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FULL LENGTH PAPER

Towards xeno-free cultures of human limbal stem cells for ocular surface reconstruction

Zala Lužnik · Claudia Breda · Vanessa Barbaro · Stefano Ferrari · Angelo Migliorati · Enzo Di Iorio · Barbara Ferrari · Carlo Griffoni · Andrea Grassetto · Hossein Mostafa Elbadawy · Marina Bertolin

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Abstract Isolated limbal epithelial stem cells (LESCs) were cultured with or without a 3T3 murine fibroblast feeder-layer (FL) in 4 different culture media on culture plates or on denuded human amniotic membrane (AM) support and fibrin gel support: (1) control medium supplemented with fetal bovine serum; (2) control medium supplemented with the synthetic serum "XerumFree^{TM} XF205" (XF); (3) CnT-20 medium supplemented with "XerumFree^{TM} XF205" (CnT-XF) and (4) CnT-20 medium supplemented with human AB serum (CnT-AB). The three xenogeneic media were compared to standard condition (control + FL) and parameters assessed included cell morphology, proliferative potential, number of passages, assessment of clonogenic and abortive colonies, life span, ΔNp63α expression and epithelial morphology on AM. During serial cultivation of LESCs, most of the tested xeno-free media supported similar numbers of cell passages, total colony number, cumulative cell doublings (CCD) rates and expression of ΔNp63α compared to control. The conditions cultivated with a FL showed a non-statistically significant higher number of cell passages and CCD rates before senescence when compared to the same conditions cultured without FL. Except for the control medium, only XF medium enabled the growth of cells on AM. The expression of ΔNp63α was comparable in all the cultures grown onto AM, when compared to the controls on fibrin gel. In conclusion, the xeno-free media enabled LESC culture both on plastic and on denuded human AM. Despite the analyses were carried out in a statistically low number of samples and need re-assessment in a larger cohort, our results suggest that the production of a completely xeno-free LESC graft could be beneficial for future clinical applications.

Keywords Limbal epitehlial stem cells · Synthetic medium · Feeder-layer · Amniotic membrane · Fibrin gel

Introduction

Severe ocular surface pathologies can cause total limbal stem cell deficiency (LSCD), a disease which

Zala Lužnik and Claudia Breda contributed equally to the work.

Z. Lužnik
Eye Hospital, University Medical Centre, Ljubljana, Slovenia

C. Breda · V. Barbaro · S. Ferrari · E. Di Iorio · B. Ferrari · C. Griffoni · A. Grassetto · M. Bertolin (✉)
Fondazione Banca degli Occhi del Veneto, c/o Padiglione G. Rama - Via Paccagnella 11, 30174 Zelarino, Venice, Italy
e-mail: marina.bertolin@fbov.it

A. Migliorati · E. Di Iorio
Department of Molecular Medicine, University of Padua, Padua, Italy

H. M. Elbadawy
Department of Pharmacology and Toxicology, College of Pharmacy, Taibah University, Almadinah Almunawwarah, Saudi Arabia

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frequently leads to painful visual loss (Baylis et al. 2011; Chan et al. 2015) and is difficult to manage. Transplantation of limbal epithelial stem cells (LESCs) is necessary to restore vision (Meller et al. 2002). In 1997, Pellegrini et al. first described the successful transplantation of ex vivo cultured limbal epithelial (LE) sheets containing LESCs from a small limbal tissue biopsy (Pellegrini et al. 1997). Since then, ex vivo expanded limbal epithelial grafts have become a promising treatment option to regenerate the corneal epithelium in patients with LSCD (De Araujo and Gomes 2015; Baylis et al. 2011; Haagdorens et al. 2016). Currently, protocols for culturing LESCs differ significantly between centers. These protocols include the use of limbal explants (explant technique) or single cell culture (suspension technique), the presence or absence of feeder-layer (FL) usage (e.g. murine 3T3-J2 FL), different LESC markers (Ramos et al. 2015; Pellegrini et al. 1999; Di Iorio et al. 2010; Rama et al. 2010), different culture media compositions, as well as different carrier scaffolds for cell expansion and transplantation (Baylis et al. 2011; Ramos et al. 2015). Human amniotic membrane (AM) (Zhao and Ma 2015; Zakaria et al. 2014; Koizumi et al. 2007) and fibrin matrix gels (Marchini et al. 2012; Pedrotti et al. 2015; Rama et al. 2001) are some of the most commonly used scaffolds. Although almost two decades have passed since the first transplantation, most clinically used culture techniques still use xenobiotic (animal-derived) products to achieve appropriate cell culturing conditions (Pellegrini et al. 2014). Standard complex culturing media therefore contain fetal bovine serum (FBS) in addition to various growth hormones and cholera toxin (Pellegrini et al. 2014). The presence of animal derived products carries the theoretical health risk of pathogen transmission (e.g. prions, viruses, bacterial endotoxins) that may further initiate host immune reactions and lead to graft failure (Baylis et al. 2011). Moreover, these culture substrates can also be biologically variable (De Araujo and Gomes 2015).

Although, there have been only a few safety reports arising from autologous or allogeneic CLET (Pellegrini et al. 2014; Trounson and McDonald 2015), as the regulatory requirements for cell-based therapies are becoming more stringent (Di Iorio et al. 2010), alternatives have been sought to replace animal derived products and toxins from the culturing procedures. Autologous human serum has already been reported as an alternative to bovine serum in complex media (Sangwan et al. 2011; Kolli et al. 2008; Nakamura et al. 2006; Shimazaki et al. 2007) or as a single growth supplement (Pathak et al. 2013; Zakaria et al. 2010, 2014) in media for LESC culture. However, patients have to be medically suitable to donate blood, and the risk of transmitting unknown infections is still not fully overcome (Baylis et al. 2011). Thus, concerns about disease transmission can be reduced by the use of a culture medium free of all animal or human derived products and other growth supplements.

In 2002 Fondazione Banca degli Occhi del Veneto (FBOV) established a Cell Factory with the aim to restore damaged corneal surfaces through transplantation of autologous cultured limbal stem cells. Corneal stem cells are isolated from 1 to 2 mm^2 limbal biopsies and cultured onto murine 3T3-J2 FL before being plated onto fibrin-glue scaffolds needed for transportation and surgical application (Di Iorio et al. 2010; Marchini et al. 2012). Since 2003, approximately 200 patients with limbal stem cell deficiency (the largest cohort ever reported) have been treated with positive outcomes in nearly 70% of cases (Di Iorio et al. 2010; Marchini et al. 2012; Pedrotti et al. 2015). As a consequence of the regulation (EC) No 1394/2007, from 2012 the production of Advanced Therapy Medicinal Products has been carried out in a GMP Cell Factory, that has recently been authorized as production site by AIFA (authorization N. aM – 14/2016, dated 8th February 2016). The aim of the present study is to optimize our current approach for LESC cell suspension technique, which is based on the culturing method described by Pellegrini et al. (1997, 1999) and still utilizes complex media supplemented with FBS and murine FL co-culture onto fibrin gel scaffolds. Our goal is to replace our medium and FL with a newer xeno-free formulation for LESCs cultured on AM using cells suspension technique and to compare the two approaches.

