Short title: **Repeated Stem Cell Expansion from a Limbal Biopsy**

Title: **Consecutive Expansion of Limbal Epithelial Stem Cells from a Single Limbal Biopsy**

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ABSTRACT

Purpose: Corneal epithelium is maintained by limbal epithelial stem cells (LESCs), the loss of which can be catastrophic for corneal transparency. Effective therapies include the transplantation of cultivated LESCs, requiring optimization of *in vitro* cultivation protocols. Unfortunately, optimization studies are hampered by the limited number of ocular tissue donors. We investigated the feasibility of obtaining more than one limbal primary culture (LPC) from the same 1-2 mm^2 limbal explant (LE).

Methods: LEs were plated and maintained until outgrowth surrounded each, being removed at this point. LPCs were allowed to reach confluence (LPC0). The same removed LE was plated again, following the same procedure, obtaining LPC1. This procedure was repeated as often as possible up to 6 times. LPCs from each passage were analysed by real time RT-PCR and immunofluorescence-microscopy.

Results: LPCs from LPC0 to LPC2 presented an heterogeneous cell population, with cells positive for LESC markers K14, K15, ABCG2 and p63, differentiated corneal epithelial cell-specific markers K3 and K12, and for the fibroblast marker S100A4. These cells had an epithelial-like morphology. In LPC3-LPC4, elongated cell morphology appeared, and the presence of LESC markers decreased, while the presence of differentiated corneal epithelial-cell and fibroblast markers increased.

Conclusion: one LE can be successfully cultivated up to three consecutive times while maintaining the LESC phenotype in the LPC cells. This protocol provides several homologous LPCs for basic research. Additionally, by using a cell-carrier, the resulting LPCs could serve reservoirs for potential autologous expanded LESC transplantations and/or for making correlations between laboratory and clinical outcomes.

Key Words: limbal stem cells ● corneal epithelium ● limbal stem cell deficiency ● limbal explants ● limbal transplantation

INTRODUCTION

The corneal surface is covered by a stratified nonkeratinized epithelium that is continually renewed by stem cells (SCs). The SCs are mainly located in the basal layer of the corneoscleral-limbal epithelium, at the junction between the cornea and the sclera-conjunctiva. Limbal epithelial SCs (LESCs) are characterized by small cell size, the absence of differentiation markers, slow cell cycle and high nucleus-to-cytoplasm ratio. LESC deficiency (LSCD) results from the dysfunction of LESC population or from an insufficient microenvironment to support their function. LSCD symptoms include chronic inflammation, decreased vision, and recurrent episodes of pain, reviewed in [1-3].

Different treatments for LSCD have been developed, including *in vitro* cultured LESC transplantation to re-establish a stable corneal epithelial phenotype, first developed by Pellegrini *et al*..[4] During the LESC expansion procedure, LESCs are isolated from healthy limbal epithelial tissues and grown on a substratum to produce a sheet of cultured LESCs that is suitable for transplantation onto the damaged ocular surface. However, a standard *in vitro* LESC expansion protocol has not been established yet. Therefore, numerous techniques have been used to culture LESCs, reviewed in [2, 5-7]. Currently, much basic research is focused on this area, with the purpose to optimize *in vitro* LESC expansion and to further understand LESC biology. Unfortunately, these necessary investigations can often be hampered by the limited number of ocular tissue donors.

Here we report the development of a protocol to obtain more than one expanded sheet of cells from the same limbal tissue sample (limbal explant, LE). This protocol will enable the preparation of several homologous experimental limbal primary cultures

(LPCs), with a high proportion of LESCs. Additionally, by using a cell carrier, this protocol could also be used in clinical applications to originate an expanded autologous LESC reserve for each patient with LSCD and/or for making correlations between laboratory findings and clinical outcomes.

MATERIALS AND METHODS

This protocol was approved by the IOBA Research Committee and the Valladolid Medical School Ethics Committee. The Tenets of Declaration of Helsinki were followed at all times.

Materials

Dulbecco Modified Eagle Medium/Ham F-12 (DMEM/F12), Hank's balanced salt solution (HBSS), gentamicin, amphotericin B, fetal bovine serum (FBS), collagenase type I, penicillin, streptomycin, non-essential amino acids 100X, 0.25% trypsin-1mM ethylenediaminetetraacetic acid (EDTA), phosphate buffer solution (PBS), Quant-iT RNA Assay kit, SuperScript® VILO™ cDNA Syntesis Kit, Qubit-fluorometre, propidium iodide, and fluorescein Alexa Fluor® 488 donkey anti-mouse and donkey anti-rabbit antibodies were purchased from Invitrogen-GIBCO (Inchinnan, UK). Epidermal growth factor (EGF), insulin, transferrin, dimethyl-sulfoxide (DMSO), hydrocortisone, sodium-selenite, Triton X-100 and donkey serum were from Sigma-Aldrich (St Louis, MO, USA). Dispase-II was from Roche Diagnostics (Basel, Switzerland), cholera toxin was from Gentaur (Kampenhout, Belgium), Tissue-Tek-OCT compound was from Sakura Tissue Tek® (Torrance, CA, USA) and β-mercaptoethanol 14.3 M was from Merck (Darmstadt, Germany). Formaldehyde,

methanol and sucrose were purchased from Panreac (Lyon, France). RNeasy® Mini Kit and RNase-Free DNase were from Qiagen (Valencia, CA, USA). TaqMan Universal PCR Master Mix and 20X Target Primers-Taqman® probes were from Applied Biosystems (Foster City, CA, USA). Trephines were from Katena products (Denville, NJ, USA).

Human Tissue Preparation

Cadaveric non-diseased human corneoscleral tissues were obtained from the Barraquer Eye Bank (Barcelona, Spain**)** and preserved in supplemented (see below) culture medium. The mean ± standard error the donor age was 81.7 ± 1.5 years (range 68-87 years). Only tissues that could be cultured within 5 days after death were used, with average of 3.2 ± 0.3 days in preserved conditions.

Human skin tissue was obtained from a certified Tissue Bank (León, Spain).

Corneoscleral and skin tissues for immunofluorescence-microscopy were fixed in 4% formaldehyde and embedded in Tissue-Tek-OCT-compound.

Limbal Primary Cultures

Corneoscleral tissues for culture were prepared using a modification of a previously described method.[8] Tissues were rinsed with HBSS containing 50 µg/ml gentamicin and 2.5 µg/ml amphotericin B. Excess sclera, conjunctiva, iris, and corneal endothelium were removed, and a central corneal button was extracted with a 7.5 mm trephine. The remaining corneoscleral rings were cut into 1-2 mm^2 LEs that were plated singly into 3.8 cm^2 polystyrene wells. After 30 min in a laminar-flow-hood, each was incubated

with 50 µl of FBS overnight at 37ºC, 5% CO_2, and 95% humidity. After that, the LEs were cultured in DMEM/F12 (1:1) culture medium supplemented with 2.5 ng/ml EGF, 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml sodium-selenite, 0.01 µg/ml hydrocortisone, 0.5% DMSO, 132.5 ng/ml cholera toxin, 50 µg/ml gentamicin, 2.5 µg/ml amphotericin, and 5% FBS. Each LE was maintained in culture until migrating cells from all LE edges originated a cell ring around it. Then it was removed and the remaining LPC was allowed to reach confluence, whereupon it was designated LPC0. When each LE was removed, it was then plated again in a new culture dish, and a similar procedure was followed to obtain the LPC1. This protocol was repeated as many times as possible to obtain LPC2, LPC3, etc.

