: TTGTTCTCTGGA TGAGCCAC R: AAGA TGA TGGTTCGGCCCTG	101
BM1-1 (D)	F: CCCCACCTAATGTGTGTGCT R: GGTCTCCAGGTAACGCACAA	108
Connexin 43 (D)	F: AGGCAAGCTCCTGGACAAAG R: CTGACTCAACTGCTGTCCCC	110
CK12 (Liu et al. 2010)	F: GCTCGGGATGGGATTG R: AGGGCAGCCTCATTCTCG	387
CK3 (Liu et al. 2010)	F: TCCGTCACAGGCACCAAC R: TCGTTTTGTTGATTTCGTCT	175

D: Rabbit primers designed in this study

Normalization to three housekeeping genes was done according to Vandesompele et al. (2002).

Statistical analysis

Statistical Analysis was performed using SPSS program (IBM SPSS Statistics Version 20, SPSS Inc. Chicago, IL, USA). Data were evaluated with the non-parametric Mann-Whitney U-test and Kruskal-Wallis test. A *p*-value of < 0.05 was considered to be statistically significant.

Results

Cell growth from explants in different culture media

The success of limbal explant culture was dependent on the attachment of the explants to PET membrane

inserts and irrespective of the media used, 80% of the explants were observed to attach. The shapes of the cells proliferating from the explants are shown in Fig. 1, were not significantly different from each other in the media used in this study. Few cells were observed to grow from the explant in the D-KSFM medium.

After 14 days on PET inserts, approximately 140,500 cells were obtained from an explant in the DMEM-F12-FBS. DMEM-F12-Pluripotin medium gave a similar cell yield, whereas the use of Epilife, KSFM, and DMEM-F12-AS resulted in significant increases in cell yield ($p < 0.0001$, $p < 0.0001$ and $p < 0.009$, respectively). Cell numbers were approximately ten-fold greater for Epilife and KSFM and three-fold for DMEM-F12-AS (Fig. 2). Among the media tested, D-KSFM provided the lowest cell yield, one-tenth of the DMEM-F12-FBS. Therefore, further experiments with this medium were not performed.

Fig. 1 Limbal epithelial explant culture on PET membrane inserts. Representative phase contrast images of limbal epithelial cells growing from limbal explants on the 8th day of culture.

a DMEM-F12-FBS, **b** DMEM-F12-Pluripotin, **c** Epilife, **d** KSFM, **e** D-KSFM, **f** DMEM-F12-AS (Light Microscope $\times 20$). Scale bar: 100 μ m

Fig. 2 Cell yield from explants on PET membrane inserts in five different cell culture media. Cells were isolated from PET membrane inserts using dispase and total cell yield per insert was counted. Mann-Whitney U-test was performed ($*p < 0.0001$). Data shown are mean $\pm$ S.E. The N values for each group are; DMEM-F12-FBS;11, DMEM-F12-Pluripotin;10, DMEM-F12-AS;3, Epilife;10, KSFM;11, D-KSFM;13

Protein expression in cornea and conjunctiva cells

Isolated corneal cells were shown to express CK3/12, but not CK14 and vimentin. Almost all conjunctival cells expressed CK14, but hardly any expressed vimentin and CK3/12 (Fig. 3).

Protein expression in cultivated limbal epithelial cells

Immunofluorescence analysis was performed with cells grown in the five media (Fig. 4). The expression

of the following proteins was investigated; CK14; a limbal cell marker, vimentin; a marker for limbal basal and stem cells and CK3/12 that is a marker for differentiated limbal cells. In all five media tested, most of the limbal epithelial cells (LEC) which proliferated from explants were CK14 (+) (85–90%) (Fig. 5). Cells grown in Epilife and DMEM-F12-AS had a higher percentage of CK14 positive cells compared to DMEM-F12-FBS ($p < 0.0001$, $p < 0.013$, respectively, two-sided posthoc analysis). The increase in CK14 expressing cells in DMEM-F12-pluripotin was not significant.

