Tittle: **Successful consecutive expansion of limbal explants using a biosafe culture medium under feeder layer-free conditions**

Marina López-Paniagua,[1,2] Teresa Nieto-Miguel,[2,1] Ana de la Mata,[1,2,a] Sara Galindo,[1,2] José M. Herreras,[1,2] Rosa M. Corrales,[2,1,b] Margarita Calonge[1,2]

1. IOBA (Institute of Applied Ophthalmobiology), University of Valladolid, Valladolid, Spain.

2. CIBER-BBN (Networking Research Center on Bioengineering, Biomaterials and Nanomedicine), Valladolid, Spain.

a. Present address: Department of Physiology and Membrane Biology, University of California, Davis, CA, USA.

b. Present address: Ocular Surface Diagnostic Innovations, LLC "OSD Innovations" (a Point Guard Partners subsidiary), Tampa, FL, USA.

Dual Corresponding Authors: Margarita Calonge and Marina López-Paniagua, IOBA, Universidad de Valladolid, Campus Miguel Delibes, Paseo de Belén 17, E-47011, Valladolid, Spain. Phone: +34-983-184750. Fax: +34-983-184762. E-mail: calonge@ioba.med.uva.es and marina@ioba.med.uva.es. http://www.ioba.es

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ABSTRACT

Purpose: Transplantation of *in vitro* cultured limbal epithelial stem cells (LESCs) is a treatment widely used for LESC deficiency. However, the number of limbal tissue donors is limited, and ~~the~~ protocols for LESC cultivation often include compounds and/or feeder layers that can induce side effects and/or increase the cost of the culture procedure. We investigated the feasibility of obtaining more than one limbal primary culture (LPC) from the same biopsy using a culture medium in which several potentially harmful compounds were replaced at the same time by biosafe supplements, allowing the LESC cultivation without feeder layers.

Materials and Methods: We established feeder layer-free LPCs with three culture media: (1) a modified supplemental hormonal epithelial medium (mod-SHEM), containing potential harmful components (cholera toxin, DMSO, and FBS), (2) IOBA-FBS, a medium with FBS but with no other harmful supplements, and (3) IOBA-HS, similar to IOBA-FBS but with human serum instead of FBS. Additionally, the same limbal explant was consecutively cultured with IOBA-HS producing three cultures. LPCs were characterized by real time RT-PCR and/or immunofluorescence.

Results**:** LPCs cultured with the three media under feeder layer-free conditions showed cuboidal cells and no significant differences in the percentage of positive cells for limbal (ABCG2, p63, and K14) and corneal (K3, K12) proteins. Except for *ABCG2,* the relative mRNA expression of the LESC markers was significantly higher when IOBA-FBS or IOBA-HS was used. LPC1 showed characteristics similar to LPC0, while LPC2 cell morphology became elongated and the expression of some LESC markers was diminished.

Conclusion: IOBA-HS enables the culturing of up to two biosafe homologous LPCs from one limbal tissue under feeder layer-free conditions. The routine use of this culture medium could

improve both the biosafety and the number of available LPCs for potential clinical transplantation, as well as decrease the expense of the culture procedure.

INTRODUCTION

The corneal epithelium is constantly renewed by a population of stem cells located in the basal layer of the corneoscleral-limbal epithelium, at the junction between the cornea and the sclera-conjunctiva. These limbal epithelial stem cells (LESCs) are characterized by slow cell cycle, absence of differentiation markers, and high proliferative potential (reviewed by [1-3]). LESC loss or dysfunction causes a pathology called LESC deficiency, mainly characterized by chronic inflammation, decreased vision, and recurrent episodes of epithelial breakdown which results in pain.[4] The cultured limbal epithelial transplantation is the treatment more widely used at present for LESC deficiency.[5,6] During the expansion procedure, LESCs are isolated from healthy limbal tissues and grown on a substratum to produce a sheet of cultured limbal epithelial cells that is suitable for transplantation onto the damaged ocular surface (reviewed by [2]). Although this procedure has been a great advance in the treatment of patients suffering from LESC deficiency, unfortunately the protocols used for *in vitro* LESC cultivation often include compounds and/or feeder layer that could induce zoonosis and/or side effects, compromising the potential clinical use of the limbal primary cultures (LPCs) obtained.

Examples of culture media components that could present problems for clinical use include cholera toxin, dimethylsulfoxide (DMSO), and fetal bovine serum (FBS). Cholera toxin is a supplement commonly used to increase *in vitro* LESC proliferation;[7] however, its use is controversial because there remains a theoretical risk to transmit disease of cholera and the availability is limited in several countries due to biological controls.[8] DMSO, used as a free radical scavenger, is another problematic component. DMSO can induce apoptosis in cultured human corneal epithelial cells and can have side effects in patients when it is used in different clinical applications.[9-11] FBS, used at

high concentration (10 – 20%), possesses proliferative and anti-differentiation properties that are helpful in establishing LPCs;[12] however, as a component derived from animal origin, it carries the potential to induce zoonosis, such as spongiform encephalopathy.[8] On the other hand, fibroblasts from animal origin are often used as feeder layer for LESCs cultivation, increasing the risk to induce zoonosis.[8] In addition, the use of feeder layers entails additional steps in the cell cultivation protocol, thus increasing the expense and consequently decreasing the number of potential candidate transplants.

In an effort to improve the biosafety and the expense of protocols used for human *in vitro* LESC culture, several research groups have recently reported different culture media compositions or substratums.[13-18] However, the removal of cholera toxin, DMSO, FBS, and feeder layer from protocols could cause a decrease of cell proliferation and compromise the balance of cell oxidative stress, decreasing the number of successful LPCs and the molecular properties of cells.

An additional problem for the preparation of *in vitro* cultured LESCs for transplantation is the limited number of limbal tissue donors available. Our research group has recently reported a protocol that provides several homologous LPCs from single human limbal explants to overcome this limitation.[19] However, the protocol was developed using a non-biosafe culture medium.

Here, we report a culture medium in which cholera toxin, DMSO, and FBS were replaced at the same time by biosafe components with similar effects. This new culture medium composition, lacking animal serum and any other potentially harmful supplements for human use, enables the culturing of up to two homologous successful biosafe LPCs from a single limbal tissue under feeder layer-free conditions, a fact that has never been previously published.

MATERIALS and METHODS

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

Materials and reagents

Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mixture (DMEM/F12), gentamicin, amphotericin B, FBS, 0.25% trypsin with 1 mM ethylenediaminetetraacetic acid (EDTA), Quant-iT RNA Assay Kit, SuperScript® VILO™ cDNA Synthesis Kit, propidium iodide, and fluorescein Alexa Fluor® 488 donkey anti-mouse and donkey anti-rabbit antibodies were purchased from Life Technologies (Carlsbad, CA, USA). Epidermal growth factor (EGF), insulin, transferrin, hydrocortisone, sodium selenite, adenine, DL-isoproterenol hydrochloride, 3, 3′, 5-triiodothyronine, and dimethylsulfoxide (DMSO) were from Sigma-Aldrich (St. Louis, MO, USA). Tissue-Tek-OCT compound was from Sakura Tissue Tek® (Torrance, CA, USA). Formaldehyde and methanol were purchased from Panreac (Lyon, France). RNeasy® Mini Kit and RNase-Free DNase were from Qiagen (Valencia, CA, USA). Trephines and HS were from Katena (Denville, NJ, USA) and Lonza (Basel, Switzerland), respectively. Cholera toxin was purchased from Gentaur (Kampenhout, Belgium). Polystyrene culture dishes and eight-well Permanox chamber slides were purchased from Nunc (Roskilde, Denmark).

Human tissue preparation

Healthy human corneoscleral tissues from deceased donors were obtained from the Barraquer Eye Bank (Barcelona, Spain). Mean ± standard error of the mean donor age was 81.8 ± 1.8 years (range 68-97 years). The samples were preserved in supplemented culture medium[18] an average of 3.2 ± 0.2 days from donor death. There were no significant differences in donor age or in the time of limbal tissues storage between different groups.