Materials and methods

All the experiments complied with the tenets of the Helsinki Declaration. Human donor corneoscleral tissues unsuitable for transplantation were used for the purposes of this research study after Fondazione Banca degli Occhi del Veneto (Venice, Italy) obtained

a signed informed consent form from the donor's next of kin and specific authorization has been granted for their use for research purposes in case they are not suitable for transplantation. Forms have been approved by the Regional Transplant Centre which is part of the National Transplant Centre. No approval from the Ethical Committee is therefore needed.

Cell culture media

The standard control medium consisted of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12 (F12) (DMEM/F12 2:1) supplemented with 10% fetal bovine serum (FBS) (all from Gibco, Invitrogen, Italy), 50 μg/ml penicillin–streptomycin (P/S), 4 mM glutamine (both from Euroclone, Italy), 5 μg/ml insulin (Humulin R, Lilly, Canada), 0.4 μg/ml hydrocortisone (Flebocortid Richter, Sanofi, Italy), 0.18 mM adenine (Adenine grade I, Pharma Waldhof GMBH, Germany), 8.1 μg/ml cholera toxin (Cholera Toxin QD, List Biological Laboratories, USA), 2 nM triiodothyronine (Liotir, IBSA, Italy) and 10 ng/ml epidermal growth factor (EGF GMP Cellgro, Cell Genix GmbH, Germany).

The standard control medium was compared to three different xeno-free culture media, as described in Table 1: (1) control with 10% XerumFree™ XF205 (TNCBIO, Italy) (XF); (2) progenitor cell targeted CnT-20 medium (CellnTec, Switzerland) supplemented with 10% of "XerumFree™ XF205" (CnT-XF); (3) CnT-20 supplemented with 1% human type AB serum (Sigma Aldrich, Italy) (CnT-AB) (Table 1). EGF was added to the XF medium condition from the first day of culture. As the CnT-20 basal medium already contains EGF, the latter was not added. Media containing "XerumFree™ XF205" synthetic serum were supplemented with 10 μg/ml P/S instead of 50 μg/ml P/S to avoid any toxic effect, as suggested by the manufacturer (TNCBIO). Media were changed every 2–3 days. The standard control medium, XF and CnT-AB media have not been tested without FL.

LESC cultures

LESCs were cultured as previously described (Corradini et al. 2012; Pellegrini et al. 1997; Barrandon and Green 1987). Briefly, 3 donor limbal rims were treated with trypsin (0.05% trypsin/0.01% EDTA, Life Technologies, Italy) at 37 °C for 3–4 cycles of 30 min each.

In the first set of experiments (n = 2), once isolated, LESCs were plated at a standard seeding concentration (20,000/cm^2) onto a FL of lethally irradiated 3T3-J2 fibroblasts (40,000/cm^2) or directly onto FL-free culture plates, if not otherwise stated. The formulation media used for cell cultures are described in Table 1. Cells were incubated at 37 °C and 5% CO_2 in humidified atmosphere and a life span assay, a standard quality control used to determine the proliferation capacity of the cells (Pellegrini et al. 1997), performed.

In the second set of experiments, isolated LESCs from the secondary culture were cultured in parallel on denuded human AM with or without FL using control, CnT-XF, CnT-AB (n = 3) and XF (n = 2) media and were compared to the control condition (LESCs cultured onto fibrin gel with FL). The cryopreserved human AMs (3 × 3 cm) were obtained from the Treviso Tissue Bank (Treviso, Italy) and were prepared as previously described (Zakaria et al. 2010). Briefly, the amniotic epithelium layer was enzymatically removed. The spongy layer side was removed mechanically by gentle scraping off the stromal matrix

Table 1 List of culture media and characteristics

Culture medium	Xeno-free	EGF, ng/mL	Base Medium	FL[a] co-culture
CONTROL[b]	No/No	10	DMEM/F12 (2:1)	Yes
XF[c]	Yes/Yes	10	DMEM/F12 (2:1)	Yes
CnT-XF	Yes/Yes	Yes (in CnT medium)	CnT-20	Yes
				No
CnT-AB	Yes/No	Yes (in CnT medium)	CnT-20	Yes

[a] FL feeder layer

[b] CONTROL medium supplemented with growth factors

[c] Contains cholera toxin and epidermal growth factor

with a cell scraper. The denuded AMs, with the basement membrane facing up, were then secured in an inter-lockable plastic ring (2.3 cm^2) system (designed and realized in FBOV), which enables complete submerged cell culture.

LESCs cultured on AM in all the media formulations were initially seeded at 35,000/cm^2 cell density. Since LESCs cultured in CnT-XF medium failed to attach and grow on AM, they were seeded at higher densities (65,000/cm^2) to obtain optimal cell growth, according to manufacturer's recommendations. Standard LESCs cultured onto fibrin gel (control condition) were prepared according to our standard protocol (Di Iorio et al. 2010) as control of AM preparation protocol. Briefly, LESCs were seeded at a density of 17,000/cm^2 onto the fibrin gel scaffold with FL and were cultured under standard conditions in control medium. At confluence, LESCs were cultured for 4–5 additional days on AM and for 1–2 days on fibrin gel scaffolds to promote stratification. The cultures were then punched (Ø 0.4; 0.13 cm^2) for histological evaluation. The remaining parts of the cultures (2.17 cm^2) were enzymatically treated to dissociate the cells. The collected cells were counted and used for Real Time qPCR analysis to determine K12 and ΔNp63α expression.

