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Ex vivo expanded autologous limbal epithelial cells on amniotic membrane using a culture medium with human serum as single supplement☆

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ABSTRACT

In patients with limbal stem cell deficiency (LSCD), transplantation of *ex vivo* expanded human limbal epithelial cells (HLECs) can restore the structural and functional integrity of the corneal surface. However, the protocol for cultivation and transplantation of HLECs differ significantly, and in most protocols growth additives such as cholera toxins, exogenous growth factors, hormones and fetal calf serum are used. In the present article, we compare for the first time human limbal epithelial cells (HLECs) cultivated on human amniotic membrane (HAM) in a complex medium (COM) including fetal bovine serum to a medium with human serum as single growth supplement (HSM), and report on our first examinations of HLECs expanded in autologous HSM and used for transplant procedures in patients with LSCD. Expanded HLECs were examined by genome-wide microarray, RT-PCR, Western blotting, and for cell viability, morphology, expression of immunohistochemical markers and colony forming efficiency. Cultivation of HLECs in HSM produced a multilayered epithelium where cells with markers associated with LESCs were detected in the basal layers. There were few transcriptional differences and comparable cell viability between cells cultivated in HSM and COM. The *p63* gene associated with LESCs were expressed 3.5 fold more in HSM compared to COM, and Western blotting confirmed a stronger p63α band in HSM cultures. The cornea-specific keratin CK12 was equally found in both culture conditions, while there were significantly more CK3 positive cells in HSM. Cells in epithelial sheets on HAM remaining after transplant surgery of patients with LSCD expressed central epithelial characteristics, and dissociated cells cultured at low density on growth-arrested fibroblasts produced clones containing $21 \pm 12\%$ cells positive for p63α ($n = 3$). In conclusion, a culture medium without growth additives derived from animals or from animal cell cultures and with human serum as single growth supplement may serve as an equivalent replacement for the commonly used complex medium for *ex vivo* expansion of HLECs on HAM.

1. Introduction

Slow-cycling limbal epithelial stem cells (LESCs) found within the basal cell layer of the limbal epithelium are responsible for continuously renewing the entire corneal epithelium, and thus ensuring a transparent cornea (Ahmad et al., 2010; Cotsarelis et al., 1989; Davanger and Evensen, 1971; Dua et al., 2005; Majo et al., 2008). When the limbal area is partially or totally damaged, limbal stem cell deficiency (LSCD) occurs, a condition characterized by corneal ingrowth of conjunctival epithelium, neovascularization, recurrent epithelial defects, scarring, chronic inflammation, pain and reduced vision (Tseng, 1996). In such cases, grafting of limbal tissue or *ex vivo* expanded human limbal epithelial cells (HLECs) can restore the structural and functional integrity of the corneal surface (Notara et al., 2010; Shortt et al., 2007). While the use of autologous limbal fragments depends on a healthy contralateral eye, *ex vivo* autologous expansion of HLECs can be used to treat

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patients with bilateral disease, as long as some healthy limbal tissue is present. Alternatively, epithelial cells from other sources such as the conjunctiva (Tanioka et al., 2006), the oral mucosa (Nishida et al., 2004), or allogenic HLECs from a cadaveric or living relative donor can be transplanted (Shortt et al., 2007), the latter requiring long-term postoperative immunosuppression (Daya et al., 2005; Shortt et al., 2007).

Since Pellegrini et al. (1997) published the clinical transplantation of *ex vivo* expanded HLECs in two patients, this technique has become a routine treatment for ocular surface reconstruction in patients with LSCD in several clinics (Ahmad et al., 2010; Sangwan et al., 2006; Shortt et al., 2007). However, the protocol for cultivation and transplantation differ significantly. These protocols include the use of explants - or cell culture, the use of mouse 3T3 feeder cell layer, as well as different carriers for cell expansion and transplantation (Di Girolamo et al., 2009; Mariappan et al., 2010; Pellegrini et al., 2010; Shortt et al., 2007). The use of HAM as substrate has been suggested to be beneficial since it is easily obtained, and serves as a strong biodegradable, hypoimmunogenic and relatively easily manipulated carrier. In addition, it facilitates the growth and expansion of HLECs without the need of 3T3 feeder cells and may have a positive influence on the long-term survival of LESCs (Lee and Tseng, 1997; Meller et al., 2002; Shortt et al., 2007, 2008).

The composition of the medium is also essential for the culture of HLECs. To achieve successful cell culturing conditions, fetal bovine serum (FBS), in addition to various hormones and growth factors, has been included in most culture methods for treatment of LSCD (Shortt et al., 2007). However, these animal derived products carry a potential risk of transmission of animal viruses, prions and foreign proteins that may initiate xenogeneic immune responses. Therefore, using a culture medium completely free of animal products could be beneficial. *Ex vivo*, 1–10% of human serum is suitable for cultivation of HLECs (Di Girolamo et al., 2007; Mariappan et al., 2010; Zakaria et al., 2010), and transplantation of HLECs expanded on HAM in an autologous serum-based media have been shown to be successful in treatment of LSCD (Kolli et al., 2010; Meller et al., 2010; Nakamura et al., 2006; Shimazaki et al., 2007). However, in these studies an epithelial media containing various growth factors, cholera toxin and hormones were used. Only one group that we are aware of has previously used a culture medium with autologous serum as single growth supplement, and they applied a contact lens-based technique (Di Girolamo et al., 2009). In the present study, *ex vivo* expanded HLECs on HAM in a commonly used complex medium containing FBS and other non-human derived products is compared to a culture medium with human serum as single growth supplement. We also report on our first examinations of *ex vivo* expanded autologous HLECs maintained on HAM in medium with autologous serum and used in transplant procedures of patients with LSCD.