Fig. 3 Protein expression in corneal, conjunctival, and cultivated limbal epithelial cells. Representative immunofluorescence staining images of corneal (a–c) conjunctival (d–f) and cultivated limbal epithelial cells (g–i). a, d, g CK14, b, e,

h vimentin, c, f, i CK3/12 marked cells. FITC (green) labeled secondary antibody. Nuclear staining was performed with DAPI (blue). Fluorescence microscope $\times 20$. Scale bar: 100 μm . (Color figure online)

36% of cells expressed vimentin in DMEM-F12-FBS, its expression was significantly lower (6–27%) in the four media tested. Vimentin-positive cells grown in Epilife and KSFM were lower than when grown in DMEM-F12-FBS ($p < 0.003$, $p < 0.001$, respectively, two-sided posthoc analysis).

Among the five media, the highest expression of CK 3/12 was in DMEM-F12-FBS (56%), and was lower in four media (13–28%) with the lowest value observed in DMEM-F12-AS.

Gene expression by real-time PCR

Gene expression of rabbit limbal cultures on PET membrane inserts were compared to gene expression in native limbal tissue. In DMEM-F12-FBS, DMEM-

F12-pluripotin, and DMEM-F12-AS media, expression of potential LESC markers *CXCR4* and *Bmi-1* were not significantly different from that in limbal tissue (Fig. 6). The expression of these two genes decreased in KSFM, the decline in *Bmi-1* was significant in Epilife. Relative to limbal tissue, *ABCG2*, and *p63* expressions were significantly decreased in all five media tested.

DMEM-F12-pluripotin and DMEM-F12-AS better maintained the progenitor characteristics of LESC, as the mRNA expression of *CXCR4*, *ABCG2*, *Bmi-1* and *p63* was higher in these media when compared to Epilife and KSFM.

The expression of three differentiation-related genes; *connexin 43*, *CK3*, *CK12*, was observed to decrease significantly in all five media tested.

MEDIA ↓	CK14	Vimentin	CK3/12
DMEM-F12	86%	35%	58%
DMEM-F12 + Pluripotin	92%	26%	27%
DMEM-F12 + AS	95%	13%	11%
Epilife	91%	17%	24%
KSFM	90%	8%	15%

Fig. 4 Representative photographs of CK14, vimentin and CK3/12 expression in cells grown from rabbit limbal explants on PET membrane inserts. Following collection of cells by cytopspin and immunofluorescent staining, five manually selected random areas were imaged and the percentage of positive cells calculated. For each representative photograph,

percent positive cells (average from five images) are given in the left-hand corner. CK14, vimentin, and CK3/12 indicate the primary antibodies used against these proteins. FITC (green) labeled secondary antibodies. Nuclear staining was performed with DAPI (blue). Fluorescence microscope magnification; $\times 20$. The bar is 100 μm . (Color figure online)

Fig. 5 Bar chart showing expression of proteins in cells grown from rabbit limbal explants on PET membrane inserts. As described in Fig. 4, the percentage of positive cells was calculated in five randomly selected areas and averaged. Protein expressing cells are given as percent of the total. The N values for each group are: DMEM-F12-FBS: 9; DMEM-F12-Pluripotin: 10; DMEM-F12-AS: 3; Epilife:10; KSFM:7. Kruskal-Wallis test, p values < 0.001 were obtained for CK14,

vimentin and CK3/12. By taking DMEM-F12-FBS as control, Mann Whitney U (post hoc test) p values were calculated for CK14, vimentin and CK3/12: * p < 0.05, ** p < 0.003, *** p < 0.001, † p < 0.0001. The p values for CK14, vimentin and CK3/12 for each medium are presented, respectively, for DMEM-F12-Pluripotin: 0.072, 0.041, 0.0001, for DMEM-F12-AS: 0.013, 0.021, 0.013, for Epilife: 0.0001, 0.003, 0.009, and for KSFM: 0.01, 0.001, 0.001

Discussion

LECs were grown from limbal explants on PET membrane inserts and the effects of culture media on cell yield, protein and gene expression was studied. The use of full thickness explants in our approach is envisioned to keep the proximity of stromal niche cells with LESC and maintain their support during outgrowth from the explants.

