Experimental Eye Research 211 (2021) 108720

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Characterisation of corneas following different time and storage methods for their use as a source of stem-like limbal epithelial cells

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ARTICLE INFO

Keywords:
Corneoscleral tissues
Sclerocorneal rims
Limbal epithelial stem cells (LESCs)
Limbal stem cell deficiency (LSCD)
Corneal storage
Corneal shortage/availability
Corneal cell cultures
Melanocytes

ABSTRACT

The transplantation of expansions of limbal epithelial stem cells (LESC) remains one of the most efficient therapies for the treatment of limbal stem cell deficiency (LSCD) to date. However, the available donor corneas are scarce, and the corneas conserved for long time, under hypothermic conditions (after 7 days) or in culture (more than 28 days), are usually discarded due to poor viability of the endothelial cells. To establish an objective criterion for the utilisation or discarding of corneas as a source of LESC, we characterized, by immunohistochemistry analysis, donor corneas conserved in different conditions and for different periods of time. We also studied the potency of LESCs isolated from these corneas and maintained in culture up to 3 cell passages. We hoped that the study of markers of LESCs present in both the corneoscleral histological sections and the cell cultures would show the adequacy of the methods used for cell isolation and how fit the LESC enrichment of the obtained cell populations to be expanded was. Thus, the expressions of markers of the cells residing in the human limbal and corneal epithelium (cytokeratin CK15 and CK12, vimentin, Collagen VII, p63α, ABCG2, Ki67, Integrin β4, ZO1, and melan A) were analysed in sections of corneoscleral tissues conserved in hypothermic conditions for 2–9 days with post-mortem time (pmt) < 8 h or for 1 day with pmt > 16 h, and in sclerocorneal rims maintained in an organ culture medium for 29 days. Cell populations isolated from donor corneoscleral tissues were also assessed based on these markers to verify the adequacy of isolation methods and the potential of expanding LESCs from these tissues. Positivity for several putative stem cell markers such as CK15 and p63α was detected in all corneoscleral tissues, although a decrease was recorded in the ones conserved for longer times. The barrier function and the ability to adhere to the extracellular matrix were maintained in all the analysed tissues. In limbal epithelial cell cultures, a simultaneous decrease in the melan A melanocyte marker and the putative stem cell markers was detected, suggesting a close relationship between the melanocytes and the limbal stem cells of the niche. Holoclones stained with putative stem cell markers were obtained from long-term, hypothermic, stored sclerocorneal rims. The results showed that the remaining sclerocorneal rims after corneal transplantation, which were conserved under hypothermic conditions for up to 7 days and would have been discarded at a first glance, still maintained their potential as a source of LESC cultures.

1. Introduction

Corneal blindness is estimated to affect 23 million people worldwide. However, due to the shortage of corneal donors, only 1 out of 70 needs can be covered (Oliva et al., 2012; Gain et al., 2016). A population of limbal epithelial stem cells (LESCs) is responsible for the maintenance of a healthy corneal epithelium throughout life by constantly supplying daughter cells, which migrate centripetally towards the central cornea to replace lost cells and differentiate as they progress from the basal to superficial epithelial layers (Thoft and Friend, 1983). LESCs reside in the

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https://doi.org/10.1016/j.exer.2021.108720
Received 29 January 2021; Received in revised form 21 July 2021; Accepted 5 August 2021
Available online 11 August 2021

basal layer of the epithelium within the corneal limbus—the vascularised and highly innervated border between the central cornea and conjunctiva. More specifically, the limbal crypts—the downward invaginations of the limbal epithelium into the limbal stroma between the palisades of Vogt—have been proposed as the LESC niche (Shortt et al., 2007; Dziasko et al., 2014; Bonnet et al., 2021). During LESC failure, the conjunctiva can invade the cornea, causing chronic inflammation, corneal opacity, vascularisation and severe discomfort, which can lead to blindness.

Current treatments for LESC deficiency rely upon transplantation of allogenic or autologous limbal cultures. Cultured LESC delivery is one of several examples of a successful adult stem cell therapy used in patients, which achieves permanent restoration of damaged tissues in 76% of cases (Baylis et al., 2011). The number of stem cells present in a limbal biopsy is in the order of hundreds and this number increases during the primary culture due to the amplification of the original stem cell population. This amplification is actually the basis for the grafts' clinical success (Rama et al., 2010; Pellegrini et al., 2011). In this context, the use of growth-arrested mouse embryonic fibroblasts (3T3-J2) as feeder cells is considered the gold-standard method for *in vitro* amplification (Barrandon et al., 2012), but the use of other support cells for LESC expansion and enrichment has also been reported. For example, human amniotic membranes (HAM) have been used as culture substrates to enhance limbal epithelial sheet growth and stemness (Lee et al., 2018; Sharma et al., 2018). González et al. demonstrated the efficiency of bone marrow stromal cell (BMSC) feeder layers for the expansion of LESC population in 3D cultures (González et al., 2016). Human limbal melanocyte feeders showed successful results in the maintenance of epithelial stem cells characteristics and suggested an active involvement in the niche's preservation (Dziasko et al., 2015).

However, several factors during extraction and conservation of the corneal tissue prior to any amplification step can influence the viability and capability of LESCs to proliferate *in vitro*, such as death-to-preservation time of the corneas, storage procedure and even the age of the donor patient (Notara et al., 2013). Considering this, the aim of this study was to assess the suitability of corneas conserved in different conditions and for different periods of time in terms of the quantity of LESCs. To achieve this, the expressions of putative stem cell markers CK15 (Yoshida et al., 2006), ΔNp63α (Pellegrini et al., 2011), ABCG2 (De Paiva et al., 2005; Schlötzer-Schrehardt and Kruse, 2005) and vimentin (Brookes et al., 2003), of differentiated epithelial cell markers CK12 and CK3 (Chaloin-Dufau et al., 1990), and of the cell proliferation marker Ki67 (Joyce et al., 1996) were evaluated. In addition, the adequacy of cell isolation methods, the evolution of the mentioned markers along cell culture passages and the presence of melanocytes were assessed.

2. Materials and methods

2.1. Classification of the study corneas by time and conditions of conservation

Human corneoscleral tissues and data from 40 to 81 year-old donors included in this study were provided by the following eye banks: the Basque Biobank www.biobancovasco.org; the Blood and Tissue Bank, Government of Catalonia; the Navarra Blood and Tissue Bank, Navarrabiomed Biobank, Navarra Health Department; and the Biobank A Coruña of SERGAS. The corneoscleral tissues were processed following standard operating procedures under the tenets of the Declaration of Helsinki with appropriate approval of the Ethical and Scientific Committees (CEISH/342/2015/ANDOLLO VICTORIANO). Information about donor tissues and their use in this study are determined in Table 1. Considering the storage method, two types of samples were obtained:

- Donor corneoscleral tissues (including the cornea and the sclerocorneal rim), preserved in hypothermic conditions. They were maintained at 4 °C in the corneal conservation medium Eusol-C (Alchimia, Ponte San Nicolò, Italy) or Optisol-GS solution (Bausch and Lomb, Alcobendas, Madrid, Spain). Likewise, two types of donor corneoscleral tissue samples were distinguished: i) Corneoscleral tissues extracted from donors less than 8 h after death (post-mortem time (pmt) < 8 h) and kept in the conservation medium for 2–9 days; ii) Corneoscleral tissues maintained in the conservation medium for only one day but extracted 16–22 h after donors' death (pmt > 16 h). The type of corneoscleral tissue sample is an important point to consider since the death-to-preservation time could affect the viability of the different corneal cell populations.
- Cultured sclerocorneal rims (after using corneal button for clinical purposes) preserved at 31 °C for 10 and 29 days in Tissue C organ culture medium (Alchimia), a medium for organ culture. The death-to-preservation time was less than 8 h (pmt < 8 h) in these samples.

We used 15 human tissue samples in all. For tissue section analysis, 11 samples were used: 8 fresh corneoscleral tissues conserved in hypothermic conditions for 2–9 days (pmt < 8 h); 2 fresh corneoscleral tissues conserved in hypothermic conditions for 1 day (pmt > 16 h); and 1 sclerocorneal rim maintained in Tissue C organ culture medium for 29 days. For cell cultures, 4 samples were used: 1 fresh corneoscleral tissue conserved in hypothermic conditions for 3 days, 1 for 5 days, 1 for 7 days and 1 sclerocorneal rim maintained in Tissue C organ culture medium for 10 days.

Fig. 1 shows a schematic representation of the procedure followed and the storage condition of each sample.

Table 1
Donor tissue information and use of each tissue in this study.

Sample Number	Age (years)	Sex	Post morten time (h)	Conservation method	Conservation time (days)	Use
1	61	Male	16	HC	1	Tissue sections
2	62	Female	22	HC	1	Tissue sections
3	N.A	N.A	<8	HC	2	Tissue sections
4	N.A	N.A	<8	HC	3	Tissue sections
5	N.A	N.A	<8	HC	4	Tissue sections
6	N.A	N.A	<8	HC	5	Tissue sections
7	N.A	N.A	<8	HC	6	Tissue sections
8	N.A	N.A	<8	HC	7	Tissue sections
9	N.A	N.A	<8	HC	8	Tissue sections
10	N.A	N.A	<8	HC	9	Tissue sections
11	N.A	N.A	<8	Cultured	29	Tissue sections
12	64	Female	3	HC	5	Cell isolation
14	66	Female	3	HC	3	Cell isolation
15	N.A	N.A	<8	HC	7	Cell isolation
16	56	Female	<8	Cultured	10	Cell isolation

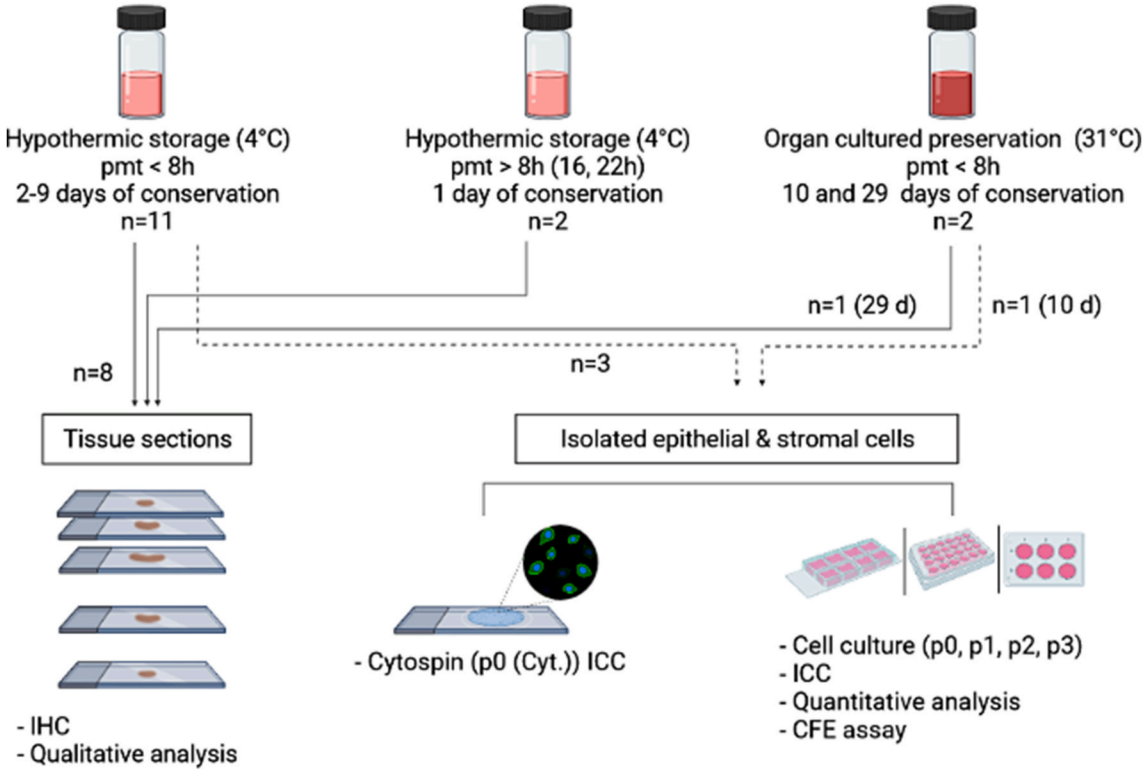

Fig. 1. Schematic representation of the experimental design showing the storage condition and the procedure followed with each of the corneoscleral samples.