2. Materials and methods

2.1. Preparation of human serum

All reagents were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. Thirty ml blood was obtained from each 4 healthy voluntary donors (comparative *ex vivo* studies) or from patients undergoing clinical transplantations of HLECs. From each donor, sufficient venous blood was drained into 10 ml vacutainer tubes without anticoagulants (BD, Plymouth, U.K.) and allowed to clot. Subsequently, the blood was centrifuged at 1800 × g for 15 min at 4 °C. The serum from each donor was collected and passed through 0.22 μm pore size filters and aliquots of the sterile serum were stored at −20 °C. For the comparative *ex vivo* studies, equal volume of serum from each of the 4 donors was pooled.

2.2. Culture medium

2.2.1. Human serum medium (HSM)

DMEM/F12 (Invitrogen, Carlsbad, CA), Penicillin/Streptomycin (100 U/ml), amphotericin B (2.5 μg/ml) and 10% pooled human serum (comparative *ex vivo* studies using HLEC derived from donor eyes) or 10% autologous serum (using HLECs derived from patients with LSCD).

2.2.2. Complex medium (COM)

DMEM/F12 (Invitrogen), Penicillin/Streptomycin (100 U/ml), amphotericin B (2.5 μg/ml), 5% FBS, EGF (2 ng/ml, R&D Systems, MN), ITS (insulin 5 μg/ml, transferrin 5 μg/ml and sodium selenite 5 ng/ml), cholera toxin A (30 ng/ml, Biomol International, LP), dimethylsulfoxid (DMSO, 0.5%), hydrocortisone (15 μM), gentamicin (50 μg/ml).

2.3. Explant culture

All experiments were conducted in accordance with the Declaration of Helsinki and all tissue harvesting was approved by the Local Committees for Medical Research Ethics. For the comparative *ex vivo* studies, human corneoscleral tissue was obtained from limbal rings of cadaveric donors, available after penetrating keratoplasty, and preserved in Optisol-GS (Baush&-Lomb Inc., NY) at 4 °C. Each ring ($n = 5$) was divided in 8 samples. Corneal limbal epithelial tissue (1.5 × 2 mm) from patients with LSCD scheduled for transplant surgery were derived from healthy limbal areas in the contralateral or in the same eye ($n = 3$). The tissue was treated with 1.1 U/ml Dispase II in Mg^{2+} and Ca^{2+}-free Hanks' balanced salt solution (HBSS) at 37 °C for 10 min, thereafter rinsed in HSM or COM (Meller et al., 2002; Raeder et al., 2007). Human amniotic membranes (HAM) were preserved according to the method described by Lee and Tseng (Lee and Tseng, 1997). A formal Institutional Review Board approval and informed consent from the donor of the HAM were obtained. The HAM was cryopreserved in 50% (v/v) glycerol and media. After thawing, a piece of the HAM was placed on a Netwell plate and sutured in six corners. The limbal biopsy was placed with the epithelium facing down on the basement membrane surface of the HAM and allowed to attach. All cultures were incubated at 37 °C and 5% CO_2. The culture medium was changed every 2–3 days. For the comparative *ex vivo* experiments, four pieces from each of the Eye Bank donor eyes were cultured in parallel in either HSM or COM. Samples from patients with LSCD were cultured in medium using autologous serum.

2.4. Colony forming assay

Colony forming assays were performed by dissociating HAM-attached HLECs and seeding at clonal concentrations (3×10^3 cell/cm^2) (Kolli et al., 2010) on a feeder layer of CRL2429 human fibroblast (ATCC, Manassas, VA) growth-arrested by 40 Gy irradiation. Colony formation was monitored daily and stained with 0.5% Rhodamine or immunostained after 10 days of culture.

2.5. Assay for viability/cell death analysis

Cell viability/death was assessed by the Annexin-V-FITC Apoptosis Detection Kit (MBL, Woburn, MA) according to manufacturer's recommendations; proportion of stained Annexin-V$^+$ and Annexin-V$^+$/Propidium iodide (PI)$^+$ cells was determined by fluorescence activated cell sorter (FACS) analysis on BD Bioscience (San Diego, CA) flow cytometer (Petrovski et al., 2007).

2.6. Immunohistochemistry

Samples were fixed in 4% fresh formaldehyde and embedded in paraffin. Three micrometer sections were cut and stained using LabVision Autostainer360 (Lab Vision Corporation, VT) and visualized using a standard peroxidase technique (UltravisionOne HRP system). The following monoclonal antibodies were used; Cytkokeratin 3 (CK3, 1:500, ImmuQuest), Cytokeratin 12 (CK12, 1:400, Santa Cruz Biotechnology, CA), p63α (1:500, Santa Cruz Biotechnology), Ki-67 (1:200, Thermo Scientific), Vimentin (1:200, NeoMarkers) and mouse anti-human ABCG2 (1:80). The positive immunoreaction of the primary antibody was detected by a secondary antibody conjugated with peroxidase-labeled polymer with diaminobenzidine (DAB) (Utheim et al., 2009) or the fluorescent markers Cy3 (1:1000) and Alexa Fluor 488 (1:500, Invitrogen). Hoechst (1:500, Invitrogen) was used for nuclear staining. Sections were also stained with hematoxylin & eosin (H&E) for morphological examination.