Human skin and tonsil tissues were obtained from a certified Tissue Bank (León, Spain). Skin, tonsil, and corneoscleral tissues for immunofluorescence microscopy were fixed in 4% formaldehyde, embedded in Tissue-Tek OCT compound, and stored at -80ºC. Cryosections of 5 µm were prepared.

LPCs

LPCs were prepared from human corneoscleral buttons using a previously described method.[19] Briefly, excess sclera, conjunctiva, iris, and corneal endothelium were removed, and the central cornea was extracted with a 7.5 mm trephine. The remaining corneoscleral rings were cut into 1-2 mm^2 limbal explants that were plated singly with the epithelium side up into 3.8 cm^2 polystyrene wells. After 30 min in a laminar flow hood, each was incubated with 50 µl of FBS or HS (depending on the culture medium in which the LPCs were to be incubated, see Table 1) overnight at 37ºC, 5% CO_2, and 95% humidity. After that, the limbal explants were cultured in three different culture media: modified supplemental hormonal epithelial medium (mod-SHEM), IOBA-FBS, and IOBA-HS (Table 1). The mod-SHEM,[19] a variant of SHEM previously used by other authors for limbal epithelial stem cell cultivation,[20-23] was used as a control. The number of limbal explants cultured from different human cadaveric donors was 190 (13 donors) in mod-SHEM, 82 (8 donors) in IOBA-FBS, and 107 (10 donors) in IOBA-HS.

Limbal explants incubated with IOBA-HS were consecutively cultured following the protocol previously described by our group.[19] Briefly, each limbal explant was removed from the culture dish when the cell outgrowth surrounded it, and the LPC was allowed to reach confluence (LPC0). The removed limbal explant was then plated again in a new culture dish to produce LPC1, and finally once more to produce LPC2.

Morphology and cell growth

Cell outgrowth from the limbal explants was monitored under a phase contrast microscope (Eclipse TS100, Nikon, Tokyo, 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 limbal explants plated. The elapsed time from limbal explant plating to LPC confluence (LPC generation time) was analyzed. 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 three or five selected cells. Three different LPCs were analyzed for each condition (n=3).

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.[19] Briefly, confluent LPCs were incubated with RNA lysis buffer. 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). 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). Subsequently, RT^2-PCR assays were performed from 20 ng of cDNA in a 7500 Real Time PCR System using oligonucleotide primers and Taqman® probes (Table 2). Assays were performed in duplicate. A non-template control was included in all experiments, and the *GAPDH* 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. LPCs cultured in mod-SHEM served as the calibrator controls and had an assigned value of 1 when LPCs cultured in IOBA-FBS and IOBA-HS culture media were compared. For IOBA-HS LPC1 and LPC2 cultures, LPC0 served as the calibrator control. 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 limbal explants (from at least three different donors) were analyzed for each condition (n=6).

Immunofluorescence microscopy

Confluent LPCs were incubated in 0.25% trypsin with 1 mM EDTA for 5 min at 37ºC and then incubated in eight-well Permanox chamber slides (12,500-25,000 cells/cm^2) overnight. Subsequently, LPC cells were fixed with cold methanol for 10 min at -20ºC. Immunofluorescence assays were performed following a previously reported protocol.[19] In brief, cryosections (5 µm) of fixed human tissues and fixed LPC cells were permeabilized (except for antibody anti-PECAM incubation), blocked, and incubated overnight at 4ºC with specific primary antibodies (Table 2). After incubation with the primary antibodies, samples were incubated 1 hour at room temperature with the corresponding secondary antibody (Alexa Fluor® donkey anti-mouse or donkey anti-rabbit). Propidium iodide was used to counterstain the cell nuclei.

Images were acquired with an inverted fluorescence microscope (DM4000B, Leica, Wetzlar). 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. Human tonsil (for anti-PECAM) and human skin (for anti-MART-1 and anti-CD11c) cryosections were used as positive controls to validate the antibodies. In addition, ABCG2, p63, K14, K15, K3, K12, and S100A4 antibodies were previously validated in human ocular tissues or cultured limbal fibroblasts by our research group.[19] Four LPCs from different limbal explants (from at least three different donors) were analyzed for each condition (n=4).

Statistical analysis

Statistical significance was determined by one-way factorial ANOVA, 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. Subsequently, all of the groups were compared between them by Student's t-test for independent samples. All values were expressed as means ± standard error of the means. P-values ≤ 0.05 were considered statistically significant.

RESULTS

LPC characteristics

Successful LPCs were obtained with the three culture media tested under feeder layer-free conditions. The percentage of confluent LPCs (mod-SHEM 32.5 ± 7.5%, IOBA-FBS 25.9 ± 5.0%, and IOBA-HS 29.0 ± 8.8%) as well as the LPC generation time (mod-SHEM 26.0 ± 2.0 days, IOBA-FBS 25.8 ± 3.1 days, and IOBA-HS 29.0 ± 2.2 days) were similar among the three culture media (Figure 1A, B).

LPC cells obtained with the three culture media had a homogeneous polygonal morphology. However, LPC cells cultivated with mod-SHEM were smaller in size (25.3 ± 1.6 µm x 36.5 ± 2.0 µm) than cells incubated with IOBA-FBS (29.0 ± 1.8 µm x 53.4 ± 3.1 µm) or IOBA-HS (30.1 ± 1.4 µm x 48.9 ± 2.3 µm) (Figure 1C). Occasionally, the cell outgrowths incubated with the three different culture media were larger and more elongated than normal and had an irregular morphology (data not shown).

Characterization of non-epithelial cells in LPCs

Markers of endothelial cells (PECAM), melanocytes (MART-1), dendritic cells (CD11c), and fibroblasts (S100A4) were analyzed by immunofluorescence microscopy to evaluate possible contamination by non-epithelial cells. Firstly, the specificity of antibodies was analyzed in positive controls. PECAM protein was detected in endothelial cells of blood vessels in human tonsil, MART-1 protein was expressed by several cells (melanocytes) in human skin epithelium, and CD11c protein was expressed by cells (dendritic cells) in the stromal region of human skin tissue (Figure 1D). The expression of these protein markers was then analyzed in healthy human ocular surface tissues. Protein expression of PECAM was detected in the limbal stroma, while MART-1 and CD11c protein expression was present in limbal epithelium, specifically in the basal and

intermediate layers, respectively (Figure 1D). These proteins were not detected in corneal tissues, as we expected.

PECAM, MART-1, and CD11c protein markers were not detected in the LPCs (Figure 1E). However, the fibroblast marker S100A4 was expressed in LPCs incubated with all three culture media. The highest percentage of S100A4-positive cells was observed with IOBA-FBS (27.8 ± 10.4%), while the lowest was detected with mod-SHEM (3.3 ± 1.9%), although the differences among LPCs incubated with the three culture media were not significant (Figure 1E, F). The expression of *S100A4* mRNA was confirmed by RT^2-PCR for LPCs incubated in all three culture media. The relative expression of *S100A4* mRNA was higher when mod-SHEM was used, although significant differences were detected only when the expression levels were compared with those found in LPCs incubated with IOBA-FBS (0.33 fold, $p \leq 0.001$; Figure 1G).

LESC marker expression in LPCs

By immunofluorescence microscopy, the percentage of cells that expressed ABCG2, p63, and K14 proteins was higher in LPCs incubated with mod-SHEM (ABCG2, 43.0 ± 10.4%; p63, 34.2 ± 16.8%; K14 59.3 ± 17.1%) than in LPCs cultivated with IOBA-FBS (ABCG2, 12.0 ± 6.6%; p63, 11.2 ± 1.3%; K14, 30.6 ± 17.9%) or IOBA-HS (ABCG2, 22.5 ± 13.9%; p63, 11.0 ± 11.0%; K14, 39.8 ± 22.7%); however, there were no significant differences among the media (Figure 2A, B). In addition, the percentages of ABCG2-, p63- and K14-positive cells in LPCs cultured with IOBA-FBS and with IOBA-HS were similar (Figure 2A, B). The percentage of cells expressing the K15 protein marker was 24.1 ± 23.9% and 13.5 ± 11.4% in LPCs cultured with mod-SHEM and IOBA-HS, respectively. This marker was not detected in the LPCs cultured with IOBA-FBS (Figure 2A, B).

