Comparison of functional limbal epithelial stem cell isolation methods

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ABBREVIATIONS

LESC: limbal epithelial stem cell

LPC: limbal primary culture

CFE: colony forming efficiency

K: keratin

LSCD: limbal stem cell deficiency syndrome

HS: human serum

TEM: Transmission electron microscopy

ABSTRACT

The transplantation of limbal epithelial stem cells (LESCs) cultured *in vitro* is a great advance in the treatment of patients suffering from LESC deficiency. However, the ~~most~~ optimal technique for LESC isolation from a healthy limbal niche has not yet been established. Our aim was to determine which isolation method renders the highest recovery of functional LESCs from the human limbus. To achieve this purpose, we compared limbal primary cultures (LPCs) obtained from explants and cell suspensions on plastic culture plates. Cell morphology was observed by phase contrast and transmission electron microscopy. LESC, corneal epithelial cell, fibroblast, endothelial cell, melanocyte, and dendritic cell markers were analyzed by real time by reverse transcription polymerase chain reaction and/or immunofluorescence. In addition, colony forming efficiency (CFE) and the presence of holoclones, meroclones, and paraclones were studied. We observed that LPC cells obtained from both methods had cuboidal morphology, desmosomes, and prominent intermediate filaments. The expression of LESC markers (K14, K15, ABCG2, p63α) was similar or higher in LPCs established through cell suspensions, except the expression of p63α mRNA, and there were no significant differences in the expression of corneal epithelial markers (K3, K12). Endothelial cell (PECAM), melanocyte (MART-1), and dendritic cell (CD11c) proteins were not detected, while fibroblast-protein (S100A4) was ~~mildly~~ detected in all LPCs. ~~On the other hand,~~ The CFE was significantly higher in LPCs from cell suspensions. Cells from confluent LPCs produced by explants generated only paraclones (100%), while the percentage of paraclones from LPCs established through cell suspensions was 90% and the remaining 10% were meroclones. In conclusion, LPCs established from cell suspensions have a cell population richer in functional LESCs than LPCs obtained from explants. These results suggest that in a clinical situation in which it is possible to choose between either of the isolation techniques from the donor limbal tissue, then the cell suspension is probably the best option as long as the cells are expanded following our culture conditions.

Key Words: cell culture ● ocular surface ● limbal stem cells ● explants ● cell suspensions

1. INTRODUCTION

The corneal surface is covered by an epithelium that is continually renewed and maintained by a population of stem cells that reside principally in the limbus, the junction between the cornea and the sclera-conjunctiva (reviewed by Notara et al., 2010a; O'Callaghan and Daniels, 2011; Ordonez and Di Girolamo, 2012). Therefore, the limbus is considered to be the main structure implicated in the maintenance of corneal homeostasis. Currently, a population of stem cells is thought to also exist in the peripheral or central cornea (reviewed by Nakamura et al., 2015; West et al., 2015), and these cells could have a similar function to that of the limbal epithelial stem cells (LESCs). ~~These~~ LESCs are characterized by their small size (Schlotzer-Schrehardt and Kruse, 2005), the absence of differentiation markers such as keratin (K) 3 and 12 (Kurpakus et al., 1990; Schermer et al., 1986), the high nucleus-to-cytoplasm ratio, slow cell cycle, and high proliferative potential (Cotsarelis et al., 1989). Limbal stem cell deficiency syndrome (LSCD) is the end-stage morbidity resulting from a critical reduction and/or dysfunction of these LESCs. It is caused by a wide variety of ocular surface disorders (chemical, thermal or mechanical injuries, contact lens wear, infections, immune-based disorders, severe dry eye syndrome, etc.) that leads to a deficient regeneration of the cornea, resulting in a corneal opacity, loss of vision, and a chronic pain syndrome (Dua et al., 2000). The *in vitro* cultured LESC transplantation (CLET), introduced by Pellegrini et al. in 1997, has been a great breakthrough in the treatment of patients suffering from LSCD. However, the best technique to isolate the LESCs from the limbal niche tissues has not been established. There are two main methods to produce limbal primary cultures (LPCs). One is the explant culture technique in which a small limbal biopsy (limbal explant), from 1 to 6 mm^2, is plated on a substratum. These biopsies include limbal epithelium and stroma, and they are often removed from either the superior or the inferior limbal ring region (reviewed by Shortt et al., 2007). The second isolation technique is the suspension culture system, in which limbal tissue is treated with enzymes to separate the stroma from the epithelium, and the isolated epithelial cells are then seeded on a substratum. Usually, this

method employs two enzymes: dispase, which digests basement membrane collagen and separates epithelial cells from the stroma, and trypsin, which separates clumps of limbal epithelial cells into a suspension of single cells. These enzymatic protocols can be performed on a limbal biopsy or in a complete limbal ring (reviewed by Burman and Sangwan, 2008; Shortt et al., 2007).

Currently, there is no agreement among the different laboratories working in this field (Gonzalez and Deng, 2013; Kawakita, 2011; Kim et al., 2004; Koizumi et al., 2002; Mariappan et al., 2014; Zhang et al., 2005; Zito-Abbad et al., 2006) in the choice of the technique used to isolate LESCs as each method has advantages and disadvantages (reviewed by Shortt et al., 2007). With the purpose of clarifying which isolation method renders the highest recovery of cells with the LESC phenotype, here we report an exhaustive comparison between LPCs obtained from both limbal explants and cell suspensions. To carry out a deep phenotypic characterization, we have performed a cell clonal capacity analysis of LPCs obtained from both techniques using a biosafe culture medium (biosafe IOBA-HS) that lacks non-human animal supplements and other potentially harmful compounds. This work will help establish a standard method to isolate LESCs from the limbal niche that will improve clinical outcomes in the treatment of ocular surface failure due to LSCD.

2. MATERIALS AND METHODS

The following protocols were approved by the IOBA Research Committee and the Valladolid Medical School Ethics Committee. Human tissues were always handled according to the Tenets of Declaration of Helsinki.

2.1. Materials and reagents

Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) - GlutaMAX^{TM} 3.1 g/l glucose-with pyruvate, DMEM - GlutaMAX^{TM} 4.5 g/l glucose-without pyruvate, Dulbecco's phosphate-buffered saline no calcium – no magnesium (DPBS), gentamicin, amphotericin B,

antibiotic-antifungal solution 1X, fetal bovine serum (FBS), 0.05% trypsin - ethylenediaminetetraacetic acid (EDTA), 0.25% trypsin-EDTA, 0.5% trypsin – EDTA, dispase II, Quant-iT RNA Assay Kit, SuperScript® VILO™ cDNA Synthesis Kit, propidium iodide, sodium bicarbonate, and insulin were purchased from Life Technologies (Inchinan, UK, http://www.lifetechnologies.com). Epidermal growth factor (EGF), transferring, hydrocortisone, adenine, DL-isoproterenol hydrochloride, 3′,5-triiodothyronine, adult bovine serum (ABS), trypan blue, poly-L-lysine, glass cylinders, mitomycin C, rhodamine B, silicone, and uranyl acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA, http://www.sigmaaldrich.com). Paraformaldehyde, ethanol, and methanol were purchased from Panreac (Lyon, France, http://www.panreac.es). Lead citrate and osmium tetroxide were from Merck (Darmstadt, Germany, http://www.menzel.de). SPURR resin was used from TAAB Laboratories (Berksire, UK, http://www.taab.co.uk) and epoxy resin from Elektron Technology (Cambridge, UK, http://www.elektron-technology.com). RNeasy® Mini Kit and RNase-Free DNase were from Qiagen (Valencia, CA, USA, http://www.qiagen.com). Trephines and human serum (HS) were from Katena (Denville, NJ, USA, http://katena.com) and Lonza (Basel, Switzerland, http://www.lonza.com), respectively. Polystyrene culture dishes and coverslips of Thermanox® were purchased from Nunc (Roskilde, Denmark, http://www.thermoscientific.com). Petri's dishes of 100 mm were from Corning (Tewksbury, MA, USA, http://www.corning.com). Cholera toxin was from Gentaur (Kanpenhoot, Belgium, http://www.gentaur.com). Glass slides were purchased from Menzel-Gläser (Braunschweig, Germany, http://www.menzel.de). OptiSol-GS solution was obtained from Bausch&Lomb (Irvine, CA, USA, http:// www.baush.com).

