Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com

Poly-L/DL-lactic acid films functionalized with collagen IV as carrier substrata for corneal epithelial stem cells

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ARTICLE INFO

Keywords:
Corneal epithelium
Collagen IV
Limbal stem cells
Polylactic acid
Tissue engineering

ABSTRACT

Limbal epithelial stem cells (LESCs) are responsible for the renewal of corneal epithelium. Cultivated limbal epithelial transplantation is the current treatment of choice for restoring the loss or dysfunction of LESCs. To perform this procedure, a substratum is necessary for *in vitro* culturing of limbal epithelial cells and their subsequent transplantation onto the ocular surface. In this work, we evaluated poly-L/DL-lactic acid 70:30 (PLA) films functionalized with type IV collagen (col IV) as potential *in vitro* carrier substrata for LESCs. We first demonstrated that PLA-col IV films were biocompatible and suitable for the proliferation of human corneal epithelial cells. Subsequently, limbal epithelial cell suspensions, isolated from human limbal rings, were cultivated using culture medium that did not contain animal components. The cells adhered significantly faster to PLA-col IV films than to tissue culture plastic (TCP). The mRNA expression levels for the LESC specific markers, K15, P63α and ABCG2 were similar or greater (significantly in the case of K15) in limbal epithelial cells cultured on PLA-col IV films than limbal epithelial cells cultured on TCP. The percentage of cells expressing the corneal (K3, K12) and the LESC (P63α, ABCG2) specific markers was similar for both substrata. These results suggest that the PLA-col IV films promoted LESC attachment and helped to maintain their undifferentiated stem cell phenotype. Consequently, these substrata offer an alternative for the transplantation of limbal cells onto the ocular surface.

1. Introduction

The limbus, located at the transition zone between the cornea and the conjunctiva, is the niche for the maintenance and proliferation of limbal epithelial stem cells (LESCs), which are responsible for corneal epithelium regeneration [1]. This limbal niche is located in the palisades of Vogt, where the LESCs correspond to 10% of the basal cells [2] and are supported by the basement membrane and the underlying connective tissue. The limbal basement membrane provides cell support and improves the adhesion, growth, and proliferation of LESCs due to the associated extracellular matrix proteins, where type IV collagen (col IV) is the most abundant protein [3].

The destruction or dysfunction of LESCs or their limbal niche leads to a clinical entity known as limbal stem cell deficiency (LSCD). LSCD is characterized by the presence of unstable corneal epithelium and subsequent ulceration, invasion of the conjunctiva in the cornea, emergence of neovascularization on the corneal surface, and persistent inflammation and chronic pain, which ultimately causes vision loss and even corneal blindness [4,5]. Corneal transplantation is often the best option for vision recovery when the cornea is damaged, but it will not be successful if corneal damage is accompanied by the absence of viable LESCs. In this situation, restoration of the stem cell population is a possible treatment for LESC deficiency and must precede corneal transplantation. Cultivated limbal epithelial transplantation (CLET) for reconstruction of the damaged ocular surface in LSCD syndrome was first reported in 1997 [6]. Subsequently, this strategy has been used by

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https://doi.org/10.1016/j.colsurfb.2019.01.054
Received 6 November 2018; Received in revised form 10 January 2019; Accepted 26 January 2019
Available online xxx
0927-7765/

many other authors [7–10]. To perform CLET, a substratum is necessary for both *in vitro* expansion of epithelial cells and the subsequent transplantation onto the ocular surface. Biological substrata such as fibrin [9,11], human amniotic membrane [10,12–14], and cellular feeder layers such as 3T3 fibroblasts [6] have been used to facilitate the expansion of corneal epithelial cells. However, as natural products, none of these agents can be standardized, and they are not optically transparent. Furthermore, the risk of disease transmission, the limited availability of these products, and the high economic cost [15] necessitate the investigation of a new substrata for LESCs.

An ideal biomaterial for proliferation and transplantation of LESCs onto the ocular surface would be biocompatible [16], biodegradable, optically clear, sturdy enough to be handled and withstand suturing, possess low immunogenicity, and would not induce cell differentiation. Moreover, these biomaterials should resemble the structure and properties of the natural LESC niche to provide an ideal environment for *in vitro* culturing of such cells. Various polymeric biomaterials have been described as alternatives to natural materials as substrates or scaffolds for stem cells. Biosynthetic materials like polycaprolactone [17], polylactic glycolic acid, chitosan and gelatin membranes [18], collagen gels [16,19–21] or elastin like polymers [22] have been used to support ocular epithelial cells. Levis et al. described the use of collagen constructs for the support of LESCs previously isolated on 3T3 fibroblasts for obtaining a stratified corneal epithelium [21]. The same group also described the use of a surface-modified contact lens to support LESCs previously expanded on 3T3 fibroblasts [23]. Other groups have also used type I collagen scaffolds, with embedded stromal fibroblasts, as substrata for LESCs [24,25].