Cell outgrowth from the LEs was monitored under a phase contrast microscope (Eclipse TS100, Nikon, Japan). A LPC was considered successful when it reached more than 80% confluence. The percentage of successful LPCs was calculated based on the number of LEs plated at each passage. The elapsed time from LE plating to LE removal and the elapsed time from LE removal to LPC confluence were analyzed. LPC generation time was considered as the total time needed for a LE to generate a successful LPC. In order to account for the variability between donors, we first calculated the average from data obtained from each donor. We then calculated the average between each of the different donors.

Limbal stromal fibroblasts were isolated and cultured.[9] Briefly, limbal epithelium was removed by 2 hours of incubation with 5 mg/ml of dispase-II at 37ºC. The limbal stroma was then digested with 2 mg/ml collagenase type I overnight at 37ºC. Finally, stromal cells were cultured up to 3 passages.

Histology Study

LEs from fresh cadaveric limbal rings and LEs cultivated for 6 consecutive passages were fixed in 4% formaldehyde, treated with sucrose (5% and 30%), and embedded in Tissue-Tek-OCT compound. Cryosections (5 μm) were stained with periodic acid-Schiff (PAS)-hematoxylin in the standard manner to examine overall morphology. Three LEs from three different donors (n=3) were analyzed for each condition.

Tissue and Cell mRNA Extraction

The epithelium from 6 limbal and 6 corneal frozen specimens were scraped and collected in RNA lysis buffer (1:100 β-mercaptoethanol-buffer RLT RNeasy® Mini Kit). Limbal stromal mRNA was isolated from 3 other specimens after scrapping off the epithelium and endothelium. The stroma was incubated with 20 μl RNA lysis buffer/mg tissue, cut, and homogenized. Lysates were collected and centrifuged at 15,000 g for 3 min. Supernatants were purified in Qiashredder columns (RNeasy® Mini Kit) following the manufacturer's recommendations.

Confluent LPCs were incubated with RNA lysis buffer (LE tissues were excluded from LPC extractions). Total mRNA from limbal and corneal epithelium, limbal stroma, and confluent LPCs was extracted by RNeasy Mini Kit, treated with RNase-Free DNase I Set, and quantified using the commercial kit Quant-iT RNA Assay and Qubit-fluorometre following the manufacturer's instructions.

Reverse Transcription (RT) and Real Time Polymerase Chain Reaction (RT^2-PCR)

Complementary DNA (cDNA) was synthesized from 1 µg of total mRNA by SuperScript® VILO™ cDNA Syntesis Kit using the Mastercycler® Personal thermocycler (Eppendorf AG, Hamburg, Germany). Two µl of cDNA (20 ng) were used for RT^2-PCR in a total volume of 20 µl containing 1 µl of 20X Target primers-Taqman® probes (Table 1), 10 µl of 2X Taqman Universal PCR Master Mix, and 7 µl of water. RT^2-PCR parameters consisted of uracil N-glycosylase activation at 50ºC for 2 min, pre-denaturation at 95ºC for 2 min, followed by 40 cycles of denaturation at 95ºC for 15 sec, and annealing and extension at 60ºC for 1 min, in a 7 500 Real Time PCR System from Applied Biosystems.[10] Assays were performed in duplicate. A non-template control was included in all experiments and the human *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* gene was used as the endogenous control. The comparative cycle threshold (Ct) method was used to analyze the results. Corneal epithelium, limbal epithelium, and LPC0 served as the calibrator controls when limbal epithelium, limbal stroma, and LPCs were analyzed, respectively. The results were reported as a fold up-regulation or fold down-regulation when the fold-change was greater or less than 1, respectively. The number of LPCs from different LEs and donors is shown in Table 2.

Immunofluorescence-Microscopy

Confluent LPCs were incubated with 0.25% trypsin-1 mM EDTA for 5 min at 37ºC and then seeded into eight-well Permanox chamber slides (12 500 cells/cm^2) overnight.

Limbal cells were fixed with cold methanol for 10 min at -20ºC. Cryosections (5 µm) of fixed human corneoscleral and skin tissues and fixed limbal cells were permeabilized with 0.3% Triton X-100 for 10 min and incubated for 1 hour with 5% donkey-serum at room temperature. Samples were incubated overnight at 4ºC with specific primary antibodies (Table 3) and incubated for 1 hour at room temperature with secondary antibody (Alexa Fluor® donkey anti-mouse 1:200 or Alexa Fluor® donkey anti-rabbit 1:300). Nuclei were counterstained with propidium-iodide (1:12000). Images were acquired with an inverted fluorescence microscope (DM4000B, Leica, Wetzlar, Germany).

Limbal cell images at 20X magnification were made of five areas randomly selected manually, and the percentage of positive cells was calculated. Negative controls included the omission of primary antibodies. Human colon carcinoma cell line (HT29MTX), a kind gift by Dr. Thécla Lesuffleur (INSERM U843, Paris, France) and human skin cryosections were used as a positive control for ATP-binding cassette sub-family G member 2 protein (ABCG2) and p63, respectively.[11,12] The Human Corneal Epithelium (HCE) cell line, kindly provided by Dr. Arto Urtti (University of Helsinki, Finland) was used as a negative control for keratin 14 (K14) and K15 and as positive controls for K3 and K12 expression. Cultured limbal fibroblasts were used as positive controls for S100 calcium-binding protein A4 (S100A4) expression.[13] The number of LPCs from different LEs and donors analyzed is shown in Table 2.

Statistical Analysis

Statistical significance was determined by one-way factorial ANOVA, except for the analysis of quantitative immunofluorescence results, where two-way factorial ANOVA

was used. Comparison between two groups was made using a Student's t-test. All values were expressed as mean ± standard error of the mean. P-values ≤ 0.05 were considered statistically significant.

RESULTS

LPC characteristics

Confluent LPCs were successfully produced from LPC0 to LPC6. The percentage of confluent LPCs tended to increase from LPC0, 32.5 ± 7.5%, to LPC4, where the highest percentage of confluent LPCs was achieved, 63.7 ± 12.4%. There were no differences in the percentage of successful cultures among the consecutive LPCs 0 through 6 (Fig. 1A), and the overall success rate was 49.8 ± 4.5%. The total number of LEs plated and confluent LPCs obtained is shown in figure 1A and 1B, respectively. The elapsed time from LE plating to LE removal and elapsed time from LE removal to LPC confluence varied in each consecutive LPC. LPC generation time increased from 26.0 ± 2.8 days in LPC0 to 35.5 ± 7.1 days in LPC6; however, there were no significant differences in generation time, and the average was 29.9 ± 1.7 days to reach confluence (Fig. 1B).

LPC Cell Morphology and Histological Study of LEs

By phase contrast microscopy, the cells of LPC0-LPC3 had a homogeneous cuboidal morphology, about 27×40 μm (Fig. 1C, D). In LPC4, the morphology of the cells became more elongated (Fig. 1C), a change that became more apparent as confluence was reached (Fig. 1D). Occasionally, this change was observed in LPC3. In LPC5 and

LPC6, the cells always had a homogeneous, elongated morphology of about 18×200 µm (Fig. 1C, D).