2.2. Human tissue preparation

Healthy human corneoscleral tissues from deceased donors were obtained from the Barraquer Eye Bank (Barcelona, Spain). The mean ± standard error of the donors' age was 74.5 ± 3.9 years (range 35-88 years). The samples were maintained in preserved conditions (IOBA-SH medium, described below, or OptiSol-GS solution) an average of 4.5 ± 0.5 days. Corneoscleral tissues were prepared using a previously described method (Lopez-Paniagua et al., 2013). Briefly, excess sclera, conjunctiva, iris, and corneal endothelium were removed, and the central cornea was extracted with a 7.5 mm trephine, obtaining corneoscleral rings. Limbal epithelial cell isolation by limbal explant or single cell suspension was randomly assigned for each corneoscleral ring to minimize the effect of biological variability in our results. Forty-five percent of the corneoscleral tissues used to provide explants were preserved in the IOBA-SH medium and 55% were stored in OptiSol-GS solution. These percentages were similar for the tissues processed through the cell suspension technique (52% were preserved in IOBA-SH and 48% in OptiSol-GS). There were no significant differences in donor age or in the time of limbal tissues storage between both groups (Table 1). For clonogenicity assays, half of a limbal ring was used to cultivate explants and the other half was used to generate the cell suspension.

2.3. Limbal explants

The remaining corneoscleral rings were cut into 1-2 mm^2 limbal explants that were plated singly into 3.8 cm^2 polystyrene wells (plastic). After 30 min in a laminar flow hood, each was incubated with 50 µl of HS overnight at 37ºC, 5% CO_2, and 95% humidity. After that, the limbal explants were cultured with biosafe IOBA-HS culture medium: DMEM/F12 (3.1 g/l glucose-with pyruvate) supplemented with 5 ng/ml EGF, 5 µg/ml insulin, 0.4 µg/ml hydrocortisone, 1 µM isoproterenol, 0.18 mM adenine, 2 nM triiodothyronine, 50 µg/ml gentamicin, 2.5 µg/ml amphotericin, and 10% HS. Each limbal explant was maintained in culture until a rim of migrating cells surrounded it (a mean of 14.5 ± 0.6 days). Then, the explants were removed and the remaining LPCs were grown until confluence (80-90%). The number of limbal explants cultured from different human cadaveric donors is shown in Table 1.

2.4. Cell suspensions

Each limbal rim was divided into two similar portions. Each portion was incubated with 1.2 U/ml of dispase II at 37°C for 2 h. The epithelial sheets were then mechanically removed, collected and treated with 0.25% trypsin-EDTA at 37°C for 10 min to isolate single cells. The isolated limbal epithelial single cell suspension was centrifuged for 5 min at 156 g, re-suspended, and then seeded at a density of $1.5x10^4$ cells/cm^2 into 3.8 cm^2 plastic well. Cultures were incubated with 300 µl of biosafe IOBA-HS culture medium at 37°C under 5% CO_2 and 95% humidity. Two hours later, 200 µl of medium were added for each LPC. The medium was changed every 2–3 days until 80-90% confluence was reached. The number of LPCs established by cell suspension technique from different human cadaveric donors is shown in Table 1.

2.5. Morphology and cell growth

Cell outgrowths were monitored under a phase contrast microscope (Eclipse TS100, Nikon, Tokyo, Japan, http://www.nikoninstruments.com). A LPC was considered successful when it reached more than 80% confluence. The percentage of successful LPCs was calculated based on the number of LPCs started from explants or from cell suspensions. The elapsed time from limbal explant or cell suspension plating to LPC confluence (LPC generation time) was determined.

To calculate the number of cells in each confluent LPC, the cells were incubated with 0.25% trypsin-EDTA at 37ºC for 10 min. Subsequently, the cell suspension was centrifuged for 5 min at 156 g and the total live and dead cells were counted by the Trypan Blue-Neubauer chamber method. Cell viability was calculated as: % viability = Number of live cells / Total number of cells x 100.

In addition, cell size was calculated. Micrographs of the LPC cells were taken from one randomly selected area, and the high and low diameters were measured in five selected cells. At least fifteen LPCs from different donors were analyzed for each condition (n=15).

2.6. Transmission electron microscopy (TEM)

Limbal epithelial cell ultrastructure was examined by TEM. The LPCs were grown on Thermanox® coverslips in the same way as when they had been grown on plastic. When confluence was reached, the LPCs were gently washed twice with DPBS and then fixed with Karnosvky's fixative at 4ºC. The samples remained in these conditions until they were washed and post-fixed in 1% osmium tetroxide for 2 h at 4ºC. They were then dehydrated in ascending grades of ethanol and embedded in SPURR or epoxy resin. Thin sections (50 nm) were cut with a glass knife (parallel and perpendicular cuts) and mounted onto grids. They were counterstained with uranyl acetate and/or lead citrate. The samples were viewed under a transmission electron microscope JEOL 1010 (Peabody, MA, USA) operating at 80 Kv (images acquired with a SC1000 Orius CCD camera, Gatan, UK) or JEOL JEM-1011 HR (Peabody, MA, USA) working at 60-100 Kv (images taken with an ES1000W CCD camera, Gatan, UK). Two LPCs from different donors were analyzed for each condition.

2.7. RNA extraction, reverse transcription (RT) and real time polymerase chain reaction (RT^2-PCR)

Total RNA extraction, RT, and RT^2-PCR were performed as previously described (Lopez-Paniagua et al., 2013). Briefly, confluent LPCs were incubated with RNA lysis buffer from RNeasy Mini Kit. Total cell RNA was extracted by RNeasy Mini Kit, treated with RNase-Free DNase I Set, and quantified using the commercial kit Quant-iT RNA Assay/Qubit-fluorometer (Invitrogen-Gibco, Inchinan, UK, http://www.b2b.invitrogen.com). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA by SuperScript® VILO™ cDNA Synthesis Kit using the Mastercycler® Personal Thermocycler (Eppendorf AG, Cologne, Germany, http://www.eppendorf.com). Subsequently, RT^2-PCR assays were performed from 20 ng of cDNA in a 7500 Real Time PCR System using the following oligonucleotide primers and Taqman® probes from Applied Biosystems (Carlsbad, CA, USA, https://www.appliedbiosystems.com): S100 calcium binding protein A4 (*S100A4*, Hs 00243201_m1), ATP-binding cassette, sub-family G, member 2 (*ABCG2*, Hs 00184979_m1), p63α *(TP63*, Hs 00978338_m1), keratin 14, (*KRT14*, Hs

00559328_m1), keratin 15 (*KRT15*, Hs 00267035_m1), keratin 3 (*KRT3*, Hs 00365080_m1), and keratin 12 (*KRT12*, Hs 00165015_m1). Assays were performed in duplicate. A non-template control was included in all experiments, and the human glyceraldehyde-3-phosphate dehydrogenase *(GAPDH,* 4352934E*)* gene was used as the endogenous control. The comparative cycle threshold (Ct) method, where the target fold = $2^{-\Delta\Delta Ct}$, was used to analyze the results. The expression of each marker in LPCs generated from limbal explants was used as the calibrator for LPCs established from cell suspensions. The results were reported as a fold up-regulation or fold down-regulation when the fold-change was greater or less than 1, respectively. Six LPCs from different donors were analyzed for each condition ~~(n=6)~~.

2.8. Immunofluorescence microscopy

Confluent LPCs were incubated in 0.25% trypsin-EDTA for 5 min at 37ºC. Subsequently, the cell suspensions obtained were centrifuged and the number of live cells was calculated by the Trypan blue-Neubauer chamber method. A total of 40,000-50,000 cells in 250 µl of DMEM-F12 were pelleted by cytocentrifugation for 10 min at 123 g with low acceleration (*Citospin 4-Shandon,* Thermo Scientific, Walthman, MA, USA). Special supports (*Shandon EZ-double cytofunnels*, Thermo Scientific) were used for centrifugation. The cells were deposited onto a 28 mm^2 area of poly-L-lysine-treated glass slides and fixed with cold methanol at -20ºC for 10 min. Immunofluorescence assays were performed following a previously reported protocol (Lopez-Paniagua et al., 2013). In brief, previously fixed LPC cells were permeabilized (except for antibody anti-PECAM incubation), blocked, and incubated overnight at 4ºC with specific primary antibodies: platelet endothelial cell adhesion molecule (PECAM/CD31, clone Ab1, 1:20) from Oncogene™-MercMillipore (Darmstadt, Germany, http://www.merckmillipore.com). The proliferation marker ki67 (clone MIB-1, 1:50) from Dako (Barcelona, Spain, http://www.dako.com). Melanoma associated antigen recognized by T cells (MART1/MelaA, clone A103, 1:50) and S100A4 (clone 1B10, 1:100) from Abcam (Cambridge, UK, http://www.abcam.com). Integrin alphaX (CD11c, clone b-ly6, 1:50) and keratin 12 (K12, clone H-60, 1:50) from Santa Cruz Biotechnology

(Heidelberg, Germany, http://www.scbt.com). ABCG2 (clone BXP-21, 1:20), keratin 14 (K14, clone RCK107, 1:50), and keratin 15 (K15, clone LHK15, 1:50) from Chemicon-Millipore (Darmstadt, Germany, http://www.millipore.com). Keratin 3 (K3, clone AE5, 1:50) from Mp Biomedical (Illkirch, France, http://www.mpbio.com), and p63α protein from Cell Signaling (Boston, MA, USA, http://www.cellsignal.com). After incubation with the primary antibodies, samples were incubated 1 hour at room temperature with the corresponding secondary antibody (Alexa Fluor® 488 donkey anti-mouse or donkey anti-rabbit, Life Technologies). Propidium iodide was used to counterstain cell nuclei.