2.7. Real-time RT-PCR

Total RNA was extracted from cells using Qiazol reagent (Qiagen, Hilden, Germany). Following DNase treatment (Ambion, Austin, TX), RNA was quantified by spectrophotometry (Nanodrop, Wilmington, DE). Reverse transcription (RT) was performed using the High Capacity cDNA Archive Kit (Applied Biosystems, Abingdon, U.K.) with 200 ng total RNA per 20 μl RT reaction. Comparative Relative Quantification was performed using the StepOnePlus Real-Time RT-PCR system (Applied Biosystems) and Taqman Gene Expression assays following protocols from the manufacturer (Applied Biosystems) (Table 1). The data were analyzed by $2^{-\Delta\Delta Ct}$ method as the fold change in gene expression and normalized to GAPDH as endogenous reference gene and relative to COM, which was arbitrarily chosen as calibrator and equals one. All samples were run in triplicates (each reaction: 2.0 μl cDNA, total volume 20 μl). The thermo cycling parameters were 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.8. Affymetrix gene expression profiling

100 ng of total RNA was subjected to the GeneChip HT One-Cycle cDNA Synthesis Kit and GeneChip® HT IVT Labeling Kit following the manufacturer's (Affymetrix, Santa Clara, CA) recommended protocol for whole transcript gene expression analysis. Labeled and fragmented single-stranded DNA was hybridized to the Affymetrix GeneChip Human Gene 1.0 ST Arrays. The signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA). The CEL files were imported into ArrayAssist Expression Software (v5.2.0; Iobion Informatics LLC, La Jolla, CA) and normalized using the RMA (Robust Multichip Average) algorithm in Array Assist to calculate relative signal values for each probe set (Utheim et al., 2009).

Table 1
Primers used for Real-time RT-PCR.

Gene name	Gene symbol	Alias	Taqman assay ID
ATP-binding cassette sub family G2	*ABCG2*	*BCRP*	HS01053790_m1
Cytokeratin 3	*KRT3*	*CK3*	HS00365080_m1
Gap junction protein α1, 43 Kda	*GJA1*	*CX43*	HS00748445_s1
Occludin	*OCLN*	–	HS00170162_m1
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	*GAPD*	HS99999905_m1
Tumor protein P63	*TP63*	*P63*	HS00978340_m1

2.9. Western blotting

Total protein was prepared from frozen samples (Invitrogen). Proteins were mixed in sample-loading buffer (Tris buffer pH 6.8, 2% SDS, 10% sucrose, and protease inhibitors), boiled for 10 min, centrifuged, and protein concentration in the clarified lysates was determined using the BCA Protein Assay kit (Termo Fisher Scientific, Rocford, IL). Equal amounts of protein in cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electrotransferred to a polyvinylidine difluoride membrane (Millipore, Billerica, MA) the membranes were blocked with 5% skim milk in PBS containing 0.1% Tween 20, incubated with the primary anti-human ABCG2 antibody (Abcam, Cambridge, U.K.) and anti-human p63α antibody (Santa Cruz) for 2 h, washed 3 times, and incubated respectively with the anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase for 1 h. Finally, the membranes were washed 3 times and protein bands were detected using enhanced chemilluminescence reagent (Amersham Biosciences, Sweden). The membrane was stripped for re-blotting with β-actin antibody as control.

2.10. Transmission electron microscopy (TEM)

The HAM-attached HLECs were fixed in 2% glutaraldehyde in cacodylate buffer (pH 7.4) overnight at 4 °C, postfixed in 1% osmium tetroxide, and dehydrated through a graded series of ethanol up to 100%. The tissues were then immersed in propylene oxide for 20 min and embedded in Epon (Electron Microscopy Sciences, Hatfield, PA). Ultra-thin sections (60–70 nm thick) were cut on a Leica Ultracut Ultramicrotome UCT (Leica, Wetzlar, Germany), stained with uranyl acetate and lead citrate and examined using a Tecnai12 transmission electron microscope (Phillips, Amsterdam, the Netherlands) (Moe et al., 2009).

2.11. Statistics

The results are presented as mean ± SD. Differences between groups were tested with one-sample *t*-tests or two-tailed independent sample *t*-tests. Data that were not normally distributed were normalized by log transformation. The significance level was set to $p < 0.05$, and data were analyzed using SPSS Version 14.0.

3. Results

3.1. Comparison of ex vivo expansion in human serum medium and complex medium

A total of 40 HCLEC explants on HAM from 5 donors were cultured in parallel in either HSM or COM (20 explants in each group). Under both culture conditions, the epithelial cells migrated from the limbal edges to form an epithelial sheet of 1–5 cell layers with basal column-shaped cells and superficial flattened squamous-like cells.