Besides the substrata mentioned above, there are other systems in which a corneal epithelial cell sheet for transplantation can be acquired without using a carrier. One of these systems is the Mebiol gel, which remains liquid at temperatures below 20 °C, and gels over 20 °C [26,27]. Yang et al. proposed the use of thermosensitive culture plates, in which changing the temperature allows for the growth of an epithelial cell sheet without any enzymatic action, keeping the synthesized extracellular matrix intact [28].

Among the various materials available, a polylactic acid derivative is one of the preferred choices. Polylactic acid is a lactic acid derivative that can take on different mechanical properties by varying its structure and altering its crystallinity degree [29]. It is known to be a biocompatible and biodegradable material of which the degradation products, mainly carbon dioxide and water, are not toxic to the organism [30]. Polylactic acid has been used in several biomedical applications, such as absorbable sutures, a transport medium for drug delivery, and tissue engineering [29,31]. Currently, the use of polylactic acid has expanded significantly in the field of bone regeneration [32–34] and combined with glycolic acid (polylactic-glycolic acid) in conjunctival and corneal reconstruction [35–37]. However, it has been demonstrated that cell adhesion to polylactic acid films is poor, and that it is necessary to functionalize the surface of this type of polymer to improve cell attachment [38,39]. Col IV is an extracellular matrix protein described in the limbal, corneal, and conjunctival epithelial basement membranes, specifically in the basement membrane of the limbal epithelium. The α1 and α2 chains of col IV have been repeatedly described as limbal zone-specific and they are not expressed in the differentiated corneal epithelial basement membrane [3,40,41]. This type of collagen has been used to select populations of LESCs based on their rapid adhesion to this protein. Additionally, cells with greater adherence to col IV mainly expressed undifferentiated LESC markers, where the expression of differentiated corneal epithelial markers was decreased [42].

Based on these alternatives, the present work aimed to replace the biological substrates and culture techniques currently used for culturing and transplanting LESCs onto the ocular surface with synthetic biopolymers, avoiding the use of any animal components, in both the substratum and in the culture medium for limbal epithelial cells. Additionally, to avoid the use of murine 3T3 fibroblasts to expand LESCs, the surface of these polymers was functionalized with a human extracellular matrix protein to promote cell adhesion to the substratum. The goal of this work was to evaluate the suitability of poly-L/DL-lactic acid 70:30 (PLA) films functionalized with col IV (PLA-col IV) as potential carrier substrata for human LESCs.

2. Material and methods

2.1. Reagents

Poly–L/DL lactic acid 70/30 (Purasorb PLDL 70/38, inherent viscosity midpoint 3.8 dl/g, Mw 850,000 Da) (PLA from now on) was purchased from Purac Biomaterials (Amsterdam, The Netherlands). Phosphate-buffered saline solution (PBS), Hanks balanced salt solution, trypsin–ethylenediamine-tetraacetic acid (EDTA) 1X, Versene, Dulbecco's modified Eagle's medium (DMEM/F12), fetal bovine serum (FBS), epidermal growth factor (EFG), human insulin, penicillin/streptomycin, gentamicin, and fungizone were purchased from Invitrogen-Gibco (Inchinan, UK). Human serum and cholera toxin were purchased from Lonza (Basel, Switzerland) and Gentaur (Kanpenhout, Belgium), respectively. The Viability-Cytotoxicity Assay Kit for Mammalian Live and Dead Cells was purchased from Biotium, Inc. (Hayward, CA, USA), and the proliferation assay kit Alamar Blue™ was purchased from AbD Serotec (Oxford, UK). Col IV from human placenta (C7521) and all the other reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) and used without further purification. The Micro BCA™ Protein Assay Kit was purchased from Thermofisher Scientific (Waltham, MA, USA).

2.2. PLA films fabrication

PLA films were obtained by solvent casting. Briefly, PLA was dissolved in $CHCl_3$ (2.5% w/v) and was poured into Petri dishes with a diameter of 12 cm. The solvent was allowed to evaporate for 3 days in a solvent-saturated atmosphere. After this time, the obtained films were stored in a vacuum and dry atmosphere until their use. The thickness of the resulting films was 100 μm.