PAS-hematoxylin staining showed that the surface of LEs before cultivation was covered by a stratified epithelium of five to six layers in the limbal region (Fig. 1E). In contrast, the surface of LEs after six consecutive expansions did not show epithelium (Fig. 1F). The quantity of stromal cells tended to decrease after six LE passages (Fig. 1F).

mRNA and Protein Expression in Cadaveric Corneal and Limbal Tissues

Human limbal and corneal tissues were used to validate the expression of specific markers of limbal epithelium (K14, K15, ABCG2, and p63), corneal epithelium (K3 and K12), and limbal fibroblasts (S100A4) by RT^2-PCR and immunofluorescence-microscopy (Fig. 2). The relative expressions of LESC mRNAs *K14* (72.38 fold), *K15* (62.64 fold), *ABCG2* (630 fold) and *p63alpha* (16.64 fold) were significantly higher (*K14* $p \leq 0.0005$ and *K15*, *ABCG2*, *p63alpha* $p \leq 0.005$) in limbal epithelium compared to corneal epithelium (Fig. 2A). K14 and K15 proteins were detected in the cytoplasm of limbal epithelial cell layers (Fig. 2D) and were not in the HCE cell line (negative control, Fig. 2F). K14 was also expressed in corneal epithelium (Fig. 2E). ABCG2 protein was detected in epithelial and stromal limbal cells (Fig. 2D) and in the HT29MTX cell line (positive control, Fig. 2F), but not in corneal epithelial cells (Fig. 2E). p63 was only expressed in the nuclei of limbal basal epithelial cells and in human skin (positive control, Fig. 2D, F).

In contrast, the relative expression of *K3* (0.38 fold) and *K12* (0.30 fold) mRNA was significantly lower in limbal than in corneal epithelium ($p \leq 0.005$ and $p \leq 0.0005$, respectively, Fig. 2B). In agreement with this, protein expression of K3 and K12, assessed by immunofluorescence-microscopy, was high in the cytoplasm of all corneal epithelial layers (Fig. 2E). K12 was also present in the suprabasal layers of limbal tissues (Fig. 2D).

The relative expression of *S100A4* mRNA was significantly higher in limbal stroma (330 fold) than in limbal epithelium ($p \leq 0.05$, Fig. 2C). S100A4 protein was mainly detected in limbal and corneal stroma (Fig. 2D, E), as well as in human cultured limbal fibroblasts (positive control, Fig. 2F).

LESC Marker Expression in LPCs

LESC markers K14, K15, ABCG2, and p63alpha were evaluated for each consecutive LPC (Fig. 3). The mRNA expression level of each LPC was compared to that in LPC0, which was assigned a value of 1. The relative expression of *K14* mRNA increased from LPC0 to LPC2 (4 fold), but the changes were not statistically significant. For LPC3, *K14* expression decreased 7×10^{-5} fold ($p \leq 0.01$, Fig 3A), and it remained low through LPC6. The *K14* mRNA relative expression was significantly higher in LPC0-LPC2 than in LPC3-LPC6 ($p \leq 0.05$, Fig 3A). The percentage of K14-positive cells decreased from LPC0, 59.3 ± 17.1%, to LPC4, 9.1 ± 5.5% (Fig. 3E, I). K14 was not detected in any of the cells in LPC5 and LPC6. The percentage of positive cells for K14 was significantly higher in LPC0-LPC2 than in LPC4-LPC6 ($p \leq 0.01$, Fig. 3E, I).

The relative expression of *K15* mRNA in LPC1 and LPC2 compared to LPC0 were 0.66 and 1.15 fold respectively. In LPC3, relative *K15* expression was only 0.008 fold, and it remained reduced through LPC6 (Fig. 3B). The percentage of K15-positive cells in LPC0 was 24.1 ± 23.8% (Fig. 3F, J). Similar percentages were present in LPC1 and LPC3, but none was detected in LPC4-LPC6.

The relative expression of *p63alpha* mRNA increased from LPC0 through LPC2 (2.25 fold), but the changes were not statistically significant (Fig. 3C). In LPC3 it decreased 0.003 fold ($p \leq 0.01$) and remained low through LPC6. The percentage of cells expressing p63 protein in LPC0, 34.2 ± 16.8%, decreased to 3.8 ± 3.8% in LPC2 ($p \leq 0.01$, Fig. 3G, K). p63 was not detectable in LPC3 through LPC6.

The relative expression of *ABCG2* mRNA was lowest in LPC0 (Fig. 3D). It tended to increase in LPC1, LPC2 and LPC3, but decreased in LPC4. For LPC4-LPC6, it tended to increase again; however, none of these changes were statistically significant. The percentage of positive cells for ABCG2 was greater in all LPCs than in LPC0 (43 ± 10.4%), except in LPC3 where only 6.8 ± 6.8% of cells expressed it (Fig. 3H, L).

In summary, all mRNA and protein LESC markers analyzed were present in LPC0-LPC2. For these passages, there were no significant differences except for a decrease in p63 and an increase in ABCG2 protein expression. Changes in LESC marker expression was often detected in LPC3 or LPC4. In LPC5 and LPC6, LESC marker mRNAs were detected; however, we did not find protein expression by immunofluorescence-microscopy.

Corneal Epithelial Cell Marker Expression in LPCs

Corneal epithelial cell markers K3 and K12 were evaluated for each consecutive LPC (Fig. 4). The mRNA expression level of each LPC was compared to that in LPC0, which was assigned a value of 1 (Fig. 4A, B). The relative expression of *K3* mRNA was lower in LPC1 and LPC2 (0.33 and 0.83 fold respectively), though the differences were not significant. However, it decreased significantly ($p \leq 0.05$) in LPC3 (0.03 fold) and remained lower through LPC6 (0.015 fold, Fig. 4A). The percentage of K3-positive cells detected by immunofluorescence-microscopy was similar in all LPCs, with an average of 59.2 ± 5.2% (Fig. 4D, G). The relative expression of *K12* mRNA was similar in LPC1 and LPC2, 5 fold more than in LPC0; however these increases were not statistically significant (Fig. 4B). Relative expression decreased dramatically in LPC3 (7×10^{-4} fold, $p \leq 0.01$) and remained lower through LPC6 (6×10^{-4} fold). While the percentage of K12-positive cells increased in LPC1 and LPC2 (approximately 40.5 ± 0.7% each), the differences compared to LPC0 (28.3 ± 4.4%) were not significant (Fig. 4E, H). For LPC3, only 5.9 ± 3.5% were positive ($p \leq 0.01$), but for LPC4 74.6 ± 10.5% were positive ($p \leq 0.01$).

Fibroblast Marker Expression in LPCs

The fibroblast marker S100A4 mRNA and protein were present in all of the LPCs (Fig. 4C, F, I). The relative expression of *S100A4* mRNA decreased from LPC0 to LPC1 ($p \leq 0.05$). Expression in LPC3 was also significantly lower than LPC0. Other changes were not statistically significant (Fig. 4C). The percentage of S100A4-positive cells was significantly higher in all LPCs compared to LPC0 ($p \leq 0.01$). The maximum percentage was 96 ± 0.7% in LPC6 (Fig. 4F, I).

DISCUSSION

In this study we developed a protocol to obtain consecutive successful LPCs from single 1-2 mm^2 cadaveric limbal samples. The resulting LPCs preserved cell morphology and maintain the LESC phenotype through LPC3-LPC4. In subsequent passages the cells became larger and more elongated. This morphological change coincided with decreased LESC marker expression and with a trend to increased protein expression of the fibroblast marker S100A4. In a similar study, Li *et al.* cultured and passaged human LEs three consecutive times. They also found a decline in limbal epithelial cell outgrowth and an increase in cell size during successive LE passages. However, cell outgrowth was not characterized.[14] Recently, Selver *et al.* characterized the outgrowth obtained during three consecutive LE passages, but only the ABCG2 marker was analysed.[15] To investigate the feasibility of increasing the number of available LPCs from a single LE, we considered it necessary to characterize them exhaustively.