3.1.1. Comparative transcriptional profiling

To determine differences between HLECs cultured in HSM and COM at the transcription level, we compared the global gene expression profile using microarray from three different donors. Intensity profiles of the $\log_2$ transformed signal values of the 28,869 transcripts (vertical columns) are shown in Fig. 1A. Of these, 188 transcripts were differentially expressed more than 2 fold change

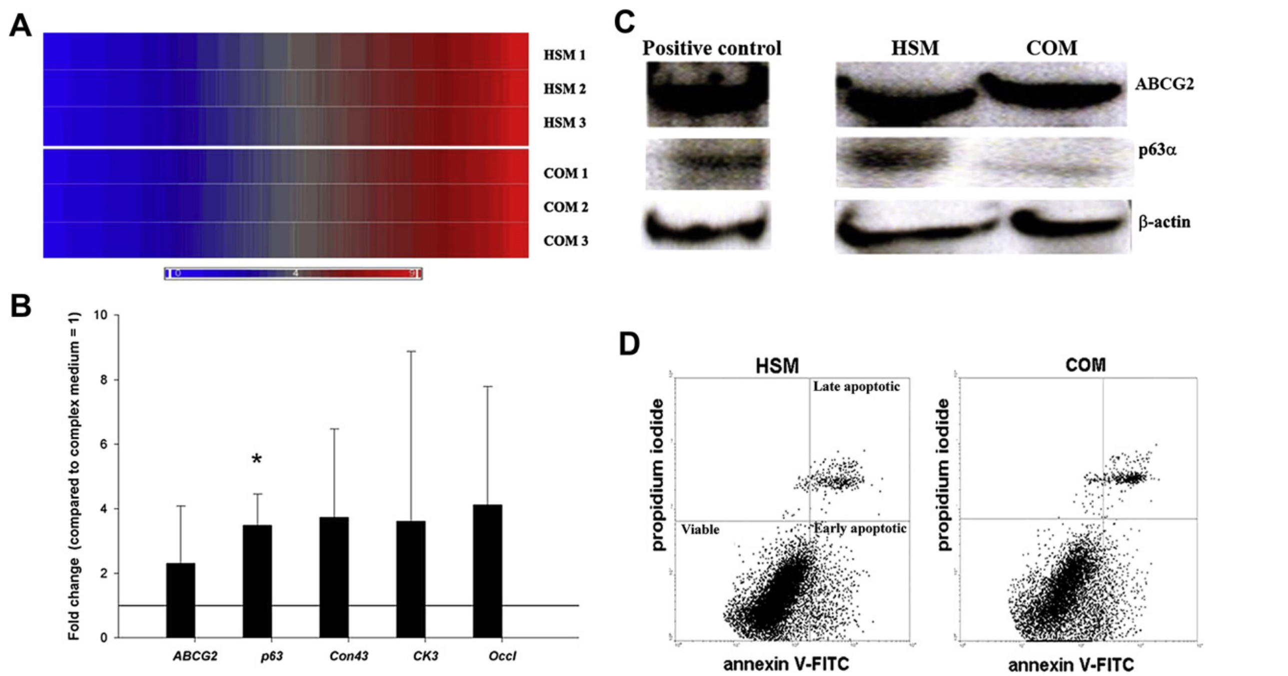

Fig. 1. Differential gene and protein expression levels of human limbal epithelial cells (HLECs) from a validated microarray study using cells from three different donors which were in parallel expanded in a culture medium with pooled human serum as single supplement (HSM) or a complex medium (COM) containing fetal bovine serum and other non-human products. Intensity profiles of the $\log_2$ transformed signal values of the 28,869 transcripts (vertical columns) are shown. Red and blue colors indicate high and low expression, respectively (A). Real-time RT-PCR analysis of the expression of selected genes associated with stemness (*ABCG2* and *p63*) and differentiated corneal epithelial cells (*connexin43*, *CK3* and *occludin*) in HLECs cultivated in HSM relative to COM, which was arbitrarily chosen as calibrator and equals one (B). Western blotting of ABCG2 and p63α in HSM and COM explant cultures. The membrane was stripped for re-blotting with β-actin antibody as control(C). Representative cell viability/death analysis of the HLECs grown in HSM and COM – distribution of viable, annexin V^+ and annexin V^+/propidium iodide$^+$ cells after 2 weeks of cultivation (D).

($n = 3$, $p < 0.01$) and 85 transcripts exhibited more than 3 fold change ($p < 0.01$) between the two culture conditions. This indicates relatively low transcriptional differences between the HSM and COM culture conditions. Table 2 shows 40 top genes comparatively expressed in HLECs cultured in HSM compared to COM which were differentially expressed more than 5 fold change ($p < 0.01$). These genes were mostly involved in extracellular matrix and protein binding activities, while a few genes were related to cell stemness and control of cornea integrity such as *ALDH1A1* and *DKK2*, respectively (Ahmad et al., 2008; Mukhopadhyay et al., 2006).

3.1.2. Cell viability/death profiling

The viability of HLECs cultured in HSM and COM was also comparable (HSM 79.7 ± 8.1 and COM 84.9 ± 5.1) with a comparable but small percentage of cells undergoing early or reversible (annexin V^+) apoptosis (HSM 12.0 ± 3.9 and COM 4.1 ± 1.1) and similarly small percentage undergoing late or irreversible (annexin V^+/PI^+) apoptosis or secondary necrosis (HSM 7.8 ± 3.7 and COM 11.0 ± 4.2) (Fig. 1D, $n = 3$, $p > 0.05$).