2.2. Dissection of donor corneoscleral tissues and sclerocorneal rims

Donor tissues were immersed in Hank's solution containing HEPES (20 mM, Sigma, St. Louis, MO, USA), 1% Penicillin-Streptomycin (Lonza, Verviers, Belgium) and 1.25 μg/ml Amphotericin B (Sigma) to wash off the remaining traces of the preservation medium. Once cleaned, the tissues were dissected under a dissection microscope (Stemi 508, Zeiss; Axiocam ERc 5s) to remove the iris, endothelium, Tenon's capsule and conjunctiva. For corneoscleral tissues, the corneal button was separated from the sclerocorneal rim by an 8.5 mm punch and discarded.

Subsequently, samples intended for histological sections were cut and included in OCT (Optimal Cutting Temperature) compound (TissueTek®, Sakura Finetek, NL) blocks with the desired orientation to obtain transversal sections of the tissues. The blocks were kept at −80 °C.

2.3. Tissue digestion and cell isolation

Samples for cell culture were enzymatically digested. Dispase II (Hoffmann-La Roche; Basel, Switzerland), at a concentration of 2.4 U/ml, in a DMEM:F12 (Lonza) medium supplemented with 5% fetal bovine serum (FBS; Lonza) was used for 2 h at 37 °C to separate the epithelium from the stroma. Supernatants containing suspended isolated cells and cell clusters from the limbal epithelium were collected, centrifuged at 200 g for 10 min and suspended in the corresponding solution to perform the following assays.

The remaining stromal tissue, after digestion with dispase, was introduced in a mixture of 4 mg/ml Collagenase A (Hoffmann-La Roche) and 0.01% Hyaluronidase (Sigma) at 37 °C overnight for the extraction of stromal cells. The supernatant obtained from this digestion was centrifuged at 200 g for 10 min and suspended in the corresponding solution to perform the following assays.

2.4. Cell culture

For the *in vitro* propagation of limbal epithelial cells collected after digestion with dispase, cells were suspended in a SHEM5 hormonal medium—containing DMEM:F12 (Lonza), 5% fetal bovine serum (FBS; Lonza), 1% Penicillin-streptomycin (Lonza), 1% N_2 supplement (Thermo Fisher Scientific; Waltham, Massachusetts, USA), 0.5 μg/ml Hydrocortisone (Sigma), 0.5% DMSO (Sigma), 8.4 ng/ml Cholera toxin (Gentaur Molecular Products, Brussels, Belgium) and 2 ng/ml EGF (Sigma)—and seeded at a density of 25000 cells/cm^2 in 24 well plates previously coated with poly-L-lysine. They were detached with 0.25% trypsin-EDTA (Sigma) when confluence was reached and reseeded in new 24 well plates.

The stromal cells extracted from the cornea after digestion with collagenase and hyaluronidase were suspended in a stromal cell medium (DMEM:F12, 10% FBS, 2.5 mM Glutamine and 1% Penicillin-streptomycin) and seeded in 6 well plates (5000 cells/cm^2). They were detached with 0.25% trypsin-EDTA when confluence was reached and reseeded in new 6 well plates.

For all cell cultures, cell media were changed every 2–3 days.

For immunocytochemical staining of cultured epithelial and stromal cells, 20000 cells/well were seeded on chambered coverslips with 8 wells for cell culture (Ibidi; Gräfelfing, Germany). The coverslips were previously coated with a poly-L-lysine solution diluted with deionised water (1:10) to facilitate cell adhesion.

For their clonal growth, the limbal epithelial cells were seeded at a density of 300 cells/cm^2 on growth-arrested 3T3 mouse fibroblasts. These cells were inactivated using 7 μg/ml Mitomycin C (Sigma) for 2.5 h at 37 °C and seeded at a density of 30000 cells/cm^2 in 6 well plates. A SHEM5 hormonal medium was used for clonal cultures.

2.5. Colony-forming efficiency (CFE) assay

To perform colony-forming efficiency (CFE) assays, plates used for clonal growth were fixed with 4% paraformaldehyde (PFA) on the 14th

day of growth. Then, the 3T3 monolayer was mechanically removed by pipetting over the cell layer surface several times, and the remaining colonies were stained with Crystal Violet for 10 min at room temperature (RT).

2.6. Immunocytochemistry and immunohistochemistry

To perform the immunocytochemical analysis of cells directly isolated from sclerocorneal rims, part of the cells obtained from tissue digestion, both epithelial and stromal cells, were washed in PBS 1X (Sigma) and fixed in 2% paraformaldehyde for 20 min. Once fixed, they were washed in PBS 1X, centrifuged for 7 min at 300 g and suspended in PBS-BSA (1%) (Sigma). The cell solution was then spun at 1300 rpm for 8 min using a Cytospin (Cytospin 4 Centrifuge; Thermo Scientific) and precipitated over slides coated with poly-L-lysine (Sigma). One hundred microlitres (100 μl) of cell suspension with a density of around 20000 cells were added per cytofunnel.

Immunocytochemistry was assessed in slides containing cells directly isolated from tissues and in 8-well chambered coverslips (Ibidi) containing the cultured limbal cells fixed in 2% paraformaldehyde in PBS for 20 min at room temperature. Fixed samples were then permeabilised for 10 min with 0.1% Triton TX-100 in PBS (PBT) and blocked for an hour with a blocking solution containing 5% BSA and 10% FBS in PBT to reduce non-specific interactions that could result in background and false-positive staining. Samples were treated with the corresponding solution of primary antibodies (Table 2) overnight at 4 °C, washed with PBT 3 times for 10 min each and stained with secondary antibodies for 2 h at room temperature in darkness. Finally, the samples were counterstained with DAPI (Sigma), diluted 1:1000 in PBS, or with 4 mg/ml Hoechst 33342 (Invitrogen, Thermo Fisher Scientific), diluted 1:1000 in PBS, at room temperature for 15 min and washed two times with PBS for 5 min each. The cells seeded on 8-well chambered coverslips (Ibidi) were maintained in PBS and the slides of directly isolated cells were mounted onto microscope slides with Fluoromount-G® mounting media (SouthernBiotech; Birmingham, England).

The same immunostaining procedure was used for tissue sections but 4% paraformaldehyde in PBS was used for the fixation step instead. All images were taken on the fluorescence microscope Apotome.2 (Zeiss; Oberkochen, Germany).

Fluorescence images of tissue sections were divided into three regions—conjunctiva, limbus and peripheral cornea—to perform a semi-quantitative analysis of the expressions of CK15, vimentin, ΔNp63α and Ki67 markers. Fluorescence ratios of the stained sections were calculated by dividing the mean fluorescence intensity by the area of the region of interest. These ratios allowed having comparable fluorescence values between the samples studied. Three sections were analysed to semi-quantitatively determine the expressions of the markers. For nuclear markers, the number of positive nuclei was counted. For cultured cells, the number of positive cells for the markers of interest and their corresponding mean intensities were automatically determined. The fluorescence analysis of both tissue sections and cell culture was performed using ImageJ software (developed by Wayne Rasband at the Research Services Branch, National Institute of Mental Health, Bethesda, MD).

Table 2
List of primary antibodies and dilutions used in the study.

Antibody	Dilution	Secondary Antibody	Commercial product
Cytokeratin 3	1:100	Goat anti-mouse IgG1	Millipore CBL218
Cytokeratin 12	1:50	Goat anti-rabbit IgG	Abcam ab185627
Cytokeratin 15	1:400	Goat anti-mouse IgG2a	Santa Cruz sc-47697
Vimentin	1:1000	Goat anti-rabbit IgG	Abcam ab16700
Collagen VII	1:400	Goat anti-mouse IgG1	Millipore MAB1345
p63α[a]	1:800	Goat anti-rabbit IgG	Cell signaling Tech. #13109
Ki67	1:400	Goat anti-mouse IgG1	Millipore MAB4190
ZO-1	1:20	Donkey anti-goat IgG	Abcam ab190085
Integrin β4	1:50	Goat anti-mouse IgG1	Abcam ab29042
Melan A	1:200	Goat anti-rabbit IgG	Abcam ab51061

[a] Since the predominant p63 in the limbus is ΔNp63, it is presumed that positive cells express ΔNp63.

2.7. Statistical analysis

Quantification results were subjected to the Kruskal–Wallis test in which the Dunn test was used for multiple comparisons. Statistical differences were set at the $p < 0.05$ level. IBM SPSS Statistics 24 (IBM, Armonk, NY, USA) software was used for this analysis.

3. Results

3.1. Histological appearance of the tissue sections

The integrity of the corneoscleral tissues between 1 and 5 days of conservation showed healthy appearance and well-conserved morphology of the epithelium, irrespective of the death-to-exeresis time being higher or lower than 8 h.

Corneoscleral tissues that had been conserved for 6 days or more showed signs of deterioration such as loss of most superficial layers of the epithelium and oedema. These signs were evident in the corneoscleral tissues on the 8th to 9th day of conservation, where a single layer of epithelial cells or even total loss of the epithelium was observed in small areas.

Cultured rims were more affected by the passage of time and showed unstructured and oedematous epithelia. Furthermore, the staining pattern of the studied proteins was not precise and appeared intermingled between the peripheral cornea and the limbus.

3.2. Analysis of corneal markers in tissue sections

Considering the integrity, death-to-exeresis time and preservation characteristics of the corneoscleral tissues, the results of the expressions of cell markers were classified into 5 groups. The first 3 groups showed the information related to fresh corneoscleral tissues (obtained less than 8 h after the donor's death (pmt < 8 h)) conserved in hypothermic conditions for different conservation periods (2–5, 6 to 7, and 8–9 days of conservation, Fig. 2). The fourth group represented the corneoscleral tissues conserved for 1 day but extracted more than 16 h post-mortem (Fig. 3 left column). The remaining group included the results pertinent to cultivated sclerocorneal rims (Fig. 3 right column).

3.2.1. CK12–CK15 cell marker expressions

The results obtained showed the expected staining patterns of both cytokeratins in all fresh corneoscleral tissues despite the different days of conservation. The expression of CK12 was limited to the suprabasal areas throughout the peripheral corneal epithelium. Its specificity decreased in tissue sections obtained from corneoscleral samples conserved for a longer time. The fluorescence pattern of CK15 was limited to the basal areas of the limbal and conjunctival epithelium (a–c of Fig. 2 and a–b of Fig. 3).

In order to observe the possible differences in CK15 staining between groups, a quantitative study of the CK15-stained area in analysed corneoscleral rim sections was performed. The staining pattern was quantified in the conjunctiva, limbus and peripheral cornea. The results showed a decreasing CK15 staining trend in the limbal area as the conservation time of the corneoscleral tissues increased from 2 to 8 days. However, its expression increased in the tissue at 9 days of conservation (Fig. 4). The corneoscleral rims conserved for 1 day (pmt > 16 h) and the ones cultured for 29 days were not included in the statistical analysis due to alterations in the markers' expressions.