2.3. PLA films functionalization with col IV

The surface functionalization of the PLA films with col IV was carried out following previously developed protocols [38,39]. In short, PLA films were treated with sodium hydroxide 0.5 M for 30 min to hydrolyze the surface of the films. After extensively rinsing with water, the films were activated with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide and N-hydroxysuccinimide (EDC and NHS) 0.1/0.2 M in PBS (pH 7.4) for 1 h. Further, films were washed twice with PBS, and col IV was covalently attached to the surface of the material, incubating the films in a PBS col IV solution at 20 μg/ml overnight. Finally, functionalized films were washed again with water and vacuum sealed until their use.

2.4. PLA-col IV films physico-chemical characterization

The physico-chemical characterization of the PLA films during the different steps of the functionalization process has already been reported elsewhere [39]. Contact angle (CA) measurements were carried out with an optical contact angle device (OCA15, Dataphysics, Germany) before and after col IV functionalization of the PLA films.

Immunofluorescence was used to determine the presence of col IV on the surface of the PLA films. In brief, the samples were blocked for 20 min at room temperature with PBS-Glycine-bovine serum albumin.

After washing with PBS-Glycine (2 × 5 min), the samples were incubated with the primary antibody (Table 2) for 45 min at 37 °C and washed with PBS-Glycine (2 × 5 min). The incubation of the secondary antibody (Table 2) was performed in the dark over 45 min at 37 °C. The samples were visualized with a Nikon E600 upright fluorescence microscope (Tokyo, Japan). Different controls were performed by incubation of either only primary or secondary antibodies.

For col IV quantification, a Micro BCA™ Protein Assay Kit was used, following the manufacturer's protocol. The experiment was conducted in triplicate with six replicas per experiment.

2.5. Human corneal epithelial (HCE) cell culture

With the aim of delimitating a culture area of 1 cm^2 and preventing the polymer film from floating, reusable cell culture inserts made of silicone (flexiPERM, Sigma Aldrich) were used. The flexiPERM was gently pressed against the dry polymer film or the control tissue culture plastic (TCP) before cell culture.

Simian virus-40-transformed HCE cells [43] (a kind gift from Arto Urtti, University of Helsinki, Finland) were used in passage 40–48 to test the polymer as cellular substratum. A total of 4×10^4 cells/cm^2 were seeded on the TCP and on each biopolymer and cultured using DMEM/F12 culture medium supplemented with 15% FBS, 0.5% DMSO, 0.1 μg/ml cholera toxin, 10 ng/ml EGF, 5 μg/ml human insulin, and antibiotics (62.5 U/ml penicillin, 62.5 mg/ml streptomycin). The cells were incubated at 37 °C, under 5% CO_2 and 95% humidified air. The culture medium was carefully changed every 2–3 days.

2.6. HCE cell viability

The viability of HCE cells grown on the PLA-col IV film was analyzed at 6 h and at 1, 2, 4, and 8 days. Cellular viability-cytotoxicity assays were performed on attached cells by using the Viability-Cytotoxicity Assay Kit for Mammalian Live and Dead Cells. The kit uses a combination of calcein-AM and ethidium homodimer (EthD-III) to dye live cells green and dead cells red. At each time point, the culture medium was gently removed and cells were washed with PBS. After staining according to the manufacturer's instructions, the cultures were rinsed with PBS prior to being viewed under a fluorescence microscope (Leica DMI 6000 B, Leica, Wetzlar, Germany). Live and dead cells were counted in 5 random fields per substratum and time. Viability was expressed as the percentage of live cells ± standard error of the mean (SEM) from 4 independent experiments.

2.7. HCE cell proliferation

Cell growth was determined by using the fluorometric non-toxic Alamar Blue™ (AB) assay, which depends on the conversion of resazurin to resorufin, a pink fluorescent dye. The reaction is based upon chemical reduction of the culture medium resulting from cell growth. The AB assay was performed after 1, 2, 4, and 8 days according to the manufacturer's instructions. After 5 h of incubation, duplicate 300 μl samples of culture medium for each test sample were transferred to a 24-well plate. Fluorescence was measured on a SpectraMAx M5 (Molecular Devices, Sunnyvale, CA, USA) at an excitation wavelength of 560 nm and emission wavelength of 590 nm, according to the specifications of the manufacturer.

A total of 4×10^4 cells/cm^2 were seeded onto TCP and the PLA-col IV film. To consider only the cells adhered to the biopolymers, the polymeric membranes were transferred to another clean well. Proliferation data were expressed as the relative cell density reached after 8 days compared to the cell density at 1 day from 4 independent experiments.

2.8. Human limbal epithelial cell isolation and culture

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.