The preferred treatment of LESC deficiency by the majority of clinical research groups is growing LEC from limbal explants on AM with subsequent transplantation to the patients. Variability among donors, the need for thorough infection testing, the unreliability of supplies are significant drawbacks in using AM (Levis and Daniels 2009). Our approach has been to

use PET membrane inserts to support the limbal explants and DMEM-F12-FBS, Selver et al. (2011) have reported that PET membrane inserts promote the growth of human LEC and enable successive passaging of outgrowth cells for 1 month.

We compared cell yield from limbal outgrowth on PET membrane inserts after 14 days in culture. Our results indicate that the cell yield from rabbit limbal explants grown on PET membrane inserts in the DMEM-F12-FBS is 14.05×10 (Schlötzer-Schrehardt and Kruse 2005) cells. This yield is not significantly different from the cell yield of explants cultured on AM in the same medium (M. Gurdal et al. unpublished results). Epilife and KSFM increased the cell yield approximately ten-fold, while, with DMEM-F12-AS, the increase was threefold. Calcium

Fig. 6 Gene expression in cells grown from rabbit limbal explants on PET membrane inserts. Expression of different stem cell and differentiation marker genes was measured by real-time PCR. The expressions were normalized to values in fresh limbal tissue. The effect of five culture media on relative fold

expression of the genes is presented. Mann-Whitney U-test was performed (* $p < 0.05$). Data shown are mean $\pm$ S.E. The N values for each group are; DMEM-F12-FBS;9, DMEM-F12-Pluripotin;11, DMEM-F12-AS;4, Epilife;8, KFSM;10

concentration of DMEM-F12-FBS (1.05 mM) is higher than of Epilife (0.06 mM) and of KFSM (0.09 mM). High extracellular calcium has been shown to affect limbal cell proliferation (Kruse and Tseng 1992) and differentiation (Kawakita et al. 2008). The high cell yield we have observed in these two media with low calcium levels may be related to the presence of EGF. Acting as a mitogenic growth factor, EGF may be responsible for the high cell yields in Epilife and KFSM. We have observed very low numbers of cells growing from explants in D-KFSM. Others have also reported the failure of cell proliferation in D-KFSM (Bray et al. 2012).

In this study, the phenotype of LEC was investigated using immunocytochemistry, and a quantitative analysis of cell phenotype was performed in different media. In all five media tested, almost all cells were CK14 (+) (85–90%). CK14 has been reported to be expressed in LESC and TACs expanding from limbal explants (Figueira et al. 2007). This result indicates that all five media support the growth of LESC from rabbit explants. In human limbal explants cultured in supplemental hormonal epithelial medium (SHEM, almost identical to Green medium), 69% of the

outgrowth cells were CK14(+) (González and Deng 2013).

Vimentin is an intermediary filament protein expressed by limbal basal epithelial cells, has been found to be positive in cells present in the limbal crypt (Shanmuganathan et al. 2007) and is widely accepted as a marker of undifferentiated cells (Ghoubay-Benallaoua et al. 2011). Vimentin is present in 36% of cells in DMEM-F12-FBS, and its expression was significantly lower in the other four media tested (lowest in KFSM; 6%). In a similar study, 37% of cells grown in Green medium from full thickness human limbal explants on plastic culture plates expressed vimentin (Ghoubay-Benallaoua et al. 2011). In a report comparing SHEM with Epilife and KFSM, vimentin has been shown to be highly expressed in all three media (65–74%) (Loureiro et al. 2013). The researchers have attributed this high vimentin expression to the presence of spindle-shaped fibroblast-like cells in their culture. In our experiments, the cells proliferating from explants were almost all polygonal in shape, ruling out fibroblast contamination in the culture of 2 weeks' duration. In a recent study in which human limbal explants were cultured utilizing

FBS containing DMEM only, cells growing proximal to the explants showed 67% vimentin positivity (Szabó et al. 2015). Our results differ from these studies in that much lower vimentin expression was observed in all four media.