The relative expression of LESC mRNA markers was evaluated by RT^2-PCR. The mRNA expression levels of LPCs cultured with IOBA-FBS and IOBA-HS were compared to LPCs

incubated with mod-SHEM culture medium, which were assigned a value of 1. The relative mRNA expression of *ABCG2* was similar in LPCs incubated with the three culture media (mod-SHEM, 1.00; IOBA-FBS, 1.48 fold; IOBA-HS, 0.95 fold; Figure 2C). However, the relative mRNA expression of *p63alpha* and *K14* was significantly higher in LPCs cultured with IOBA-HS (4.09 fold and 4.69 fold, respectively) than in LPCs grown with mod-SHEM ($p \leq 0.05$, Figure 2C). On the other hand, *K15* mRNA relative expression was higher for cells cultured in IOBA-FBS (14.27 fold) or IOBA-HS (13.23 fold) compared with mod-SHEM, although the difference was significant only for the IOBA-FBS culture medium ($p \leq 0.05$; Figure 2C). In addition, there were no significant differences for the relative mRNA expression of *p63alpha*, *K14,* and *K15* among LPCs grown with IOBA-FBS (*p63alpha,* 3.77 fold; *K14*, 3.84 fold; *K15* 14.27 fold) and IOBA-HS (*p63alpha,* 4.09 fold; *K14*, 4.69 fold; *K15* 13.23 fold (Figure 2C).

Corneal epithelial cell marker expression in LPCs

By immunofluorescence microscopy, there were no significant differences in the percentage of K3-positive cells in LPCs cultured with mod-SHEM (56.2 ± 7.7%), IOBA-FBS (52.3 ± 8.7%), and IOBA-HS (68.5 ± 8.7%). Likewise, the percentage of K12-positive cells was similar in the LPCs incubated with the three culture media (mod-SHEM, 28.3 ± 4.4%; IOBA-FBS, 18.0 ± 9.2%, IOBA-HS, 27.1 ± 13.6%; Figure 3A, B).

The mRNA expression of LPCs cultured with IOBA-FBS and IOBA-HS culture media was compared to that in LPCs cultured with mod-SHEM culture medium (Figure 3C). In contrast to the presence of K3 protein, the relative expression of *K3* mRNA was significantly higher when mod-SHEM was used ($p \leq 0.05$). *K3* mRNA expression level was similar in LPCs incubated with IOBA-FBS and IOBA-HS culture media (0.38 and 0.23 fold, respectively; Figure 3C). On the other hand, the relative expression of *K12* mRNA was lower in LPCs cultured with mod-SHEM than in LPCs incubated with IOBA-FBS (2.99 fold) and with IOBA-HS (6.99 fold), although the differences were not significant (Figure 3C).

Consecutive expansion of a single limbal explant using IOBA-HS culture medium

LPC characteristics

Three confluent LPCs (LPC0 to LPC2) were obtained from consecutive expansion of single limbal explants cultured with IOBA-HS under feeder layer-free conditions (Figure 4A). The percentage of confluent LPCs increased significantly from LPC0 (29.0 ± 8.8%) to LPC1 (85.9 ± 6.8%) ($p \leq 0.01$), and then decreased in LPC2 (60.5 ± 15.2%), although not significantly (Figure 4A). The LPC total generation time was significantly higher in LPC0 (28.9 ± 2.2 days) than in the consecutive LPCs (LPC1, 17.1 ± 1.2 days, $p \leq 0.01$; LPC2, 16.9 ± 1.6 days, $p \leq 0.001$; Figure 4B).

The cell outgrowths from limbal explants had a homogeneous cuboidal morphology in LPC0 and LPC1 (Figure 4C). Quite often, cell outgrowth continued with similar morphology in LPC2, although sometimes the cell morphology was more elongated.

LESC marker expression in consecutive LPCs

The percentage of cells positive for the LESC marker ABCG2 increased significantly from LPC0 (22.5 ± 13.9%) to LPC1 (94.7 ± 2.0%). While it decreased significantly in LPC2 (58.3 ± 21.4%; $p \leq 0.05$; Figure 5A, B), it remained significantly higher in LPC2 than in LPC0 ($p \leq 0.05$, Figure 5B). p63 protein expression was only detected in LPC0 (11.0 ± 11.0%; Figure 5A, B). The percentage of K14-positive cells was similar in all the consecutive LPCs, with an average of 37.8 ± 2.3% (Figure 5A, B). The percentage of K15-positive cells increased from LPC0 (13.5 ± 11.4%) to LPC1 (29.4 ± 17%), but the difference was not significant. It then decreased to an undetectable level in LPC2 ($p \leq 0.05$; Figure 5A, B).

The mRNA expression level for LESC marker proteins in LPC1 and LPC2 was compared to that in LPC0, which was assigned a value of 1. The relative expression of *ABCG2* mRNA decreased from LPC0 to LPC1 (0.70 fold), and increased in LPC2 (1.65 fold), but the changes were not statistically significant (Figure 5C). The relative expression of *p63alpha* mRNA decreased

significantly from LPC0 through LPC2 (0.006 fold) (LPC0 *vs* LPC1, $p \leq 0.05$; LPC0 *vs* LPC2, $p \leq 0.01$; Figure 5C). The relative expression of *K14* mRNA increased from LPC0 to LPC1 (1.31 fold), but the difference was not significant. It then decreased significantly to 0.07 fold in LPC2 (LPC0 *vs* LPC2, $p \leq 0.05$; LPC1 *vs* LPC2, $p \leq 0.001$; Figure 5C). The relative expression of *K15* mRNA was similar in LPC0 and LPC1 (with an average of 1.08 fold). It then decreased to 0.04 fold in LPC2 (LPC0 *vs* LPC2, $p \leq 0.05$; LPC1 *vs* LPC2, $p \leq 0.001$; Figure 5C).

Corneal epithelial cell marker expression in consecutive LPCs

By immunofluorescence microscopy, the percentage of K3-positive cells was 68.5 ± 8.7% in LPC0. It increased to 83.4 ± 2.1% in LPC1, but the change was not significant ($p > 0.05$, Figure 6A, B). It then decreased significantly to 47.9 ± 9.3% in LPC2 ($p \leq 0.05$, Figure 6A, B). The percentage of K12-positive cells was similar in all LPCs, with an average of 29.4 ± 1.6%.

The mRNA expression level of each LPC was compared to that in LPC0. The increase in relative expression of *K3* mRNA from LP0 to LPC1 (1.7 fold) was not significant, but the decrease in LPC2 was (0.07 fold, $p \leq 0.001$, Figure 6C). The mRNA relative expression of *K12* was similar in LPC0 and LPC1 (0.9 fold), but then decreased significantly in LPC2 (0.002 fold, $p \leq 0.001$, Figure 6C).

Fibroblast marker expression in consecutive LPCs

The fibroblast marker S100A4 protein and mRNA were present in all LPCs (Figure 6). The percentage of S100A4-positive cells increased significantly from LPC0 (16.2 ± 8.6%) to LPC1 (74.0 ± 12.0%, $p \leq 0.001$), and decreased significantly in LPC2 (44.6 ± 16.5%; $p \leq 0.05$; Figure 6A, B). However, the percentage of S100A4-positive cells continued to be significantly higher in LPC2 than in LPC0 ($p \leq 0.05$, Figure 6B). The relative expression of *S100A4* mRNA decreased significantly from LPC0 to LPC2 (0.25 fold, $p \leq 0.001$, Figure 6C).

DISCUSSION

Cultured limbal epithelial transplantation has been a great breakthrough in the treatment of patients suffering from LESC deficiency [17,24-27] However, the protocols used for LESC cultivation include commonly culture media[28] and/or feeder layers[28] that can induce zoonosis and/or side effects, compromising the biosafety of the LPCs and therefore their clinical application. In addition, the use of feeder layers increases the cost of the LESC culture procedure considerably and since the availability of limbal tissue donors is restricted, the number of potential transplants can subsequently decrease.