Images were acquired with an inverted fluorescence microscope (DM4000B, Leica, Wetzlar, http://www.leica-microsystems.com). LPC cell images at 20X magnification were taken of five randomly selected areas, and the percentage of positive cells was calculated. Negative controls included the omission of primary antibodies. All antibodies were previously validated in different positive controls by our research group (Lopez-Paniagua et al., 2013). Four LPCs from different donors were analyzed for each condition by immunofluorescence microscopy.

2.9. Maintenance of 3T3/J2 mouse fibroblasts as a feeder layer

For clonogenicity assays, 3T3/J2 mouse fibroblasts from ATCC (CCL-92) were used as a feeder layer. They were cultured with DMEM-GlutaMAX™ (4.5 g/l glucose, without pyruvate) supplemented with antibiotics-antifungal solution 1X (100 U/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin), and 10% ABS upon reaching 80% confluence. Then, mitosis was arrested by adding 10 µg/ml of mitomycin C to the fibroblast culture medium (specified above) for 2 h. Growth-arrested 3T3/J2 fibroblast were plated at seeding densities of $2.7x10^4$ cells/cm^2 on 100-mm dishes.

2.10. Colony forming efficiency

Limbal epithelial cells from confluent LPC established from explants or cell suspensions (n=5 or n=8 different donors, respectively), were placed on a 3T3/J2 fibroblast feeder layer at a seeding

density of 20 cells/cm^2. Cultures were kept at 37ºC, 5% CO_2 and 95% relative humidity in DMEM/F12-GlutaMAX™ + DMEM-GlutaMAX™ 4.5 g/l glucose and without pyruvate (1:3) supplemented with antibiotic-antifungal solution 1X (100 U/ml of penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin), insulin 5 µg/ml, transferring 5 µg/ml, hydrocortisone 0.4 µg/ml, EGF 10 ng/ml, cholera toxin 8.5 ng/ml, sodium bicarbonate 0.075%, adenine 0.18 mM, triiodothyronine 2 nM, and FBS 10%. Cultures were kept until day 10 or 11. Then the colonies were washed and fixed with 2% paraformaldehyde for 15 min at room temperature. Subsequently, the cells were rehydrated with DPBS and stained with 2% rhodamine B for 3 min at room temperature. Finally, the colonies were counted and the colony forming efficiency (CFE) was calculated using the equation: CFE (%) = Number of colonies / Number of cells seeded x 100.

2.11. Clonal analysis

For clonal analysis, co-cultures of 3T3/J2 fibroblasts with limbal epithelial cells were established following the same protocol as for the CFE study described above. However, for these experiments, the cells were maintained for 8-9 days, instead of 10-11 days, until the colony diameters were 700–900 µm. Subsequently, the cultures were incubated with 0.05% trypsin–EDTA for 2 min at 37ºC to remove the 3T3/J2 fibroblasts. After trypsinization of the feeder layer, 10 randomly selected colonies were individually trypsinized using cloning cylinders, silicone, and 0.5% trypsin-EDTA for 5 min at room temperature. Single colonies were then transferred to secondary 100-mm dishes, treated with 3T3/J2 fibroblasts as a feeder layer, and grown for an additional 13 days. Then, the colonies were washed and fixed with 2% paraformaldehyde for 15 min at room temperature. Subsequently, the cells were rehydrated with DPBS and stained with 2% rhodamine B for 3 min at room temperature. Finally, the terminal and non-terminal colonies were counted. Transferred clones that gave rise to <5% terminal colonies were defined as “holoclones”. For transferred clones that formed no colonies or only terminal colonies, the clones were classified as “paraclones”. When more than 5% but less than 100% of the colonies were terminal, the clones

were classified as “meroclones”. Holoclones are originated from undifferentiated cells, while meroclones and paraclones are derived from transit amplifying and differentiated cells, respectively (Barrandon and Green, 1987). Four LPCs from different donors were analyzed for each condition by clonal analysis ~~(n=4)~~.

2.12. Statistical analysis

Statistical significance was determined by Student’s t-test for independent samples, except for RT^2-PCR results. For RT^2-PCR result analysis, the calibrator-control group (value 1) was compared with the remaining groups by Student’s t-test for one sample. All values were expressed as means ± standard error of the means (SEM). P-values ≤ 0.05 were considered statistically significant.

3. RESULTS

3.1. LPC characteristics and cell morphology

Confluent LPCs were successfully established from both explants and cell suspensions. Limbal explants yielded a significantly lower percentage of successful LPCs than did cell suspensions and required a longer LPC generation time (Table 1). In addition, the total number of cells in confluent LPCs from explants was significantly lower than in those from cell suspensions (Table 1), while the cell viability was similar for the two isolation procedures (Table 1).

By phase contrast microscopy, we observed that the LPC cells obtained from both isolation techniques had a homogeneous cuboidal morphology (Fig. 1A, B). However, the cells of the LPCs generated from explants were significantly larger (44.42 ± 1.90 µm x 28.50 ± 0.64 µm) than the cells generated from cell suspensions (28.04 ± 1.61 µm x 18.62 ± 0.99 µm) (Fig. 1A, B). Cell morphology was also analyzed by TEM. The cells obtained from either technique had a sparse cytoplasm containing few mitochondria and ribosomes, a nucleus with a well-defined nucleoli (Fig.

1C, G), numerous intercellular junctions of which most were desmosomes (Fig. 1D, H), and prominent intermediate filaments (Fig. 2E, I). In addition, primary lysosomes (Fig. 1C, G) and microvilli (Fig. 1F, J) were observed in both cases.

3.2. LESC marker expression in LPCs

Relative mRNA expression of *K14* was similar when the LPCs were obtained from explants and cell suspensions (0.86 fold, Fig. 2A). However, the percentage of cells positive for this protein was significantly lower for LPCs derived by the explant technique (62.26 ± 13.26%) than the cell suspension technique (99.34 ± 0.42%, p=0.031, Fig. 2B, C).

On the other hand, the mRNA expression of *K15* was significantly lower in LPCs obtained from explants than from cell suspensions (1.8 fold, p=0.018, Fig. 2A). Moreover, the percentage of cells that expressed the K15 protein was also significantly lower in the LPCs generated from limbal explants (18.70 ± 10.40%) than from cell suspensions (51.89 ± 5.10%, p=0.029 Fig. 2B, C).

The relative mRNA expression of *ABCG2* was similar in LPCs obtained by both isolation techniques (Fig. 2A). On the other hand, the protein ABCG2 was expressed by 57.33 ± 20.54% of the cells cultured from explants. Although this percentage was higher in LPCs obtained from cell suspensions (85.47% ± 8.53), the difference was not significant (Fig. 2B, C).

Finally, the expression of p63α was analyzed. The mRNA expression of this marker was significantly higher in LPCs obtained by the explant technique than the cell suspensions (0.79 fold, p=0.030, Fig. 2A). However, the p63α protein was not detected in LPCs established from explants, while the 42.76 ± 14.50% of the cells in the LPCs cultured from cell suspensions expressed this protein (Fig. 2B, C).

3.3. Corneal epithelial cell marker expression in LPCs

The mRNA expression of *K3* was lower in LPCs derived from explants compared to cell suspensions (3.27 fold); however the difference was not statistically significant (Fig. 3A). The percentage of positive cells for K3 protein was similar in the LPCs obtained using both cell

isolation techniques (62.85% ± 11.53% from explants, 57.79% ± 14.74 from cell suspensions, Fig. 3B, C).

On the other hand, the relative expression of K12 mRNA was similar in LPCs established from explants and cell suspensions (1.25 fold, Fig. 3A). In addition, the percentage of cells positive for this marker was similar for the two techniques (65.63% ± 1.68 for explants, 68.70% ± 23.15% for cell suspensions, Fig. 3B, C).