Similar fluorescence ratios were quantified in all tissue samples for the conjunctiva and peripheral cornea.

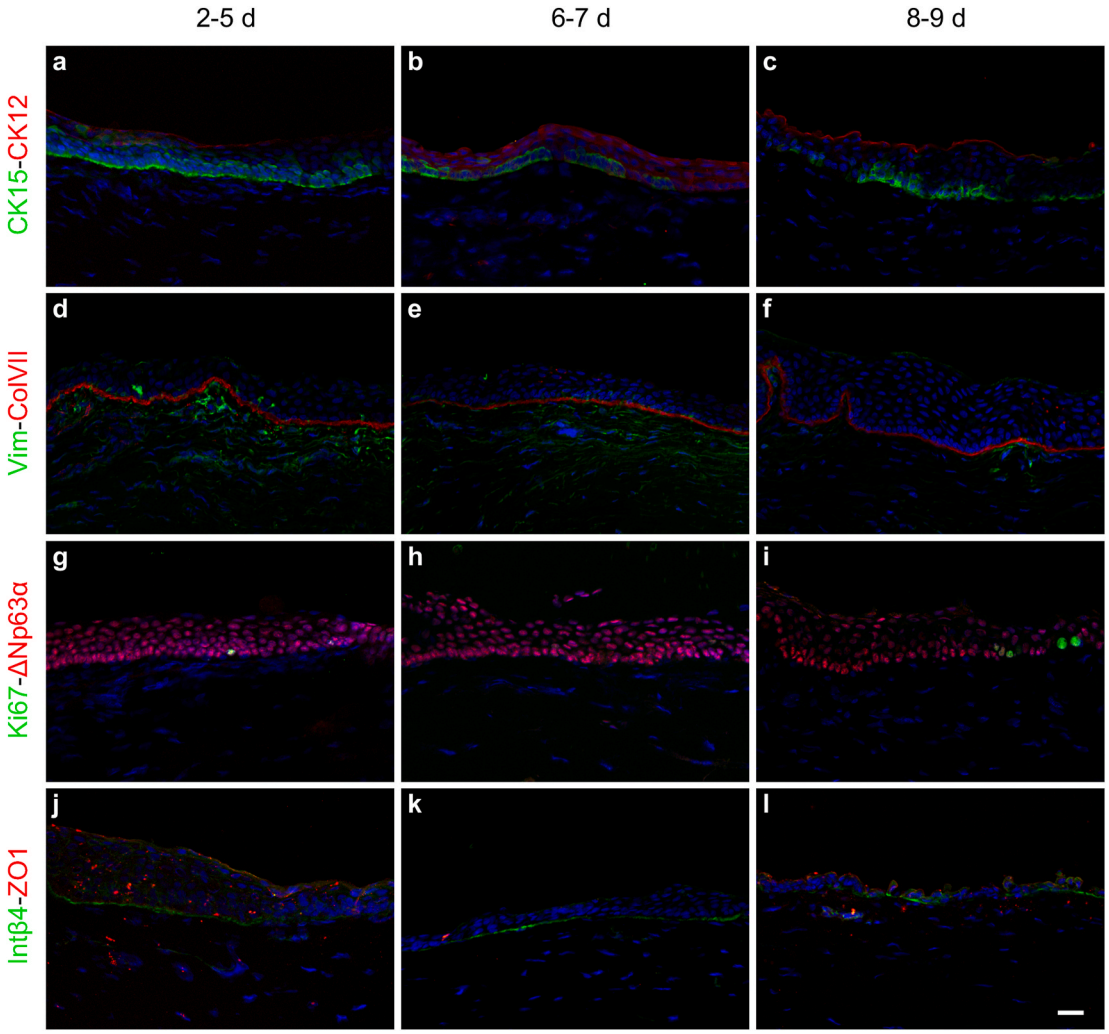

Fig. 2. Expressions of different markers in the limbal area of corneoscleral tissues stored in hypothermic conditions for 2–9 days (pmt < 8 h). The samples used during the study were classified in 3 groups (2–5, 6–7 and 8–9 days) represented in one column each. After 6 or 7 days, the expression of the putative stem cell markers (CK15, Vim, ΔNp63α) diminished. Differentiation (CK12), adhesion (ZO-1, integrin β4, Collagen VII), and proliferation (Ki67) markers were maintained in all groups.Vimentin labelling was notably lost in the tissues of more than 5 days of conservation. All the images were taken with 20 x magnification objective (scale bar corresponds to 25 μm).

3.2.2. Vimentin–collagen VII cell marker expressions

Because vimentin is a marker of fibroblastic cells, vimentin-positive staining was observed for stromal keratocytes in all analysed tissue sections. Concerning the epithelium, a positive staining pattern of vimentin in the sections conserved for 2–5 days was observed in the basal area of the limbal and conjunctival epithelium (d of Fig. 2). However, a significant loss of specific staining in the basal area of the limbus was registered in the tissues conserved for 6–7 days (e of Fig. 2). This loss was even more noticeable in the corneoscleral rims conserved for 8–9 days (f of Fig. 2), in which the staining became completely negative for all the cells of the basal limbal epithelium. For corneoscleral rims conserved for 1 day with pmt >16 h, positive labelling was detected in specific cells of the limbus (c of Fig. 3). Finally, complete absence of expression in the epithelium of cultivated sclerocorneal rims was observed. The only vimentin-positive cells of this last group were located in the conjunctiva (d of Fig. 3).

The expression of collagen VII was similar and homogeneous for the entire epithelial extent in all the samples, clearly delimiting the junction between the epithelium and the stroma (d–f of Fig. 2 and c–d of Fig. 3).

The specific staining of vimentin in the peripheral corneal epithelium was quantitatively analysed in the corneoscleral rims conserved for 3–5 days. The positive staining pattern was displaced towards the limit of the limbus with the peripheral cornea as tissue preservation days increased (Fig. 5). Thus, an increasing trend of vimentin expression in the peripheral cornea and a decreasing trend in the limbus were registered over time. The expression registered in the conjunctiva was variable and did not follow any clear pattern.

Vimentin was also combined with CK15 and CK3 to observe possible colocalisation of these markers. The results for vimentin and CK15 showed colocalisation in specific areas of the sclerocorneal transition epithelium. However, CK3 and vimentin were not registered together; the expression of CK3 was limited to the superficial areas of the limbal and conjunctival epithelia whereas vimentin stained the basal cells of both epithelia (Fig. 6).

3.2.3. Vimentin–melan A cell marker expressions

After the identification of vimentin positive cells in the corneal epithelium of the corneoscleral tissues conserved for 3–5 days, the melanocyte marker melan A was combined with this protein to observe possible colocalisations. The staining pattern of melan A was limited to a few cells located in the limbal crypts (Fig. 7). Vimentin and melan A colocalised in some cells, but not all vimentin-positive cells were classified as melanocytes. The cells that expressed any of these markers exhibited a ramified morphology.

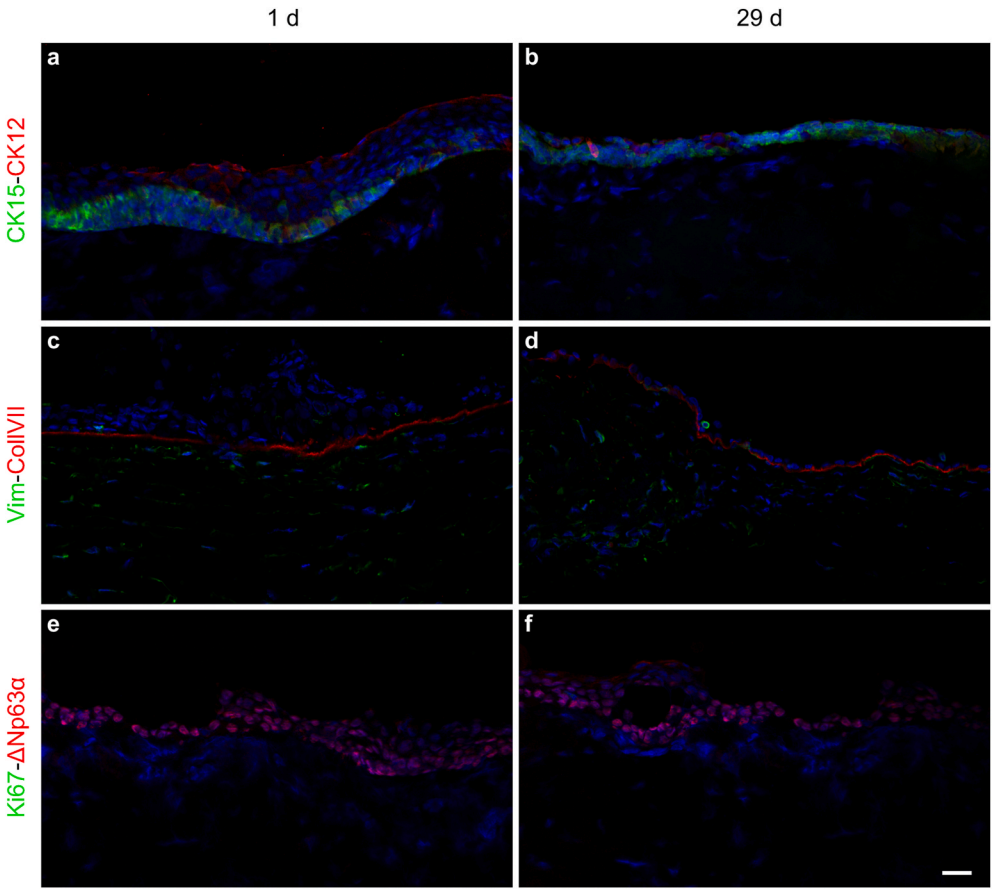

Fig. 3. Expressions of different markers in the limbal area of the corneoscleral tissue stored in hypothermic conditions for only 1 day with pmt >16 h, and in the culture medium for 29 days. Both conditions showed similar expression patterns of non-differentiation markers (CK15, Vim and ΔNp63α),but the expressions decreased in the cultured tissue. Moreover, the corneoscleral sample cultured for 29 days showed great tissue deterioration. All the images were taken with a 20 x magnification objective (scale bar corresponds to 25 μm).

3.2.4. *ΔNp63α–Ki67 cell marker expressions*

The staining pattern of ΔNp63α was almost identical in all corneoscleral rim sections. The limbal or conjunctival basal area of the epithelium contained the most intensely marked cells; even so, cells in the suprabasal areas as well as in the peripheral cornea were slightly labelled too (g–i of Fig. 2 and e–f of Fig. 3).

Besides quantifying the number of ΔNp63α-positive cells, the percentage of "bright" ΔNp63α cells was specifically recorded, as they were supposed to be the ones with higher stemness potential. The results showed that all the corneoscleral rims expressed ΔNp63α regardless of their preservation time. The presence of ΔNp63α "bright" cells corresponded mainly to the limbus and conjunctiva (Fig. 4).

Cells with high mitotic activity, that is Ki67-positive cells, were observed in all the groups (g–i of Fig. 2) except for the corneoscleral tissues conserved for 1 day with pmt >16 h and the ones cultured for 29 days (e–f of Fig. 3). The number of Ki67-positive cells was also determined in each tissue section of the samples conserved for 2–9 days. The presence of Ki67-positive cells was registered in all these tissue samples, especially in the limbal area of corneoscleral tissues preserved for a few days, but it was not possible to determine any clear trend (Fig. 4).

3.2.5. *Integrin β4–ZO-1 cell marker expressions*

The results of the corneoscleral rims conserved for 1 day with pmt >16 h and the ones cultured for 29 days were omitted for integrin β4 and ZO-1 markers due to their deteriorated state.