3.1.3. Expression of stem cell – and differentiation associated markers

Next, we performed Real-time RT-PCR, western blot and immunohistochemical analysis of selected markers associated with stemness and differentiation in HLECs. Even though no specific markers for LESCs have been identified (Joseph et al., 2004; Schlotzer-Schrehardt and Kruse, 2005), the ATP-binding cassette transporter G2 (ABCG2), which is the molecular determinant of the side population phenotype (Zhou et al., 2001), is currently one of the leading candidate LESC markers (Watanabe et al., 2004). In addition, the transcription factor p63 (Yang et al., 1999), and in particular the isoform ΔNp63α that is by far the most abundant in the corneal epithelium (Robertson et al., 2008), has been linked to stemness and also clinical outcome after transplantation of HLECs (Rama et al., 2010; Schlotzer-Schrehardt and Kruse, 2005). We found that there were a tendency for higher *ABCG2* expression ($p = 0.18$) and a 3.5 fold increased *p63* expression after cultivation in HSM than in COM, respectively ($n = 5$) (Fig. 1B). Western blot results confirmed an increase in p63α protein expression of explants cultured in HSM compared to COM (Fig. 1C). Immunohistochemical analysis revealed abundant ABCG2 staining of the plasma membrane in both HSM and COM cultures, and even though there were a significant increase in the p63α mRNA expression, no significant difference in the number of cells with nuclear p63α staining was found (65 ± 22 and 79 ± 13% of HSM and COM cultures, respectively, $n = 3$) (Fig. 2). Immunohistochemical analysis of the intermediate filament Vimentin, that also is found in LESCs (Schlotzer-Schrehardt and Kruse, 2005), showed equal expression patterns of basally located cells co-stained with p63α in the two culture conditions (Fig. 2), and the proliferation marker Ki-67 did not indicate any major differences in the proliferative capacity of HLECs cultivated in HSM and COM, with a Ki-67 index of 28 ± 4% and 28 ± 8%, respectively ($n = 3$) (Fig. 2).

Of the differentiation-associated markers (Schlotzer-Schrehardt and Kruse, 2005), Real-time RT-PCR showed a tendency for that both the gap-junction marker *connexin 43* ($p = 0.09$) and the tight-junction marker *occludin* ($p = 0.13$) were upregulated in cultures with HSM compared to COM, however these analysis did not reach statistical significance (Fig. 1B, $n = 5$). While the amount of cells positive for the differentiation marker cytokeratin 12 (CK 12) was not statistically different between HSM and COM (48 ± 21% and 54 ± 23%, respectively, $n = 3$), CK3 positive cells were almost absent in the COM cultures (2 ± 4%), whereas 13 ± 2% of the cells in the

Table 2
Most highly overexpressed transcripts (fold change ≥ 5, and $p < 0.01$, $n = 3$) of human limbal epithelial cells (HLECs) cultured with human serum as single growth supplement (HSM) compared to a complex medium (COM) containing fetal bovine serum and other non-human products. If a gene was represented with more than one probe in Affymetrix gene expression profiling list, the average of differential expression was selected. Values represent median regulation of gene expression in HSM cultures compared to COM.

Gene symbol	Gene description	Fold change	Regulation	Molecular function
POSTN	Periostin specific factor	59	Up	Protein binding
TAGLN	Transgelin	23	Up	Actin binding
BGN	Biglycan	20	Up	Extracellular matrix constituent
COL3A1	Collagen type III	20	Up	Extracellular matrix constituent
COL1A2	Collagen type 1	19	Up	Extracellular matrix constituent
SPP1	Secreted phosphoprotein1	15	Down	Cytokine activity
CDH11	Cadherin 11	13	Up	Calcium ion binding
TNC	Tenascin C	11	Up	Receptor binding
DCN	Decorin	11	Up	Protein binding
COL6A3	Collagen type VI	11	Up	Serine inhibitor
PDGFRA	PDGF receptor	11	Up	Nucleotide binding
IGFBP5	IGF- binding protein 5	11	Up	Insulin-like growth factor binding
COL1A1	Collagen, type I, alpha 1	11	Up	Extracellular matrix constituent
FABP4	Fatty acid binding protein	10	Up	Transporter activity
VCAN	Versican	10	Up	Calcium ion binding
C1S	Complement component	9	Up	Rhodopsin-like receptor activity
SULF1	Sulfatase 1	8	Up	Arylsulfatase activity
THBS2	Thrombospondin 2	8	Up	Cell adhesion
FBN1	Fibrillin 1	8	Up	Transmembrane receptor activity
GLIPR1	GLI pathogenesis-related1	8	Up	–
CCDC80	Coil domain containing 80	8	Up	–
MFAP5	Fibrillar associated protein 5	8	Up	Extracellular matrix constituent
CDH2	Cadherin 2 (N-cadherin)	8	Up	Calcium ion binding binding
PRRX1	Paired related homeobox 1	8	Up	Transcription factor activity
RGS4	Regulator of protein signal 4	7	Up	Signal transducer activity
AEBP1	AE binding protein 1	7	Up	Transcription factor activity
FAP	Fibroblast activation protein	7	Up	Metalloendopeptidase
CCL2	Chemokine ligand 2	7	Up	G-protein-coupled receptor
COL12A1	Collagen, type XII, alpha 1	7	Up	Structural molecule activity
AMTN	Amelotin	7	Up	Protein binding
COL5A1	Collagen type V	7	Up	Heparin binding
DKK2	Dickkopf homolog 2	7	Up	Multicellular development
FBLN5	Fibulin 5	7	Up	Transmembrane receptor activity
HMCN1	Hemicentin 1	7	Up	Transmembrane receptor
ACTG2	Actin gamma 2	6	Up	Nucleotide binding
MMP13	Matrix metallopeptidase	6	Up	Metalloendopeptidase activity
LUM	Lumican	6	Up	Extracellular matrix constituent
TGFB2	Transforming growth factor 2	5	Up	Growth factor activity
THY1	Thy-1 cell surface antigen	5	Up	Protein binding
ALDH1A1	Aldehyde dehydrogenase 1	5	Down	Cellular enzyme activity

HSM cultures, mostly in superficial layers, stained for this corneal epithelial marker ($n = 3$, $p < 0.05$) (Fig. 2) even though RT-PCR data did not show any upregulation of CK3 in HSM (Fig. 1B).