3.4. Non-epithelial cells in LPCs

The limbal fibroblast S100A4 marker was expressed in LPCs generated by both cell isolation protocols. The relative mRNA expression of *S100A4* was significantly higher in LPCs derived from explants ($p \leq 0.001$, Fig. 4A). The percentage of cells positive for the protein was higher in LPCs obtained from explants, although the differences were not significant (Fig. 4B, C). Protein markers for endothelial cells (PECAM), melanocytes (MART-1), and dendritic cells (CD11c) were not detected in any of the LPCs (Fig. 4C).

3.5. Cell proliferation marker Ki67 expression in LPCs

The percentage of cells positive for the proliferation marker Ki67 was similar in LPCs established from explants (64.67% ± 8.56%) and cell suspensions (56.55% ± 9.15%, Fig. 5A).

3.6. CFE in LPCs

The CFE was analyzed to determine the number of cells that could originate new colonies. The CFE was significantly higher in LPCs established from cell suspensions (8.31% ± 1.37%) than from explants (1.00% ± 0.23%, $p=0.019$, Fig. 5B and C).

3.7. Clonal analysis: holoclones, meroclones, and paraclones

The cells derived from confluent LPCs produced by explants generated only paraclones (100%, Table 2, Fig. 6A, B, E). However, the percentage of paraclones derived from LPCs established from cell suspensions was 90% ± 4.0% (Table 2, Fig. 6A, C, F), and the remaining 10% ± 4.0% were meroclones (Table 2, Fig. 6A, D, G). Therefore, the percentage of meroclones was significantly

higher in the LPCs obtained from cell suspensions compared to explants ($p \leq 0.05$). In addition, paraclones generated from limbal explant-derived LPCs originated no colonies (Fig. 6E), while the paraclones generated from LPCs derived from cell suspensions originated new colonies (Fig. 6F), although all of them were terminal. None of the LPC-derived cells produced holoclones.

4. DISCUSSION

In this study, we compared the two most commonly used techniques for the isolation of epithelial cells from the limbal niche: limbal explants and cell suspensions (Shortt et al., 2007). Several investigators prefer the explant culture technique because limbal explants are easy to prepare and only a small donor biopsy is necessary (reviewed by Burman and Sangwan, 2008; Shortt et al., 2007). Moreover, the native niche of LESCs is not disrupted during the culture, and therefore it might help to better maintain the phenotype of LESCs *in vitro* (Gonzalez and Deng, 2013). However, disadvantages of this technique include low reproducibility and low proliferative rate of the limbal epithelial cells because the cells must migrate from the more basal layers of the limbal epithelial tissue towards the substratum (Gonzalez and Deng, 2013). In contrast, the cell suspension protocol can facilitate cell isolation from deeper limbal epithelial layers, reduce contamination with non-epithelial cells (fibroblasts, endothelial cells, dendritic cells, etc.), and the culture conditions are more reproducible. The main disadvantages of LESC isolation through cell suspensions are that it is more time-consuming and that the enzymatic treatment could potentially affect the integrity of the limbal cells.

The experimental differences between these two cell isolation techniques have previously been studied by several research groups (Gonzalez and Deng, 2013; Kawakita, 2011; Kim et al., 2004; Koizumi et al., 2002; Mariappan et al., 2014; Zhang et al., 2005; Zito-Abbad et al., 2006). However, in terms of outcomes, up to now, no significant differences between them have been reported. It should be pointed out that the functional analyses of cell clonogenicity presented here have never been made to compare the performance of both cell isolation techniques. Here, we compared both

explant and cell suspension techniques by performing cell characterizations and cell clonal analyses in parallel.

To achieve this aim, 208 LPCs were established by outgrowth from limbal explants and 61 were established from limbal cell suspensions. An average of nine explants were plated from each limbal half-ring, and 40% (3-4 LPCs per half-ring) reached confluence. However, only an average of two cell suspensions could be cultured from the same quantity of limbal tissue (limbal half-ring). In this case, the percentage of LPCs that reached confluence was 95% (2 LPCs). Therefore, an average of four confluent LPCs were established from limbal half-ring explants while two were established from cell suspensions. The low percentage of cultures that reached confluence when they were established from explants was most likely affected by the specific limbal ring region from where the explants were taken. Utheim et al. reported that LESCs are mainly located in the superior and the inferior limbal ring area (Utheim et al., 2009); however, we cultured explants from the whole limbal ring. Recently, our research group reported that when limbal explants taken exclusively from the superior area of the limbal ring were cultured on plastic dishes with biosafe IOBA-HS, the percentage of confluent LPC increased to 58% (de la Mata et al., 2013), although ~~However~~, these results cannot be strictly compared with the outcomes shown here because the areas of cultivation that the cells had to cover to achieve confluence were different.

The time to reach confluence was significantly higher for LPCs obtained from explants, 19 days, compared to those from cell suspensions, 13 days. This is consistent with the observations made by Kim et al. who showed that LPCs from explants needed 14-21 days to reach confluence, while those from cell suspensions required only 10-14 days (Kim et al., 2004). However in their study, two limbal explants were plated together, providing two different growth points in the same well, and the cell suspensions were established on 3T3/J2 fibroblast feeder layers (Kim et al., 2004). In addition, the tissue donors' age and the time of tissues storage before cell cultivation were lower than those of our experiments. All these factors can negatively affect the time needed to obtain confluent LPCs (James et al., 2001; Notara et al., 2012; Vemuganti et al., 2004). Nevertheless, we

did not see any negative effect of these factors in the elapsed time from the plating to the achievement of confluence for either explant or suspension cultures. The culture medium used by Kim et al. was supplemented with FBS, cholera toxin, and DMSO. Therefore, we think that our biosafe IOBA-HS culture medium could improve limbal epithelial cell growth from explants and cell suspensions (Lopez-Paniagua et al., 2104); however more experiments would be necessary to test this aspect. The different elapsed times to reach confluence from explants and from cell suspensions could be explained by a putatively greater presence of cells with a slow cell cycle in LPCs obtained from explants (Kim et al., 2004), which is a typical characteristic of LESCs (Cotsarelis et al., 1989). However, we think that this difference is correlated with the time required for cells to migrate from the limbal explants to the plastic plates (9 days), a period of time that is not necessary for the cell suspension protocol.

The number of cells present in confluent LPCs was higher in the ones generated from cell suspensions. In agreement with this, the size of the cells was significantly smaller in the confluent LPCs established through cell suspensions than those obtained from explants. This is consistent with a report by Kim et al. who found that LPC cells from cell suspensions were smaller than those derived from explants (Kim et al., 2004), suggesting a higher number of LESCs in these LPCs (Budak et al., 2005; Schlotzer-Schrehardt and Kruse, 2005; Zhou et al., 2001).

Cell ultrastructure was analyzed by TEM. Through this technique, we observed the similarity of cell morphology in LPCs derived from explants and from cell suspensions. The cells had cytoplasm containing few mitochondria and ribosomes, both typical characteristics of undifferentiated cells, as Schlotzer-Schrehardt and Kruse (2005) previously reported. However, the cells had nuclei with distinct nucleoli, high quantity of intermediate filaments, and desmosomes, which are common features of differentiated cells. These characteristics suggest that the LPCs generated from explants and from cell suspensions contained a cell population rich in transient cells (Schlotzer-Schrehardt and Kruse, 2005).

LPC cells had a typical epithelial cell morphology when they were established through both cell isolation techniques, expressing specific epithelial cell markers such as keratins (Moll et al., 2008) along with the absence of endothelial (PECAM) (Ma et al., 1999), melanocyte (MART-1) (Higa et al., 2005), and dendritic cell (CD11C) markers (Chen et al., 2007; Mayer et al., 2007). In contrast, LPCs obtained through both cell isolation techniques expressed the S100A4 marker that is specific for fibroblasts (Ryan et al., 2003). This marker was detected in greater amounts in LPCs generated from explants, probably because fibroblasts could migrate from the stroma present in the explants to the substratum during cultivation. In agreement, Mariappan et al. showed that LPC cells obtained from explants and cell suspensions expressed other specific cell stromal markers, such as nestin and vimentin (Mariappan et al., 2014). However, they also reported the expression of melanocyte markers in these LPCs, concluding that both isolation techniques generated primary cell cultures containing both epithelial and other cells from the limbal niche (Mariappan et al., 2014). With our results, we postulate that both techniques enable the establishment of LPCs mainly composed of epithelial cells that coexist with limbal stromal fibroblasts (S100A4-positve cells), maintaining *in vitro* the native interactions between LESCs and stromal cells. This interaction likely supports LESC growth without the presence of a 3T3/J2 fibroblast feeder layer. In addition, our data suggest that disruption of cell-cell interactions that occurs in the limbal tissue cell suspension technique is not an impediment for effective LESC culture.