To overcome these limitations, we propose a culture medium composition, IOBA-HS, that does not have animal serum or other potentially harmful supplements and that enables the culturing of two homologous biosafe LPCs from the same limbal tissue. A similar culture medium was recently reported by Stasi *et al*. They showed that it allowed culturing of LESCs *in vitro* using fibroblasts as support for the epithelial cells.[29] However until now, a culture medium without animal serum and other potentially harmful components has not been used for consecutive expansion of limbal explants. In addition, we tested the ability of IOBA-HS culture medium to support LESC culture without a fibroblast substratum.

The use of cholera toxin potentially enables the transmission of cholera to a patient, though the possibility is remote.[29-31] Here, we replaced the cholera toxin in the mod-SHEM by isoproterenol, a component that increases *in vitro* keratinocyte proliferation by raising the intracellular concentration of cyclic AMP, as does cholera toxin, through activation of adenylate cyclase.[7] However, the cell effect of isoproterenol is quickly reversed while cholera toxin exerts an irreversible action on the cyclase.[7,32] To supplement the action of isoproterenol, we added triiodothyronine and adenine to the IOBA-FBS and IOBA-HS culture media.[29-31,33]

DMSO can induce low viability and high levels of apoptosis in cultured corneal epithelial cells [9] as well as side effects when used in clinical applications.[11,34] Therefore, we replaced DMSO in the mod-SHEM with transferrin and selenium in our designed culture media[35,36] increasing the biological safety while not compromising the balance of oxidative stress.

Animal blood serum can induce zoonotic disease transmission. Previously, *in vitro* investigations and clinical trials have shown that FBS can be replaced by HS while maintaining the morphology, proliferative, and differentiation stages of LPC cells,[25,37] as well as the clinical outcomes of LESC transplantation.[25] Therefore, we replaced the FBS in IOBA-FBS with HS, to create the IOBA-HS composition.

For the three culture media that we tested, the human limbal explants exhibited a relatively low performance as expressed by the percentage of LPCs that reached the confluent stage. We think that this fact was most likely due to the advanced age and cadaveric origin of the tissue donors, as other authors have reported.[38,39]

LPC cells showed typical epithelial cell morphology for all three of the tested culture media under feeder layer-free conditions, expressing specific epithelial markers such as cytokeratins[40] along with the absence of endothelial (PECAM),[41] melanocytic (MART-1),[42] and dendritic cell (CD11c) markers.[43] The results suggest that these LPCs were mainly composed of epithelial cells. In contrast, LPC cells obtained with the three culture media expressed the S100A4 marker,[44] mainly when the culture medium was supplemented with FBS, and occasionally the cells had an elongated morphology, both typical characteristics of fibroblasts. In a similar way, our research group as well as others have reported sporadic fibroblast growth in LPCs obtained with other culture media.[19,37] Therefore, we believe that the composition of the new IOBA-HS culture medium does not especially promote the growth of fibroblasts.

Until now, no marker has been definitively associated with LESCs. However, the presence of LESC-associated markers ABCG2, p63, K14, and K15, together with the absence of corneal epithelial-differentiation markers K3 and K12, can be used to identify putative LESCs.[3,45,46] In our study, we detected no significant differences among LPCs cultured with mod-SHEM, IOBA-FBS, or IOBA-HS for the percentage of cells that expressed LESC (ABCG2, p63, and K14) or corneal epithelial cell proteins (K3 and K12). In contrast, the relative mRNA expression of the LESC markers was similar or significantly higher in LPCs incubated with IOBA-FBS or IOBA-HS than in LPCs cultured with mod-SHEM. In addition, the lowest relative mRNA expression of the *K3* marker was present in LPCs grown with IOBA-FBS or IOBA-HS. These results suggest that a culture medium in which cholera toxin and DMSO are replaced by isoproterenol and transferrin-selenite can originate LPCs with similar or better characteristics than a culture medium supplemented with those components.

To determine if FBS and HS could induce different effects, IOBA-FBS and IOBA-HS culture media were compared. Nakamura *et al.* showed that human limbal epithelial cells cultivated with cholera toxin-supplemented culture medium plus FBS or HS had similar *in vitro* characteristic.[37] Consistent with these results, we observed that the expression of S100A4, ABCG2, p63, K14, K3, and K12 markers was similar in LPCs incubated with IOBA-FBS or IOBA-HS. This suggests that FBS and HS produce similar *in vitro* actions when a cholera toxin- and DMSO-free culture medium was used. However, K15 protein was only expressed by LPC cells when HS was used, suggesting that HS could better maintain the undifferentiated cell stage of LESCs than FBS.

In a similar way, Takagi *et al.* analyzed the colony forming efficiency of oral epithelial cells cultured in a medium supplemented with isoproterenol plus HS or FBS. They showed that cholera toxin can be replaced by isoproterenol in the culture medium for *in vitro* cultivation of epithelial cells, especially when HS is added.[30] Recently, Stasi *et al.* compared two culture media that were identical except for presence of FBS and cholera toxin in one and HS and isoproterenol in the other.

They showed that the HS-isoproterenol culture medium produced a higher percentage of holoclones than the other culture medium, supporting our findings for the combination of HS and isoproterenol for cultured LESCs.[29]

Due to the good outcomes obtained with IOBA-HS culture medium under feeder layer-free conditions and the benefits that it provides over mod-SHEM and IOBA-FBS, we investigated the feasibility of culturing the same limbal explants consecutively with this culture medium and without feeder layers. In similar studies, limbal explants were repeatedly cultured; [19,47,48] however the culture media in those studies were supplemented with cholera toxin, DMSO, and/or FBS.

We showed that a single limbal explant can be cultured up to three consecutive times (LPC0-LPC3) with IOBA-HS culture medium and feeder layer-free conditions, though only LPC0 and LPC1 cell populations maintained the LESC phenotype. The phenotypic LPC characterization confirmed the protein and mRNA expression of all analyzed LESCs and corneal markers in LPC0 and LPC1, except for protein p63, which was only detected in LPC0. Except for K14 and K12 protein expression, changes in the expression of LESC and corneal markers were detected in LPC2. The percentage of p63-, K15-, and K3-positive cells and the relative mRNA expression of these markers decreased in LPC2. These results were consistent with the changes from epithelial-like morphology in LP0-LPC1 to elongated morphology in LPC2, suggesting a change in the LPC cell population. The markers suggest the presence of mixed undifferentiated and differentiated limbal epithelial cells in LPC0-LPC1 that then change to a limbal fibroblast-like cell population in LPC2. These results were confirmed by the increase of cells positive for S100A4-fibroblast protein marker from LPC0 to LPC2.

Like S100A4, the percentage of cells positive for ABCG2 and the relative expression of *ABCG2* mRNA increased from LPC0 to LPC2. The ABCG2-positive cells in LPC0 and LPC1 were probably due to the presence of LESCs, which is consistent with the expression of p63, K14, and

K15. However, ABCG2 is also expressed by limbal stromal cells.[49] This likely explains the high expression of this marker in LPC2 cells with elongated morphology and the higher percentage of S100A4-positive cells.[19]

The presence of differentiated and undifferentiated epithelial cells in the confluent LPC0 and LPC1 could be explained by the migration of some LESCs from the explant onto the substratum. According to some authors,[47,50,51] the migration of LESCs into the stroma would make them unable to colonize the culture substratum in the last limbal explant passage. In LPC2, limbal fibroblasts and/or mesenchymal cells could migrate from the limbal stroma onto the culture dish to establish LPCs composed of cells with elongated morphology and the absence of p63 and K15 protein markers. A similar phenomenon was shown by our group when a culture medium with cholera toxin, DMSO, and FBS was used; however, the change in the cell population was detected in LPC3-LPC4.[19] Several authors have reported that *in vitro* cultured keratinocytes cannot be subcultured without cholera toxin, whereas keratinocytes subcultivation was possible in the presence of cholera toxin.[7,52] Therefore, our results could suggest that the "early" change observed in the LPC cell population might be due to the absence of cholera toxin in the IOBA-HS culture medium.