We initially expanded 190 cadaveric LEs, of which 32.5% reached a confluent state. This relative low expansion index is explained by the used of cadaveric instead of fresh tissue, as well as by the advanced age of donors.[16,17] James *et al*. found a decreasing trend in growth potential of limbal tissues with increasing donor age,[16] and Vemuganti *et al.* showed that the growth potential of fresh tissues was much higher compared to cadaveric tissues.[17]

To overcome the problem of the high age of tissue donors for research, we designed a different culture medium. The composition of culture medium included components that increase cell proliferation such as FBS,[18] insulin, EGF,[19] and hydrocortisone.[20] It also included components such as transferrin,[21] selenium,[22] and DMSO[23] to reduce cell

damage caused by oxygen radicals. Another important component of this culture medium was cholera-toxin, that increases cell proliferation in keratinocytes, especially those derived from advanced age donors, and allows growth through serial cell transfers.[24,25] We used a culture medium with a relatively high concentration of cholera-toxin and found that limbal cell expansion from single LEs for six consecutive passages is possible. For hypothetical clinical purposes, it is likely that a lower concentration of cholera toxin will be sufficient because the age of the donors is usually less than 60 years.

To study changes in the cell population of successive LPCs, we characterized them phenotypically by RT^2-PCR and immunofluorescence-microscopy. There are no definitive markers for the identification of LESCs, but rather the presence and absence of a combination of characteristics. Thus to distinguish LESCs and transitional cell populations in the limbal niche from the fully differentiated cell population present in the cornea, we used several morphological features. These included the combination of small cell size and the positive or negative expression of specific genes and proteins, reviewed in [1-3]. Many investigators have reported that K3 and K12 are specifically expressed in corneal epithelial cells, defining them as markers of corneal epithelial differentiation.[26-28] On the other hand, the expression of K14, K15, ABCG2, and p63alpha markers have been described as higher in the limbal basal epithelial cell layer than in the central corneal epithelium.[28-32] In this work, our results are consistent with this. However, some authors have reported the presence of K5/K14 protein pair in the basal cells of both limbal and corneal rabbit epithelia[33] and ABCG2 protein in the SCs of the limbal stroma.[34] Our results are also consistent with this. On the other hand, S100A4 has been widely established as a specific marker of fibroblasts and of the

epithelial-mesenchymal transition phenomenon.[13] We found low expression of this marker in limbal and corneal stromal tissues, but abundant expression in limbal fibroblast primary cultures, as expected.

In LPC characterization, RT^2-PCR confirmed a considerable expression of both LESC and corneal markers from LPC0 through LPC2 and lower levels in the subsequent cultures. One exception was *ABCG2*, for which the expression in LPC1-LPC2 was similar to that in LPC5-LPC6. In contrast, the percentage of cells positive for corneal epithelial cell markers K3 and K12 detected by immunofluorescence-microscopy was high in the majority of LPCs. This apparent discrepancy can be explained because RT^2-PCR analyzes the relative amount of mRNA while immunofluorescence-microscopy analyzes the percentage of positive cells and not the amount of protein expressed in each sample. Therefore the results of RT^2-PCR cannot be directly compared with the results of immunofluorescence assays, as already pointed out by other authors.[35]

The percentage of cells that expressed LESC protein markers K14, K15, and p63 decreased from LPC0 to LPC2, while the percentage of cells that expressed corneal epithelial protein markers increased in these LPCs. However, the percent changes of positive cells were not significant between LPC0 and LPC2 for any of the LESC and corneal markers except for p63alpha. On the other hand, a low percentage of cells positive for S100A4 were detected in LPC0-LPC2, suggesting the presence of a small population of fibroblast or mesenchymal cells[13] that may be interacting with the epithelial cells in order to preserve the LESC properties.[36] Conversely, the percentage of positive cells for S100A4 was very high from LPC4 to LPC6. This suggests that limbal epithelial cells that migrated from LEs to the culture plate maintained the characteristics

of LESCs in LPC0-LPC2, but lost these characteristics in LPC3.These results are consistent with those of Li *et al*., who suggested that LESCs indeed migrated from the LE to the substratum during the *in vitro* expansion, but the percentage of progenitor cells, determined by clonogenicity assays, progressively declined in the LPCs after each LE passage due to intrastromal invasion by LESCs.[14] These results, together with the changes from epithelial-like morphology in LPC0-LPC3 to intermediate epithelial-elongated morphology in LPC4, and then finally to elongated morphology in LPC6, indicate a change in the LPC cell population to a more differentiated fibroblast-like population. This is in accordance with observations by Li *et al.* and with the "LESC cores" remaining in their natural niche after LE cultivation.[14,15] These core LESCs support the outward migrating progeny. Both authors observed that the number of epithelial cell layers on the LE surface progressively decreased after each passage, a finding that is consistent with our results. In addition, Li *et al.* observed that the basement membrane components were partially dissolved and broken down in the limbus and peripheral cornea after 2 weeks of LE culture.[14] Previously, Kawakita *et al.* showed that LESCs invade the limbal stroma in cultured rabbit LEs.[37] In the same way, Li *et al.* demonstrated two fates for LESCs in human LEs during cultivation. The first one was the migration of LESCs from the explants to the amniotic membrane, and the second one was an intrastromal invasion.[14] Recently, Tan *et al.* reported that intrastromal invasion by LESCs in human LEs is a universal phenomenon as it occurs under different culture conditions.[38]

We postulate that the presence of undifferentiated epithelial cells in confluent LPC0-LPC2 cultures is explained by the first fate, migration of LESCs from the LE onto the polystyrene-substratum. This fate could be performed by the "LESC cores"

reported by Selver *et al*..[15] The second fate, migration of LESCs into the stroma, would make them unavailable for colonization of the culture substratum in LPC4-LPC6. For these cultures, limbal fibroblasts and/or mesenchymal cells could migrate from the limbal stroma onto the culture dish to establish primary cultures of cells with elongate morphology that express S100A4, K3, K12, and ABCG2 proteins. In agreement with Selver *et al.* we observed a gradual loss of morphological features and size in LEs during consecutive passages, suggesting a stromal degradation that matched the frequent contamination of the LPCs by fibroblasts.[15]

Mesenchymal-SCs are present underneath the limbal basement membrane,[39] and a recent report showed that K3/K12 are expressed in rabbit bone marrow mesenchymal SCs.[40] In addition, another recent report showed that human mesenchymal SCs from adult adipose tissue expressed a moderate amount of K3 and K12 markers.[35] Probably, human limbal mesenchymal SCs also express K3 and K12. Although further research will be necessary to confirm this in human cells, we believe that some of the positive cells for K3 and K12 found in our LPCs may be of mesenchymal origin. ABCG2, a marker for limbal epithelial cells, was highly expressed in the first LPCs. This is probably due to the presence of LESCs, also shown by the presence of cells positive for K14, K15, and p63alpha. However, ABCG2 protein was also detected in LPCs with elongated cells in LPC4. Du *et al*. reported that, similar to other adult tissues, SCs in the corneal and limbal stromas express the ABCG2 marker.[34] This likely explains the presence of ABCG2 protein in our LPC4.