3.2. *HLECs from patients with LSCD expanded ex vivo in autologous human serum medium and used for transplant surgery*

Two weeks after the autologous limbal biopsy, the HLECs expanded in autologous HSM on HAM had grown to form a sheet covering most of the culture plate. Examination of epithelial sheets on HAM remaining after transplant procedures revealed that cells expanded in autologous HSM expressed central epithelial characteristics, including flat superficial cells, basal cells with a more cuboidal shape attached to the HAM basement membrane and numerous desmosomal junctions between adjacent epithelial cells (Fig. 3A–C). In a separate experiment, we tested whether expanded HLECs retained a population of colony forming cells. HAM-attached HLECs were dissociated and cultivated at low density on growth-arrested human fibroblasts. Of the three epithelial sheets tested, epithelial clones (Kolli et al., 2010; Pellegrini et al., 1999) with a smooth outline appeared in all cases after 10 days of culture (Fig. 3D). In these clones, 21 ± 12% ($n = 3$) of the cells were p63α positive (Fig. 3E).

4. Discussion

In establishing tissue equivalents for transplantation, the ideal method should 1) be approved and safe with respect to disease transmission and 2) be able to recapitulate the tissue of origin after integration. For the corneal epithelium, the latter should include both LESCs with ability of self-renewal and targeted differentiation, as well as differentiated epithelial cells able to protect the ocular surface (Rama et al., 2010; Schlotzer-Schrehardt and Kruse, 2005; Shortt et al., 2008). Our culture medium uses human serum as single growth supplement, and is free of both animal derived products and other growth supplements such as exogenous growth factors, hormones and cholera toxin. The present article indicates that HLECs can be expanded on HAM *ex vivo* using this novel method without losing the potential to generate a healthy epithelium. Although there are no exact ways to identify LESCs at present (Lyngholm et al., 2008; Robertson et al., 2008; Schlotzer-Schrehardt and Kruse, 2005), there were several indications of LESCs present in the HLECs expanded with the current protocol, including 1) expression of markers found in LESCs such as p63α, ABCG2 and Vimentin, 2) a multilayered epithelium with flattened apical cells and basal cuboidal-shaped cells attached to the HAM basement membrane with intercellular junctional complexes, and 3) retained colony forming capacity.