We also analyzed LESCs and corneal epithelial cell markers. The expression of LESC proteins (K14, K15, ABCG2, p63α) was similar or higher in LPCs established through cell suspensions than from explants. These results suggest that the LPCs originating from cell suspensions have a greater number of cells with the LESC phenotype than those established from explants. Consistent with our results, ~~firstly~~, Zhang et al. showed that rabbit limbal cells cultured from cell suspensions expressed more deltaNp63 protein than LPCs established from limbal explants (Zhang et al., 2005). Subsequently, González et al. reported that the percentage of p63α positive cells was higher in human LPCs obtained from cell suspension than from explants (Gonzalez and Deng, 2013).

However, Kim et al. and Mariappan et al. reported that the protein expression of p63 was similar in human LPCs obtained by both cell isolation techniques (Kim et al., 2004; Mariappan et al., 2014). These contradictory results could be due to the different culture conditions used in each study. In addition, these two research groups analyzed all of the p63 protein isoforms (Kim et al., 2004; Mariappan et al., 2014) while Zhang et al., González et al., and our research group specifically studied the expression of deltaNp63 or p63α isoforms. On the other hand, we have not observed significant differences in either the relative mRNA expression or in the percentage of cells positive for the corneal epithelial cell markers K3 and K12 between the LPC established from explants and cell suspensions, in accordance with previous results reported by several authors (Gonzalez and Deng, 2013; Kim et al., 2004).

The results obtained during the cell characterization suggest that LPCs established from both isolation techniques had a heterogeneous cell population with differentiated and undifferentiated cells. In addition, looking at the percentage of cells that expressed LESCs and corneal epithelial cell proteins, it is clear that, at least in the LPCs established from cell suspensions, some cells expressed both types of cell markers at the same time. These results suggest the presence of transient cells, which is consistent with the results obtained by electron microscopy.

To analyze the cell clonogenicity in the LPCs established from explants and cell suspensions, we studied the CFE and the cell capacity to form holoclones, meroclones, and paraclones (Barrandon and Green, 1987). We observed that the CFE was significantly higher in LPCs established from cell suspensions (8%) than from explants (1%), showing the presence of more functional cells in LPCs establish from cell suspension technique. These results are in agreement with the higher expression of LESC markers in LPCs obtained from cell suspensions because the LESCs have more proliferative and clonogenicity capacity than differentiated corneal epithelial cells (Ebato et al., 1988; Pellegrini et al., 1999). The CFE in our cultures, mainly those established from cell suspensions, was similar to that in LPCs expanded on 3T3/J2 fibroblasts with a culture medium supplemented with FBS and cholera toxin (Notara et al., 2007; Notara et al., 2010b). On

the other hand, we observed that all colonies from confluent LPCs established through explants were paraclones, while this percentage decreased to 90% when the LPC were obtained from cell suspensions. The percentage of meroclones established from LPCs obtained from cell suspension was 10%, while none of the culture conditions studied preserved holoclone capacities. Pellegrini et al. showed that the majority of the clones, 70%, established from LPCs cultured from cell suspensions were classified as meroclones, while holoclones and paraclones constituted 20% and 10%, respectively (Pellegrini et al., 1999). Notara et al. also reported a high percentage of meroclones from LPCs obtained from cell suspensions (Notara et al., 2010b). However, both studies used limbal tissues from young donors and 3T3/J2 fibroblasts as feeders. Because several authors have reported an increased trend in growth potential of limbal tissues with decreasing donor age (James et al., 2001; Notara et al., 2012; Vemuganti et al., 2004), we think that the use of young tissues could have increased the number of holoclones grown. In addition, we think that the use of 3T3/J2 fibroblasts as the feeder layer could have increased the number of holoclones grown because fibroblasts stimulate the maintenance of undifferentiated cells *in vitro* (Chen et al., 2011; Espana et al., 2003). Moreover, some research groups have reported that in the absence of 3T3/J2 fibroblasts, cell suspensions produced no colony growth (Gonzalez and Deng, 2013). However, this was not the case in our study, perhaps because we used a new culture medium composition that promotes *in vitro* LESC cultivation (Lopez-Paniagua et al., 2014). On the other hand, Pellegrini et al. (1999) cultured cells only from the superior limbal region, increasing in this way the possibility of culturing LESCs *in vitro* (Notara et al., 2012; Utheim et al., 2009).

Our results show that LPCs obtained from cell suspensions had a mixture of differentiated and functional transient cells (Table 3). In addition, we have demonstrated that under our culture conditions, the LPC cells established from cell suspensions had more LESCs and transient cell characteristics than when derived from explant cultures (Table 3).-This is in accordance with the smallest cell size, the highest expression for LESC protein markers (K14, K15, ABCG2, and p63α), and the highest cell clonogenicity observed in the LPCs obtained from cell suspensions (Table 3).

We hypothesize that a small number of LESCs could remain in the tissue after limbal explant cultivation (Li et al., 2007; Lopez-Paniagua et al., 2013), and that these stem cells would need more time to migrate from the limbal explant to the substratum. However, we acknowledge that longer explant cultivation times could increase the presence of fibroblasts in the LPCs (Lopez-Paniagua et al., 2013).

On the whole, our results suggest that LPCs obtained from cell suspensions are likely to have a higher clinical success rate than LPCs established through explants. Previously, Shimazaki et al. (2007) reported a corneal epithelialization success rate of 50% for transplants derived from explants cultures. This rate was increased to 73% when cultures were established through cell suspensions (Shimazaki et al., 2007). This difference could be explained by the difference in the underlying culture conditions between the two groups. On the other hand, Nakamura et al. used both techniques and observed that both methods achieved 100% success (Nakamura et al., 2006). More recently, different groups reported success rates of 60% – 80% based on expansion from explants (Rama et al., 2010; Ramirez et al., 2015; Zakaria et al., 2014) and from cell suspensions (Shortt et al., 2008). Currently, it is very difficult to establish which culture method is the most successful in terms of clinical outcomes because of the different protocols that have been used and the different disease etiologies (Baylis et al., 2011; Zhao and Ma, 2015). To clarify this question, Rama et al. (2010) established that a LPC should be considered suitable for ocular surface treatment when more than 3% of cultured cells are positive for the p63 protein marker. From this point of view, our LPCs established through the cell suspension technique would be suitable for LSCD treatment.

5. CONCLUSION

In conclusion, we report that under our culture conditions LPCs established from cell suspensions have a cell population richer in functional LESCs than when produced by the explant culture technique. These results suggest that in a clinical situation in which it is possible to choose

between either of the two isolation techniques from the donor limbal tissue, then the cell suspension is probably the best option as long as the cells are expanded according to our culture conditions.

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8. FIGURE LEGENDS

Figure 1. Cell size and morphology in LPCs established from explants and cell suspensions.

(A) The cell size was calculated measuring the major and minor cell diameters. The cells were significantly larger in the LPCs obtained from explants. Values were expressed as means ± standard error of the means (SEM), n=15. (B) Representative images of confluent LPCs from explants or cell suspensions taken by contrast phase microscopy (n=15). Scale bar: 100 μm. The cell morphology was homogeneous and cuboidal in both culture conditions. (C - J) Representative micrographs of LPCs generated from limbal explants and cell suspensions taken from transmission electron microscopy (n=2). Scale bar: (C, E, F, G, I, and J) 4 μm or (D and H) 400 nm. There were no differences between cells obtained from limbal (C - F) explants and cells retrieved from (G - J) cell suspensions. (C and G) well defined nucleoli and primary lysosomes, (D and H) desmosomes, and (E and I) intermediate filaments were present. (F and J) LPC cells derived by both techniques had microvilli. Abbreviations: LPC, limbal primary culture; Ø, diameter. ***$p \leq 0.001$.

Figure 2. LESC marker expression in LPCs established from explants and cell suspensions.

(A) The relative mRNA expression of *K14* and *ABCG2* was similar for both techniques, while *K15* was more highly expressed in LPCs generated from cell suspensions and *p63α* was more highly expressed in LPCs generated from limbal explants. These differences were statistically significant (n=6). (B, C) The percentage of positive cells for K14, K15, and ABCG2 proteins was higher in the

LPCs obtained from cell suspensions; however, these differences were only significant for K14 and K15 (n=4). The p63α protein was only detected in the LPCs generated from cell suspensions (n=4). (C) Representative immunofluorescence microscopy images for K14, K15, ABCG2, and p63α proteins in LPCs generated from limbal explants and single cell suspensions. Scale bar: 50 µm. K14, K15, and ABCG2 protein expression are shown in green, while p63 is shown in yellow (red and green fluorescence co-localization). Nuclei were counterstained with propidium iodide (red). Abbreviations: LPC, limbal primary culture; LESC, limbal epithelial stem cells. All values were expressed as means ± standard error of the means (SEM). *$p \leq 0.05$.