For the first time, we have demonstrated that a culture medium (IOBA-HS) that lacks non-human animal serum and other potentially harmful compounds can be used to originate LPCs with similar or better characteristics than those obtained using a culture medium supplemented with potentially harmful components. Moreover, IOBA-HS enables the preparation of two homologous LPCs from the same limbal explant that maintained the limbal phenotype under feeder layer-free conditions. The routine use of this culture medium could improve both the biosafety and the number of available LPCs for potential clinical transplantation, as well as decrease the expense of the culture procedure.

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DECLARATION OF INTERESTS

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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FIGURE LEGENDS

Figure 1. Characteristics of confluent LPCs obtained with mod-SHEM, IOBA-FBS and IOBA-HS culture media. **(A)** There were no significant differences in the percentage of confluent LPCs obtained with the three culture media tested. **(B)** The LPC generation times were similar among the three culture media. **(C)** Representative images captured by phase contrast microscope. Scale bar: 100 µm. The LPCs incubated with the three culture media showed cuboidal and homogeneous cell morphology, typical of epithelial cells. **(D)** Representative immunofluorescence microscopy images of control, corneal, and limbal tissues (nuclei in red). Scale bar: 50 µm. The magnification for images without scale bars are the same as those with them. Anti-PECAM, anti-MART-1, and anti-CD11c antibodies successfully detected the specific proteins (in green) in positive control samples. Protein expression of PECAM was detected in the limbal stroma, while that of MART-1 and CD11c were detected in intermediate and basal limbal epithelial layers, respectively. **(E)** Representative images of immunofluorescence microscopy (nuclei in red). Scale bar: 50 µm. LPCs incubated with mod-SHEM, IOBA-FBS, and IOBA-HS did not expressed PECAM, MART-1, or CD11c protein markers; however, S100A4 protein was detected in LPCs cultured with all three culture media. **(F)** Percentage of cells positive for S100A4 protein (n=4). **(G)** RT^2-PCR results for *S100A4* marker (n=6). Relative expression of *S100A4* mRNA was detected when all three culture media were used. Abbreviations: LPC, limbal primary culture; FBS, fetal bovine serum; HS, human serum.

Figure 2. LESC marker expression in LPCs cultured with mod-SHEM, IOBA-FBS, and IOBA-HS culture media. **(A)** Representative images of immunofluorescence microscopy. Scale bar: 50 µm. Nuclei were counterstained with propidium iodide (red). ABCG2, K14, and K15 protein expression is shown in green, while p63 is shown in yellow (red and green fluorescence co-localization). **(B)** Percentage of positive cells for each analyzed marker (n=4). There were no significant differences in the percentage of positive cells for ABCG2, p63, and K14 LESC protein markers when the

different culture media were used. However, the K15 marker was not detected in the LPCs cultured with IOBA-FBS. **(C)** RT^2-PCR results (n=6). The relative mRNA expression of the LESC markers analyzed was significantly highest when IOBA-FBS or IOBA-HS was used, except for *ABCG2*, which mRNA expression was similar with the three culture media. Abbreviations: LESC, limbal epithelial stem cells; LPC, limbal primary culture; FBS, fetal bovine serum; HS, human serum. * $p \leq 0.05$.

Figure 3. Corneal epithelial differentiated cell markers expression in LPCs cultured with mod-SHEM, IOBA-FBS and IOBA-HS culture media. **(A)** Representative images of immunofluorescence technique. Scale bar: 50 µm. Nuclei were counterstained with propidium iodide (red). K3 and K12 protein expression is shown in green. **(B)** Immunofluorescence result. Percentage of positive cells for each analyzed marker (n=4). There were no significant differences in the percentage of positive cells for K3 and K12 protein marker when the three culture media were used. **(C)** RT^2-PCR results (n=6). The mRNA relative expression of *K3* was significant higher when mod-SHEM was used, while significant differences were not detected in the mRNA relative expression of *K12*. Abbreviations: LPC, limbal primary culture; FBS, fetal bovine serum; HS, human serum. * $p \leq 0.05$.

Figure 4. Characteristics of consecutive LPCs obtained from single limbal explants cultured with IOBA-HS culture medium. **(A)** The percentage of confluent LPCs increased significantly from LPC0 to LPC1. **(B)** The LPC total generation time decreased significantly from LPC0 to LPC1-LPC2. **(C)** Representative images captured by phase contrast microscope. Scale bar: 100 µm. Cell outgrowths showed a homogeneous cuboidal morphology in LPC0 and LPC1. Abbreviations: LPC, limbal primary culture; N, number of donors; Np, number of limbal explant plated for the different consecutive passages; Nc, number of confluent LPCs obtained in the different consecutive limbal explant passages. ** $p \leq 0.01$ *** $p \leq 0.001$.

Figure 5. LESC marker expression in consecutive LPCs cultured with IOBA-HS culture medium. **(A)** Representative images of immunofluorescence microscopy. Scale bar: 50 µm. Nuclei were counterstained with propidium iodide (red). ABCG2, K14, and K15 protein expression are shown in green, while p63 is shown in yellow (red and green fluorescence co-localization). **(B)** Percentage of positive cells for each analyzed marker (n=4). The percentage of positive cells for ABCG2 protein marker increased significantly from LPC0 to LPC1, but then decreased significantly in LPC2. P63 protein marker was only detected in LPC0. The percentage of positive cells for K14 was similar in all LPCs. The percentage of K15-positive cells decreased significantly in LPC2. **(C)** RT^2-PCR results (n=6). Except for *ABCG2*, the relative mRNA expression of all the analyzed LESC markers was significantly higher in LPC0-LPC1 than in LPC2. Abbreviations: LPC, limbal primary culture; HS, human serum; LESC, limbal epithelial stem cells. * $p \leq 0.05$; *** $p \leq 0.001$.

Figure 6. Differentiated corneal epithelial cell and fibroblast marker expression in consecutive LPCs cultured with IOBA-HS culture medium. **(A)** Representative images of immunofluorescence microscopy. Scale bar: 50 µm. Nuclei were counterstained with propidium iodide (red). K3, K12, and S100A4 protein expression is shown in green. **(B)** Percentage of positive cells for each analyzed marker (n=4). The percentage of positive cells for K3 decreased significantly in LPC2, while K12 was similar in all of the consecutive LPCs. The percentage of positive cells for S100A4 protein marker increased significantly from LPC0 to LPC1, then decreased significantly in LPC2. **(C)** RT^2-PCR results (n=6). The mRNA relative expression of the corneal markers *K3* and *K12* and of the fibroblast marker *S100A4* decreased significantly in LPC2 (n=6). Abbreviations: LPC, limbal primary culture; HS, human serum. * $p \leq 0.05$; *** $p \leq 0.001$.

Table 1. Composition of control (mod-SHEM) and experimental (IOBA-FBS and IOBA-HS) culture media for *in vitro* limbal epithelial stem cells expansion.

Compounds	mod-SHEM	IOBA-FBS	IOBA-SH
Gentamicin	50 µg/ml	50 µg/ml	50 µg/ml
Amphotericin	2.5 µg/ml	2.5 µg/ml	2.5 µg/ml
Insulin	10 µg/ml	10 µg/ml	10 µg/ml
Transferrin	5.5 µg/ml	5.5 µg/ml	5.5 µg/ml
Sodium selenite	5 ng/ml	5 ng/ml	5 ng/ml
Hydrocortisone	0.01 µg/ml	0.5 µg/ml	0.5 µg/ml
EGF	2.5 ng/ml	2.5 ng/ml	2.5 ng/ml
Cholera toxin	132.5 ng/ml	-	-
DMSO	0.5%	-	-
Isoproterenol	-	1 µM	1 µM
Adenine	-	0.18 mM	0.18 mM
Triiodothyronine	-	2 nM	2 nM
Fetal Bovine Serum	5%	10%	-
Human Serum	-	-	10%

Supplements were added to DMEM/F12 (1:1) to yield the final concentrations shown. All supplements were from human, recombinant or chemical origin.

Table 2. Taqman® probes used for RT²-PCR and primary antibodies used for immunodetection.