Both CK3 and CK12 are cytoskeletal proteins reported to be present in the cornea and indicate corneal differentiation (Baylis et al. 2011). CK3 is also present in the human limbal epithelium in a suprabasal localization (Schlötzer-Schrehardt and Kruse 2005). In an immunofluorescence study, rabbit limbal explants on AM showed CK3 and CK14 protein expression on upper suprabasal and lower basal layers of the explant, respectively (Wang et al. 2003). We have used an antibody that recognizes both CK3 and CK12 proteins to probe their presence on cells growing from explants. CK3/12 protein expression was 56% in cells in DMEM-F12-FBS and lower in Epilife and KSFM. Our CK3/12 protein expression in Epilife and KSFM is significantly lower than that reported for CK3 by Loureiro et al. for these media (46.5 and 65% respectively) (Loureiro et al. 2013). Cells in DMEM-F12-AS show the highest CK14 and lowest CK3/12 protein expression, indicating that DMEM-F12-AS is much more effective in supporting the limbal stem cell phenotype. In a recent study by Pathak et al. (2016), the expression of cytokeratin 3, 12 and 14 proteins was not significantly different between SHEM and DMEM-F12 with autologous serum.

When compared to data obtained from human limbal explants in media of similar composition, our results indicate that almost all cells from rabbit limbal explants are CK14(+), while CK3/12 and vimentin protein expression are low, pointing to the effect of PET membrane or a species difference.

Real-Time PCR quantified the expression of four possible LESC marker genes and three differentiation-related genes in cells growing from explants on PET inserts. The expression of *ABCG2*, *p63*, and *Bmi-1* were low in Epilife and KSFM, whereas a reduction in *CXCR4* was observed in KSFM. The decrease in mRNA expression of *CK3* and *CK12* paralleled the decline in protein levels for cells in KSFM and Epilife. The results of a study comparing the effects of Epilife and KSFM on gene expression in human cells growing from explants on plastic reported higher levels of *ABCG2* and *p63*, contrasting with our results (Loureiro et al. 2013). Another study has shown that human

limbal explants cultured on AM in SHEM, *ABCG2*, $\Delta Np63$ expressions decreased as cells proliferated away from the explant (Kolli et al. 2008).

In LESC growing from explants on PET inserts maintained in DMEM-F12-FBS, DMEM-F12-pluripotin, and DMEM-F12-AS, the expression of two stem cell markers *ABCG2*, *p63* were lower, whereas expression of *CXCR4* and *Bmi-1* did not differ from limbal tissue levels. Pluripotin is a small molecule that has been reported to maintain the self-renewal properties of embryonic stem cells in the absence of feeder cells (Pieters et al. 2012). Pluripotin increased the protein expression of $\Delta Np63$ and *ABCG2* and decreased *CK3/12* in rabbit LEC (Duan et al. 2012).

ABCG2 is a membrane transport protein, and its dye exclusion activity enables the separation of side population cells which are designated as LESC (Budak et al. 2005). In DMEM-F12 supplemented with hormones and cholera toxin, human cells proliferating from explants on AM maintained *ABCG2* gene expression at the limbal tissue level (Pauklin et al. 2011). Another study using the same medium reported 46% *ABCG2*(+) cells by immunocytochemistry (Loureiro et al. 2013), whereas in a different report, the use of cholera toxin free Green medium resulted in the total loss of *ABCG2* protein expression (Ghoubay-Benallaoua et al. 2011).

The transcription factor *p63* is a *p53* protein homolog that has been initially identified as a keratinocyte stem cell marker (Pellegrini et al. 2001). It has been shown to be expressed in cells that have migrated from limbal explants; this suggests that *p63* marks both LESC and TACs (Joseph et al. 2004). Qu et al. have reported that in human LSC grown from explants, *p63* mRNA and protein expression was decreased in 100% confluent cells compared to cells growing exponentially (Qu et al. 2015). Confluency attained after 2 weeks of culture in outgrowths from explants may be the cause of the decrease in expression of *p63* and other cell marker genes in our experiments.

Bmi-1 is a protein that has a role in self-renewal of adult stem cells (Bhattacharya et al. 2015). It was shown to be expressed in rabbit LESC and regarded as a possible marker for these cells (Umemoto et al. 2006).