Fibroblast cultures

3T3-J2 murine fibroblasts, NIH-3T3 murine fibroblasts and human corneal/limbal stromal fibroblasts (obtained from human cadaveric donor biopsies) were plated onto T75 flasks at a seeding density of 170,000/flask and serially cultivated using the following five media formulations: (1) the standard medium DMEM supplemented with 10% bovine calf serum (BCS) (Gibco, Invitrogen, Italy), 4 mM glutamine and 50 μg/ml P/S (control); (2) DMEM supplemented with 10% XerumFree™ XF205, 4 mM glutamine and 10 μg/ml P/S; (3) CnT-20 medium supplemented with 10 μg/ml P/S and 10% BCS; and (4) CnT-20 medium supplemented with 10 μg/ml P/S and (5) 1% human type AB serum. The media were changed three times a week. Morphology and growth were determined under light microscope and cells passaged at 70–80% confluence. Comparison to the control condition was carried out following the analysis of parameters such as cell viability, cell morphology and number of passages.

Cell biology-based quality control tests

In the first set of experiments, the different conditions were cultured performing the life span test, a standard assay to determine proliferation capacity of the cells (Pellegrini et al. 1997): the different cultures were serially cultivated at the same seeding concentrations until senescence, at every passage a colony forming efficiency (CFE) assay was performed. Comparison between the different growing conditions was carried out following the analysis of parameters collected from, such as number of passages, cumulative cell doubling (CCD) rate before senescence, percentage of clonogenic colonies.

In order to perform the CFE assay, LESCs were plated at each passage into 10-cm plates and cultivated in standard conditions (in control medium and co-cultured with 2×10^6 growth-arrested 3T3-J2 FL). CFE cultures were fixed on the 12th day using 1:100 Cristal violet (Sigma Aldrich, Italy). Colonies were examined and counted under a light microscope. Morphology and types of colonies (clonogenic or abortive) were identified as previously described (Barrandon and Green 1987). The total number of colonies was calculated as percentage of seeded cells that formed any colony (clonogenic and abortive) (CFE (%) = total number of colonies grown/number of cells seeded × 100). The percentage of clonogenic colonies was calculated as the number of clonogenic colonies/total number of colonies × 100.

Clonal analysis was performed on 100 clones obtained from the same donor. Fifty clones were isolated from cultures cultivated with XF media and the results were compared with 50 clones cultivated in standard condition (control). The classification of the clonal type was performed as previously described (Barrandon and Green 1987).

The cell generation number (cumulative cell doubling; CCD) was calculated using the equation below (Pellegrini et al. 1997, 1999):

$$n = \frac{(\log Y - \log X)}{0.332}$$

where Y represents the final cell count and X the number of clonogenic-cells, calculated from the CFE results, which were determined separately for each passage, as described earlier.

In order to complete the proliferation capacity analysis of the different cultures, a Real-time qPCR-

Table 2 Primers and probes

Gene symbol	Sequence
GAPDH Fw	5′-CCACTCCTCCACCTTTGACG-3′
GAPDH Rev	5′-CATGAGGTCCACCACCCTGT-3′
GAPDH Probe	5′-[TET]TTGCCCTCAACGACCACTTT[TAM]-3′
ΔNp63α Fw	5′-GCATTGTCAGTTTCTTAGCGAG-3′
ΔNp63α Rev	5′-CCATGGAGTAATGCTCAATCTG-3′
ΔNp63α Probe	5′-[6FAM]GGACTATTTCACGACCCAGG[BHQ1]-3′
ck12 for	5′-AGAACCACGAGGATGAGCTC-3′
ck12 rev	5′-TGCTCAGCGATGGTTTCATAC-3′
ck12 probe	5′-FAM-AGGCGAGGTCAGCGTAGAAATGG-BHQ1-3′

based analysis was performed to detect the expression of the stem cell marker ΔNp63α (as described in the following paragraph). Total RNA was extracted from cells at the second and fourth passage in culture in the first set of experiments and analyzed for expression of ΔNp63α. Similarly, LESCs cultured on AM and on fibrin gel were analyzed for ΔNp63α and K12 expression (Di Iorio et al. 2005, 2010).

Histology

The punched human AM cultures were fixed with 4% paraformaldehyde (PFA; CremCruz, Santa Cruz Biotechnology, INC) over night, washed in sucrose solutions (at 7, 5, 15 and 30% sucrose), embedded in OCT (Cryobloc, Diapat) and stored at −80 °C. The frozen samples were cut into 15–20 µm sections, permeabilized, stained with DRAQ5(R) for nuclear staining (Cell Signaling Technology) and closed with Vectashield® mounting medium. Fluorescent images were taken by ZEISS Confocal Microscope (ZEISS; LSM510_META).

Real-time qPCR assays

RNAs were extracted and purified using the RNeasy Micro kit (Qiagen, Milan, Italy). The purity of the RNA preparations was verified by measuring its absorbance ratio at 260/280; 500 ng of RNA were used to synthesize cDNA with random hexanucleotide primers and MoMULV reverse transcriptase (Applied Biosystem, Foster City, CA) at 42 °C for 1 h. cDNA was then amplified in an AB7900 real-time PCR detection system (Applied Biosystem) using a PCR reaction mixture containing 2.5 µl of Reaction Buffer 10X, 2 mM of $MgCl_2$, 0.2 mM of dNTPs, 1.25 U of Hot Start Taq polymerase, 0.2 pmol/µl of each Taqman probe, 0.4 pmol/µl of each primer and 40 ng of cDNA, in a total volume of 20 µl. The same mixture was used in combination with different primers and probes (Table 2). For relative gene expression analysis (Rel-qPCR), differences in relative expression of target genes were analyzed using the $2 - \Delta\Delta Ct$ method. GAPDH was used as internal control gene. The efficiency of target amplification and reference amplification (GAPDH) was opportunely measured and resulted comparable.

Statistical analysis

The results were expressed as the mean percentage of positive cells ± SEM. The non-parametric Kruskal–Wallis test was used to compare differences between groups. The level of significance (p) was set at <0.05 for all experiments.

Results

Xeno-free media validation during serial cultivation of LESCs

In the first set of experiments, xeno-free media formulations were evaluated by performing life span on culture plates, with or without FL, and compared to our control and to the CnT-AB condition, which has already been reported to enable limbal explant cultures on human AM without FL (Zakaria et al. 2014). Parameters that we checked included cell morphology (primary cells cultured in control medium is generally characterized by rounded colonies and with very regular borders, formed by small cells—around 6 µm

in diameter—with reduced cytoplasm and typical regular polygonal morphology), clonogenicity (by means of CFE assay and clonal analysis), proliferative capacity and expression of ΔNp63α (by means of Real Time qPCR).