	Taqman® probes for RT²-PCR		Primary antibodies for immunofluorescence			
Marker name	**Gene symbol**	**Assay Applied Biosystem ID***	**Category**	**Clone**	**Source****	**Working dilution**
ATP-binding cassette, sub-family G, member 2 (ABCG2)	*ABCG2*	Hs 00184979_m1	Mouse monoclonal	BXP-21	MercMillipore	1:20
Alpha isoform of nucler protein 63 (p63alpha)	*TP63 (p63)*	Hs 00978338_m1	-	-	-	
Nuclear protein 63 (p63)	-	-	Mouse monoclonal	4A4	Santa Cruz Biotechnology	1:50
Keratin 14 (K14)	*KRT14*	Hs 00559328_m1	Mouse monoclonal	RCK107	MercMillipore	1:50
Keratin 15 (K15)	*KRT15*	Hs 00267035_m1	Mouse monoclonal	LHK15	MercMillipore	1:50
Keratin 3 (K3)	*KRT3*	Hs 00365080_m1	Mouse monoclonal	AE5	Mp Biomedical	1:50
Keratin 12 (K12)	*KRT12*	Hs 00165015_m1	Rabbit polyclonal	H-60	Santa Cruz Biotechnology	1:50
S100 calcium bilding protein A4 (S100A4)	*S100A4*	Hs 00243201 m1	Mouse monoclonal	1B10	Abcam	1:100
glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*	4352934E	-	-	-	-
Platelet endothelial cell adhesion molecule (PECAM/CD31)	-	-	Mouse	Ab-1	MercMillipore	1:20
Melanoma associated antigen recognized by T cells (MART1/MelaA)	-	-	Mouse monoclonal	A103	Abcam	1:50
Integrin alpax (CD11c)	-	-	Mouse monoclonal	b-ly6	Santa Cruz Biotechnology	1:100

*Identification number from Applied Biosystems (Foster City, CA, USA).
**MercMillipore (Billerica, MA, USA), Santa Cruz Biotechnology (Heidelberg, Germany), Mp biomedical (Illkirch, France), Abcam (Cambridge, MA, USA).

Figure 1.

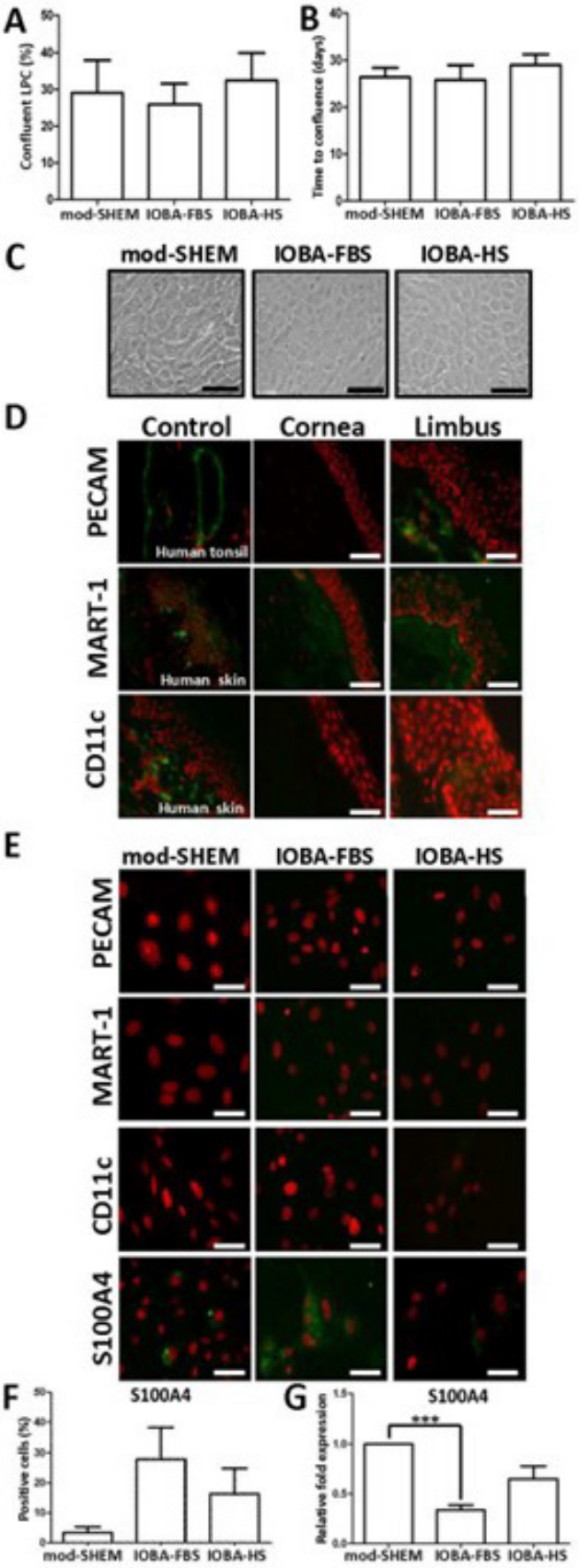

A
Confluent LPC (%)
mod-SHEM
IOBA-FBS
IOBA-HS
B
Time to confluence (days)
mod-SHEM
IOBA-FBS
IOBA-HS
C
mod-SHEM
IOBA-FBS
IOBA-HS
D
Control
Cornea
Limbus
PECAM
Human tonsil
MART-1
Human skin
CD11c
Human skin
E
mod-SHEM
IOBA-FBS
IOBA-HS
PECAM
MART-1
CD11c
S100A4
F
S100A4
Positive cells (%)
mod-SHEM
IOBA-FBS
IOBA-HS
G
S100A4
***
Relative fold expression
mod-SHEM
IOBA-FBS
IOBA-HS

Figure 2.