Cadaveric human corneoscleral buttons were obtained under informed research consent from Barraquer Eye Bank of Barcelona (Spain). The average age ± SEM of the donors was 75.6 ± 2.5 years. The buttons were stored at 4 °C and were used within 3 ± 0.2 days of the donor's death.

Human limbal epithelial cells were grown from single cellular suspensions obtained from corneoscleral buttons, as described previously [44]. Briefly, the corneoscleral tissue from 11 different donors was rinsed with Hanks balanced salt solution containing 50 μg/ml gentamicin and 2.5 μg/ml fungizone. The excess of conjunctiva, iris, and corneal endothelium tissues were carefully removed, and a 7.5 mm trephine was used to isolate the central cornea from the limbus. Then, limbal rings were excised in two pieces and each piece was incubated at 37 °C for 2 h with dispase enzyme (1.2 U/ml), which digests the basement membrane collagen and separates epithelial cells from the stroma. Then, the limbal epithelial cells were collected and treated with trypsin (0.25%) for 10 min at 37 °C to separate them into a suspension of single cells. The single cells were seeded on PLA-col IV and TCP at a density of 4×10^4 cells/cm^2. To improve the cell adhesion on the substrata, cells were maintained for 2 h with 200 μl (in 1cm^2) of culture medium, based on DMEM/F12 supplemented with 10% human serum, 5 ng/ml EGF, 5 μg/ml human insulin, 0.4 μg/ml hydrocortisone, 1 μM isoproterenol, 0.18 mM adenine, 2 nM triiodothyronine, 50 μg/ml gentamicin, and 2.5 μg/ml fungizone [18]. After 2 h, a total of 500 μl of culture medium were added, and the total amount of 1 ml/cm^2 was added when cells were attached.

All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was freshly replaced every 2–3 days, and the cultures were monitored using a phase contrast microscope (Eclipse TS100, Nikon). The time when single cells started to adhere and reach confluence was recorded.

2.9. Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Real-time RT-PCR was performed to determine the expression levels of specific corneal and limbal genes. Cytokeratins 3 (K3) and 12 (K12) are differentiated corneal epithelial cell cytoskeletal markers [45–47]. Cytokeratin 15 (K15) [13], the transcription factor P63α (P63α) [48], and the ATP-binding cassette transporter G2 (ABCG2) [49] are LESC markers [50]. Limbal epithelial cells grown from the single cell suspension were harvested at confluence. Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) according to specifications of the manufacturer. The RNA concentration was measured by a fluorometric method using the Quant-iT RNA assay and treated with RNase-Free DNase Set (Qiagen).

Total RNA (1 μg) was retrotranscribed to cDNA using SuperScript® Vilo™ cDNA Synthesis Kit (Invitrogen-Gibco, UK). The RNA was mixed with 5X Vilo Reaction Mix and 10X Superscript Enzyme Mix (7:1:2) and thermocycled at 25 °C for 10 min, at 42 °C for 120 min, and finally at 85 °C for 5 min. PCR amplifications using specific probes (Table 1) were performed in a PCR 7500 Real Time PCR System (Applied Biosystems, Carlsbad, CA, USA) according to specifications of the manufacturer.

All experiments were performed in duplicate. A non-template negative control was included in all experiments to evaluate DNA and RT-PCR contamination of the reagents. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for each reac-

Table 1
Taqman probes used for real-time PCR analysis.

Gene name	Symbol	Identification number of Applied Biosystems
Glyceraldehide-3-phosphate dehydrogenase	GAPDH	4352934E
ATP-binding cassette transporter G2	ABCG2	Hs00184979_m1
Cytokeratin 3 (K3)	K3	Hs00365080_m1
Cytokeratin 12 (K12)	K12	Hs00165015_m1
Cytokeratin 15 (K15)	K15	Hs00267035_m1
Transcription factor P63α	P63α	Hs 00978338_m1

Table 2
Primary and secondary antibodies used for immunofluorescence staining.