Cell morphology

All the LESCs cultivated in CnT-20 basal media (CnT-XF ± FL and CnT-AB) displayed a simple squamous epithelial cell growth, with small, cuboidal shaped cells being observed even after several passages. On the contrary, the cells cultured in the control and in XF media grew in colonies that gradually fused, thus generating a stratified epithelium (Fig. 1). Cells cultivated in XF media maintained a clear smaller round shape compared to controls. For all the tested conditions, sub-confluence at standard seeding concentrations was reached 1–2 days earlier (5 days) compared to the standard control condition (6–7 days).

Analysis of clonogenicity

A change both in the total number and morphology of the colonies was observed in the CFEs cultivated with the xeno-free media (Fig. 2a–c), with very few colonies showing morphological signs of differentiation. The commonly observed difference (based on the morphology of cells) between clonogenic and abortive colonies was therefore not evaluated, thus invalidating the assay. For this reason, in order to obtain proper life span data, CFEs were cultivated in control medium in all the life span testing experiments. Since the CFEs cultivated in XF medium could only be partially analyzed, due to the impossibility of discriminating between clonogenic and abortive colonies, to further evaluate the direct effect of this synthetic serum on LESC cultures, a life span ($n = 2$) for this condition was performed with two CFEs in parallel, one cultured in control and the second in XF medium (Fig. 2b, c) with FL. The CCD rates obtained from both CFEs were: 35 ± 0.0 for XF

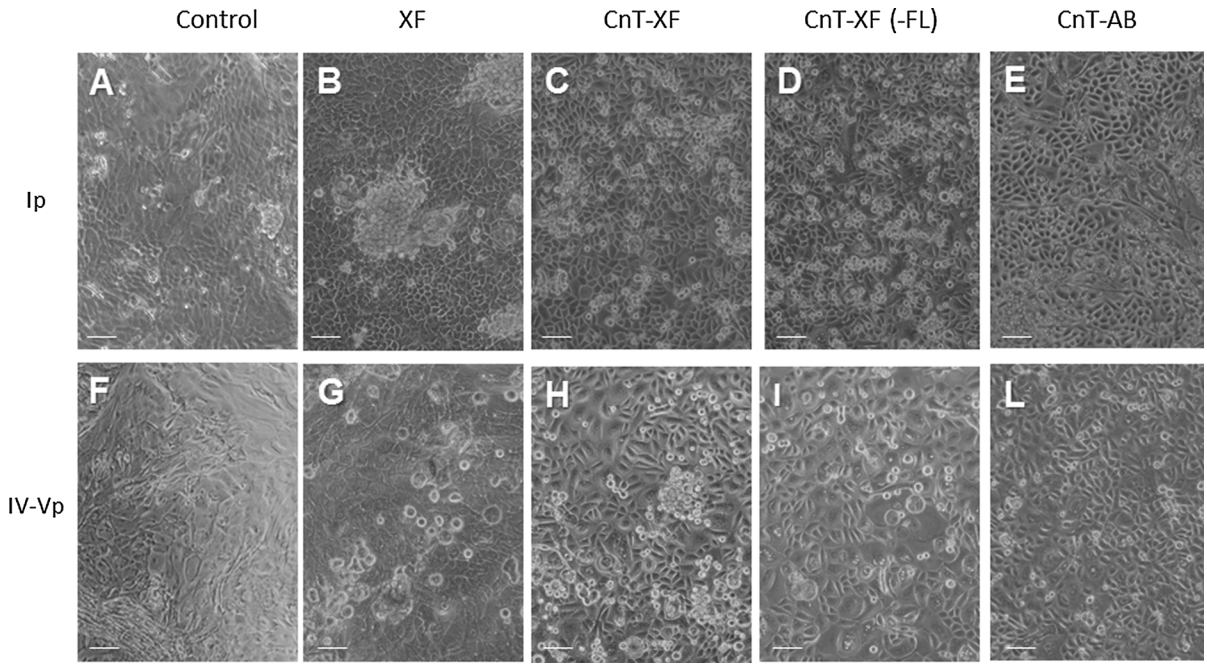

Fig. 1 Cell morphology during serial cultivation passages (*p*) in the control, XF and CnT-XF conditions. All the LESCs cultivated in CnT-20 basal media (CnT-XF ± FL and CnT-AB) displayed a simple squamous epithelial cell growth, with small, cuboidal shaped cells being observed even after several passages (**c–d–e–h–i–l**). On the contrary, the cells cultured in the control and in XF media grew in colonies that gradually fused, thus generating a stratified epithelium (**a–b–f–g**); the cell cultivated in XF media maintained an evident smaller round shape respect to the control. The small stemness-like morphology of cells is preserved even after several passages in culture. Magnification: 10×, *Scale bars* 100 μm