CXCR4 is the receptor of *CXCL12*/*SDF1* (C-X-C chemokine ligand 12/stromal derived factor 1). Initially observed in porcine limbal side population cells

(Akinci et al. 2009), this protein has recently been reported as an important component of the limbal stem cell niche (Szabó et al. 2015). While limbal stem cells are positive for SDF-1, stromal niche cells have been shown to be positive for vimentin and CXCR (Pieters et al. 2012; Schermer et al. 1986). The close physical association of LESC with niche cells through SDF-1/CXCR4 signaling is proposed to be the key components of the limbal niche. In a recent study comparing cells grown from human limbal explants on AM versus plastic, CXCR4 expression was found to be significantly lower in cells on AM (Lužnik et al. 2016).

Our approach culturing rabbit limbal tissue in DMEM-F12 based cholera toxin free medium with minimal xenogeneic medium additives may have resulted in the expansion of a group of cells that express vimentin at low levels, whereas limbal stem cell markers CXCR4 and Bmi-1 are represented at the limbal tissue level. This suggests that the culture conditions mentioned above favor the proliferation of a subset of cells belonging to the limbal niche in addition to limbal stem cells. Due to the difficulty in finding antibodies that recognize rabbit CXCR4 and Bmi-1, we were not able to perform immunocytochemistry for these two proteins.

Our results may be explained by a model in which different subpopulations of cells originating from the explants; while almost all cells express CK14, one group may be expressing low ABCG2, while CXCR4 and Bmi-1 are maintained at limbal tissue levels, the other group presenting with a typical side population phenotype with high ABCG2 expression. This proposition is supported by a recent study in which single-cell quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to show that in rabbit limbal tissue, the side population cells are formed by at least two immature cell populations with endothelial- or mesenchymal-like phenotypes (Kameishi et al. 2016).

LESCs have been propagated successfully in media containing human serum instead of FBS, with positive long-term clinical outcomes for patients with LSCD (Kolli et al. 2010), but only one study has compared the effects of DMEM-F12 with human serum with SLEM, focusing on two stem cell protein markers and a few limbal stem cell genes (Shahdadfar et al. 2012). We present an analysis of protein and gene expression of LESC grown in DMEM-F12 with autologous serum; these results are novel for rabbit cells, and our data are in line with the observations of

Shahdadfar et al. in that DMEM-F12-AS supports high CK14, low CK3/CK12, and vimentin protein expression. In this medium, mRNA expression of *CXCR4*, *ABCG2*, *Bmi-1* and *p63* were also maintained at limbal tissue levels.

In summary, our work contributes to the field by presenting an analysis of protein and gene expression of LESC grown in DMEM-F12 with autologous serum. DMEM-F12-AS better maintains the progenitor characteristics of LESC, as the mRNA expression of *CXCR4*, *ABCG2*, *Bmi-1* and *p63* was higher when compared to Epilife and KFSM.

In conclusion, our study shows that rabbit LECs can be cultivated on PET inserts using DMEM-F12 with autologous serum without a requirement for AM or feeder cells. With further study, this may be applied to human cells grown from limbal explants and can be utilized in clinical treatment. We believe that this study advances our understanding of the behavior of stem cells *in vitro* and *in vivo*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

- Akinci MM, Turner H, Taveras M, Wolosin JM (2009) Differential gene expression in the pig limbal side population: implications for stem cell cycling, replication, and survival. *Investig Ophthalmol Vis Sci* 50:5630–5638. <https://doi.org/10.1167/iops.09-3791>
- Baylis O, Figueiredo F, Henein C, Lako M, Ahmad S (2011) 13 years of cultured limbal epithelial cell therapy: a review of the outcomes. *J Cell Biochem* 112:993–1002. <https://doi.org/10.1002/jcb.23028>
- Bhattacharya R, Banerjee Mustafa S, Street M, Dey A, Dwivedi SKD (2015) Bmi-1: At the crossroads of physiological and pathological biology. *Genes Dis* 2:225–239. <https://doi.org/10.1016/j.gendis.2015.04.001>
- Bray LJ, Heazlewood CF, Atkinson K, Huttmacher DW, Harkin DG (2012) Evaluation of methods for cultivating limbal mesenchymal stromal cells. *Cytotherapy* 14:936–947. <https://doi.org/10.3109/14653249.2012.684379>
- Budak MT, Alpdogan OS, Zhou M, Lavker RM, Akinci MM, Wolosin JM (2005) Ocular surface epithelia contain