We showed that LPCs from LPC0 through LPC2 were composed of a heterogeneous cell population, with cells positive for LESC-specific markers K14, K15, ABCG2, and p63, for central corneal epithelial specific markers K3 and K12, and for fibroblast

marker S100A4. This indicates a mixture of undifferentiated LESCs, differentiated epithelial cells, and some fibroblasts. From LPC4, the presence of differentiated epithelial cells and fibroblast increased, while the presence of LESCs significantly decreased. Therefore, LPC0-LPC2 could be used as homologous LPC standards for basic research to increase the number of available human LPCs, to optimize *in vitro* LESCs expansion, and to further the understanding of LESC biology (Fig. 5). Additionally, our results suggest that by using a carrier, this protocol potentially could be adapted for application in clinical practice. Usually, human amniotic membrane and/or mice-embryonic-3T3 fibroblasts are used as support for *in vitro* LESC expansion.[7] However; human amniotic membrane is an expensive tissue difficult to obtain due to its human origin, thus limiting its use to those studies where it is indispensable. On the other hand, we support the idea that the use of compounds from animal-origin could compromise the potential clinic application of this protocol and, according, we did not use mice-embryonic 3T3 fibroblast as cell substratum.

In vitro expanded LESC transplantation can be hampered by culture failure, graft transport problems, surgical mishaps, or by postoperative complications 1 - 6 weeks after placement on the recipient's ocular surface. Any of these complications could result in early graft failure.[41] With the establishment of successive LPCs from a single LE, these issues could be quickly resolved with a new transplant of expanded autologous LESCs from the same initial biopsy. This would avoid the necessity of taking another biopsy or the use of allogenic tissue with the implied risk of rejection. This procedure could be used to originate an expanded autologous LESC reserve for each patient (Fig. 5). Additionally, LPCs not needed for transplantation could be analyzed. The results could then be correlated with the success or failure of the clinical

application[41] resulting in a better knowledge base from which future decisions regarding the likelihood of success could be made.

CONCLUSION

Single cadaveric LEs of 1-2 mm^2 can be successfully expanded up to three consecutive passages to obtain LPCs that maintain the LESC phenotype. This procedure can be adopted for basic research to increase the number of available human LPCs, making it then easier to investigate and correlate the molecular properties with clinical outcome. In addition, this report could have an indirect clinical impact, establishing a protocol to make reserve autologous LPCs that could be used in case of failure at any step of cell expansion, during the surgical procedure, or as a result of early postoperative complications. Further investigations are required to develop this potential clinical application.

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Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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FIGURE CAPTIONS

Figure 1. Limbal cell outgrowth from LEs, confluent LPCs and histological study of LEs during successive passages. **(A)** There were no significant differences in the percentage of successful LPCs from successive cultures of the LEs. The percentages were calculated considering the variability among donors. **(B)** The interval between LE plating and removal (green) was similar for all consecutive LE passages. Likewise, there were no significant differences in the elapsed time from LE removal and LPC confluence (purple) for the consecutive LE passages. The times were calculated considering the variability among donors. **(C)** Representative images of limbal cell outgrowth from LEs and **(D)** from confluent LPCs. The cuboidal shape in LPC0-LPC3 evolved to a more elongated morphology in LPC4, LPC5, and LPC6. Magnification 20X. **(E)** Representative images of PAS-hematoxylin staining for three limbal explants before cultivation and **(F)** for three different limbal explants after six consecutive passages**.** The surface of LEs before cultivation was covered by a stratified epithelium, however LEs after six consecutive expansions did not show epithelium. Abbreviations: LPC, limbal primary culture; LE, limbal explants; N, number of donors; Np, number of LEs plated for the different consecutive passages; Nc, number of confluent LPCs obtained in the different consecutive LE passages.

Figure 2. Analysis of specific marker expression in cadaveric limbal and corneal tissues. **(A)** RT^2-PCR showed that relative expression of the LESC specific markers *K14, K15, ABCG2*, and *p63alpha* were significantly higher in limbal epithelium than in corneal epithelium. **(B)** Corneal epithelial cell specific markers *K3* and *K12* mRNA was significantly higher in corneal than in limbal epithelium. **(C)** Fibroblast marker *S100A4*

mRNA was significantly higher in limbal stroma than in limbal epithelium. Corneal epithelial mRNA levels for each marker were used as calibrator controls in **(A)** and **(B)**. Limbal epithelial S100A4 mRNA served as calibrator control in **(C)**. Representative immunofluorescent staining profiles for K14, K15, ABCG2, p63, K3, K12, and S100A4 in cryosections of **(D)** human limbus**, (E)** human cornea, and **(F)** different positive and negative control cell lines and tissues. Nuclei were counterstained with propidium iodide (red). LESC markers K15 (green), ABCG2 (green) and p63 (yellow, colocalization of green and red fluorescence) were highly expressed in limbal epithelium. The LESC marker K14 (green) was expressed in both corneal and limbal epithelia. Corneal epithelial cell markers K3 and K12 (green) were highly expressed in corneal epithelium. S100A4 (green) was expressed in corneal and limbal stroma. Magnification 40X. Abbreviations: LESC, limbal epithelial stem cells; HCE, human corneal epithelium cell line; HT29MTX, colon adrenocarcinoma grade II human cell line; *$p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$.

Figure 3. LESC marker expression in consecutive LPCs. RT^2-PCR profiles showed that the relative expressions of *K14* **(A)**, *K15* **(B)** and *p63alpha* **(C)** mRNAs was higher from LPC0, the calibrator control, through LPC2 than in LPC3 and subsequent passages. There were no significant differences in the relative expression of *ABCG2* **(D)** mRNA among consecutive LPCs. For immunofluorescent microscopy staining, the percentage of cells positive for K14 **(E)**, K15 **(F)**, p63 **(G)** and ABCG2 **(H)**. There was a decreasing trend in the percentage of positive cells for LESC markers in the consecutive LPCs, except for ABCG2. K14, K15, and p63alpha were not expressed in LPC5 and LPC6. ABCG2 protein expression was not analyzed in LPC5 and LPC6

because the number of samples collected was not sufficient. Nuclei were counterstained with propidium iodide (red). Representative images (40X) for expression of K14 **(I)**, K15 **(J)**, p63 **(K),** and ABCG2 **(L)** in different LPCs (green fluorescence, except for p63 where colocalization of green and red fluorescence was showed as yellow). Abbreviations: LPCs, limbal primary cultures; * $p \leq 0.05$; ** $p \leq 0.01$.

Figure 4. Corneal epithelial cell and fibroblast marker expression in consecutive LPCs. Corneal epithelial cell markers: RT^2-PCR showed that the relative expression of *K3* **(A)** and *K12* **(B)** mRNAs was significantly higher in LPC0, the calibrator control, than in LPC3 through LPC6. There were no significant differences in percentage of positive cells for K3 **(D)**, while the percentage of positive cells for K12 was significantly lower in LPC3 and higher in LPC4 than in LPC0-LPC2 **(E)**. Expression of K12 protein was not analyzed in LPC5 and LPC6 because the collected samples were not sufficient. Fibroblast marker: RT^2-PCR profiles showed that the relative expression of S100A4 mRNA **(C)** was significantly higher in LPC0, the calibrator control, than in LPC1 and LPC3. For immunofluorescent microscopy staining, the percentage of positive cells for S100A4 **(F)** increased from LPC0 to LPC6. Representative images (40X) to K3 **(G)**, K12 **(H)** and S100A4 **(I)** in different LPCs (green). Nuclei were counterstained with propidium iodide (red). Abbreviations: LPCs, limbal primary cultures; * $p \leq 0.05$; ** $p \leq 0.01$.