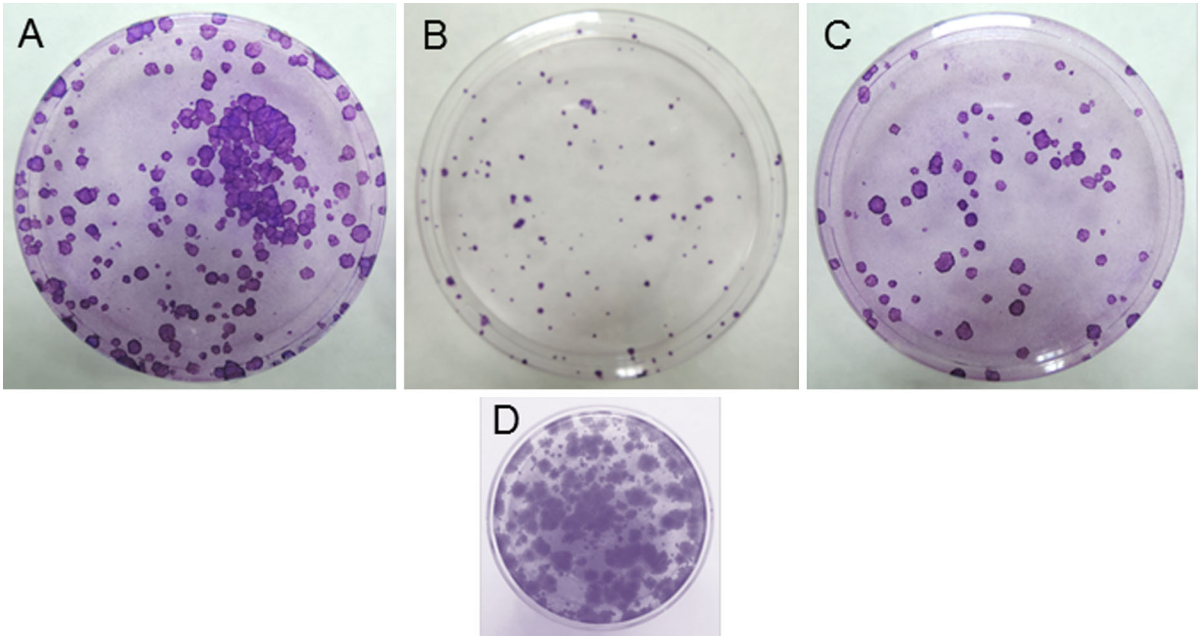

Fig. 2 Colony forming efficiency assay. CFEs obtained during lifespan cultured in different media (100 Ø plates): CFE obtained from the control condition and cultured in control medium (**a**); CFEs obtained from the XF condition and cultured: in XF medium (**b**) and in control medium (**c**). Colonies observed in CFEs cultured in the tested xeno-free medium exhibited smaller dimensions (**b**) compared to the CFEs cultured in the standard control medium (**c**). The commonly observed difference (based on the morphology of cells) between clonogenic and abortive colonies was therefore not evaluated. Representative image of a CFE (60 Ø plate) obtained from the CnT-XF condition and cultured in CnT-XF medium (**d**), unusable for life span analysis, since neither the segregation between colonies (absence of smooth bordered perimeters), nor evaluation between clonogenic and abortive colonies were allowed

medium and 36.5 ± 8.1 for control medium, versus 25 ± 2.8 for CFE of control condition. Both CFEs obtained from the XF condition suggested a different capacity in the maintenance of the culture proliferative properties. On the contrary, the total colony forming capacity calculated at consecutive passages was higher for the CFE of XF condition cultured in the control medium. To further evaluate the observed difference, a clonal analysis was performed. Classification of 50 isolated single cell clonal types from the XF condition revealed 50% less of paraclones and 16% more of meroclones than in control condition, while the percentage of holoclones was the same (2%). However, the single clones grew less in the xeno-free medium due to the low cell-density culture (Fig. 3). Again, 50% less of total number of colonies was observed in the CFE assay. The observed discrepancy demonstrates the negative effect of low cell mass culture that affected proper LESC survival in XF medium.

Proliferative capacity

During the serial cultivation of LESCs, the mean of cell passages and CCD rates of control condition did not statistically differ compared to the tested conditions, even regardless of the presence/absence of FL: the passage rate of control condition was 6.75 ± 0.65 and CCD was 32.4 ± 3.2, compared to CnT-XF without FL characterized by 8.5 ± 0.35 passages and 38.5 ± 3.18 CCD, CnT-AB with FL with 9.5 ± 0.35 passages and 33.2 ± 4.81 CCD, XF with FL condition with 9 ± 0.71 passages and 44.4 ± 2.58 CCD and CnT-XF with FL with 10.5 ± 0.35 passages and 44.5 ± 3.18 CCD. No statistically significant difference was observed (Fig. 4a) between the tested conditions. The same results were obtained during the analysis of the total colony-forming capacity (Fig. 4b) and the percentages of abortive colonies (data not shown), performed at different passages of the life span tests; i.e., the total colony-forming capacity at the second passage was

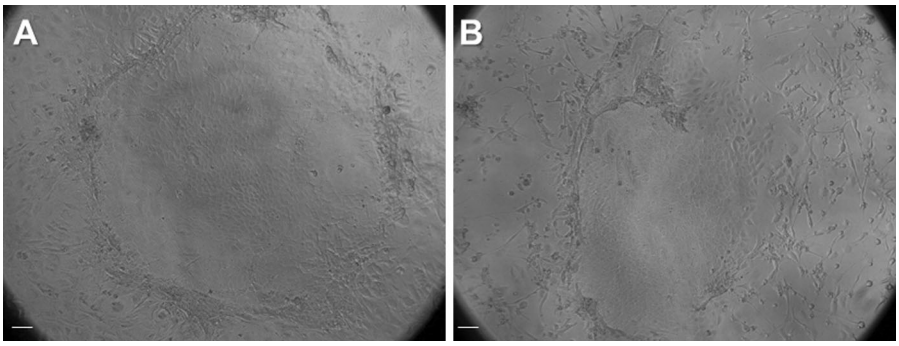

Fig. 3 Clonal analysis. Scoring of single cell cultures/well under the microscope for the presence of holoclone/meroclone/paraclone-type single colony. **a** The standard morphology of a holoclone-type single colony grown in control medium (control condition); **b** a holoclone-type single colony in XF medium condition, exhibiting lower growth, irregular borders and detachment of FL cells. Magnification: 5×, *Scale bars* 100 μm

29.40 ± 7.81 for the control condition, 28.80 ± 6.20 for CnT-XF without FL, 49.50 ± 19.50 for CnT-AB with FL, 42.25 ± 5.25 for XF with FL and 52.40 ± 4.40 for CnT-XF with FL. In sharp contrast, during serial cultivation of LESCs, the rate of total colony-forming capacity obtained was 52.40 ± 4.40 for the condition with FL and 28.80 ± 6.20 for the same condition without FL (Fig. 4b).

Expression of ΔNp63α

The mean ΔNp63α values obtained at the second and fourth passage of LESCs in the control condition were compared with all the tested conditions (Fig. 4c). During the second passage, the expression value of ΔNp63α was quantified as +275% ± 85 for CnT-XF without FL, +95% ± 55 for CnT-AB with FL, +39% ± 1 for XF with FL and +239% ± 192 for CnT-XF with FL, compared to the control condition. During the fourth passage, the expression value of ΔNp63α was quantified as +110% ± 16 for CnT-XF without FL, +231% ± 75 for CnT-AB with FL, +210% ± 135 for XF with FL and +386% ± 110 for CnT-XF with FL, compared to the control condition. By comparing the expression profile of ΔNp63α at the second and at the fourth passage, when co-cultivated with FL, an increased expression was observed, whereas in the condition without FL co-culture, a decrease was observed. The same pattern (a decrease) was observed in the control condition. Although we have shown that xeno-free conditions CnT-XF enabled serial cultivation of LESCs without the support of a FL, the low expression of ΔNp63α in the old passages showed that FL still plays an important role in the maintenance of the LESCs, thus preventing the premature culture senescence.

Evaluation of xeno-free media in LESC cultures grown on human AM

The xeno-free conditions CnT-XF, XF and CnT-AB (the last one already reported to enable limbal explant culture on human AM without FL), were further tested for suspension LESCs culture on human AM, with or without FL. Comparisons were made with the control standard conditions (cells cultivated in parallel on human AM with and without FL in control medium and cells cultivated on fibrin gel with FL in control medium).