A homogeneous staining pattern of integrin β4 was observed in the basal area along the epithelium of the tissues conserved 2–5 days (j of Fig. 2), whereas ZO-1 was localized in the epithelial surface. However, the integrin β4 expression became discontinuous and extended across the whole epithelial thickness over time (tissues conserved for 6–7 days) (k of Fig. 2), so that it appeared interlaced with ZO-1 in the corneoscleral tissues conserved for 8–9 days (i of Fig. 2).

3.3. *Isolation and culture of limbal epithelial cells*

The suitability of the methodologies used for the isolation of cells from corneoscleral tissues conserved in hypothermic or culture conditions, as well as for the *in vitro* cell expansion of the isolated cells, was evaluated by a series of immunocytochemical procedures. The evolution of the expressions of the cell markers was evaluated for the different culture passages.

Epithelial cells directly isolated from the corneal limbal region were precipitated using a Cytospin, and the cell markers were analysed. CK15-, vimentin- and ΔNp63α-positive cells were observed in directly precipitated epithelial cells (Fig. 8, a, f). When limbal epithelial cells isolated from corneoscleral tissues were cultured for 12 ± 2 days (p0 passage), they maintained high staining for the CK15 marker (b of Fig. 8). However, the expression of this protein decreased as the cells were subcultured: from 40% of cells stained with CK15 in the cells precipitated from the Cytospin, to 10% of cells stained with CK15 in cultured passage 0, to total absence of its expression at the second passage of cultures (c of Fig. 8). Statistical differences were observed between p0 (Cytospin) and p1, p2 and p3 of cultured cells for CK15 ($p = 0.005$) and vimentin ($p = 0.037$) cell markers (e of Fig. 8). On the contrary, the expression of the vimentin cell marker in cells isolated from corneoscleral tissues and centrifuged with the Cytospin was limited to a few cells (around 7% of the total cells). In p0 passage cultures, vimentin-positive cells showed an elongated and branched morphology (b of Fig. 8), with some of them enclosing CK15-positive cells. These positive cells observed in epithelial cultures at early passages presented a different morphology from the cells marked with vimentin in epithelial cultures of second and later passages (c, d of Fig. 8). The latter cells

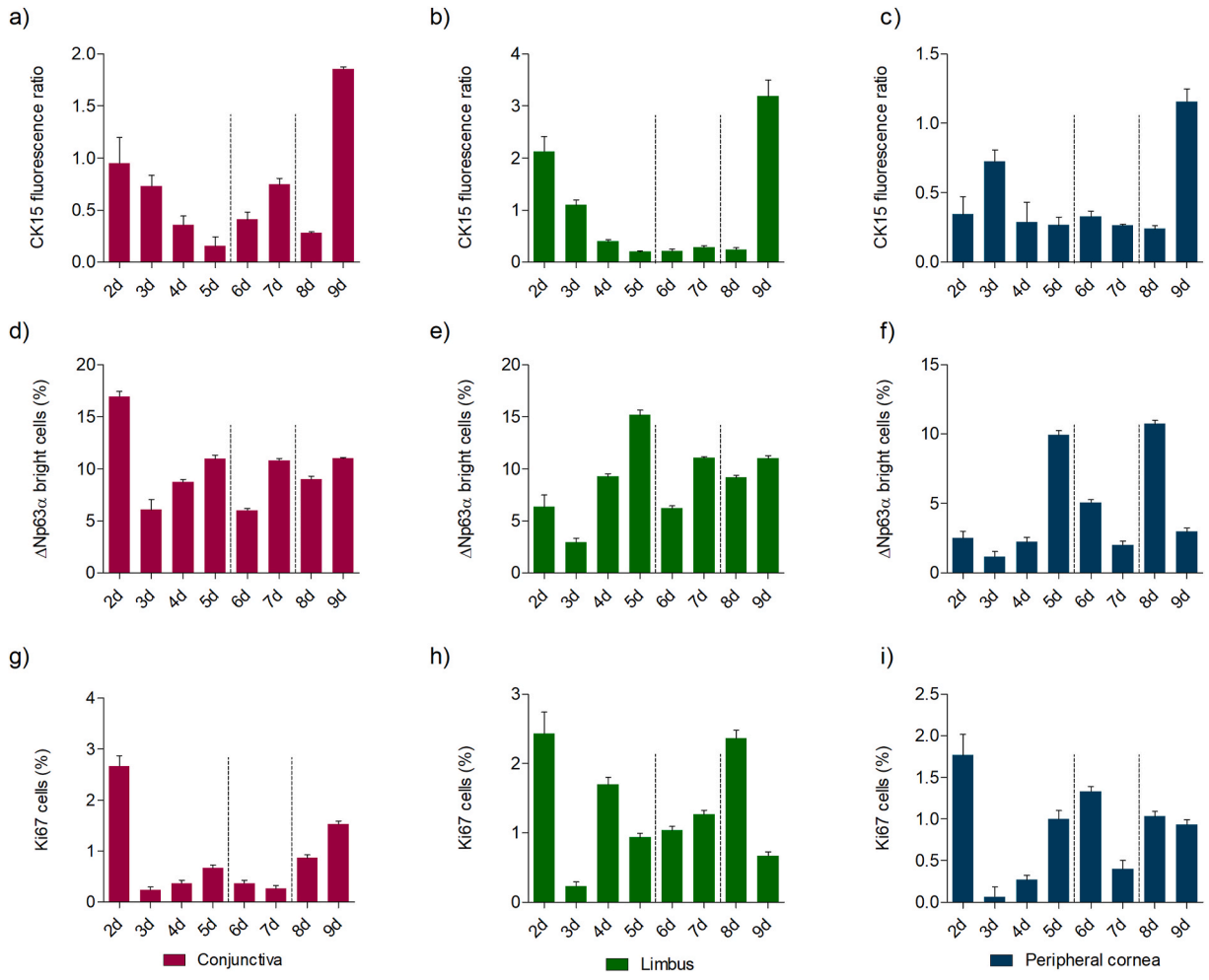

Fig. 4. Quantification of fluorescence staining of CK15 (a–c) and the percentage of ΔNp63α- "bright" (d–f) and Ki67-positive cells in the corneoscleral tissues (g–i). The specific expression of CK15 was observed in the limbal region of all samples. As time of conservation increased, the expression reduced and the distribution of CK15 became more unspecific. At short storage time (up to 5 days), ΔNp63α "bright" cells were more abundant in the limbal region than in the peripheral cornea. All corneoscleral tissues presented Ki67-positive cells but with no clear trend.

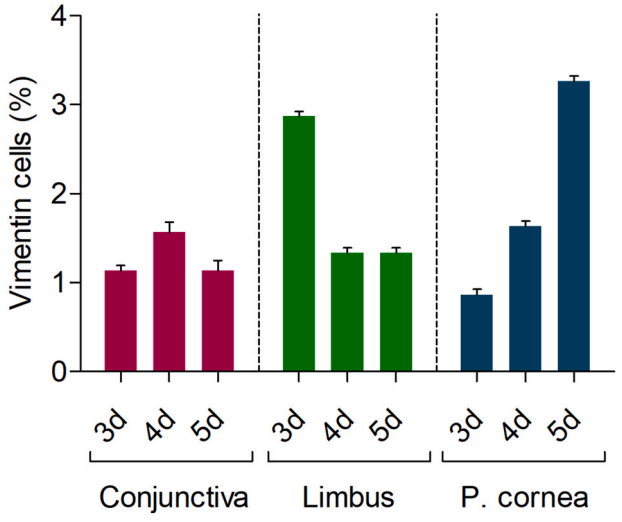

Fig. 5. Quantification of vimentin fluorescence marker in the three corneoscleral regions for the samples conserved for 3–5 days. The graphs show the displacement of vimentin staining from the limbal region to the peripheral cornea as conservation time increases.

showed a morphology consistent with the differentiated stromal cells but not with the epithelial cell phenotype.

Epithelial cells obtained just after tissue digestion from both fresh and cultured corneoscleral tissues showed ΔNp63α-positive staining. The evolution of the staining pattern was the same in cells from all tissues: the number of bright cells decreased drastically from one passage to another while the number of cells with no or insignificant signal increased (f, g, h and i of Fig. 8). The staining pattern decreased from 20% to 6% of counted bright cells from the passage of cells directly precipitated from the Cytospin to the p0 culture. From the p0 passage onwards, the number of ΔNp63α-positive cells did not exceed 5%. Statistical differences between p0 (Cytospin) and p1, p2 and p3 of cultured cells were recorded ($p = 0.043$) (j of Fig. 8).

3.4. Isolation and culture of limbal stromal cells

Directly isolated or cultured stromal cell populations were very pure and the double CK15 –vimentin staining revealed a lack of CK15 expression and a predominance of the vimentin marker. In addition, the cells were specifically aligned throughout the passages (Fig. 9).

3.5. Presence of melanocytes in the epithelial cell culture

Morphological differences were observed in vimentin-positive cells observed in epithelial and stromal cell cultures of p0 passage. The cells

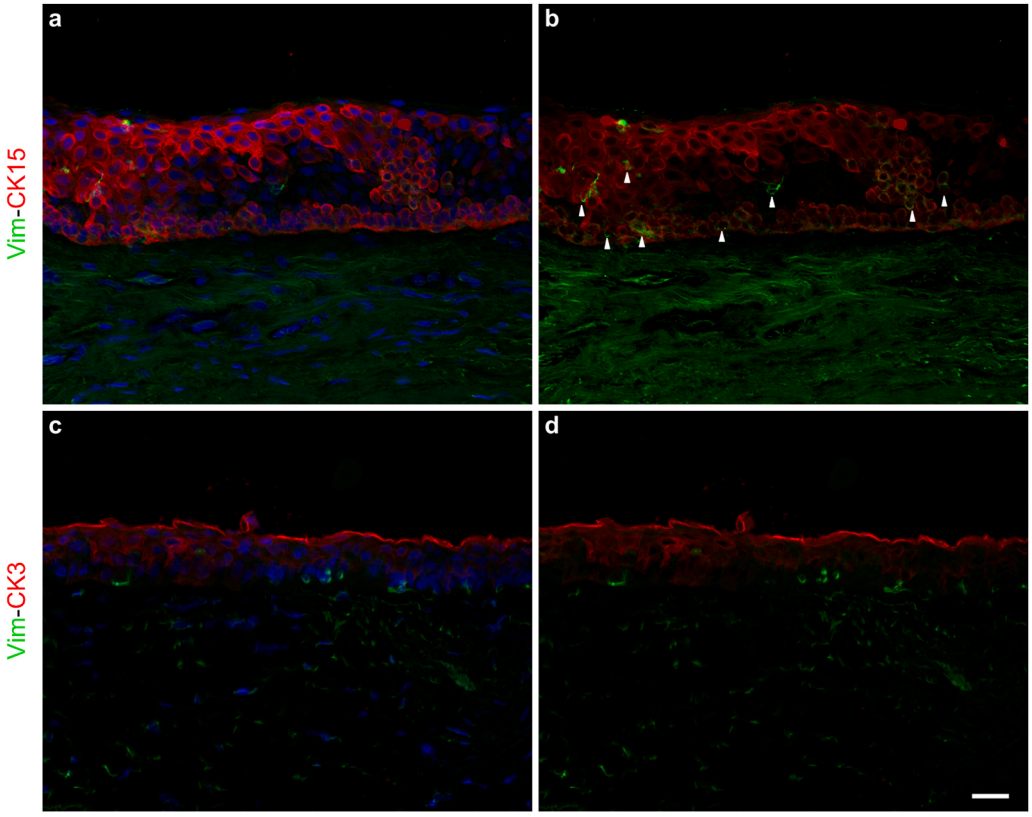

Fig. 6. Colocalisation of vimentin with CK3 and CK15 (differentiation and non-differentiation markers, respectively). Images on the left (a and c) show vimentin, CK15 or CK3 and DAPI nuclear staining. Images on the right (b and d) are the same as those on the left without the nuclear staining. No overlap between vimentin and CK3 was observed whereas some vimentin-positive cells colocalised with CK15-positive cells at the limbal epithelium (cells marked with white arrowheads). All the images were taken with a 20 x magnification objective (scale bar corresponds to 25 μm).

stained with vimentin in the epithelial culture showed a dendritic phenotype similar to that of melanocytes, whose positivity to vimentin was already registered in histological sections (Fig. 10). This cell phenotype was observed in limbal epithelial cell cultures seeded above the 3T3 cell feeder layer. These ramified cells were especially observed around forming colonies of epithelial cells (Fig. 11).