Figure 5. Potential uses for successful consecutive LPCs. One LE of 1-2 mm^2 can be successfully expanded maintaining epithelial morphology and LESC phenotype up to three consecutive times (LPC0, LPC1, and LPC2). These can be used to increase the

number of available human LPCs for basic research. In addition, this method could also be adapted to expand LESCs *in vitro* for autologous transplantation. *In vitro* expanded LESC transplantation can be hampered by failure in the LESC culture, graft transport, surgery, or by early (1-6 weeks) postoperative complications on the recipient's ocular surface. These complications could be quickly solved with a new transplant of expanded autologous LESCs from the same initial biopsy. This procedure could be used to originate a reserve of expanded autologous LESCs for each patient. If not needed for the patient, the reserve LPCs could be analyzed for properties that correlate with clinical results. Abbreviations: LE, limbal explant; LPC, limbal primary culture; LESCs, limbal epithelial stem cells.

TABLES

Table 1. Oligonucleotide primers and probes used for RT^2-PCR.

Gene name	Gene symbol	Assay Applied Biosystem ID*
Keratin 3	*KRT3*	Hs 00365080_m1
Keratin 12	*KRT12*	Hs 00165015_m1
Keratin 14	*KRT14*	Hs 00559328_m1
Keratin 15	*KRT15*	Hs 00267035_m1
ATP-binding cassette, sub-familiy G, member 2	*ABCG2*	Hs 00184979_m1
P63alpha	*TP63*	Hs 00978338_m1
S100 calcium binding protein A4	*S100A4*	Hs 00243201_m1
Human glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	4352934E

*Identification number from Applied Biosystems (Foster City, CA, USA,).

Table 2. Number of limbal primary cultures (LPCs) from different limbal explants and donors analyzed by RT^2-PCR and immunofluorescence-microscopy.

	RT^2-PCR		**Immunofluorescence-microscopy**	
LPCs	**Explants (n)**	**Donors (n)**	**Explants (n)**	**Donors (n)**
LPC0 to LPC3	6	3	4	3
LPC4	4	3	4	3
LPC5	4	3	3*	3*
LCP6	3	2	3*	3*

*K12 and ABCG2 markers were not analyzed in LPC5 and LPC6 because the number of samples collected was not enough to perform the assays.

Table 3. Antibodies used for immunodetection assays.

Antibody	**Category**	**Clone**	**Source***	**Working dilution**
Keratin 3	Mouse monoclonal	AE5	Mp Biomedical	1:50
Keratin 12	Rabbit polyclonal	H-60	Santa Cruz Biotechnology	1:50
Keratin 14	Mouse monoclonal	RCK107	Chemicon (Millipore)	1:50
Keratin 15	Mouse monoclonal	LHK15	Chemicon (Millipore)	1:50
ABCG2	Mouse monoclonal	BXP-21	Chemicon (Millipore)	1:20
P63	Mouse monoclonal	4A4	Santa Cruz Biotechnology	1:50
S100A4	Mouse monoclonal	1B10	Abcam	1:100

*Mp biomedical (Illkirch, France), Santa Cruz Biotechnology (Heidelberg, Germany), Millipore (Billerica, MA, USA), Abcam (Cambridge, MA, USA).

FIGURES

Figure 1.

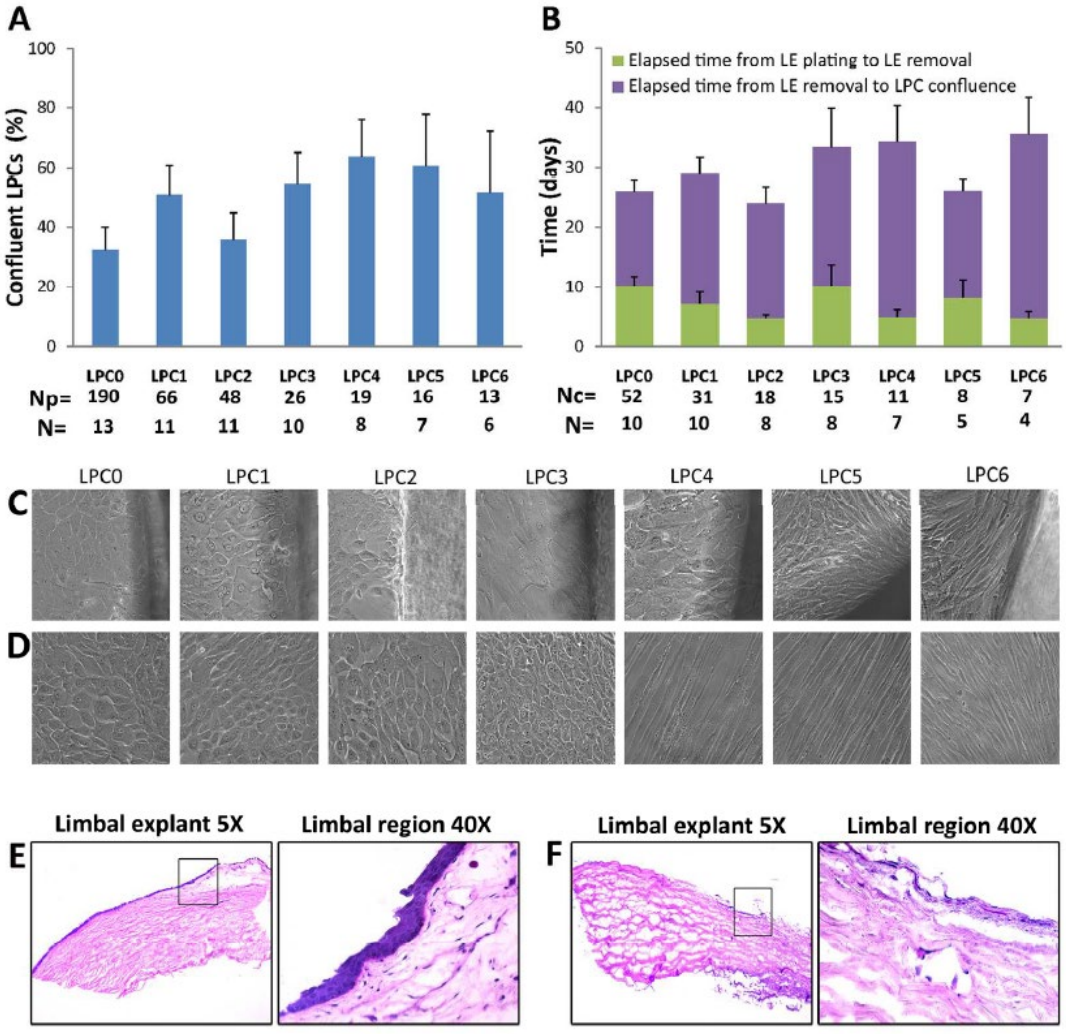


Figure 2.