Cell morphology and proliferation

In CnT-XF and CnT-AB conditions, irrespective of FL co-culture, detached, vacuolated cells were observed on the surface of the AM. In contrast, in both XF medium conditions, cells produced a well-stratified thick cell layer and reached confluence 1–2 days before the control condition, with no fibroblast-like cells being observed (Fig. 5). For these conditions, epithelial growth was observed, with similar epithelial stratification being found following histology examination (Fig. 5). Few cell layers were found after histology. Confluence was reached at the same time (although the seeding concentration was twice the concentration used on the fibrin gel scaffold), except for the LESCs cultivated in control media on AM without FL, where the confluence was reached very slowly, with presence of fibroblast-like cells.

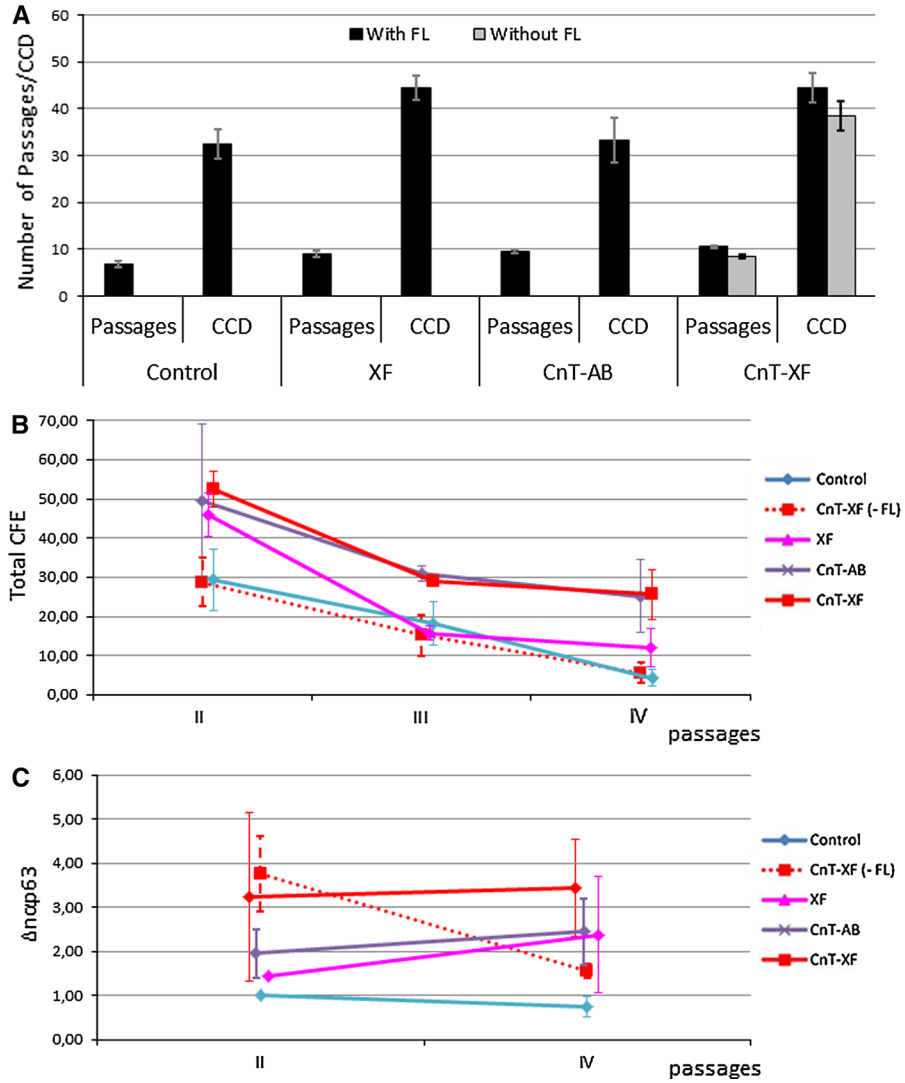

Fig. 4 Proliferative capacity, total colony-forming efficiency results and ΔNp63α expression of LESCs in different media with and without FL. **a** With a FL, during serial cultivation (life span) the best culturing performance was observed in CnT-XF condition with FL: 10.5 ± 0.35 passages, 44.5 ± 3.18 CCD; followed by XF condition: 9 ± 0.71 passages, 44.4 ± 2.58 CCD; and CnT-AB condition: 9.5 ± 0.35 passages, 33.2 ± 4.81 CCD, compared to the control condition (mean passage rate 6.75 ± 0.65; CCD 32.4 ± 3.2). Without a FL, even cells cultivated in CnT-XF medium showed comparable or slightly better life span results than control condition with: 8.5 ± 0.35 passages, 38.5 ± 3.18 CCD. **b** The mean total CFE data during serial cultivation. Limbal cells cultured in xeno-free media with a FL showed superior total colony-forming capacity results compared to the control condition and the same conditions without a FL. **c** A low expression of ΔNp63α in control condition was detected both in the second and fourth passage

Expression of ΔNp63α and K12

The expression of ΔNp63α and K12 was further analyzed in xeno-free conditions that enabled stratified epithelial cell growth (XF -FL compared to the control conditions) (Fig. 6). The expression value of ΔNp63α in cells cultivated on AM was compared to the control condition (ΔNp63α fixed to 1) cultivated on the fibrin gel scaffold and it resulted equal to 2.47 ± 0.85 for control condition on AM + FL, 1.35 ± 0.38 for control condition on AM–FL and 2.44 ± 0.52 for xeno-free condition on AM XF -FL. The xeno-free condition XF without FL showed a positive expression level of the corneal differentiation