Antibodies	Category	Catalog No.	Source	Working dilution
PRIMARY				
Cytokeratin AE5 (K3)	Mouse (monoclonal)	69143	Mp Biomedical (Illkirch, France)	1:50
Cytokeratin 12 (K12)	Rabbit (polyclonal)	25722	Sta Cruz (Heidelberg, Germany)	1:50
Cytokeratin 15 (K15)	Mouse (monoclonal)	CBL272	Chemicon/Millipore (Billerica, MA, USA)	1:50
ABCG2	Mouse (monoclonal)	MAB4146	Chemicon/Millipore (Billerica, MA, USA)	1:20
P63α	Rabbit (polyclonal)	4892	Cell Signaling (Boston, MA, USA)	1:50
Col4 A	Mouse (monoclonal)		Santa Cruz (Heidelberg, Germany)	1:100
SECONDARY				
Anti-mouse IgG Alexa fluor 488	Donkey		Invitrogen (Inchinnan, UK)	1:200
Anti-rabbit IgG Alexa fluor 488	Donkey		Invitrogen (Inchinnan, UK)	1:300
Anti-mouse IgG Alexa fluor 488	Goat		Invitrogen (Inchinnan, UK)	1:250

tion. The comparative cycle threshold (Ct) method (Applied Biosystems User Bulletin, No.2; P/N 4,303,859) was used to analyze the results [51]. Six independent experiments from different donors were carried out.

2.10. Immunofluorescence staining

Cultured cells were harvested by incubation in Versene for 30 min at 37 °C. A total of 50,000 cells in 250 µl of DMEM-F12 were pelleted by cytocentrifugation for 10 min at 800 rpm using low acceleration. Cells were deposited onto a 28 mm^2 area of a poly-L-lysine–treated glass slide and fixed with cold methanol at -20 °C for 10 min.

For immunofluorescence staining, the cells were incubated for 1 h at room temperature with blocking buffer (5% donkey serum in PBS). The cells were then incubated with primary antibody (Table 2) in a humidified chamber at 4 °C overnight. Afterwards, they were rinsed with PBS and incubated with the secondary antibody (Table 2) in the dark at 37 °C for 1 h. Nuclei were counterstained with propidium iodide and the samples were analyzed under fluorescent microscope (Leica DMI 6000 B, Leica).

Positively and negatively stained cells in 5 random fields of 4 independent limbal epithelial cultures were counted. The percentage of positive cells was calculated for each field, and the mean percentage of positive cells for each marker was determined.

2.11. Statistical analysis

Statistical significance was determined by one-way factorial ANOVA. Comparison between two groups was made using Student's t-test. All values were expressed as means ± SEM. P-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Functionalization of p PLA films with col IV

The PLA and PLA-col IV substrata were obtained as transparent films (Fig. 1, left). The functionalization process with col IV did not alter the transparency, handleability and suturability of the films. The physico-chemical characterization and optimization of the different steps of the functionalization process had been described elsewhere. CA measurements were performed to evaluate the changes in hydrophobicity before and after the functionalization with col IV. The CA of a pristine PLA is 78.8 ± 1.9°, as expected for a slightly hydrophobic polymer. However, the contact angle decreased to a value of 53.0 ± 11.0° upon covalent binding of col IV. Immunofluorescence was performed to investigate the presence of immobilized col IV in the surface of the materials (Fig. 1, right). Both controls using only the primary or secondary antibodies, and in samples of pristine PLA, showed no fluorescence, indicating the selectivity of the proposed method for the detection of immobilized col IV on the surface of the PLA-col IV films.

The quantification of col IV in the surface of the materials was performed by a microBCA assay. PLA discs of 1.5 cm of diameter were

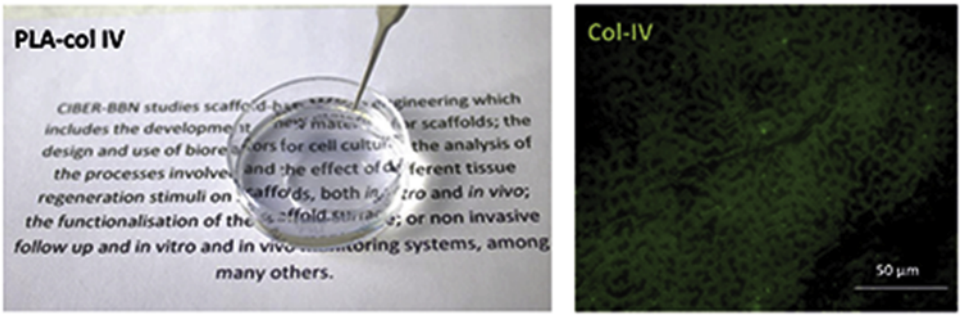

Fig. 1. PLA-col IV films. Photography of PLA-col IV film (Left). Immunofluorescence staining for collagen type IV of PLA-col IV film (magnification 40x) (Right). Scale 50 µm. PLA-col IV: poly-L/DL-lactic acid 70:30 biopolymers functionalized with type IV collagen.

functionalized following the previously described protocol. The results showed that 8.3 ± 1.3 μg/cm^2 of col IV were grafted to the surface of the material, which corresponds to a grafting yield of 73.5%.