The presence of melanocytes was also registered using the melan A marker in epithelial cultures. Positive melan A staining was registered especially in p0 passages of cultured cells from the analysed corneoscleral tissues. Following the same trend as the undifferentiation markers ΔNp63α and CK15, melan A staining decreased considerably throughout all passages (Fig. 11).

3.6. *Presence of limbal putative stem cell markers in cell colonies formed in the epithelial cell culture of a sclerocorneal rim conserved for 7 days*

The presence of limbal stem cell markers was also assessed in epithelial cultures of a sclerocorneal rim conserved for 7 days in hypothermic conditions. Isolated cells in the culture expressed CK15 and ABCG2 undifferentiation markers after several culture passages (Fig. 12). Furthermore, isolated cells seeded at clonal dilution were able to grow, forming holoclone-like colonies, which demonstrated the presence in the culture of cells with self-renewing capability.

4. Discussion

Because of the shortage of donor corneas for both research and stem cell therapy purposes, it was important to investigate how the storage time and conditions (culture media and temperature) and conservation procedures of corneoscleral tissues affect their quality in terms of the quantity of limbal stem cells. These cells are essential for the treatment of limbal stem cell deficiency, a disease caused by the lack of stem cells in the limbus or damage to the limbal niche, resulting in partial or complete loss of the corneal regenerative ability (Le et al., 2018).

The way the corneas are handled and preserved may play an important role in successful *ex vivo* limbal cell transplant. The two main methods of corneoscleral tissue storage prior to corneal transplantation are organ culture, in which corneas are stored at 31 °C in a medium for organ culture of donor corneas (Pels and Rijneveld, 2009), and hypothermic storage in commercially available media such as Optisol-GS or Eusol-C, in which corneas are stored at 2–8 °C (Kanavi et al., 2015).

Raeder et al. showed that the layered structure of corneal epithelial cells was better preserved and the number of dead cells reduced when the cells are kept at 23 °C in organ culture conditions rather than in Optisol-GS at 5 °C (Raeder et al., 2007). Kim et al. examined how time variables were related to *in vitro* limbal epithelial growth from corneas stored at 4 °C in Optisol-GS or an organ culture medium (Kim et al., 2004). They found that “fresher” tissues with low death-to-enucleation and low death-to-storage times were more likely to produce successful culture growth. The aim of the present study was to verify the presence or lack of limbal stem cells, their amount and exact location in the epithelium of sclerocorneal rims preserved in hypothermic conditions or organ culture media, and their potential to grow in culture and become a source of stem-like limbal epithelial cells for limbal cell transplantation. Several fluorescence non-differentiation markers as well as colony-forming efficiency assays were used for such purposes.

CK15 is a basal type I cytokeratin, a specific epithelial cell cytoskeletal protein present in stratified epithelia. It is part of the intermediate filaments and has been proposed as a potential putative marker of limbal stem cells (Yoshida et al., 2006). The expression pattern of this cytokeratin in corneoscleral tissues preserved for 2–5 days was very clear and delimited the beginning of the transition from the peripheral cornea to the sclerocorneal limbus. The fluorescence ratio recorded for this marker decreased progressively as the preservation time of fresh corneoscleral tissues increased from 2 to 8 days. However, its expression was lost in cultured tissues preserved for 29 days and in fresh tissues preserved for more than 7 days, evolving towards a less specific expression intermingled with CK12 staining. The noticeable increase in

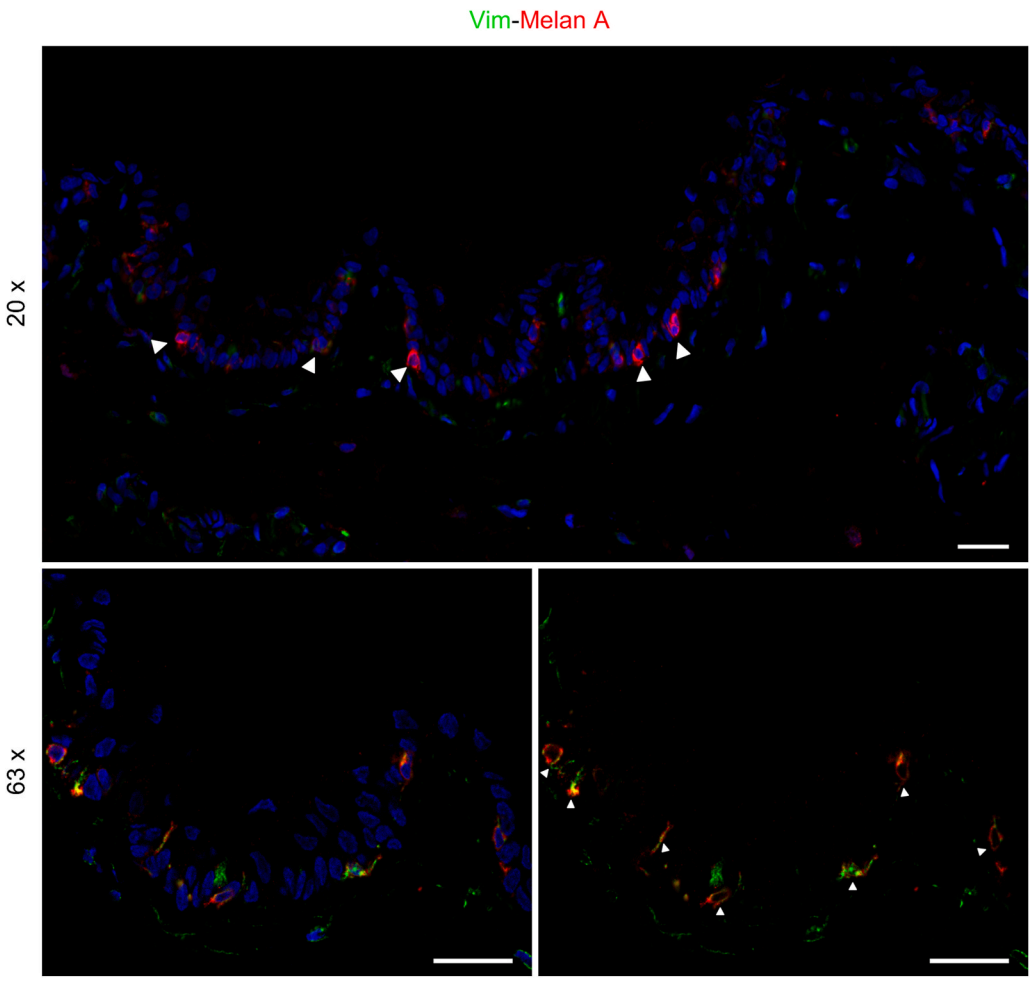

Fig. 7. Expressions of vimentin- and melan A-positive cells in a histological section of the limbal area of a corneoscleral tissue conserved for 3 days. The image above was taken with a 20 x magnification objective (scale bar corresponds to 25 μm) and shows the specific localization of melan A-positive cells (cells marked with white arrowheads) in the limbal crypts. The images below were taken with 63 x magnification objective (scale bars correspond to 25 μm). Both images correspond to the same tissue section with or without the nuclear staining with Hoechst 33342. Vimentin colocalised with melan A-positive cells (cells marked with white arrowheads) but not all vimentin-positive cells in the epithelium stained for melan A.

CK15 fluorescence ratios registered in three areas (conjunctiva, limbus and peripheral cornea) in the tissues preserved for 9 days and in the cultured sample was the result of the detachment of the epithelial cells and the progressive deterioration of the epithelium. In general, the morphological alterations recorded in long-stored donor tissue sections were alike to those already described: sloughing and loss of the superficial layers of the epithelium (Greenbaum et al., 2004). This damage caused the altered quantification of CK15 marker.

CK12 and CK3 are documented as cornea-specific cytokeratins specifically related to differentiated epithelial cells. Their expression can be observed throughout the whole corneal epithelium, although their pattern of expression varies among areas. They are present across the whole epithelial thickness in the central cornea; their pattern is limited to the superficial layers of the epithelium in the limbus, and they are absent in the conjunctiva (Merjava et al., 2011). In our study, CK12 staining was recorded throughout the suprabasal areas of the peripheral corneal epithelium. The loss of specificity of this marker was also detected as the conservation time of the corneoscleral tissues increased. Considering that CK12 forms a dimer with CK3 and that both are considered specific corneal epithelial cell markers, these results are in line with the ones reported by Joseph et al. who observed a decrease in CK3 staining as the duration of donor corneal samples in culture increased (Joseph et al., 2004).

The presence of vimentin in the corneoscleral epithelium was also assessed. Vimentin is a cytoskeletal protein classified under type III intermediate filament family. It is considered a specific marker of cells with mesenchymal origin such as keratocytes. Nonetheless, the expression of this protein has also been linked with a specific cell population in the limbal niche (Schlötzer-Schrehardt and Kruse, 2005). Vimentin staining of specific basal epithelial cells of the limbus and conjunctiva strikingly disappeared in the corneoscleral tissues conserved for 7 days or more, narrowing down the quantification of its expression in only the samples conserved for 3–5 days, which were the ones that showed the most remarkable staining. As conservation time of the tissue samples increased, a simultaneous decrease and increase in the vimentin expression in the limbus and the peripheral cornea, respectively, were registered. These results could be related to the highly motile vimentin-positive cells reported by Castro-Muñozledo et al., who suggested that some vimentin-positive cells located in the limbal epithelium could be early differentiated and highly motile epithelial progenitors that migrate from the limbal zone towards the peripheral and central cornea (Castro-Muñozledo et al., 2017).

Vimentin was combined with the differentiation marker CK3, the non-differentiation protein CK15 and the melanocytic marker melan A to analyse possible colocalisations. On the one hand, the individual cells stained with vimentin in the limbal epithelium were consistent with the undifferentiated cell phenotype. Lauweryns et al. already indicated the expression of vimentin in corneal epithelial cells with stemness characteristics, as they identified a subpopulation of transitional cells co-expressing CK19 and vimentin (Lauweryns et al., 1993). On the other hand, the colocalisation of some vimentin cells with melan A marker indicated that some of the vimentin-positive cells of the epithelium corresponded to melanocytes (Polisetti et al., 2020a, b).