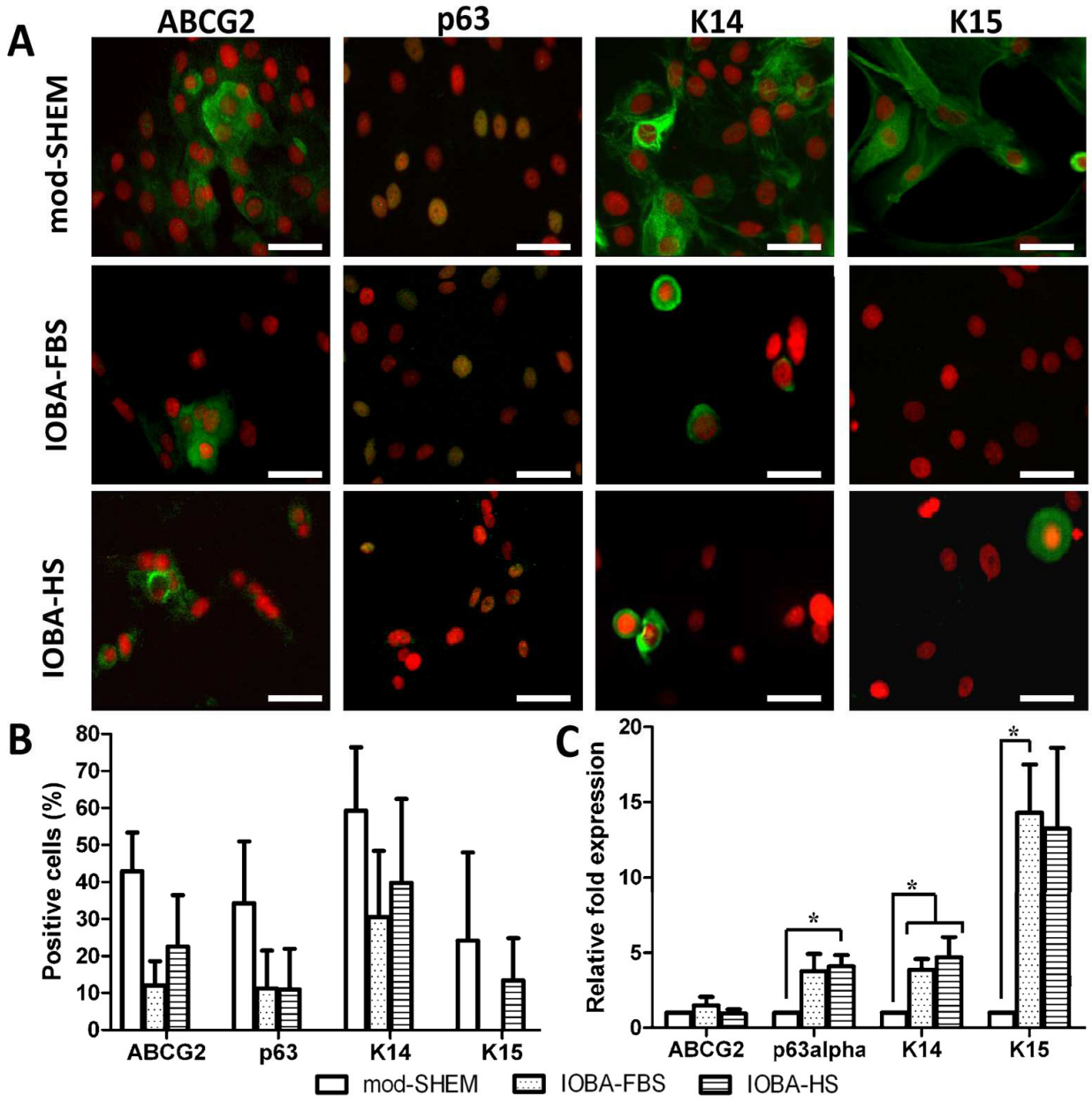

A
ABCG2
p63
K14
K15
mod-SHEM
IOBA-FBS
IOBA-HS
B
Positive cells (%)
80
70
60
50
40
30
20
10
0
ABCG2
p63
K14
K15
C
Relative fold expression
20
15
10
5
0
*
*
*
ABCG2
p63alpha
K14
K15
mod-SHEM
IOBA-FBS
IOBA-HS

Figure 3.

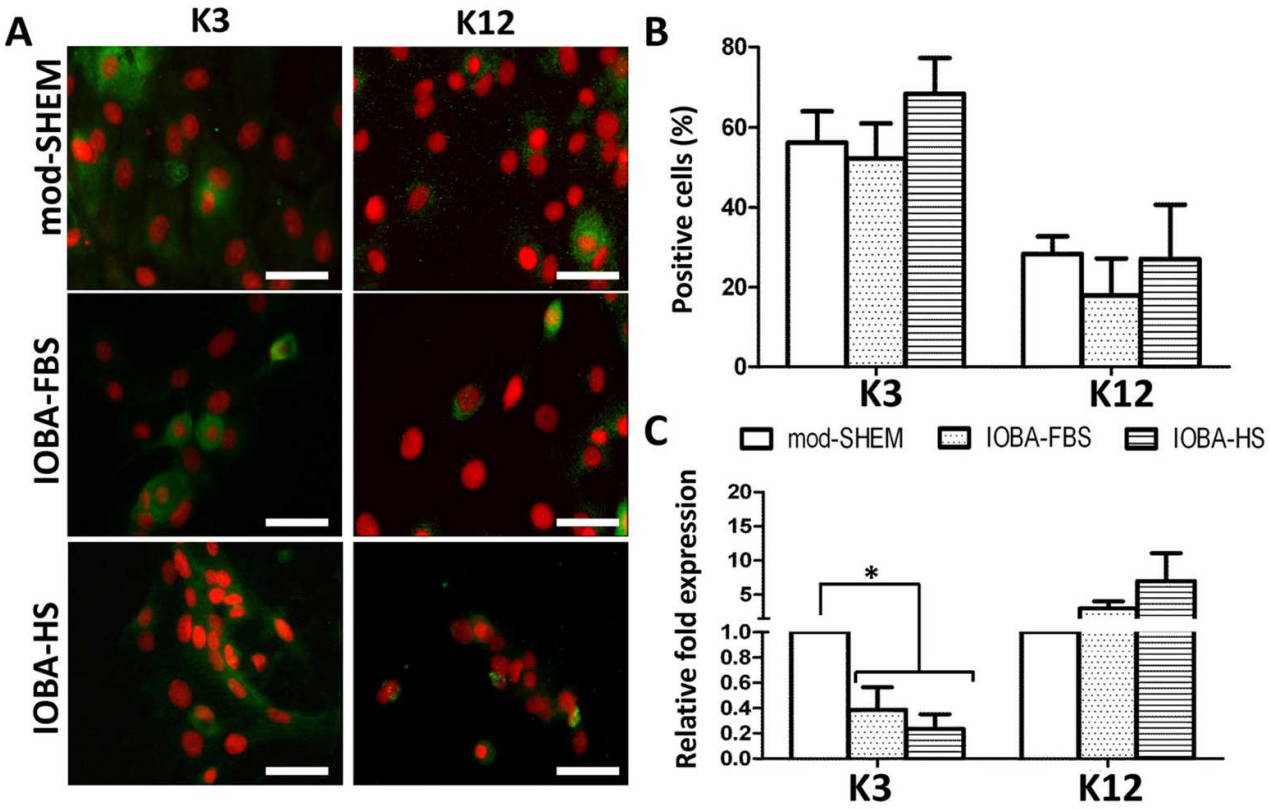

A
K3
K12
mod-SHEM
IOBA-FBS
IOBA-HS
B
Positive cells (%)
80
60
40
20
0
K3
K12
mod-SHEM
IOBA-FBS
IOBA-HS
C
Relative fold expression
20
15
10
5
1.0
0.8
0.6
0.4
0.2
0.0
*
K3
K12

Figure 4.

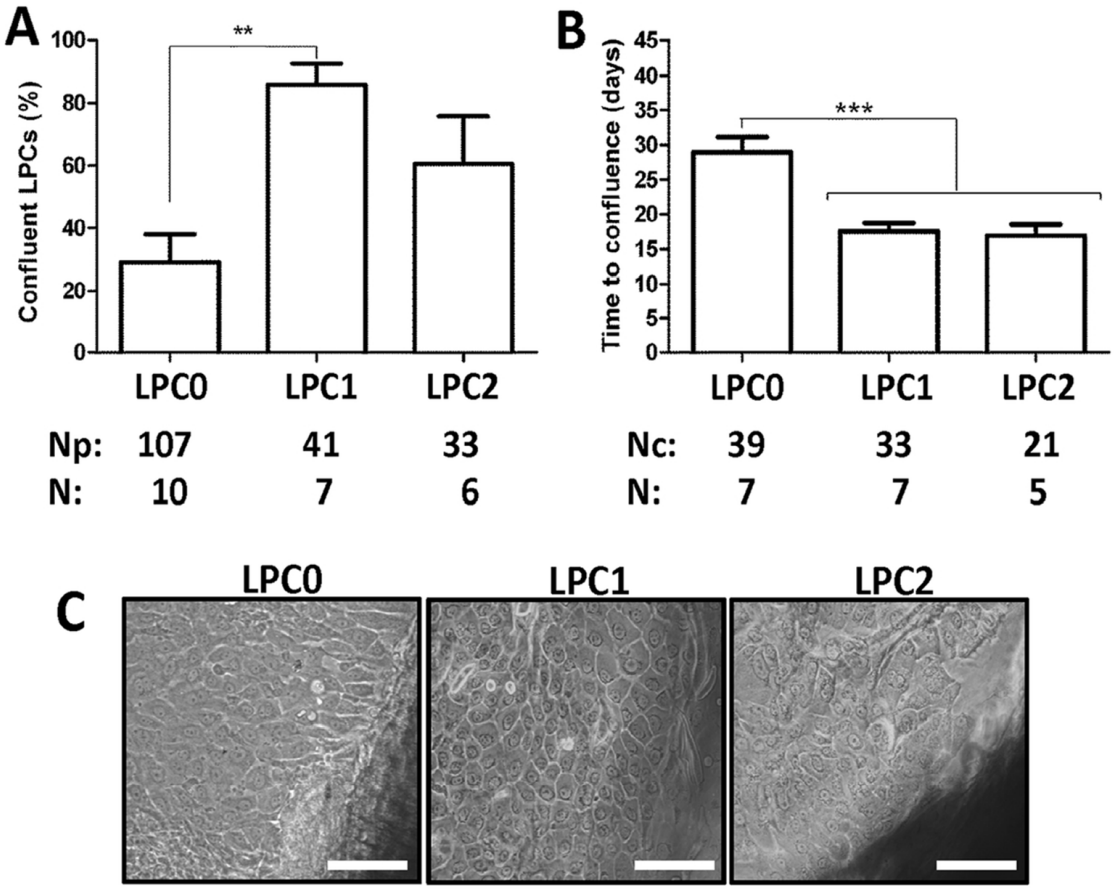

A
Confluent LPCs (%)
100
80
60
40
20
0
**
LPC0
LPC1
LPC2
Np: 107 41 33
N: 10 7 6
B
Time to confluence (days)
45
40
35
30
25
20
15
10
5
0
***
LPC0
LPC1
LPC2
Nc: 39 33 21
N: 7 7 5
C
LPC0
LPC1
LPC2

Figure 5.