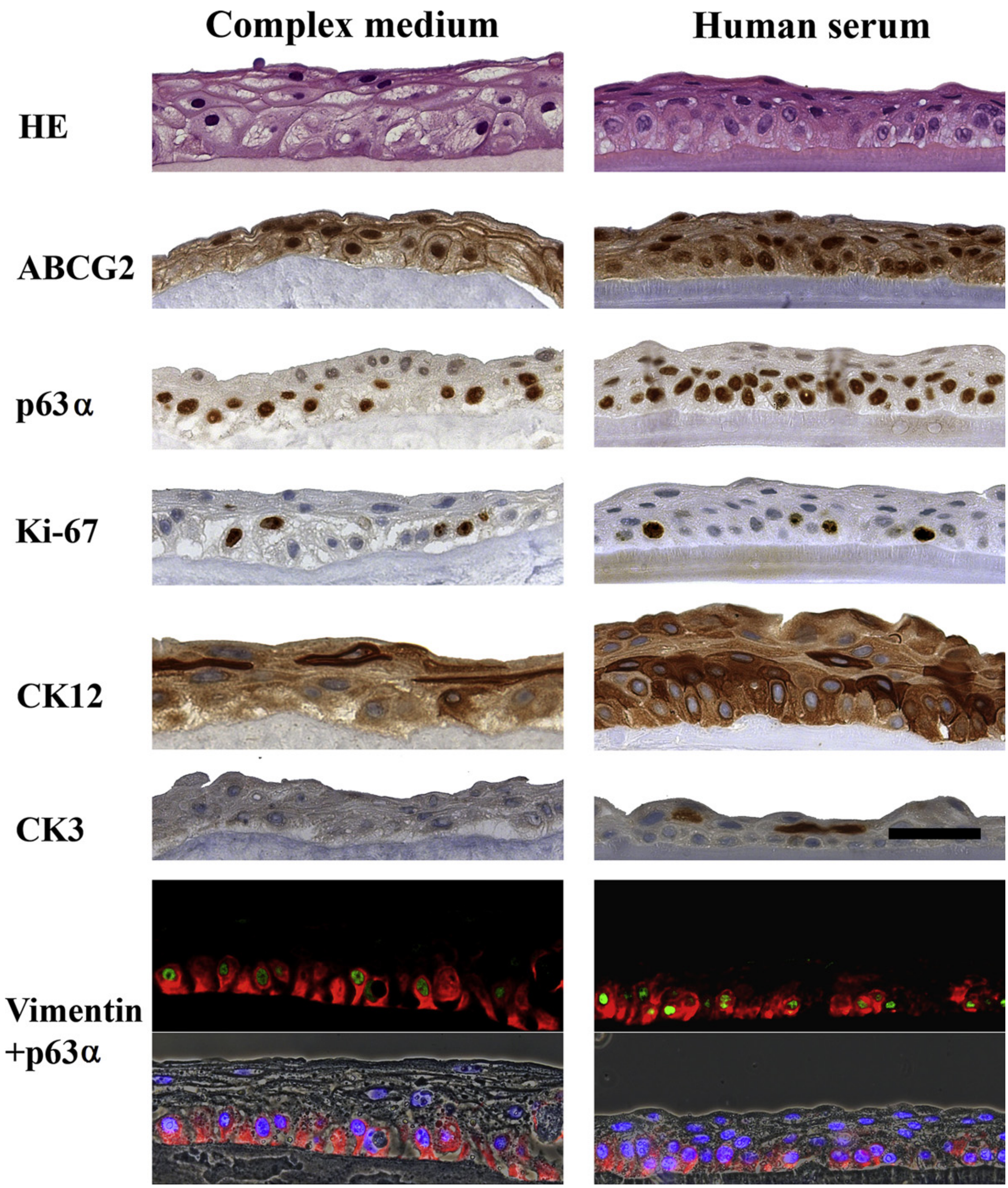

Fig. 2. Immunohistochemical comparison of human limbal epithelial cells (HLECs) cultivated in complex medium with fetal bovine serum and other non-human products (COM) to a medium with pooled human serum as single growth supplement (HSM). The cultures demonstrated similar immunoreactivity to all markers except the differentiated corneal epithelial marker CK3, which was significantly more expressed in HSM. Lower panel: Vimentin (red), p63α (green) and nuclear Hoechst staining (blue). Scale bar: 50 μm.

Previous clinical protocols for transplantation of *ex vivo* expanded HLECs have relied almost exclusively on a complex FBS-supplemented medium (Baylis et al., 2011; Nakamura et al., 2004; Pellegrini et al., 2010; Shortt et al., 2007). However, to decrease the chance of disease transmission and to omit unnecessary additives, the use of autologous serum and culture conditions free of any animal derived products has been suggested (Schwab et al., 2006). It has been shown that uptake of FBS protein by stem cells is an active process that leads to an intracellular accumulation of bovine antigen, even when the FBS concentration in the expansion medium is as low as 2% (Gregory et al., 2006). Indeed, stem cell transplantation failure has been noted as a consequence of immune attack on FBS proteins carried by transplanted cells expanded in FBS (Sundin et al., 2007). In addition, recombinant proteins produced by mammalian cells under expansion in the presence of FBS calls for secure and approved culture control for cell therapy. On the other hand, the use of selected batches of FBS may eliminate the variability of autologous serum, and this might increase the reproducibility of the cultures (Pellegrini et al., 2010). Use of autologous serum for expansion of HLECs was first introduced and proved its efficacy in a setting where the cells were expanded on plastic contact lenses (Di Girolamo et al., 2009), and a mixture of complex medium and human serum has been shown to support HLEC expansion on HAM (Kolli et al., 2010; Meller et al., 2010;