3.2. Limbal epithelial cell adhesion to bare PLA films

Taking into account that we had previously observed poor cell adhesion to bare PLA substrata using different cell types [38,39] we decided to first test the adhesion capacity of limbal epithelial cells to bare PLA films in order to evaluate if the PLA films without col IV could serve as control substrata for the subsequent studies. To that end, limbal epithelial cells isolated from human limbal rings were cultured on bare PLA films and on TCP. As we expected, limbal epithelial cells did not adhere to the surface of PLA films whereas they did adhere to TCP after 24–48 h (data not shown). Consequently, PLA films without col IV were not used as control substrata in any experiment.

3.3. HCE cell viability and proliferation on PLA-col IV films

HCE cells were grown onto membranes composed of PLA-col IV and TCP as control substratum.

The cell viability was around 90% (Table 3) for the 8-day study period, and no significant differences were observed between both substrata.

HCE cell proliferation on each substratum was assessed by AB testing at 1, 2, 4, and 8 days in culture. The relative cell density reached at 8 days onto TCP (as control substratum) was significantly higher than the cell density on PLA-col IV membranes at 8 days ($p < 0.05$) (Fig. 2).

Table 3
Cell viability of the human corneal epithelial cell line (HCE).

Substrata	Cell viability (%)			
	1 day	2 days	4 days	8 days
TCP	98 ± 0.002	99 ± 0.001	97 ± 0.010	99 ± 0.007
PLA-col IV	98 ± 0.007	99 ± 0.004	95 ± 0.010	85 ± 0.030

TCP: tissue culture plastic; PLA-col IV: poly-L/DL-lactic acid 70:30 biopolymers functionalized with type IV collagen. Mean ± SEM based upon cell counts of 5 random fields of 4 independent experiments.

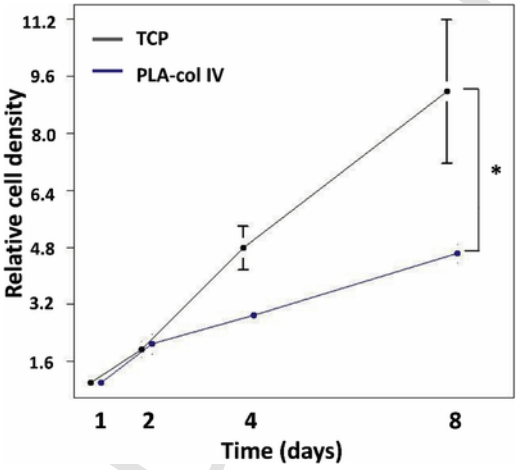

Fig. 2. PLA-col IV films are suitable for the proliferation of HCE cells. Plot representing the relative cell density reached on TCP (gray, up) and PLA-col IV (blue, down) along 8 days, compare to the density at day 1. Plot represents the average ± SEM (N = 4 independent experiments). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. PLA-col IV: poly-L/DL-lactic acid 70:30 biopolymers functionalized with type IV collagen; HCE: human corneal epithelium; TCP: tissue culture plastic.

3.4. Limbal epithelial cell isolation and expansion on PLA-col IV films

A population of limbal epithelial cells was cultured on different films of PLA-col IV and TCP as control substratum. For both substrata, the outgrowth was developed as a monolayer of cells. The cells were morphologically homogeneous, presenting a polygonal structure typical of epithelial cells.

Cell adhesion of the single cell suspension obtained from human limbal rings started after 2 h from seeding on PLA-col IV, and after 24–48 hours when they were cultured on TCP (Fig. 3A), observing a significant difference ($p < 0.01$). Cellular confluence (1 cm^2) was observed at 9.4 ± 1.0 days on PLA-col IV, and after 10.1 ± 0.9 days on TCP (Fig. 3B). No significant differences were found to reach confluence on PLA-col IV and TCP at the different time points. However, adhesion was better on the PLA-col IV films than on the control substratum. These results suggest that the substratum PLA-col IV improves the human limbal epithelial cell adhesion and did not affect the growth of limbal epithelial cells compared to TCP.

3.5. Characterization of the limbal epithelial cell population obtained on PLA-coI IV films

The relative expression of cytokeratins K3 and K12 (as markers of differentiated corneal epithelial cells) and the expression of K15, the transcription factor P63α and the transport protein ABCG2 (as LESC markers) was analyzed by real-time PCR in limbal primary cultures cultivated on PLA-col IV films and TCP.