Another putative marker used for limbal stem cells staining was ΔNp63α, the predominant isoform of the transcription factor p63 in the corneoscleral limbus. ΔNp63α is expressed in the basal cells of stratified

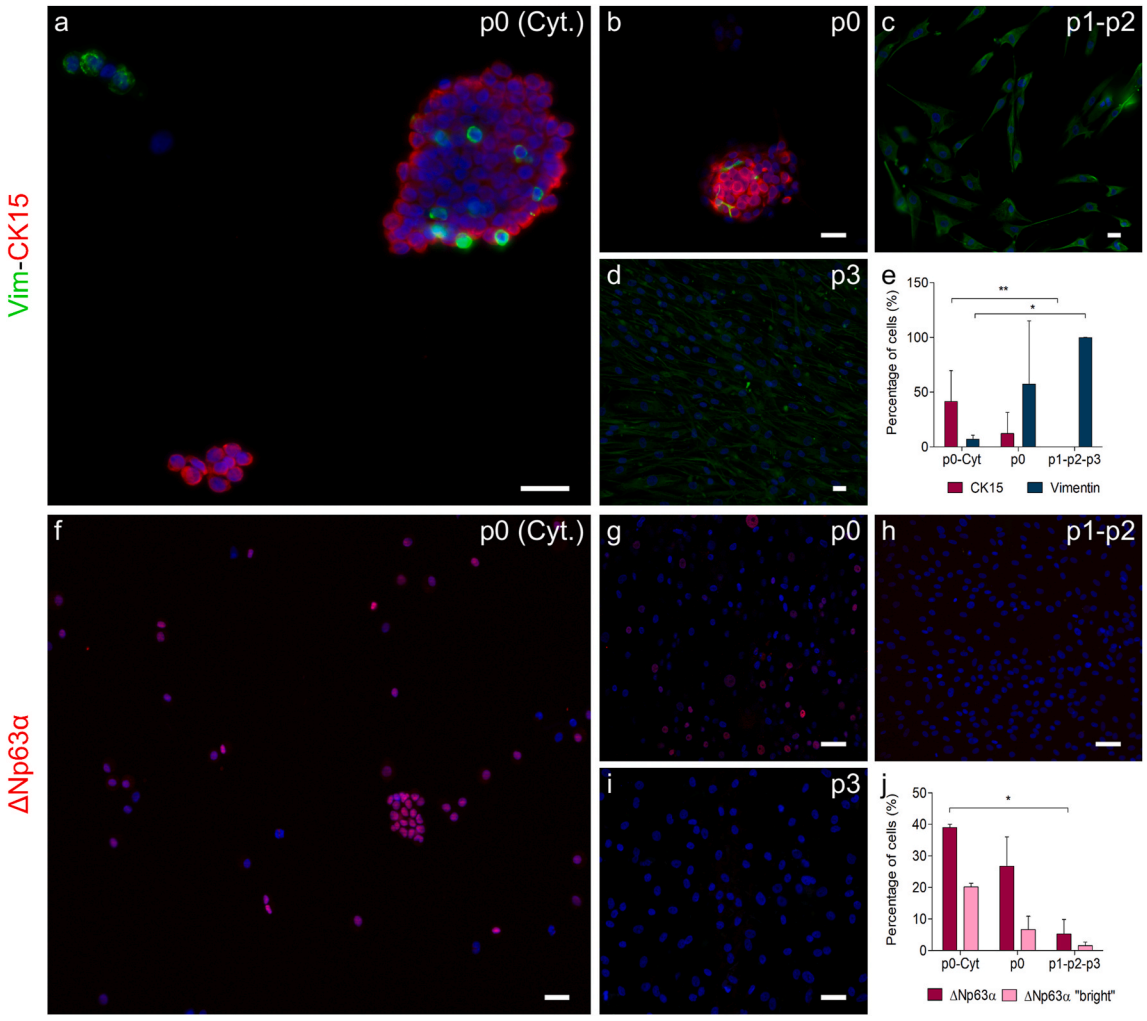

Fig. 8. Expressions of CK15, vimentin and ΔNp63α markers in limbal epithelial cell directly isolated after tissue enzymatic digestion (a and f, p0 Cytospin) or cultured during several cell passages p0 (b, g), p1, p2 (c, h) and p3 (d, i). The images a and f show that the cells extracted directly after tissue digestion highly expressed the putative non-differentiation markers CK15 and ΔNp63α. An exponential decrease in the undifferentiation markers and a predominant vimentin staining were registered throughout the passages (images b, c and d). The graphs show on the one hand the evolution of the percentage of CK15- and vimentin-positive cells (graph e) and on the other hand the expression of ΔNp63α bright cells from several corneoscleral tissues throughout the passages (graph j). Statistical differences were recorded between p0 (Cytospin) and p1, p2 and p3 of cultured epithelial cells for the three markers. All the images were taken with a 20 x magnification objective (scale bars correspond to 25 μm).

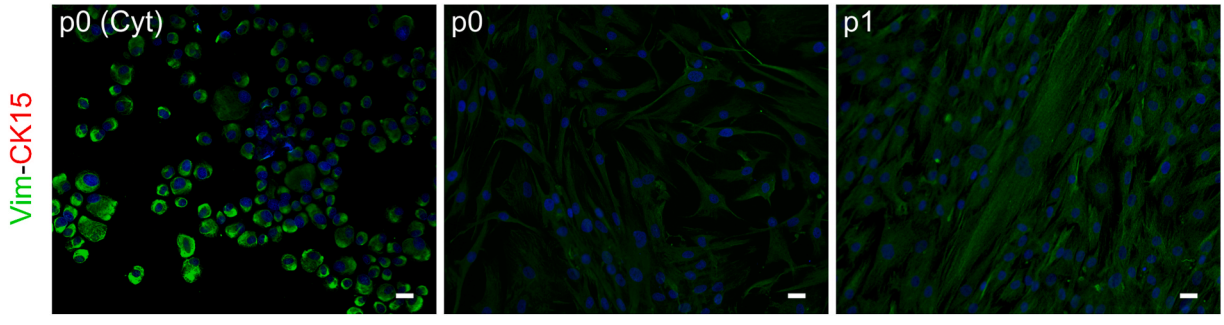

Fig. 9. Expressions of vimentin and CK15 markers in limbal stromal cells directly isolated after tissue digestion (p0 Cytospin) and p0 and p1 of cultured stromal cells. Cell cultures were characterised by complete absence of the CK15 epithelial marker. As the passage of the cell culture increased, the cell orientation and morphology tended to change. All the images were taken with a 20 x magnification objective (scale bars correspond to 25 μm).

epithelia and is highly related to limbal stem cells (Kawasaki et al., 2006). It is expressed as nuclear staining especially in the cells of the basal region of the limbal epithelium, but it can also be observed in more suprabasal areas of both the corneal and conjunctival epithelium (Melino et al., 2015). Our results indicated that the conservation time of the corneoscleral tissues was not the most critical factor affecting the abundance of ΔNp63α-positive cells, especially ΔNp63α "bright" cells, whose expression corresponds to potentially undifferentiated

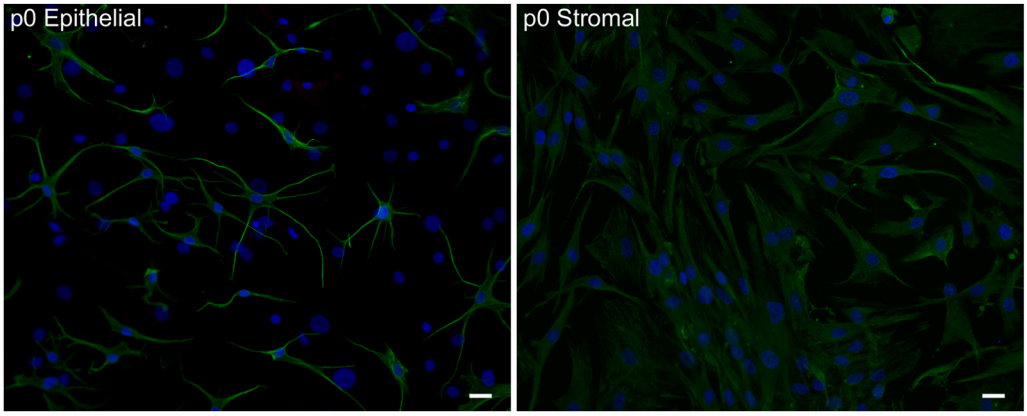

Fig. 10. Expression of the vimentin marker in limbal epithelial (a) and stromal (b) cell cultures. Both cultures corresponded to p0 passage. Both images were taken with a 20 x magnification objective (scale bars correspond to 25 μm).

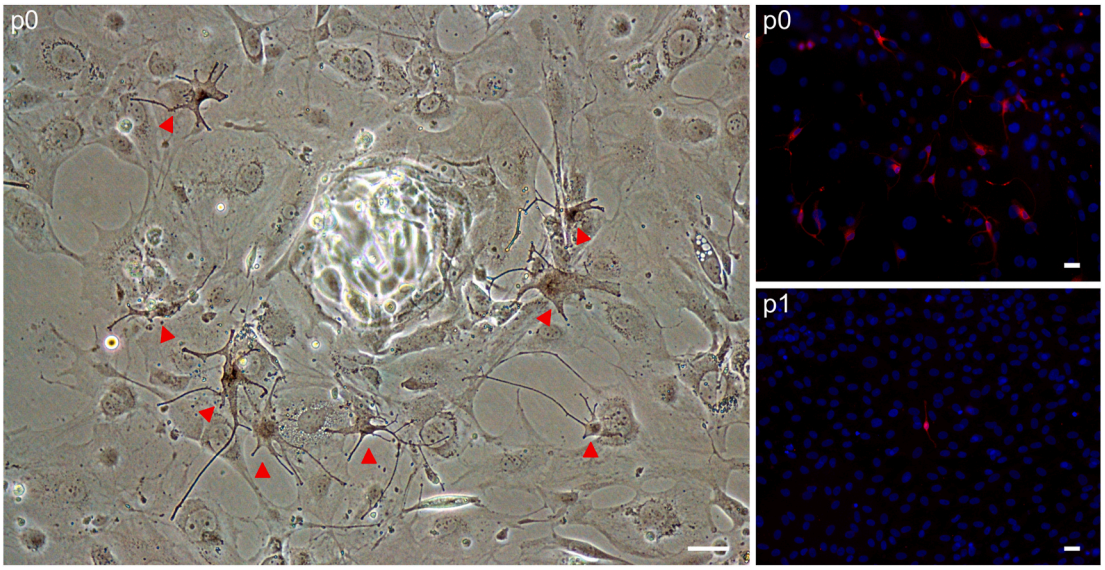

Fig. 11. Limbal epithelial cell culture of passage p0 and passage p1. The image on the left shows epithelial cells of passages p0 and seeded above a feeder layer of 3T3 cells. Cells marked with red arrowheads correspond to the melanocytes that enclosed the forming epithelial stem cell colony. The image was taken with a 20 x magnification objective (scale bar corresponds to 25 μm). The images on the right show the expression of the melan A marker in epithelial cells of passages p0 and p1. Both images were taken with a 20 x magnification objective (scale bars correspond to 25 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

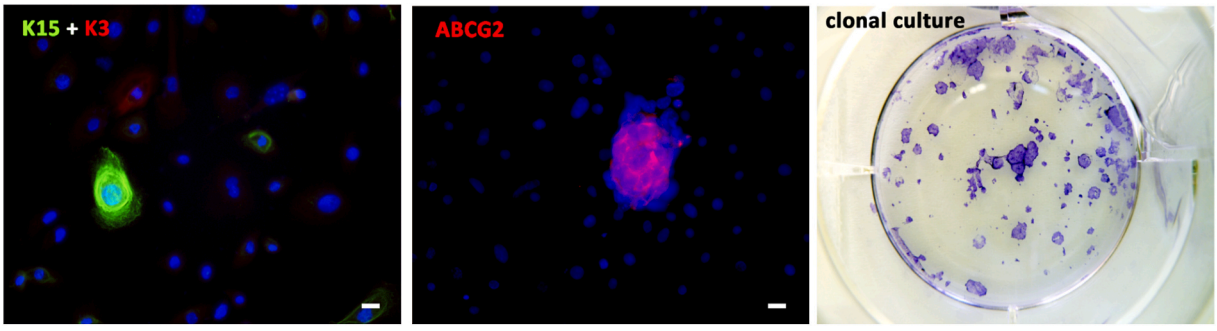

Fig. 12. Expressions of CK15 and ABCG2 undifferentiation markers in the second passage of the limbal epithelial cell culture (first and second images) and clonal culture of epithelial cells stained with violet crystal (third image). First and second images were taken with a 20 x magnification objective (scale bars correspond to 25 μm). Clonal cultures demonstrated cell growth into holoclone-like colonies. Images reproduced from Hernáez-Moya et al. (2015), with permission of the authors. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stem/progenitor cells (Rama et al., 2010). Its staining was registered in all the corneal tissues, prevailing in the basal area of the limbal epithelium.