- ABCG2-dependent side population cells exhibiting features associated with stem cells. *J Cell Sci* 118:1715–1724. <https://doi.org/10.1242/jcs.02279>
- de Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li D-Q (2005) ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells* 23:63–73. <https://doi.org/10.1634/stemcells.2004-0093>
- Duan H, Wang Y, Yang L et al (2012) Pluripotin enhances the expansion of rabbit limbal epithelial stem/progenitor cells in vitro. *Exp Eye Res* 100:52–58. <https://doi.org/10.1016/j.exer.2012.04.012>
- Dziasko MA, Daniels JT (2016) Anatomical features and cell-cell interactions in the human limbal epithelial stem cell niche. *Ocul Surf* 14:322–330. <https://doi.org/10.1016/j.jtos.2016.04.002>
- Figueira EC, Di Girolamo N, Coroneo MT, Wakefield D (2007) The phenotype of limbal epithelial stem cells. *Investig Ophthalmol Vis Sci* 48:144–156. <https://doi.org/10.1167/iovs.06-0346>
- Ghoubay-Benallaoua D, Basli E, Goldschmidt P et al (2011) Human epithelial cell cultures from superficial limbal explants. *Mol Vis* 17:341–354
- González S, Deng SX (2013) Presence of native limbal stromal cells increases the expansion efficiency of limbal stem/progenitor cells in culture. *Exp Eye Res* 116:169–176. <https://doi.org/10.1016/j.exer.2013.08.020>
- Joe AW, Yeung SN (2014) Concise review: identifying limbal stem cells: classical concepts and new challenges. *Stem Cells Transl Med* 3:318–322. <https://doi.org/10.5966/sctm.2013-0137>
- Joseph A, Powell-Richards AOR, Shanmuganathan VA, Dua HS (2004) Epithelial cell characteristics of cultured human limbal explants. *Br J Ophthalmol* 88:393–398. <https://doi.org/10.1136/bjo.2003.018481>
- Jung S, Panchalingam KM, Rosenberg L, Behie LA (2012) Ex vivo expansion of human mesenchymal stem cells in defined serum-free media. *Stem Cells Int* 2012:123030. <https://doi.org/10.1155/2012/123030>
- Kameishi S, Umemoto T, Matsuzaki Y et al (2016) Characterization of rabbit limbal epithelial side population cells using RNA sequencing and single-cell qRT-PCR. *Biochem Biophys Res Commun* 473:704–709. <https://doi.org/10.1016/j.bbrc.2015.10.155>
- Kawakita T, Shimmura S, Hornia A, Higa K, Tseng SCG (2008) Stratified epithelial sheets engineered from a single adult murine corneal/limbal progenitor cell. *J Cell Mol Med* 12:1303–1316. <https://doi.org/10.1111/j.1582-4934.2008.00297.x>
- Kinzebach S, Bieback K (2013) Expansion of mesenchymal stem/stromal cells under xenogenic-free culture conditions. *Adv Biochem Eng Biotechnol* 129:33–57. https://doi.org/10.1007/10_2012_134
- Kolli S, Lako M, Figueiredo F, Mudhar H, Ahmad S (2008) Loss of corneal epithelial stem cell properties in outgrowths from human limbal explants cultured on intact amniotic membrane. *Regener Med* 3:329–342. <https://doi.org/10.2217/17460751.3.3.329>
- Kolli S, Ahmad S, Lako M, Figueiredo F (2010) Successful clinical implementation of corneal epithelial stem cell therapy for treatment of unilateral limbal stem cell deficiency. *Stem Cells* 28:597–610. <https://doi.org/10.1002/stem.276>