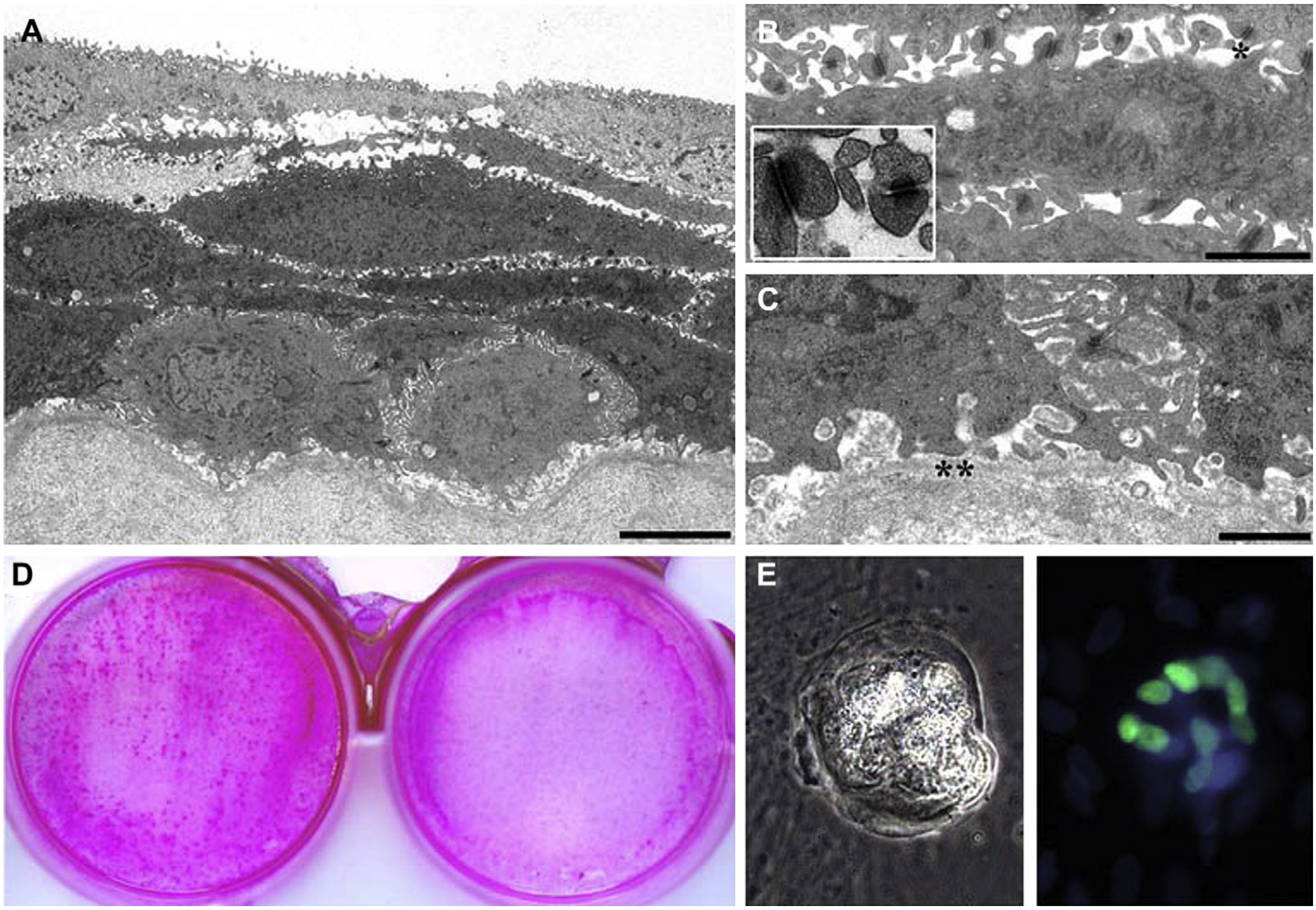

Fig. 3. Transmission electron micrographs of limbal epithelial cells (HLECs) expanded on human amniotic membrane (HAM) in a culture medium with autologous human serum medium (HSM) as single supplement. The HLECs consisted of a healthy and well-differentiated cell layer similar in appearance to normal corneal epithelium, including a basement membrane like-material, basal cuboidal-like cells and flat squamous like superficial cells (A). Neighboring cells were attached by numerous desmosomal junctions (*) and showed abundant basal interdigitation (B) Higher magnification of desmosomes (B inset). Basal cells attached to the HAM basement membrane with hemidesmosomal-like junctions (**) (C). HLECs co-cultured at clonal density with growth-arrested human fibroblasts using HSM (D, left petridish with HLECs and right with only feeder cells). Early epithelial holoclone-like colony stained for p63α (green) and Hoechst (blue, nuclear) after 10 days of culture (E). Scale bars A: 5 μm, B–C: 1 μm.

Nakamura et al., 2006; Shimazaki et al., 2007). In our study, we found that HLECs expanded on HAM in medium with human serum as the single growth supplement developed the same morphology of an epithelial sheet, a low transcriptional difference, and similar cell viability/death patterns compared to cultivation in the commonly used complex medium. Still, a key question is whether the culture medium with human serum as a single supplement retains cells with properties of LESCs.

The most common protocol to ensure the presence of LESCs in the cell population prior to transplantation is cultivation of isolated cells on 3T3 feeder cells and to study the formation of holoclones. Recently, Rama et al. showed that even though the formation of holoclones is the "gold standard" to identify LESCs, the percentage of p63 bright cells is indicative of the clinical outcome after transplantation to patients with LSCD (Rama et al., 2010). This transcription factor could thus be used to detect viable LESCs prior to transplantation. In our study, HSM cultivation increased the expression, both on the mRNA level and on the protein level, of p63 in the expanded HLECs compared to COM cultures, while there were no statistical differences between the numbers of p63α positive cells. In addition, our microarray data indicated a 5 times downregulation of *ALDH1A1* in HSM compared to COM, and it is known that ALDH(dim) human epithelial cells expresses significantly higher levels of ΔNp63 and ABCG2 as well as having a greater colony forming efficiency (CFE) when compared to ALDH(bright) cells (Ahmad et al., 2008). Together, these data indicate that HSM is at least comparable to COM in retaining cells with some central markers of LESCs during *ex vivo* expansion of HLECs on HAM.

Furthermore, markers of corneal epithelial cells including cytokeratin 3 and 12, were more or similarly expressed, respectively, in the HSM compared to the COM cultures. Thus, HSM is not inferior to COM in the ability to start proper terminal differentiation of the cell types needed to protect the ocular surface. These data are in agreement with the paper of Nakamura et al. showing that human serum is equal to FBS in *ex vivo* cultivation of HLECs using a medium also containing several hormones and growth factors (Nakamura et al., 2006), even though this study did not investigate the expression of stemness markers (p63/ABCG2) or the differentiation marker CK3. In addition, there is evidence that other populations of stem cells, such as human mesenchymal stem cells (MSCs), show enhanced stability in gene expression when expanded using autologous serum compared to FBS (Shahdadfar et al., 2005), and that autologous serum has a greater tendency than FBS to maintain MSCs in an unmethylated state (Dahl et al., 2008). Interestingly, a recent study has also indicated that even a serum-free protocol is efficient in expansion of HLECs (Lekhanont et al., 2009). Further studies mapping the epigenetic status of HLECs cultivated in a medium containing no serum, HSM and COM are therefore interesting subjects for further research.

In conclusion, there is an obvious strive for development of culture conditions with a reduced content of animal exogenic products and omission of foreign feeder cell types for *ex vivo* expansion of HLECs for therapeutic use in human patients. We here show that HLECs can be expanded on HAM *ex vivo* using a culture medium with human serum as single growth supplement. The expanded epithelial tissue contain cells with central properties of both undifferentiated LESCs and differentiated corneal epithelial cells compared to a commonly used complex medium including FBS and other non-human derived products. Although the effect of medium should be studied in more detail, omission of such products may reduce the immunogenicity of the transplanted tissue and also safeguard against the transfer of infectious diseases and therefore ultimately affect the postoperative outcome and the safety of the procedure.

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