In order to compare the expression levels of the different markers (Table 1) between TCP and PLA-col IV, the mRNA relative expression of the different markers was analyzed by using the expression of K3 in cells cultured on TCP as calibrator (Fig. 4A). In this case, the relative expression of K15 on PLA-col IV (1122.1 ± 86.6 fold) was significantly higher ($p < 0.01$) than the expression on TCP (713.4 ± 197.4 fold). Furthermore, the expression of K15 was significantly higher than the expression of all the rest of the markers ($p < 0.001$). In case of the transcription factor P63α, no differences were observed between TCP (95.3 ± 13.8 fold) and PLA-col IV (97.3 ± 11.7 fold).

On the other hand, the expression of K12 was significantly higher ($p < 0.05$) in the biopolymer (439.8 ± 135.5 fold) than in TCP (178.3 ± 80.6 fold). Moreover, K12 expression was higher than the ABCG2 and K3 expression when limbal epithelial cells were cultured on PLA-col IV.

Subsequently, the expression of K3 in cells cultured on PLA-col IV was used as calibrator to compare the expression levels of the different markers in cells cultured on PLA-col IV films (Fig. 4B). The relative expression of K15 in cells cultured on PLA-col IV (68.5 ± 5.3 fold) was significantly higher ($p < 0.001$) than the expression of K3 (1 ± 0 fold), K12 (26.8 ± 8.3 fold), ABCG2 (1.4 ± 0.4 fold) and P63α (5.9 ± 0.7 fold). The relative expression of P63α (5.9 ± 0.7 fold) was also significantly higher ($p < 0.01$) than the K3 and ABCG2 expression in cells cultured on the tested biopolymer. Moreover, K12 expression was significantly higher ($p < 0.05$) than the K3 expression when limbal epithelial cells were cultured on PLA-col IV. These expression levels detected in the cells cultured on PLA-col IV films indicate the presence of a stem-cell-rich limbal epithelial cell population.

The expression of the same markers in cells cultured on PLA-col IV films and TCP was also studied by immunofluorescence. In this case, the results were expressed in percentage of positive cells for each marker (Fig. 5). The specific limbal epithelial cytokeratin K15 was expressed in about 29.4% ± 0.1 of cells on PLA-col IV, which is significantly lower that the percentage of cells that expressed K3 ($p < 0.01$), K12 ($p < 0.001$) and ABCG2 ($p < 0.01$). However, no significant differences were found between the expression of ABCG2 compared with