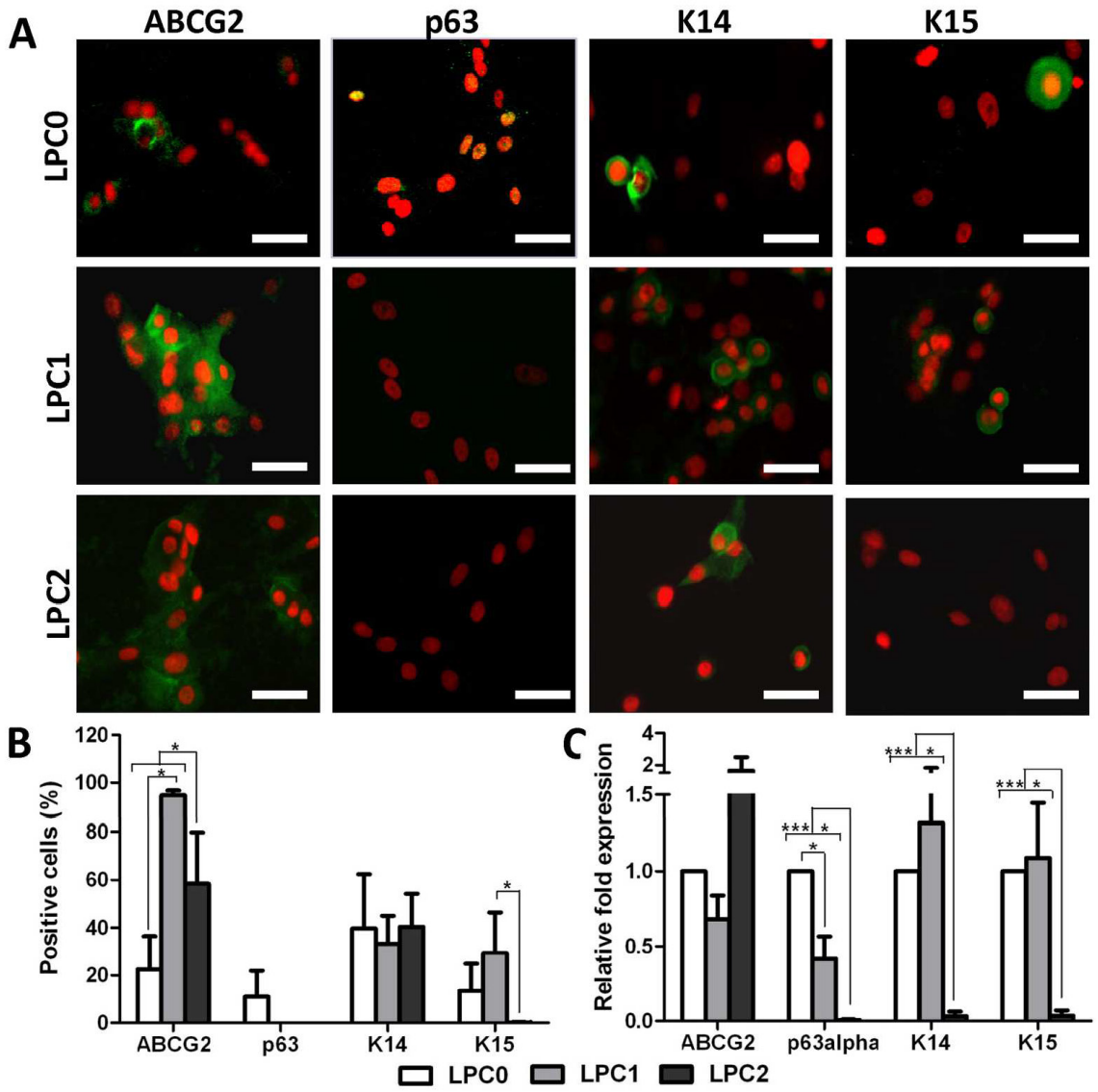
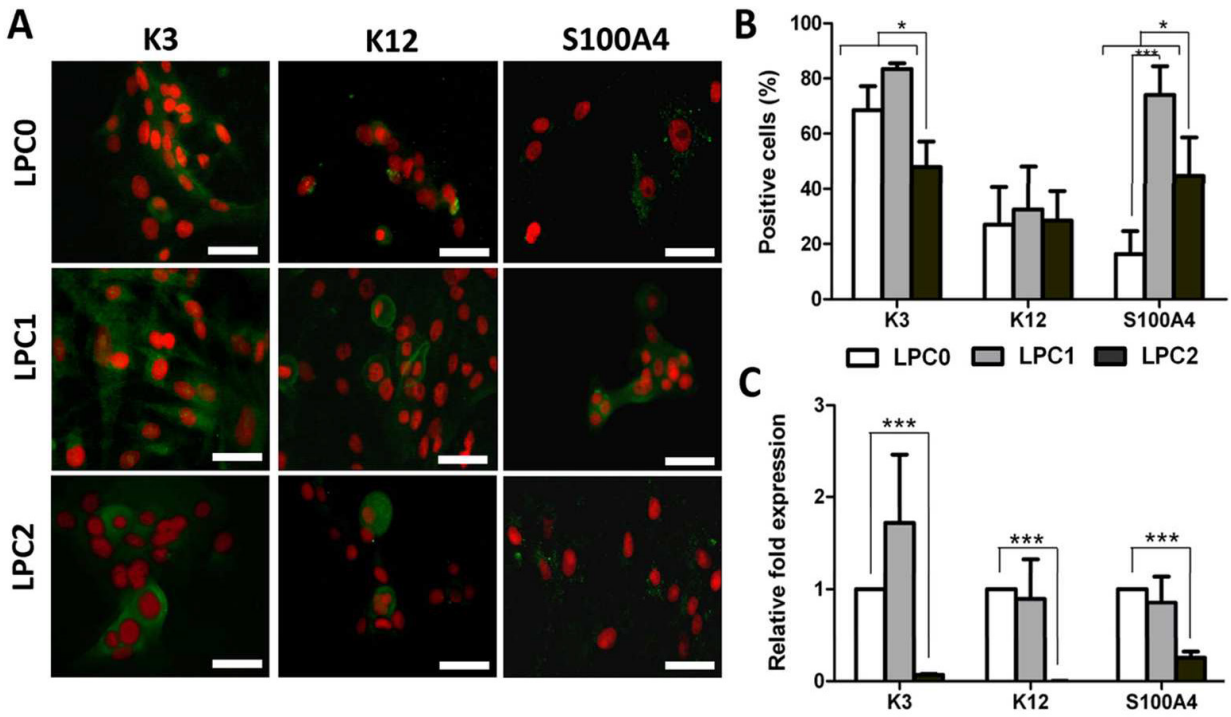

A
ABCG2
p63
K14
K15
LPC0
LPC1
LPC2
B
Positive cells (%)
120
100
80
60
40
20
0
ABCG2
p63
K14
K15
C
Relative fold expression
4
2
1.5
1.0
0.5
0.0
ABCG2
p63alpha
K14
K15
LPC0
LPC1
LPC2

Figure 6.


A
K3
K12
S100A4
LPC0
LPC1
LPC2
B
Positive cells (%)
100
80
60
40
20
0
*
***
*
K3
K12
S100A4
LPC0
LPC1
LPC2
C
Relative fold expression
3
2
1
0
***
***
***
K3
K12
S100A4