Figure 3. Corneal epithelial cell marker expression in LPCs established from explants and cell suspensions. (A) The relative mRNA expression of *K3* and *K12* was higher in the LPCs established from cell suspensions, although the differences were not statistically significant (n=6). (B, C) On the other hand, the percentage of positive cells for K3 and K12 markers was similar in the LPCs obtained by each technique (n=4). (C) Representative images of immunofluorescence assays to detect K3 and K12 proteins (green fluorescence) in LPCs established from limbal explants and single cell suspensions. Scale bar: 50 µm. Nuclei were counterstained with propidium iodide (red). Abbreviations: LPC, limbal primary culture. All values were expressed as means ± standard error of the means (SEM).

Figure 4. Detection of non-epithelial cells in LPCs established from explants and cell suspensions. (A) The relative mRNA expression of *S100A4* was significantly higher in LPCs derived from explants (n=6). (B, C) The S100A4 protein was detected in cells derived by both cell isolation protocols (n=4). (C) Specific proteins for endothelial cells (PECAM), melanocytes (MART-1), and dendritic cells (CD11c) were not detected in the LPCs obtained by either cell isolation protocols (n=4). (C) Representative images of immunofluorescence microscopy assays for PECAM, MART-1, CD11c, and S100A4 proteins (green fluorescence). Scale bar: 50 µm. Nuclei

were counterstained with propidium iodide (red). Abbreviations: LPC, limbal primary culture. All values were expressed as means ± standard error of the means (SEM). ***p≤0.001.

Figure 5. Cell proliferation marker Ki67 expression and colony forming efficiency (CFE) in LPCs established from explants and cell suspensions. (A) The percentage of cells positive for Ki67 protein was similar for both cell isolation techniques (n=4). (B, C) The CFE was significantly higher in the LPCs established from cells suspension (n=8) than from explants (n=5). (C) Representative images of colonies fixed and stained with rhodamine B. Abbreviations: LPC, limbal primary culture. All values were expressed as means ± standard error of the means (SEM). *p≤0.05.

Figure 6. Clonal analysis of LPCs established from explants and cell suspensions. (A) The cells from confluent LPCs obtained from explants generated only paraclones (100%), while the cells from confluent LPCs established from cell suspension originated paraclones and meroclones. Values were expressed as means ± standard error of the means (SEM), n=4. (B) Paraclone obtained from a LPCs derived from an explant. (C) Paraclone and (D) meroclone obtained from LPCs generated from cell suspensions. (E) Colonies formed from a paraclone found in a LPC established from explants. Colonies formed from a (F) paraclone and from a (G) meroclone found in a LPC established from the cell suspension technique. The paraclones from LPCs generated from explants originated no colonies. However, the paraclones from LPCs derived from cell suspensions enabled the generation of new colonies, although all of them were terminal. (B, C, and D) Representative phase contrast microscopy images. Scale bar: 500 μm. (E, F, and G) Representative images of colonies fixed and stained with rhodamine B. Abbreviations: LPC, limbal primary culture. *p≤0.05; ***p≤0.0001.