- Kruse FE, Tseng SC (1992) Proliferative and differentiative response of corneal and limbal epithelium to extracellular calcium in serum-free clonal cultures. *J Cell Physiol* 151:347–360. <https://doi.org/10.1002/jcp.1041510216>
- Levis H, Daniels JT (2009) New technologies in limbal epithelial stem cell transplantation. *Curr Opin Biotechnol* 20:593–597. <https://doi.org/10.1016/j.copbio.2009.09.002>
- Liu Y, Ding Y, Ma P et al (2010) Enhancement of long-term proliferative capacity of rabbit corneal epithelial cells by embryonic stem cell conditioned medium. *Tissue Eng Part C Methods* 16:793–802. <https://doi.org/10.1089/ten.tec.2009.0380>
- Loureiro RR, Cristovam PC, Martins CM et al (2013) Comparison of culture media for ex vivo cultivation of limbal epithelial progenitor cells. *Mol Vis* 19:69–77. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3559094&tool=pmcentrez&rendertype=abstract>
- Lužnik Z, Hawlina M, Maličev E et al (2016) Effect of cryopreserved amniotic membrane orientation on the expression of limbal mesenchymal and epithelial stem cell markers in prolonged limbal explant cultures. *PLoS ONE* 11:e0164408. <https://doi.org/10.1371/journal.pone.0164408>
- Mamo S, Gal AB, Polgar Z, Dinnyes A (2008) Expression profiles of the pluripotency marker gene POU5F1 and validation of reference genes in rabbit oocytes and preimplantation stage embryos. *BMC Mol Biol* 9:67. <https://doi.org/10.1186/1471-2199-9-67>
- O'Brien LE, Bilder D (2013) Beyond the niche: tissue-level coordination of stem cell dynamics. *Annu Rev Cell Dev Biol* 29:107–136. <https://doi.org/10.1146/annurev-cellbio-101512-122319>
- Pathak M, Olstad OK, Drolsum L et al (2016) The effect of culture medium and carrier on explant culture of human limbal epithelium: a comparison of ultrastructure, keratin profile and gene expression. *Exp Eye Res* 153:122–132. <https://doi.org/10.1016/j.exer.2016.09.012>
- Pauklin M, Thomasen H, Pester A, Steuhl K-P, Meller D (2011) Expression of pluripotency and multipotency factors in human ocular surface tissues. *Curr Eye Res* 36:1086–1097. <https://doi.org/10.3109/02713683.2011.608238>
- Pellegrini G, Dellambra E, Golisano O et al (2001) P63 Identifies keratinocyte stem cells. *Proc Natl Acad Sci USA* 98:3156–3161. <https://doi.org/10.1073/pnas.061032098>
- Pellegrini G, De Luca M, Arsenijevic Y (2007) Towards therapeutic application of ocular stem cells. *Semin Cell Dev Biol* 18:805–818. <https://doi.org/10.1016/j.semdb.2007.09.011>
- Pellegrini G, Rama P, Di Rocco A, Panaras A, De Luca M (2014) Concise review: hurdles in a successful example of limbal stem cell-based regenerative medicine. *Stem Cells* 32:26–34. <https://doi.org/10.1002/stem.1517>
- Pieters T, Haenebalcke L, Hochepeid T et al (2012) Efficient and user-friendly pluripotin-based derivation of mouse embryonic stem cells. *Stem Cell Rev* 8:768–778. <https://doi.org/10.1007/s12015-011-9323-x>
- Qu Y, Chi W, Hua X et al (2015) Unique expression pattern and functional role of periostin in human limbal stem cells. *PLoS ONE* 10:e0117139. <https://doi.org/10.1371/journal.pone.0117139>