The expression pattern of integrin β4 and ZO-1 adhesion proteins was also assessed to complete the analysis of tissue sections. Integrin β4 is involved in the formation of hemidesmosomes, therefore it constitutes an adhesion marker between the epithelium and the underlying extracellular matrix. This protein shows a polarized distribution in the basal cells of the limbal and corneal epithelium (Schlötzer-Schrehardt and Kruse, 2005). ZO-1 is a protein involved in the formation of tight junctions between cells and it is found mainly in the apical area of the epithelia. It is also implicated in the barrier effect of the corneal epithelium (Sugrue and Zieske, 1997). Both markers were seen along the whole corneoscleral epithelium. Integrin β4 staining was limited to the basal area (basement membrane) and ZO-1 was present in the superficial epithelium (tight junctions). The staining pattern got blurred in the corneoscleral tissues preserved for longer times for both markers, but their expression was maintained even in the most deteriorated epithelia. This confirmed that the barrier function and the ability to adhere to the extracellular matrix were maintained in all the analysed tissues.

Regarding cell cultures, the patterns observed in the corneoscleral tissue sections were similarly maintained in isolated cell populations in their passage 0. CK15-positive labelling of the epithelial cells directly precipitated from the Cytospin was consistent with the areas marked with this protein in the histological sections. Some vimentin-positive cells were also observed in directly isolated cells, as observed in the limbal area of the corneoscleral sections. The high percentage of CK15-positive cells (around 40%) extracted by tissue digestion and the lack of expression of this marker in stromal cell cultures verified the adequacy of the cell isolation methods, which generated quite pure epithelial and stromal populations. Whilst the expression of CK15 cytokeratin decreased throughout the passages, vimentin staining increased, with the vimentin-positive cells showing a morphology that resembled the differentiated stromal cell phenotype. This means that a small number of stromal cells can rapidly proliferate and overcome epithelial cultures if the cell culture does not favour the preservation of epithelial stem cells. Epithelial cells obtained just after tissue digestion from both fresh and cultured corneoscleral tissues showed ΔNp63α-positive staining irrespective of their conservation or culture time. This is consistent with ΔNp63α staining observed in tissue sections preserved for different time periods. However, the decrease in ΔNp63α levels was especially registered throughout all cell passages. The gradual decrease in p63 throughout the culture time was already reported in human limbal explant cultures. Joseph et al. registered a decrease in the number of p63-positive cells maintained for 1–3 weeks in culture. This result, together with their observation of p63 staining in the limbus and central cornea, suggested that p63 is a marker for both stem cells and transient amplifying cells. They concluded that the decrease in the positivity of this marker was related to the decline in the proliferative capacity of the cells after reaching confluence (Joseph et al., 2004). This loss of cells with high fluorescence intensity in *in vitro* cultures but not in corneal sections suggested that certain characteristics of stemness could be adequately maintained in both storage conditions before tissue digestion and that the loss of expression of markers had more to do with the *in vitro* cell culture.

Concerning the isolated stromal population, no CK15-positive staining was observed in directly isolated or cultivated stromal cells, and the vimentin marker was by far the predominant marker observed in these cultures. The specific orientation of these cells in culture was noticeable. Almost all the cells were oriented in the same sense and direction, as if they wanted to simulate their organized disposition in the corneal stroma (Espana and Birk, 2020).

Regarding the relationship between melanocytes and limbal epithelial stem cells, a very specific relationship of both cell types was suggested, based on the following highly significant observations: positive staining of melan A cells was detected in the limbal crypts; the ramified cell phenotype congruent with melanocytes was observed around forming limbal epithelial colonies in cell cultures; and the concurrent decrease throughout the culture passages of ΔNp63α-, CK15- and melan A-positive cells was registered. As suggested by Dziasko et al. a close relationship exists between the melanocytes and the limbal stem cells of the niche (Dziasko et al., 2014, Dziasko and Daniels, 2016), where the former seem to play a protective role in these potentially undifferentiated cells (Dziasko et al., 2015; Polisetti et al., 2016). This close relationship between stem cells and melanocytes and the putative role of melanocytes in preserving stemness are consistent with the simultaneous decrease in both markers. Besides, this could be related to the loss of vimentin expression in the corneal epithelium of the tissues conserved for longer times, as the colocalisations of vimentin and melan A in epithelial cultures of early passages confirmed that some vimentin-positive cells of the epithelium corresponded to melanocytes. The colocalisation of vimentin and melan A markers has already been reported. Polisseti et al. showed relatively pure cultures of melanocytes that stained positive for melan A and vimentin (Polisetti et al., 2020a). Besides, they were able to distinguish different cell types that express vimentin, since they observed positive vimentin staining colocalised with melan A corresponding to melanocytes in the basal epithelial layer of the sclerocorneal limbus, but the vimentin-positive stromal cells of the subepithelial layers lacked expression of the melanocytic marker (Polisetti et al., 2020b).

Even so, not all vimentin-positive cells stained for melan A. Apart from the possibility of being highly motile stem progenitors, the ramified cells observed in the epithelium could also be related to Langerhans cells. Like melanocytes, Langerhans cells are known to reside in the limbal basal layer and both stain positive for vimentin (De Waal et al., 1984; Higa et al., 2005; Si et al., 1993; Vantrappen et al., 1985).

Finally, because of the shortage of available corneal tissues, we considered it of interest to investigate the potential of expanding LESCs from long-term hypothermically stored sclerocorneal rims. We successfully obtained epithelial cultures containing holoclone-like cell colonies positive for the non-differentiation marker ABCG2. The potential of limbal primary cultures to grow in holoclones, instead of meroclones and paraclones, has been considered a means of quality control (Pellegrini et al., 2011), because holoclone-forming cells have all the hallmarks of stem cells, such as self-renewing capability and large proliferative potential.

The conclusions drawn from the study were mostly derived from the observations of the expressions of undifferentiated and differentiated cell markers in fluorescence images. Depending on the conservation time, increasing or decreasing expression patterns of the markers consistent with the deterioration of tissue samples were observed.

The low number of corneas available was translated into a low number of replicates for each study group, which was one of the biggest limitations to performing a detailed quantitative analysis. The shortage of donors and the high demand for corneas complicated the acquisition of tissues for this study.

Samples were classified considering the storage method, and the days of preservation and the pmt, which indicated the time that had elapsed since the death of the donor to the preservation of the tissue.. Two groups were considered based on the pmt: samples with pmt <8 h and samples with pmt >16 h. However, there is no generally established consensus on how to classify samples, and some authors lean towards not establishing such limits and being guided by the fulfilment of certain quality criteria such as donor age, storage time in organ culture, epithelial integrity, and opacity or clarity for the use of corneal grafts (Armitage et al., 2014; Sugar et al., 2009). No conclusions are drawn about the effects of donor age on graft quality because of the low number of samples available for this study. Advanced age has been related to a greater loss of endothelial cells that could compromise the success of transplants (Cruz et al., 2017). However, the Cornea Donor Study reported a similar survival rate of corneas for over a 5-year follow-up period after transplantation from donors aged 66–75 years old and

younger donors (Gal et al., 2008). Notara et al. studied the impact of the age of donor samples on the structural and phenotypic characteristics of the limbal niche. They did not observe differences in the expression of markers with respect to the age of the donors, which indicated that the cells maintained their stemness potential. However, their results indicated a significant decrease in the proliferative capacity of limbal epithelial cells from older donors, especially donors aged over 60 years (Notara et al., 2013). As long as this shortage of corneal tissues continues, it may not be prudent to set any age limit since establishing a maximum donor age could reduce the percentage of discarded corneas even more.

In summary, we have demonstrated that although a decrease in the pattern of several non-differentiation markers was recorded in the histological sections of long-preserved corneoscleral tissues, positivity for the putative stem cell markers was detected in all tissue samples, including in the corneoscleral tissue conserved in hypothermic conditions that exceeded the short desirable post-mortem time for tissue extraction. We have shown that the remaining limbal rims after corneal transplantation, which had been conserved in hypothermic conditions for as long as 7 days and should normally have been ignored, can be a valuable source of LESC cultures for research and even for stem cell therapy.

We hope that this study would in some way reduce the discard rate of tissues and thus increase the available donor pool, since disposable grafts could be used for the isolation of stem cells that could be expanded *in vitro*.

In addition, the results related to vimentin labelling suggested the need for a more exhaustive study to determine which cell type corresponds to the positivity of this marker in the corneal limbal epithelium, which would help to clarify the specific cells residing in the limbus and their interactions with the limbal stem/progenitor cells. The understanding of their behaviour could help to develop new culture conditions that would preserve the phenotype and function of the limbal stem/progenitor cells, before their use for treatment of limbal stem cell diseases.

Funding

This research study has been funded by grants from the Department of Heath of the Basque Government (RIS3, 2019222049) and the University of the Basque Country UPV/EHU-Instituto Clinico Quirurgico de Oftalmologia ICQO (US19/18). C. R–V. has received fellowship support from the University of the Basque Country UPV/EHU.

Declaration of competing interest

No sponsor or funding organisation had any role in the design or conduct of this research. None of the authors had any conflict of interest.

Acknowledgements

The authors thank the Biobank A Coruña of SERGAS, the Navarra Blood and Tissue Bank, Navarrabiomed Biobank from Navarra Health Department, the Blood and Tissue Bank from the Government of Catalonia, and the Basque Biobank (www.biobancovasco.org) for having provided them with the biological samples and/or clinical data necessary for the development of this research.

The authors thank SGIker (UPV/EHU/ERDF, EU) for their technical and human support with imaging and microscopy. They also gratefully acknowledge the technical support and assistance provided by Maria Jesus Fernandez-Martín and Cristina Tobillas.

References

Armitage, W.J., Jones, M.N.A., Zambrano, I., Carley, F., Tole, D.M., 2014. The suitability of corneas stored by organ culture for penetrating keratoplasty and influence of donor and recipient factors on 5-year graft survival. Invest. Ophthalmol. Vis. Sci. 55, 784–791. https://doi.org/10.1167/iovs.13-13386.

Barrandon, Y., Grasset, N., Zaffalon, A., Gorostidi, F., Claudinot, S., Lathion Droz-Georget, S., Nanba, D., Rochat, A., 2012. Capturing epidermal stemness for regenerative medicine. Semin. Cell Dev. Biol. 23, 937–944. https://doi.org/10.1016/j.semcdb.2012.09.011.

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.

Bonnet, C., González, S., Roberts, J.S., Robertson, S.Y.T., Ruiz, M., Zheng, J., Deng, S.X., 2021. Human limbal epithelial stem cell regulation, bioengineering and function. Prog. Retin. Eye Res. 100956. https://doi.org/10.1016/j.preteyeres.2021.100956.

Brookes, N.H., Loh, I.P., Clover, G.M., Poole, C.A., Sherwin, T., 2003. Involvement of corneal nerves in the progression of keratoconus. Exp. Eye Res. 77, 515–524. https://doi.org/10.1016/S0014-4835(03)00148-9.