9. FIGURES

Figure 1

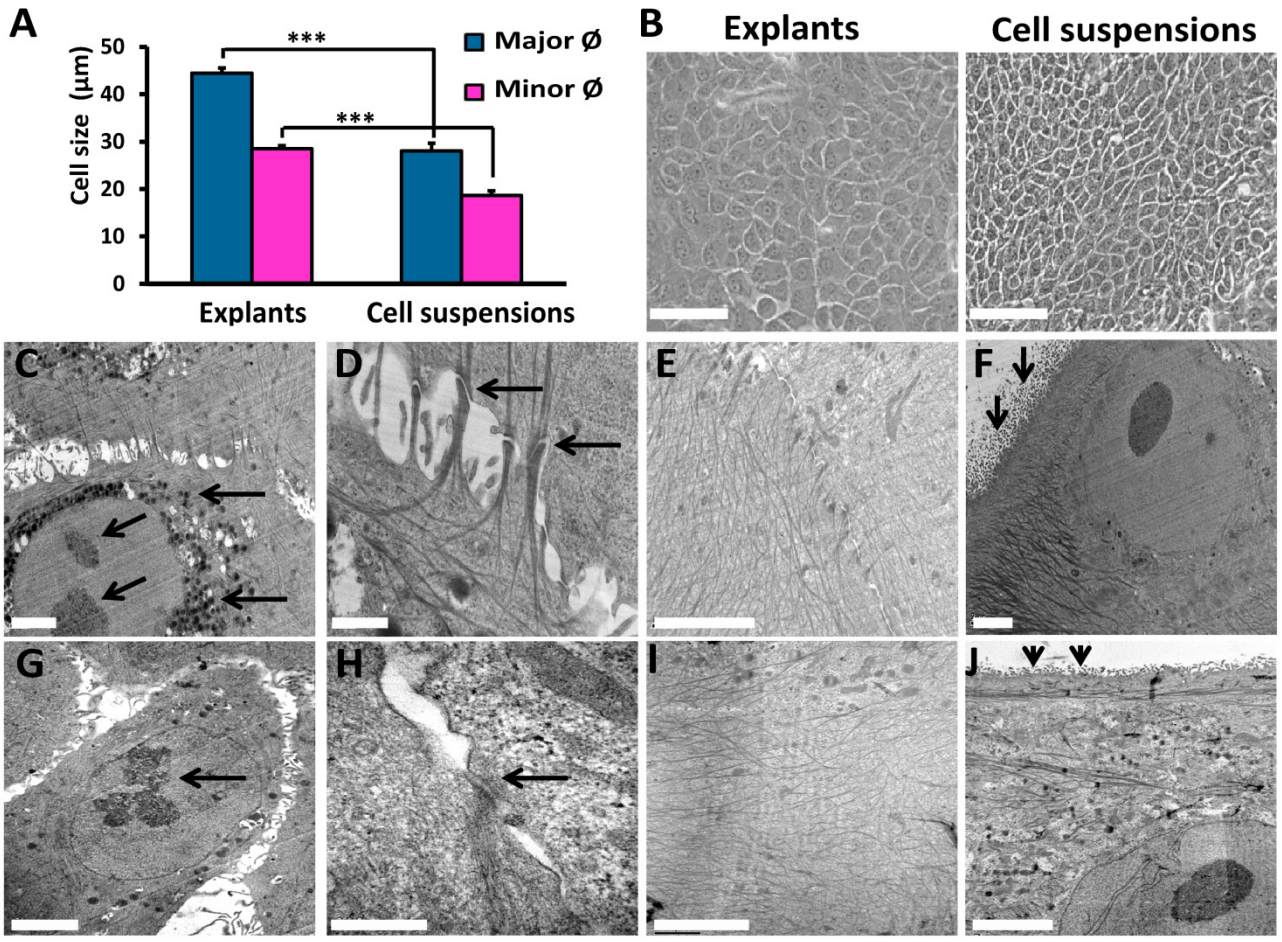


Figure 2

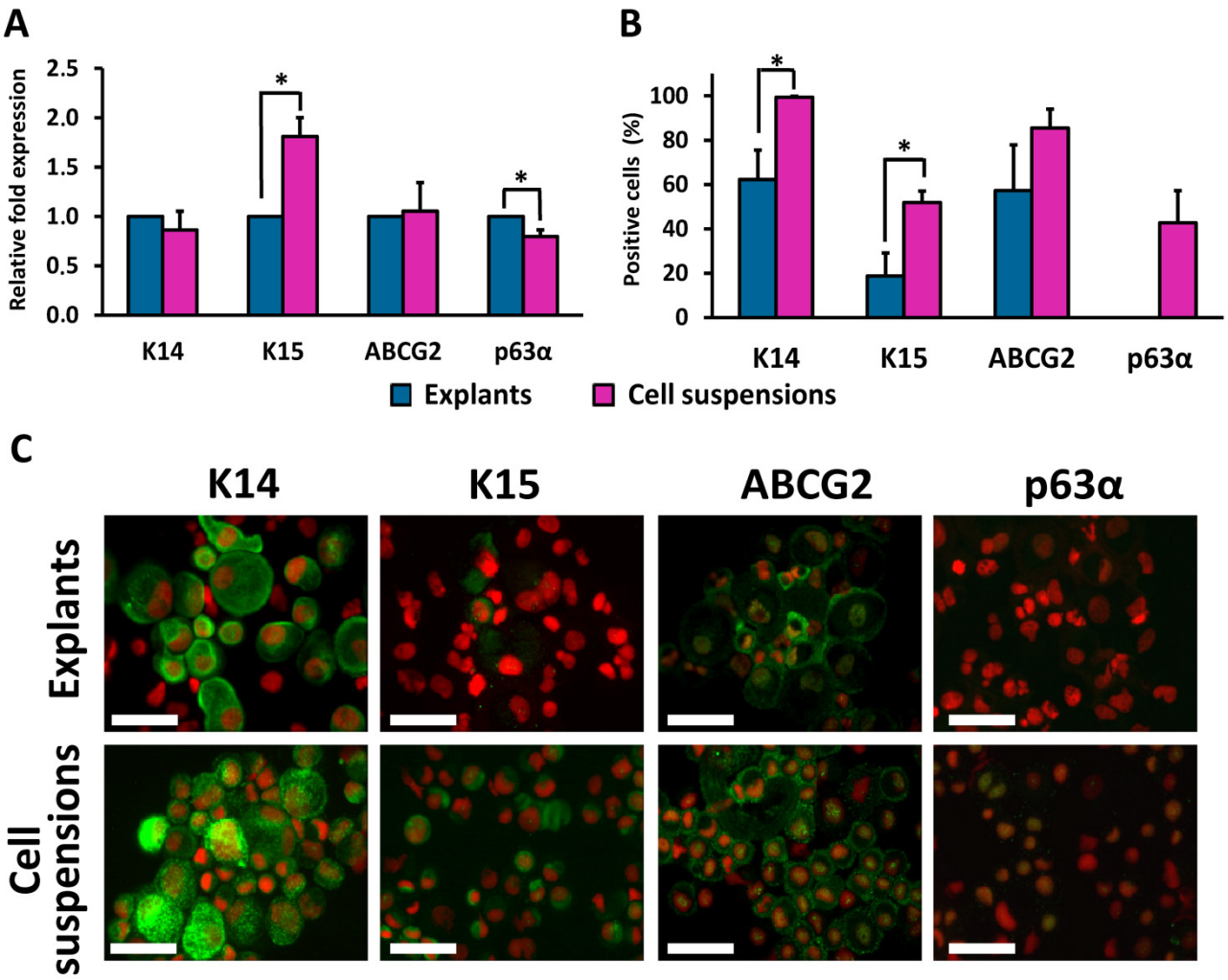

A
Relative fold expression
2.5
2.0
1.5
1.0
0.5
0.0
*
*
K14
K15
ABCG2
p63α
B
Positive cells (%)
100
80
60
40
20
0
*
*
K14
K15
ABCG2
p63α
Explants
Cell suspensions
C
K14
K15
ABCG2
p63α
Explants
Cell suspensions

Figure 3

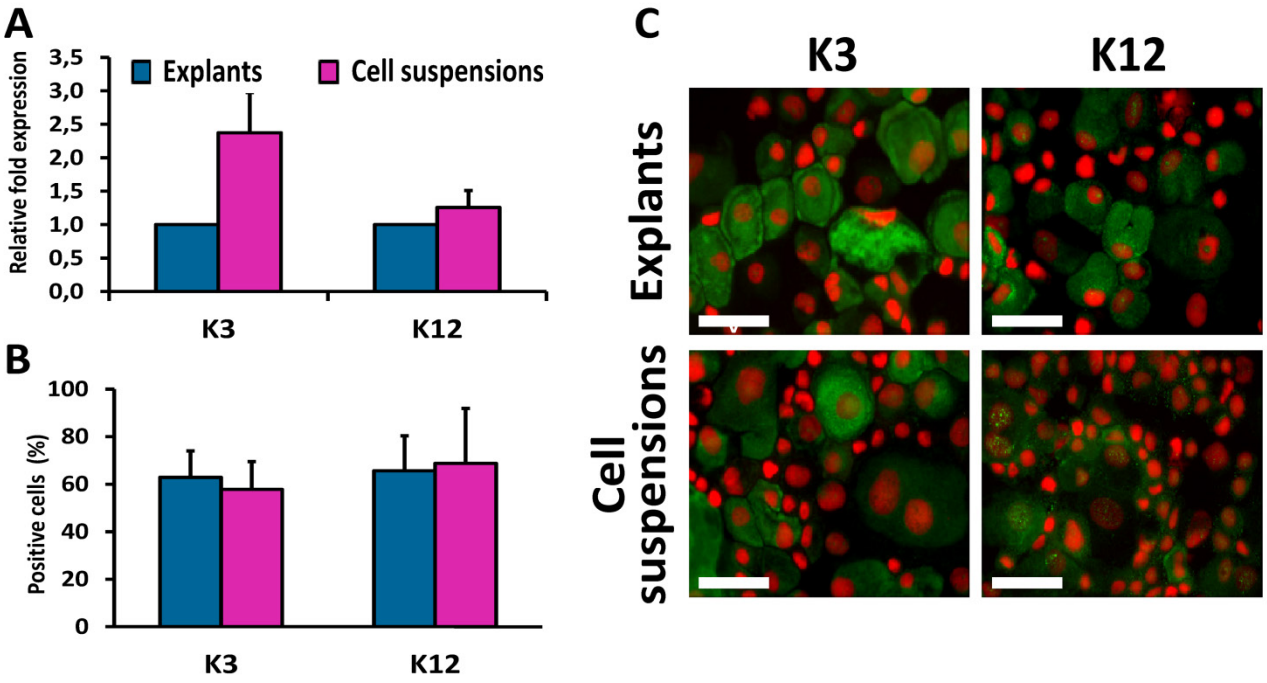

A
Relative fold expression
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Explants
Cell suspensions
K3
K12
B
Positive cells (%)
100
80
60
40
20
0
K3
K12
C
K3
K12
Explants
Cell suspensions

Figure 4

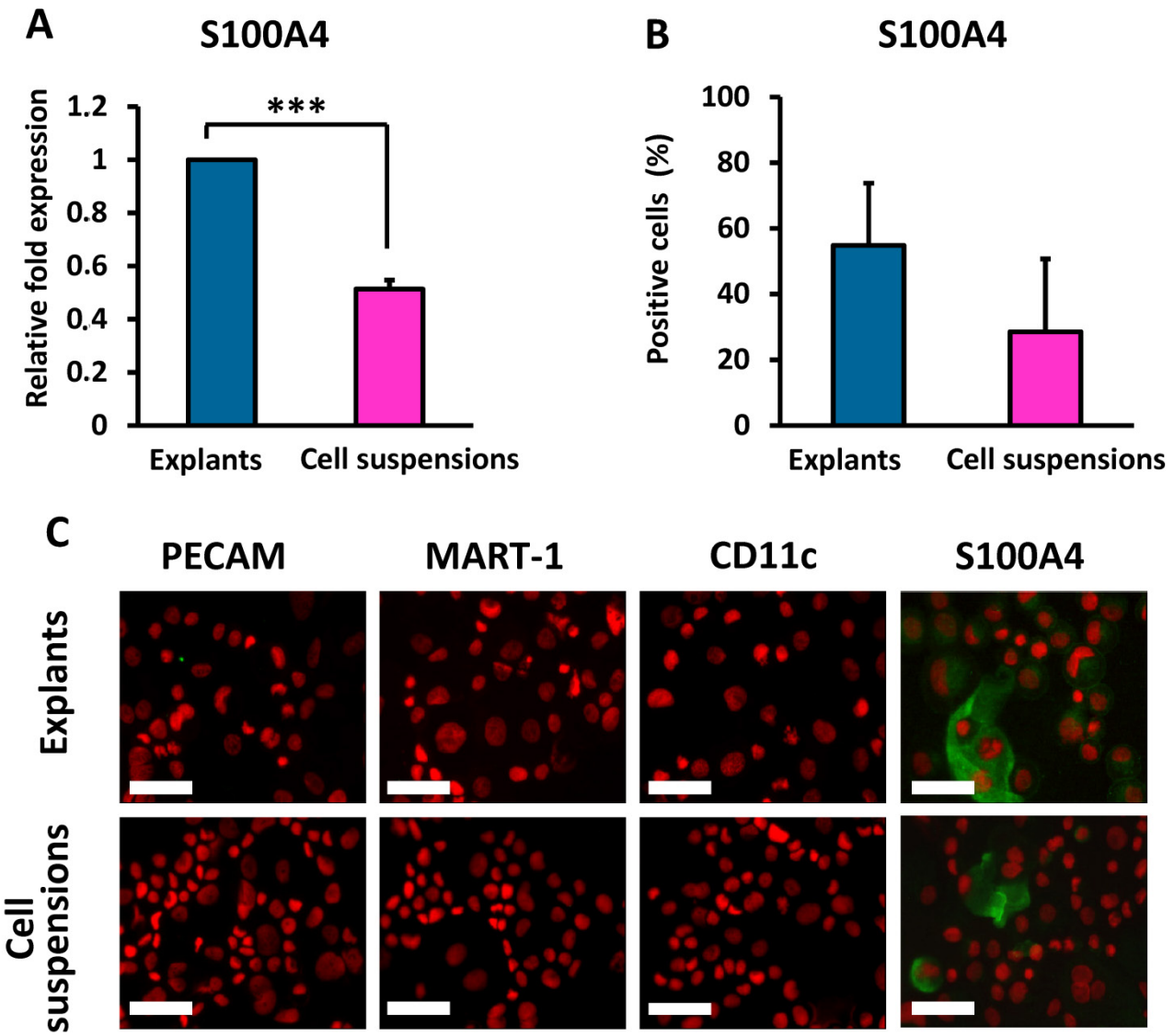

A
S100A4
Relative fold expression
1.2
1
0.8
0.6
0.4
0.2
0
***
Explants
Cell suspensions
B
S100A4
Positive cells (%)
100
80
60
40
20
0
Explants
Cell suspensions
C
PECAM
MART-1
CD11c
S100A4
Explants
Cell suspensions