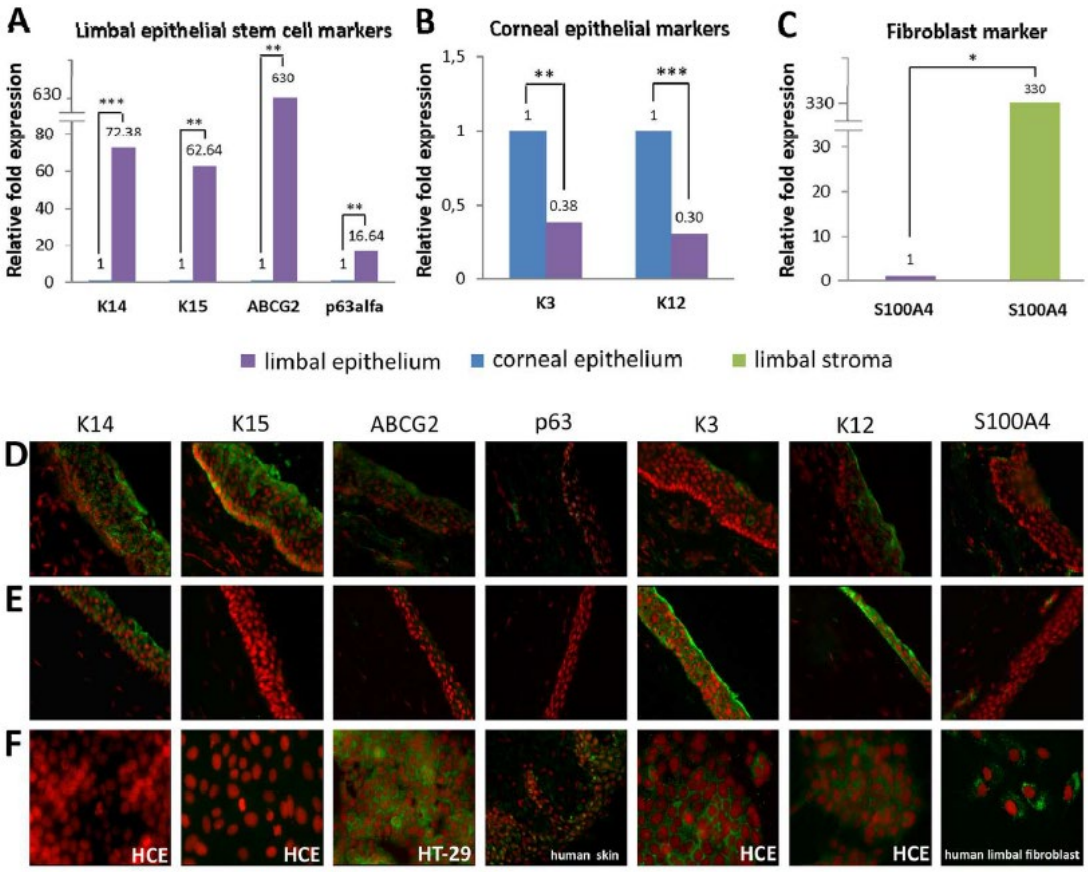

A
Limbal epithelial stem cell markers
Relative fold expression
630
80
60
40
20
0
***
72.38
**
62.64
**
630
**
16.64
1
K14
K15
ABCG2
p63alfa
B
Corneal epithelial markers
1,5
1
0,5
0
**
***
1
0.38
0.30
K3
K12
C
Fibroblast marker
330
30
20
10
0
*
330
1
S100A4
S100A4
limbal epithelium
corneal epithelium
limbal stroma
K14
K15
ABCG2
p63
K3
K12
S100A4
D
E
F
HCE
HCE
HT-29
human skin
HCE
HCE
human limbal fibroblast

Figure 3.

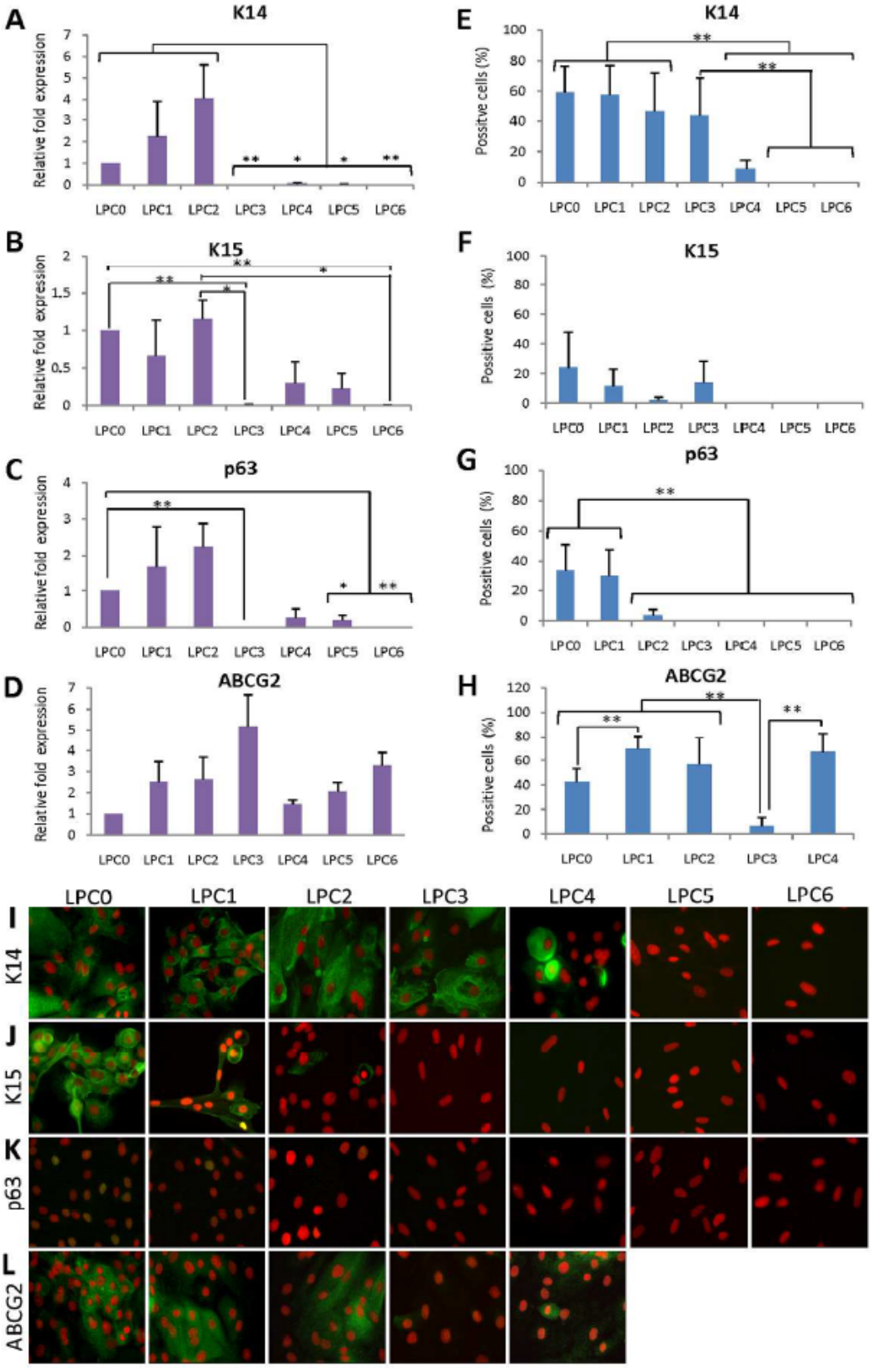

A
K14
Relative fold expression
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
E
K14
Possitve cells (%)
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
B
K15
Relative fold expression
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
F
K15
Positive cells (%)
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
C
p63
Relative fold expression
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
G
p63
Positive cells (%)
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
D
ABCG2
Relative fold expression
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
H
ABCG2
Positive cells (%)
LPC0 LPC1 LPC2 LPC3 LPC4
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
I K14
J K15
K p63
L ABCG2

Figure 4.