- Rheinwald JG, Green H (1975) Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 6:331–343. <http://www.ncbi.nlm.nih.gov/pubmed/1052771>. Accessed 13 April 2015
- Schermer A, Galvin S, Sun TT (1986) Differentiation-related expression of a major 64 K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 103:49–62. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2113783&tool=pmcentrez&rendertype=abstract>. Accessed 05 June 2015
- Schlötzer-Schrehardt U, Kruse FE (2005) Identification and characterization of limbal stem cells. *Exp Eye Res* 81:247–264. <https://doi.org/10.1016/j.exer.2005.02.016>
- Selver OB, Barash A, Ahmed M, Wolosin JM (2011) ABCG2-dependent dye exclusion activity and clonal potential in epithelial cells continuously growing for 1 month from limbal explants. *Investig Ophthalmol Vis Sci* 52:4330–4337. <https://doi.org/10.1167/iovs.10-5897>
- Seol D, Choe H, Zheng H et al (2011) Selection of reference genes for normalization of quantitative real-time PCR in organ culture of the rat and rabbit intervertebral disc. *BMC Res Notes* 4:162. <https://doi.org/10.1186/1756-0500-4-162>
- Shahdadfar A, Haug K, Pathak M et al (2012) Ex vivo expanded autologous limbal epithelial cells on amniotic membrane using a culture medium with human serum as single supplement. *Exp Eye Res* 97:1–9. <https://doi.org/10.1016/j.exer.2012.01.013>
- Shanmuganathan VA, Foster T, Kulkarni BB et al (2007) Morphological characteristics of the limbal epithelial crypt. *Br J Ophthalmol* 91:514–519. <https://doi.org/10.1136/bjo.2006.102640>
- Sharma RR, Pollock K, Hubel A, McKenna D (2014) Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. *Transfusion* 54:1418–1437. <https://doi.org/10.1111/trf.12421>
- Szabó DJ, Noer A, Nagymihály R et al (2015) Long-term cultures of human cornea limbal explants form 3D structures ex vivo—implications for tissue engineering and clinical applications. Ljubimov AV (ed) *PLoS One* 10:e0143053. <https://doi.org/10.1371/journal.pone.0143053>
- Tseng SC (1989) Concept and application of limbal stem cells. *Eye (Lond)* 3:141–157. <https://doi.org/10.1038/eye.1989.22>
- Tseng SCG, Chen S-Y, Shen Y-C, Chen W-L, Hu F-R (2010) Critical appraisal of ex vivo expansion of human limbal epithelial stem cells. *Curr Mol Med* 10:841–850. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3190238&tool=pmcentrez&rendertype=abstract>. Accessed 6 June 2015
- Umemoto T, Yamato M, Nishida K, Yang J, Tano Y, Okano T (2006) Limbal epithelial side-population cells have stem cell-like properties, including quiescent state. *Stem Cells* 24:86–94. <https://doi.org/10.1634/stemcells.2005-0064>
- Vandesompele J, De Preter K, Pattyn F et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:RESEARCH0034. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=126239&tool=pmcentrez&rendertype=abstract>. Accessed 27 July 2014
- Wang D-Y, Hsueh Y-J, Yang VC, Chen J-K (2003) Propagation and phenotypic preservation of rabbit limbal epithelial cells on amniotic membrane. *Investig Ophthalmol Vis Sci* 44:4698–4704. <http://www.ncbi.nlm.nih.gov/pubmed/14578389>. Accessed 12 Dec 2014
- Xie H-T, Chen S-Y, Li G-G, Tseng SCG (2011) Limbal epithelial stem/progenitor cells attract stromal niche cells by SDF-1/CXCR4 signaling to prevent differentiation. *Stem Cells* 29:1874–1885. <https://doi.org/10.1002/stem.743>
- Yoon JJ (2014) Limbal stem cells: central concepts of corneal epithelial homeostasis. *World J Stem Cells* 6:391. <https://doi.org/10.4252/wjsc.v6.i4.391>
- Yoon J, Ismail S, Sherwin T (2014) Limbal stem cells: central concepts of corneal epithelial homeostasis. *World J Stem Cells* 6:391–403. <https://doi.org/10.4252/wjsc.v6.i4.391>
- Zhao Y, Ma L (2015) Systematic review and meta-analysis on transplantation of ex vivo cultivated limbal epithelial stem cell on amniotic membrane in limbal stem cell deficiency. *Cornea* 34:592–600. <https://doi.org/10.1097/ICO.0000000000000398>