Figure 5

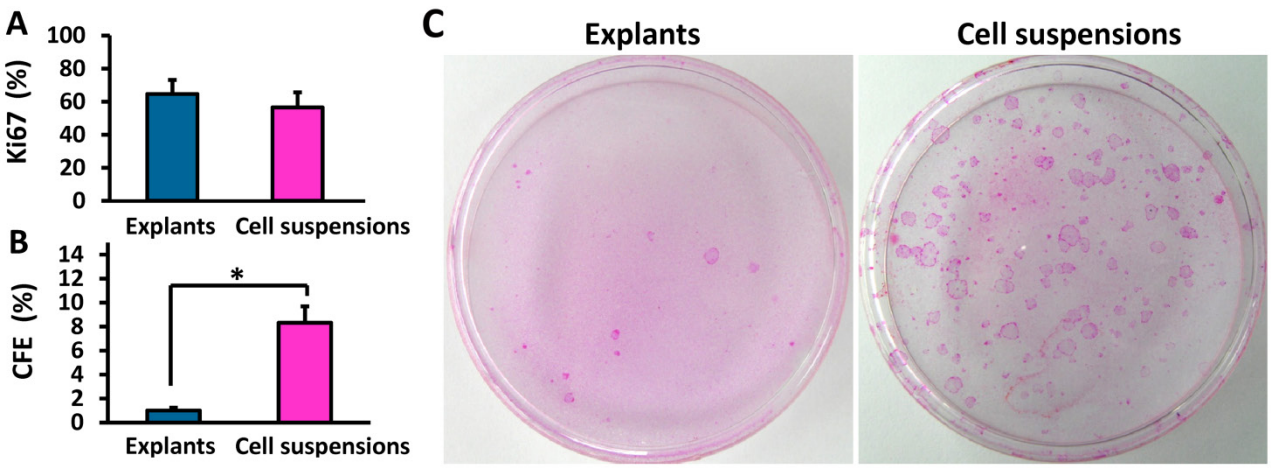
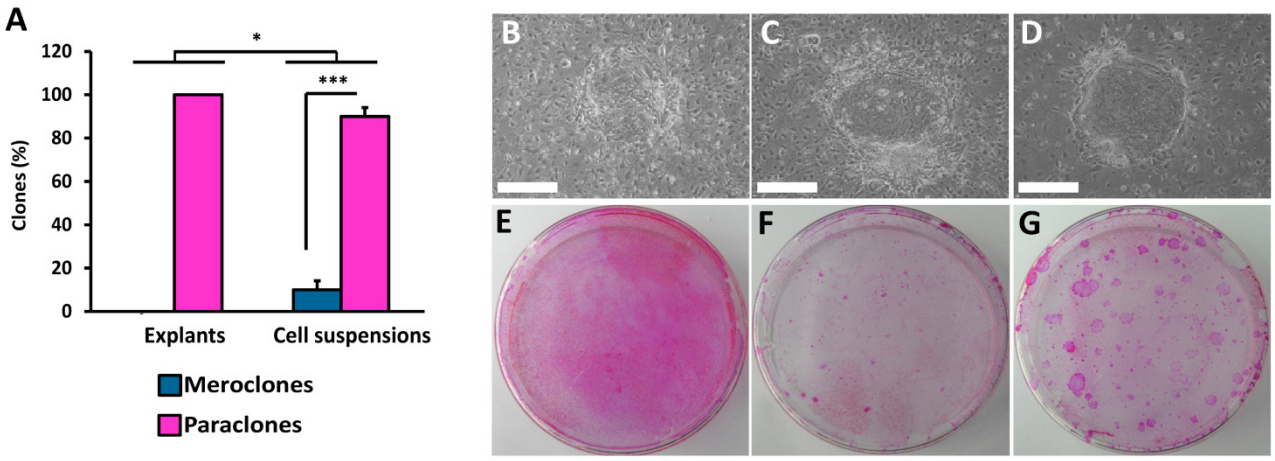

A
Ki67 (%)
100
80
60
40
20
0
Explants
Cell suspensions
B
CFE (%)
14
12
10
8
6
4
2
0
*
Explants
Cell suspensions
C
Explants
Cell suspensions

Figure 6


A
120
100
80
60
40
20
0
Clones (%)
*
***
Explants
Cell suspensions
Meroclones
Paraclones
B
C
D
E
F
G

TABLES

Table 1. Limbal primary cultures (LPC) generated from different cell isolation techniques.

Characteristics	Explants	Cell suspensions	*p*-value
Number of donors	18	19	-
Donor age (years)	70.5 ± 5.4	73.8 ± 5.2	p>0.05
Time of limbal tissue storage (days)	5.20 ± 0.65	5.06 ± 0.60	p>0.05
Number of started LPC	208	61	-
Number of successful LPC	91	51	-
Percentage of successful LPC	40.03% ± 6.08%	94.73% ± 5.26%	p≤0.001
LPC generation time (days)	19.61 ± 0.79	13.10 ± 0.69	p≤0.001
Total number of cells in confluent LPC	177,525 ± 8,805	283,134 ± 22,059	p≤0.001
Cell viability in confluent LPC	93.99% ± 1.50%	94.80% ± 0.99%	p>0.05

Time of limbal tissue storage: the elapsed time between donor death and the onset of cell expansion.
Successful LPCs: LPCs that reached more than 80% confluence.
LPC generation time: the elapsed time from limbal explant or cell suspension plating to LPC confluence.

Table 2. Results from clonal analysis: holoclones, meroclones, and paraclones.

	Donor	Trypsinized colony size (µm)	*	Holoclone	Meroclone	Paraclone
Limbal explants	**1**	822 ± 136	0/10	0%	0%	100%
	2	942 ±100	1/10	0%	0%	100%
	3	586 ± 62	0/10	0%	0%	100%
	4	688 ± 68	0/10	0%	0%	100%
	Mean ± SEM	760 ± 78	0.2/10	0%	0%	100%
Cell suspensions	**1**	753 ± 54	3/10	0%	20%	80%
	2	852 ± 104	3/10	0%	10%	90%
	3	904 ± 58	3/10	0%	10%	90%
	4	859 ± 74	1/10	0%	0%	100%
	Mean ± SEM	842 ± 32	2.5/10	0%	10 ± 4%	90% ± 4%

*Number of colonies that originated new colonies/number of colonies trypsinized

Table 3. Characteristics of limbal primary cultures (LPC) generated from two different cell isolation techniques: explant and cell suspensions.

Characteristics	Explants	Cell suspensions	Advantage
Confluent LPC	40%	94%	Cell suspension
Time for confluence (days)	20	13	Cell suspension
Number of cells in confluent LPC	175,000	285,000	Cell suspension
Cell viability	94%	94%	Similar
Cell morphology and size (µm)	45x28, cuboidal	28x18, cuboidal	Cell suspension
Presence of defined nucleoli, lysosomes, intermediate filaments, desmosomes, and microvilli	Yes	Yes	Similar
LESC marker expression	Minor or similar	Major or similar	Cell suspension
Corneal epithelial marker expression	Minor or similar	Major or similar	Explant
Presence of endothelial cell, melanocyte, and dendritic cell marker expression	No	No	Similar
Fibroblast marker expression	Major	Minor	Cell suspension
Proliferative cells	65%	56%	Similar
CFE	1%	8%	Cell suspension
Presence of: **• Holoclones** **• Meroclones** **• Paraclones**	 No No 100%	 No 10% 90%	Cell suspension

CFE: Colony forming efficiency