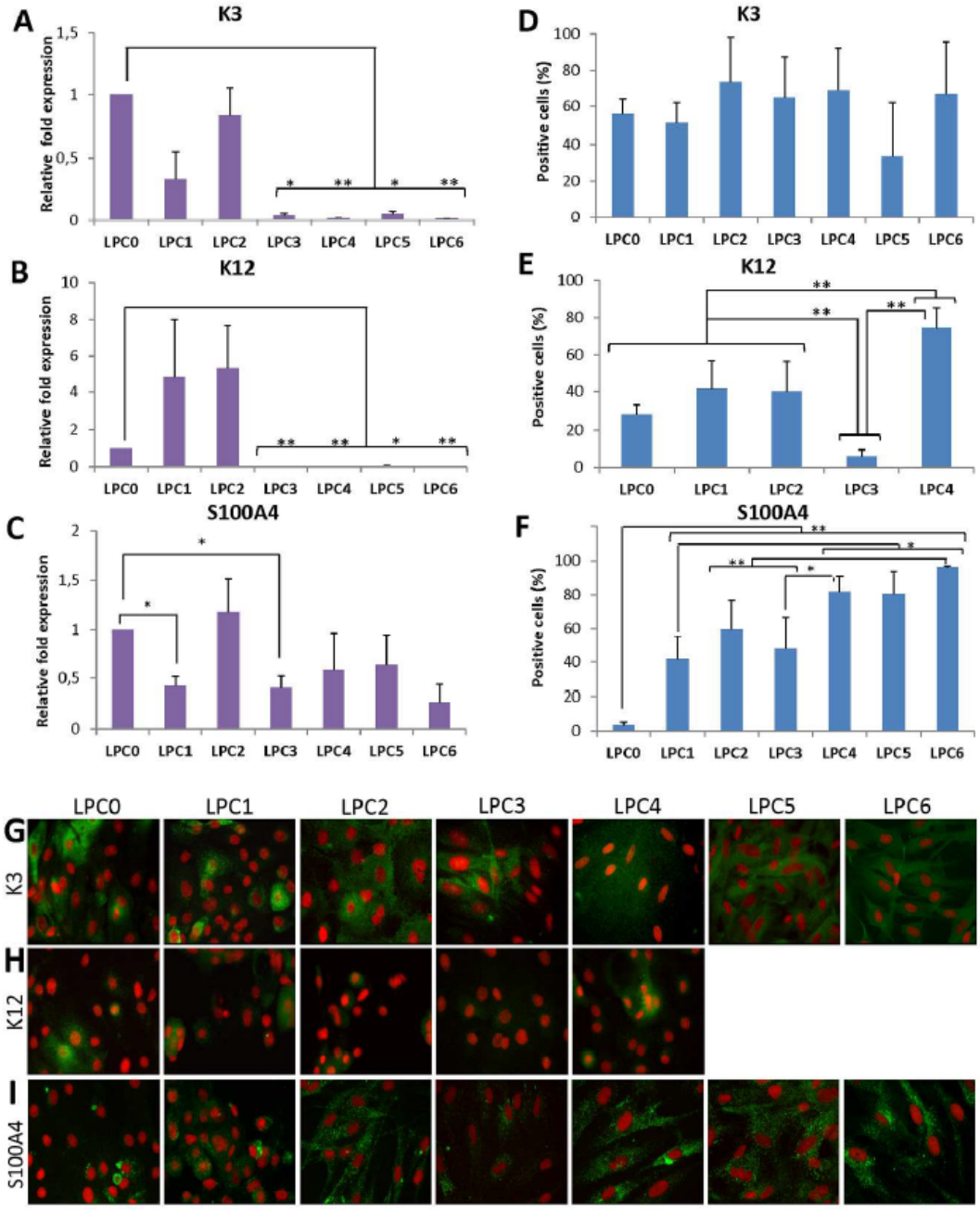
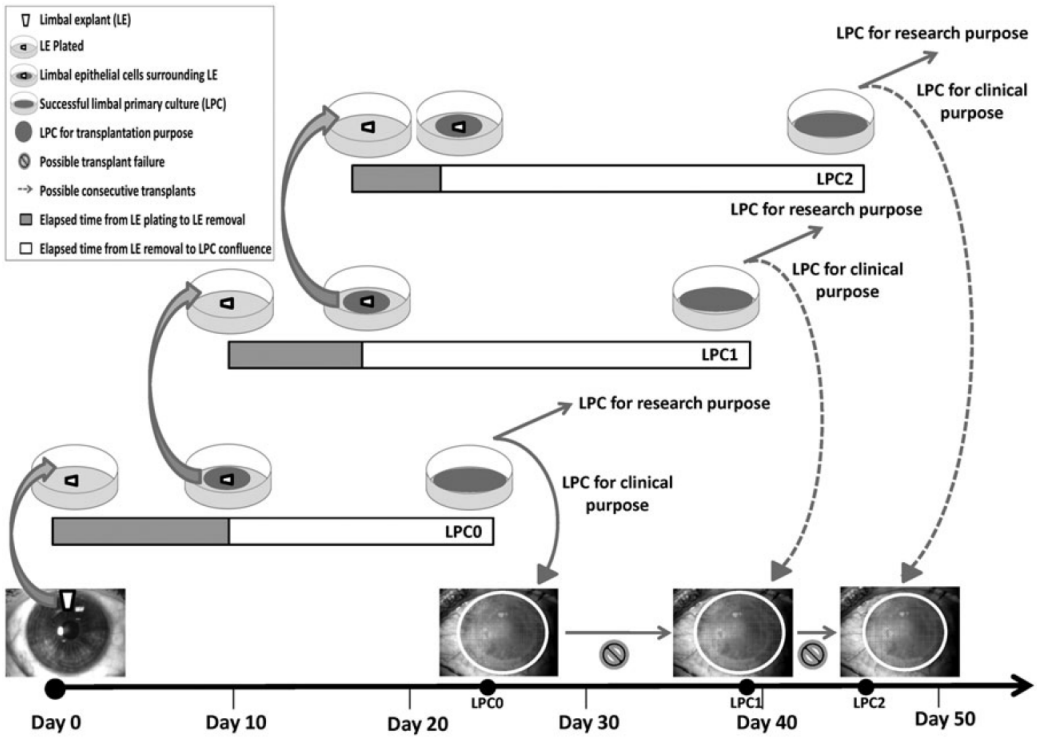

A
K3
Relative fold expression
1,5
1
0,5
0
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
B
K12
Relative fold expression
10
8
6
4
2
0
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
C
S100A4
Relative fold expression
2
1,5
1
0,5
0
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
D
K3
Positive cells (%)
100
80
60
40
20
0
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
E
K12
Positive cells (%)
100
80
60
40
20
0
LPC0 LPC1 LPC2 LPC3 LPC4
F
S100A4
Positive cells (%)
100
80
60
40
20
0
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
LPC0 LPC1 LPC2 LPC3 LPC4 LPC5 LPC6
G
K3
H
K12
I
S100A4

Figure 5.


Limbal explant (LE)
LE Plated
Limbal epithelial cells surrounding LE
Successful limbal primary culture (LPC)
LPC for transplantation purpose
Possible transplant failure
Possible consecutive transplants
Elapsed time from LE plating to LE removal
Elapsed time from LE removal to LPC confluence
LPC for research purpose
LPC for clinical purpose
LPC2
LPC for research purpose
LPC for clinical purpose
LPC1
LPC for research purpose
LPC for clinical purpose
LPC0
LPC0
LPC1
LPC2
Day 0
Day 10
Day 20
Day 30
Day 40
Day 50