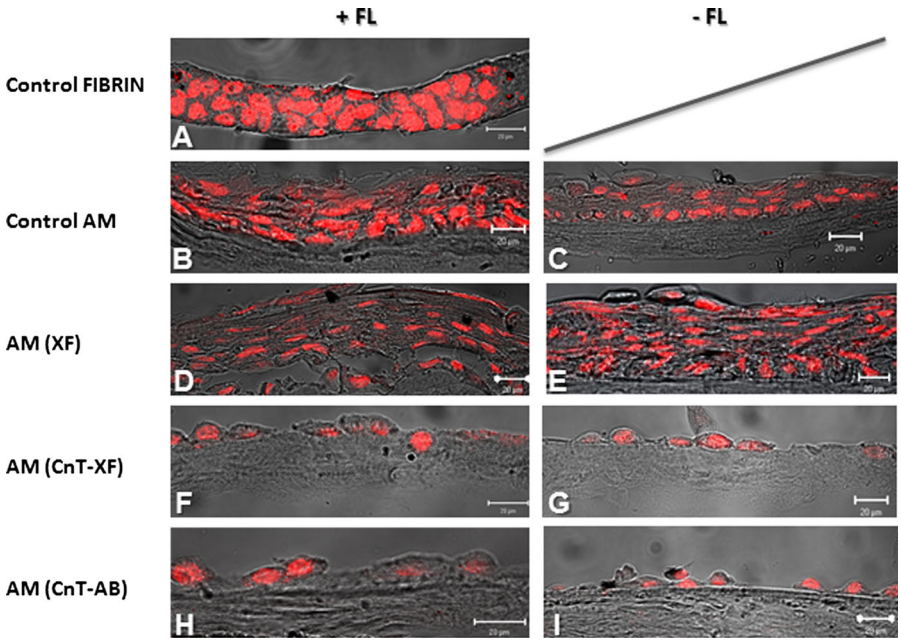

Fig. 5 Histological sections of limbal epithelia grown on AM and fibrin gel scaffolds. All the cultured epithelia are stained with DRAQ5. Cell growth was observed in XF conditions, both in the presence (**d**) and absence (**e**) of FL. CnT-20 basal medium was tested for cell cultivation on human AM but cell proliferation was not observed, regardless of the presence (**f**, **h**) or absence (**g**, **i**) of FL and the type of supplemented serum, XerumFree^{TM} XF205 (**f**, **g**) or AB human serum (**h**, **i**)

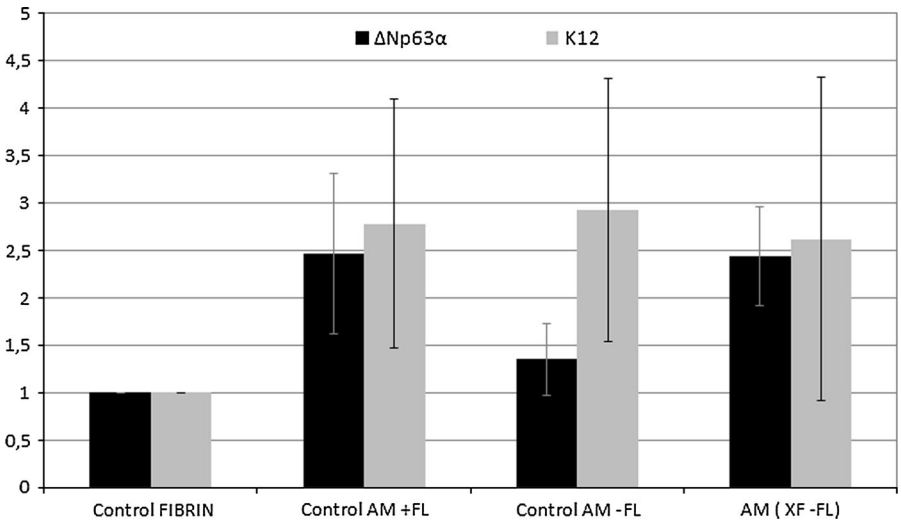

Fig. 6 The expression profile of ΔNp63α and K12. LESCs cultivated with the xeno-free condition (XF–FL) showed similar ΔNp63α expression values to the control conditions, thus confirming that the amount of limbal stem cells was preserved even if grown on AM without FL and with xeno-free media. The condition XF without FL showed an expression level of the corneal differentiation marker K12 similar to the control condition grown on AM

marker K12 (2.62 ± 1.71) compared to the value of the control condition grown on fibrin gel (fixed to 1).

Toxic effect of xeno-free media on fibroblasts

Since in all LESC cultures cultivated in xeno-free media an unhealthy FL was observed (more elongated cells with premature detaching), a toxicity test was performed. It was evident that all the tested media did not allow the survival of 3T3-J2 cells line: in the first passage cell partially attached and did not grow (Fig. 7), only the cells cultured in Cnt-20 medium with BCS serum showed a slower growth, with a different morphology. In order to investigate the toxicity of these media, we performed a test by using a NIH-3T3 cell line (which showed signs of toxicity at the first passage, similarly to 3T3-J2 cells) and human fibroblasts (slower, cells proliferation, the confluence was reached in 10 days against the 7–8 days of the control, with only 2–3 passages in culture).

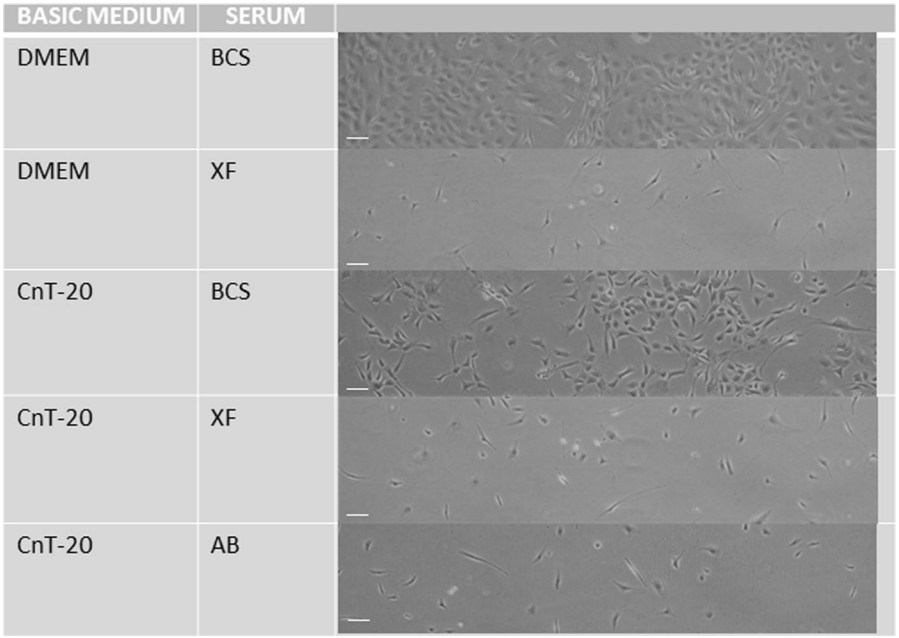

Fig. 7 Toxicity test on 3T3-J2 fibroblast cells. The tested media did not allow to culture 3T3-J2 cells. Magnification: 10×, *Scale bars* 100 μm

The xeno-free media confirmed a higher selectivity for LESCs in contrast to the standard medium, with the beneficial effect of reducing the exogenous (murine 3T3-J2 fibroblast cells) as well as the endogenous limbal fibroblast contamination in LESC cultures.