Castro-Muñozledo, F., Meza-Aguilar, D.G., Domínguez-Castillo, R., Hernández-Zequinely, V., Sánchez-Guzmán, E., 2017. Vimentin as a marker of early differentiating, highly motile corneal epithelial cells. J. Cell. Physiol. 232, 818–830. https://doi.org/10.1002/jcp.25487.

Chaloin-Dufau, C., Dhouailly, D., Sun, T.T., 1990. Appearance of the keratin pair K3/K12 during embryonic and adult corneal epithelial differentiation in the chick and in the rabbit. Cell Differ. Dev. 32, 97–108. https://doi.org/10.1016/0922-3371(90)90103-4.

Cruz, G.K.P., Azevedo, I.C. de, Carvalho, D.P., Vitor, A.F., Santos, V.E.P., Ferreira Júnior, M.A., 2017. Clinical and epidemiological aspects of cornea transplant patients of a reference hospital. Rev. Lat. Am. Enfermagem 25, e2897. https://doi.org/10.1590/1518-8345.1537.2897 e2897.

De Paiva, C.S., Chen, Z., Corrales, R.M., Pflugfelder, S.C., Li, D.Q., 2005. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. Stem Cell. 23, 63–73. https://doi.org/10.1634/stemcells.2004-0093.

De Waal, R.M.W., Semeijn, J.T., Cornelissen, I.M.H., Ramaekers, F.C.S., 1984. Epidermal Langerhans cells contain intermediate-sized filaments of the vimentin type: an immunocytologic study. J. Invest. Dermatol. 82, 602–604. https://doi.org/10.1111/1523-1747.ep12523577.

Dziasko, M.A., Armer, H.E., Levis, H.J., Shortt, A.J., Tuft, S., Daniels, J.T., 2014. Localisation of epithelial cells capable of holoclone formation *in vitro* and direct interaction with stromal cells in the native human limbal crypt. PloS One 9, 1–9. https://doi.org/10.1371/journal.pone.0094283.

Dziasko, M.A., Tuft, S.J., Daniels, J.T., 2015. Limbal melanocytes support limbal epithelial stem cells in 2D and 3D microenvironments. Exp. Eye Res. 138, 70–79. https://doi.org/10.1016/j.exer.2015.06.026.

Dziasko, M.A., Daniels, J.T., 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.

Espana, E.M., Birk, D.E., 2020. Composition, structure and function of the corneal stroma. Exp. Eye Res. 198, 108137. https://doi.org/10.1016/j.exer.2020.108137.

Gain, P., Jullienne, R., H, e Z., Aldossary, M., Acquart, S., Cognasse, F., Thuret, G., 2016. Global survey of corneal transplantation and eye banking. JAMA Ophthalmol 134, 167–173. http://doi:10.1001/jamaophthalmol.2015.4776.

Gal, R.L., Dontchev, M., Beck, R.W., Cornea Donor Study Investigator Group, 2008. The effect of donor age on corneal transplantation outcome: results of the cornea donor study. Ophthalmology 115, 620–626. https://doi.org/10.1016/j.ophtha.2008.01.003 e6.

González, S., Mei, H., Nakatsu, M.N., Baclagon, E.R., Deng, S.X., 2016. A 3D culture system enhances the ability of human bone marrow stromal cells to support the growth of limbal stem/progenitor cells. Stem Cell Res. 16, 358–364. https://doi.org/10.1016/j.scr.2016.02.018.

Greenbaum, A., Hasany, S.M., Rootman, D., 2004. Optisol vs Dexsol as storage media for preservation of human corneal epithelium. Eye 18, 519–524. https://doi.org/10.1038/sj.eye.6700693.

Hernáez-Moya, R., Etxebarria, J., Asumendi, A., Boyano, M.D., Andollo, N., 2015. Corneal rims following hypothermic storage for seven days can serve as a potential source of stem-like limbal epithelial cells. Updates on Histology 9–13.

Higa, K., Shimmura, S., Miyashita, H., Shimazaki, J., Tsubota, K., 2005. Melanocytes in the corneal limbus interact with K19-positive basal epithelial cells. Exp. Eye Res. 81, 218–223. https://doi.org/10.1016/j.exer.2005.01.023.

Joseph, A., Powell-Richards, A.O.R., Shanmuganathan, V.A., Dua, H.S., 2004. Epithelial cell characteristics of cultured human limbal explants. Br. J. Ophthalmol. 88, 393–398. https://doi.org/10.1136/bjo.2003.018481.

Joyce, N.C., Meklir, B., Joyce, S.J., Zieske, J.D., 1996. Cell cycle protein expression and proliferative status in human corneal cells. Invest. Ophthalmol. Vis. Sci. 37, 645–655.

Kanavi, M.R., Javadi, M.A., Chamani, T., Fahim, P., Javadi, F., 2015. Comparing quantitative and qualitative indices of the donated corneas maintained in Optisol-GS with those kept in Eusol-C. Cell Tissue Bank. 16, 243–247. https://doi.org/10.1007/s10561-014-9466-5.

Kawasaki, S., Tanioka, H., Yamasaki, K., Connon, C.J., Kinoshita, S., 2006. Expression and tissue distribution of p63 isoforms in human ocular surface epithelia. Exp. Eye Res. 82, 293–299. https://doi.org/10.1016/j.exer.2005.07.001.

Kim, H.S., Song, X.J., De Paiva, C.S., Chen, Z., Pflugfelder, S.C., Li, D.Q., 2004. Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures. Exp. Eye Res. 79, 41–49. https://doi.org/10.1016/j.exer.2004.02.015.

Lauweryns, B., van den Oord, J.J., De Vos, R., Missotten, L., 1993. A new epithelial cell type in the human cornea. Invest. Ophthalmol. Vis. Sci. 34, 1983–1990.

Lee, H.J., Nam, S.M., Choi, S.K., Seo, K.Y., Kim, H.O., Chung, S.H., 2018. Comparative study of substrate free and amniotic membrane scaffolds for cultivation of limbal epithelial sheet. Sci. Rep. 8, 14628. https://doi.org/10.1038/s41598-018-32914-0.

Le, Q., Xu, J., Deng, S.X., 2018. The diagnosis of limbal stem cell deficiency. Ocul. Surf. 16, 58–69. https://doi.org/10.1016/j.jtos.2017.11.002.

Melino, G., Memmi, E.M., Pelicci, P.G., Bernassola, F., 2015. Maintaining epithelial stemness with p63. Sci. Signal. 8, Re9. https://doi.org/10.1126/scisignal.aaa1033.

Merjava, S., Neuwirth, A., Tanzerova, M., Jirsova, K., 2011. The spectrum of cytokeratins expressed in the adult human cornea, limbus and perilimbal conjunctiva. Histol. Histopathol. 26, 323–331. https://doi.org/10.14670/HH-26.323.

Notara, M., Shortt, A.J., O'Callaghan, A.R., Daniels, J.T., 2013. The impact of age on the physical and cellular properties of the human limbal stem cell niche. Age (Dordr). 35, 289–300. https://doi.org/10.1007/s11357-011-9359-5.

Oliva, M.S., Schottman, T., Gulati, M., 2012. Turning the tide of corneal blindness. Indian J. Ophthalmol. 60, 423–427. https://doi.org/10.4103/0301-4738.100540.

Pellegrini, G., Rama, P., De Luca, M., 2011. Vision from the right stem. Trends Mol. Med. 17, 1–7. https://doi.org/10.1016/j.molmed.2010.10.003.

Pels, E., Rijneveld, W.J., 2009. Organ culture preservation for corneal tissue. In: Developments in Ophthalmology, pp. 31–46. https://doi.org/10.1159/000223837.

Polisetti, N., Zenkel, M., Menzel-Severing, J., Kruse, F.E., Schlötzer-Schrehardt, U., 2016. Cell adhesion molecules and stem cell-niche-interactions in the limbal stem cell niche. Stem Cell. 34, 203–219. https://doi.org/10.1002/stem.2191.

Polisetti, N., Gießl, A., Li, S., Sorokin, L., Kruse, F.E., Schlötzer-Schrehardt, U., 2020a. Laminin-511-E8 promotes efficient in vitro expansion of human limbal melanocytes. Sci. Rep. 10, 11074. https://doi.org/10.1038/s41598-020-68120-0.

Polisetti, N., Schlötzer-Schrehardt, U., Reinhard, T., Schlunck, G., 2020b. Isolation and enrichment of melanocytes from human corneal limbus using CD117 (c-Kit) as selection marker. Sci. Rep. 10, 17588. https://doi.org/10.1038/s41598-020-748691.

Raeder, S., Utheim, T.P., Utheim, Ø.A., et al., 2007. Effects of organ culture and optisol-GS storage on structural integrity, phenotypes, and apoptosis in cultured corneal epithelium. Invest. Ophthalmol. Vis. Sci. 48, 5484–5493. https://doi.org/10.1167/iovs.07-0494.

Rama, P., Matuska, S., Paganoni, G., Spinelli, A., De Luca, M., Pellegrini, G., 2010. Limbal stem-cell therapy and long-term corneal regeneration. N. Engl. J. Med. 363, 147–155. https://doi.org/10.1056/NEJMoa0905955.

Schlötzer-Schrehardt, U., Kruse, F.E., 2005. Identification and characterization of limbal stem cells. Exp. Eye Res. 81, 247–264. https://doi.org/10.1016/j.exer.2005.02.016.

Sharma, N., Mohanty, S., Jhanji, V., Vajpayee, R.B., 2018. Amniotic membrane transplantation with or without autologous cultivated limbal stem cell transplantation for the management of partial limbal stem cell deficiency. Clin. Ophthalmol. 12, 2103–2106. https://doi.org/10.2147/OPTH.S181035.

Shortt, A.J., Secker, G.A., Munro, P.M., Khaw, P.T., Tuft, S.J., Daniels, J.T., 2007. Characterization of the limbal epithelial stem cell niche: novel imaging techniques permit in vivo observation and targeted biopsy of limbal epithelial stem cells. Stem Cell. 25, 1402–1409. https://doi.org/10.1634/stemcells.2006-0580.

Si, S.P., Tsou, H.C., Lee, X., Peacocke, M., 1993. Cultured human melanocytes express the intermediate filament vimentin. J. Invest. Dermatol. 101, 383–386. https://doi.org/10.1111/1523-1747.ep12365595.

Sugar, J., Montoya, M., Dontchev, M., Tanner, J.P., Beck, R., Gal, R., Gallagher, S., Gaster, R., Heck, E., Holland, E.J., Kollman, C., Malling, J., Mannis, M.J., Woody, J., Group, G.C.D.S.I., 2009. Donor risk factors for graft failure in the cornea donor study. Cornea 28, 981–985. https://doi.org/10.1097/ICO.0b013e3181a0a3e6.

Sugrue, S.P., Zieske, J.D., 1997. ZO1 in corneal epithelium: association to the zonula occludens and adherens junctions. Exp. Eye Res. 64, 11–20. https://doi.org/10.1006/exer.1996.0175.

Thoft, R., Friend, J., 1983. The X, Y, Z hypothesis of corneal epithelial maintenance. Invest. Ophthalmol. Vis. Sci. 10, 1442–1443.

Vantrappen, L., Geboes, K., Missotten, L., Maudgal, P.C., Desmet, V., 1985. Lymphocytes and Langerhans cells in the normal human cornea. Invest. Ophthalmol. Vis. Sci. 26, 220–225.

Yoshida, S., Shimmura, S., Kawakita, T., Miyashita, H., Den, S., Shimazaki, J., Tsubota, K., 2006. Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. Investig. Ophthalmol. Vis. Sci. 47, 4780–4786. https://doi.org/10.1167/iovs.06-0574.