Discussion

In the context of GMP regulations, several attempts have been made to replace potentially harmful xenogeneic materials from LESC cultures. Xeno-free culture protocols currently use either explant (Zakaria et al. 2010, 2014; Sangwan et al. 2011; Kolli et al. 2008; Nakamura et al. 2006; Shimazaki et al. 2007) or single cells suspension limbal culture technique, applying at least human serum or human fibroblast cell-line to the xenogeneic-free media (Stasi et al. 2014; Notara et al. 2007).

As an alternative, 'synthetic' media have been proposed, although they might lack some of the undefined important physiological cell stimulators found in the serum (Pellegrini et al. 2014), which can influence in vitro stem cell expansion and preservation. The culturing techniques are critical for proper expansion and prevention of LESC differentiation in vitro, especially as the amount of LESC in the cultured limbal epithelial grafts is crucial for successful long-term clinical outcomes (Di Iorio et al. 2010). To the best of our knowledge, our study is the first to demonstrate LESC expansion and long-term survival in two xeno-free culturing conditions with and without the presence of feeder layers. Moreover, the xeno-free media were further evaluated for cell culture growth on denuded human AM.

The XerumFreeTM syntetic serum was tested for the first time during LESCs serial cultivation, by adding it in our control medium (in order to replace FBS) and in CnT-20 medium (a corneal progenitor cell targeted and fully defined medium), both with and without FL. We also tested CnT-20 medium supplemented with the clinically approved 1% human AB serum. Zakaria et al. (2010, 2014) already use CnT-20 medium in the clinical application of human AM grafts, without FL, using the biopsy explant technique. Although human serum is reported to successfully replace bovine serum in clinical studies using LESC culture (Baylis et al. 2011), certain medical conditions (e.g. viral infection transmission and prion transmission) still exists and the biologic variability of the serum components still hinder the use of human serum altogether. Here, we report for the first time the

replacement of human AB serum with a fully defined additive for optimal serum free cell suspension limbal culture (XerumFree™ XF205).

As already described for cell cultures in Epilife medium (Loureiro et al. 2013), we also observed that cells cultivated in CnT-20 and/or XerumFree™ XF205 supplemented media, were morphologically more uniform, with a decrease in epithelial stratification rate. Furthermore, the small stemness-like morphology of cells is preserved even after several passages in culture. Thus, we conclude that the defined XerumFree™ XF205 'synthetic' supplements and the tested media support progenitor cell survival, and prevent differentiation of transient amplifying cells (TACs) towards terminally differentiated cells. We might speculate that the cells that would normally undergo terminal differentiation do not survive in these conditions, thus explaining the apoptotic cells that were observed in the higher passages.

The observed morphological culture changes (progenitor-like appearance) were also in agreement with the functional stem cell evaluation tests and molecular tests, as shown by the results of the lifespan tests. No statistical differences in number of cell passages, total number of colonies, cell population doublings and ΔNp63α expression were obtained in all conditions with xeno-free media. Another issue that we have faced was that none of the xeno-free media conditions enabled propagation or normal survival of feeder cells (both human and murine fibroblasts). However, feeder cells seemed to be still sufficiently metabolically active when used as feeder layer in LESC cultures in xeno-free media.

Here we showed that the defined XerumFree™ XF205 'synthetic' supplements could successfully replace FBS or human serum and, in some conditions, also FL. Serum was thought to be essential for cultivation of epithelial cells as it stimulated epithelial growth (Osei-Bempong et al. 2009) and stratification and had a significant influence on the calcium concentration in the cultured media (Loureiro et al. 2013). On the contrary, the defined 'synthetic' media exhibited low calcium concentration. This can prevent cell differentiation, but it might interact with cell proliferation, as it is well known that high calcium concentrations in culture produce stimulatory effect on cell proliferation due to its interactions with growth factors. In consistence with this, we observed that the limbal cells needed to be cultivated in sufficient seeding concentrations to obtain enough paracrine signals to survive in the serum-free conditions, as well as for performing of CFE assay and clonal analysis.

While there are still no specific molecular limbal stem cells markers, functional evaluation of LESC preservation in culture by serial cultivation testing or clonal analysis are still the gold-standard tests. However, as we demonstrated in this study, xeno-free cultivation methods complicate proper assessment of these quality control tests (CFE assay), as the interpretation is based on clonal morphology assessment and cultivation of single cells. We showed that the number and the morphology of cultured cells grown in xeno-free media changed, making discrimination between clonogenic and abortive colonies difficult. Therefore, the tested xeno-free media could not be used for CFE assay.

The next step was to evaluate the xeno-free conditions for single cell suspension culture on denuded human AM. When we tested the CnT-20 basal medium, cell proliferation was not observed, irrespective of FL co-culture, and of the type of supplemented serum (XerumFree™ XF205 or AB human serum). The low calcium levels and the absence of additional undefined growth factors in serum might be the reason why it was not possible to cultivate LESCs on human AM. Further experiments are needed to clarify this issue.

On the contrary, cell growth was present in XF medium conditions, with no significant histological differences being observed between conditions with and without FL co-culture. Furthermore, the histology of these two conditions was similar to the epithelial cell growth on AM in control conditions (control with and without FL, respectively) and the Real-Time qPCR analysis showed no statistical differences in ΔNp63α expression. Therefore, the xeno-free condition XF without FL showed to be optimal for LESCs grown on AM. Moreover, the higher selectivity for epithelial cell culture could be used for reducing the exogenous as well as the potential endogenous limbal fibroblast contamination of the final LESC for transplantation purposes.

The low number of analyzed samples (N = 2/3) did not allow to highlight the quality improvements in the tested conditions. According to these statistical limits, the obtained data may only be used to indicate there seem no to be obvious and large differences between conditions. Therefore our results showed that it could

be possible to obtain a xeno-free graft on AM by using single cell suspension limbal culture, and further studies are needed to better investigate differences and improvements of the conditions here described. Further studies are needed to characterize if these cultures could be clinically used.

Conclusion

Currently, all of the previously reported protocols for LESC cultures make use of xenogeneic and allogenic components (human/bovine serum and FL) to support the growth of cells for transplantation. Because of the theoretical health risks, we sought a xeno-free culturing method to support the growth and survival of limbal epithelial cells and LESCs in culture. In this study, we compared different culture conditions assessing morphological and functional evaluation of stem cell preservation in culture, as well as the expression of stem cell markers. To the best of our knowledge, this is the first report that demonstrates that LESCs can be preserved in a fully xeno-free culturing condition, thus facilitating the translation into clinical application.

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