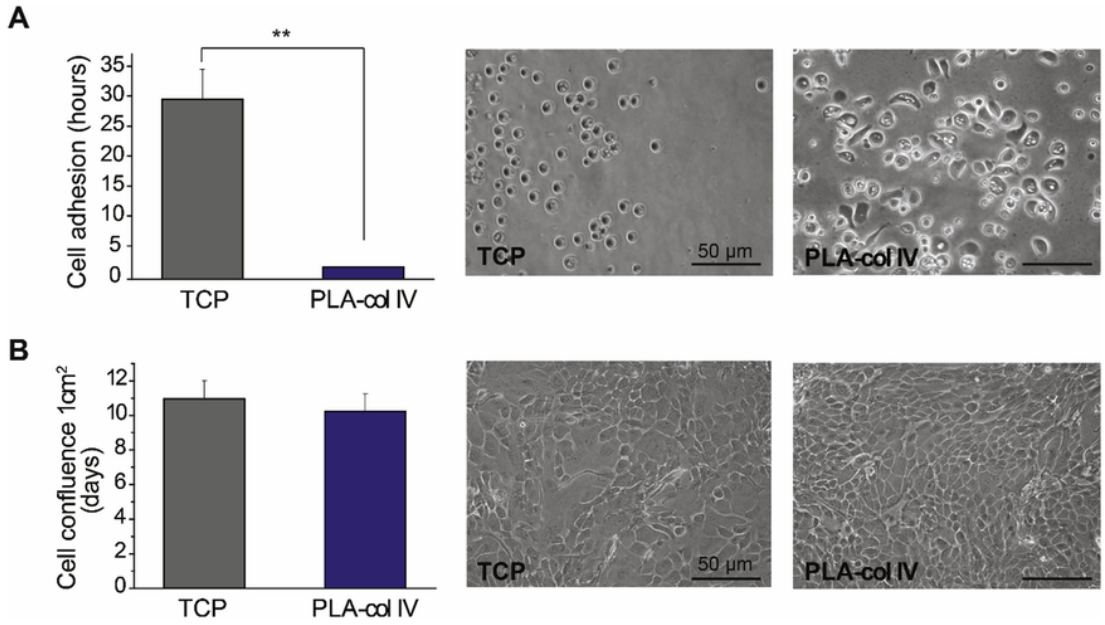

Fig. 3. PLA-col IV films improve limbal epithelial cell adhesion compared to TCP. A, left) Histogram representing the human LESCs adhesion time (hours) after seeding on PLA-col IV (blue, right bar) and TCP (gray, left bar); A, right) Representative bright field images (magnification ×40) of human LESCs plated on TCP and PLA-col IV after 2 h. B, left) Histogram representing the time (days) needed for human LESCs to reach confluence ($1cm^2$) on PLA-col IV (blue, right bar) and TCP (gray, left bar); B, right) Representative bright field images (magnification ×40) of confluent cultures of human LESCs on TCP and PLA-col IV. Plot represents the average ± SEM of cell cultures obtained from 11 different donors (N = 11). Scale 50 µm. *p < 0.05, **p < 0.01, ***p < 0.001. PLA-col IV: poly-L/DL-lactic acid 70:30 biopolymers functionalized with type IV collagen; TCP: tissue culture plastic.

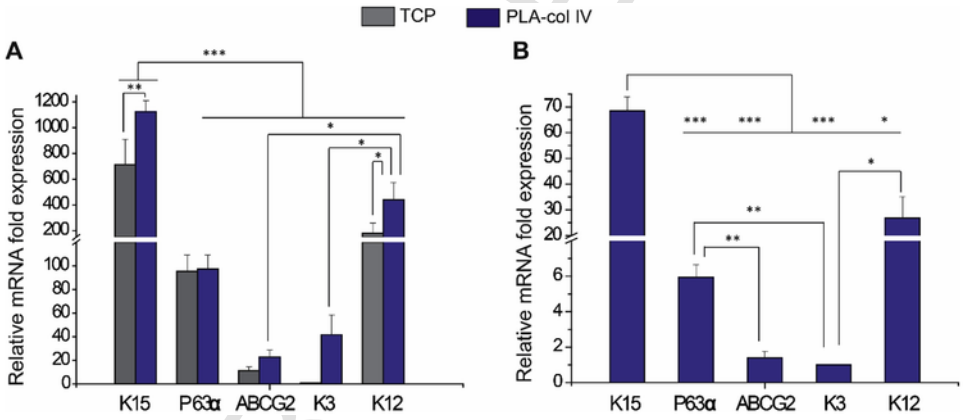

Fig. 4. Human LESCs cultivated on PLA-col IV films maintain the phenotype (at mRNA expression level) of the LESC population. Relative mRNA expression levels of limbal (K15, P63α and ABCG2) and corneal (K3 and K12) epithelial cells markers using the expression of K3 on TCP (gray, left bars in A) (A) or K3 expression on PLA-col IV (blue) (B) as calibrators. N = 4 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. PLA-col IV: poly-L/DL-lactic acid 70:30 biopolymers functionalized with type IV collagen; TCP: tissue culture plastic; LESC: limbal epithelial stem cell.

K3 and K12, showing 78.3% ± 0.1 positive cells for ABCG2, and 80.9% ± 0.1 and 96.2% ± 0.0 positive cells for K3 and K12, respectively (Fig. 5). Otherwise, a high percentage of cells expressed the transcription factor P63α (36.9% ± 0.1), described in limbal epithelial stem cells.

4. Discussion

In the present work, PLA biopolymers, functionalized with col IV, were evaluated as potential carrier substrata for the culture and further transplantation of LESCs to the ocular surface. These PLA-col IV films ensured enough flexibility and transparency to make this biomaterial ideal for use in tissue engineering. In addition, PLA is biocompatible and biodegradable, and it is known that its degradation products are non-toxic. Several analogs of PLA have been used in different biomedical applications as well as in advanced therapy drugs in tissue engineering. In the field of ophthalmology, some members of the PLA family (pure or combined with other materials) are being used in preclinical studies [37]. However, it is known that PLA alone is not adequate for the adhesion of some cell types [38,39].

To improve cell attachment to PLA, we functionalized the films with a human extracellular matrix protein. It has been described that the limbal epithelial basal membrane has several extracellular matrix proteins. These proteins are secreted by stromal and limbal epithelial cells, and they are essential for the adhesion of LESCs. Among all the proteins, col IV α1 and α2 chains have been described as the specific proteins in the limbal area, and they are not present in the differentiated corneal epithelial basal membrane [3,40,41]. Additionally, type IV col has been used to select LESC populations based on their fast attachment to these surfaces. It has also been demonstrated that cells containing higher col IV-adhesion properties mainly expressed LESC

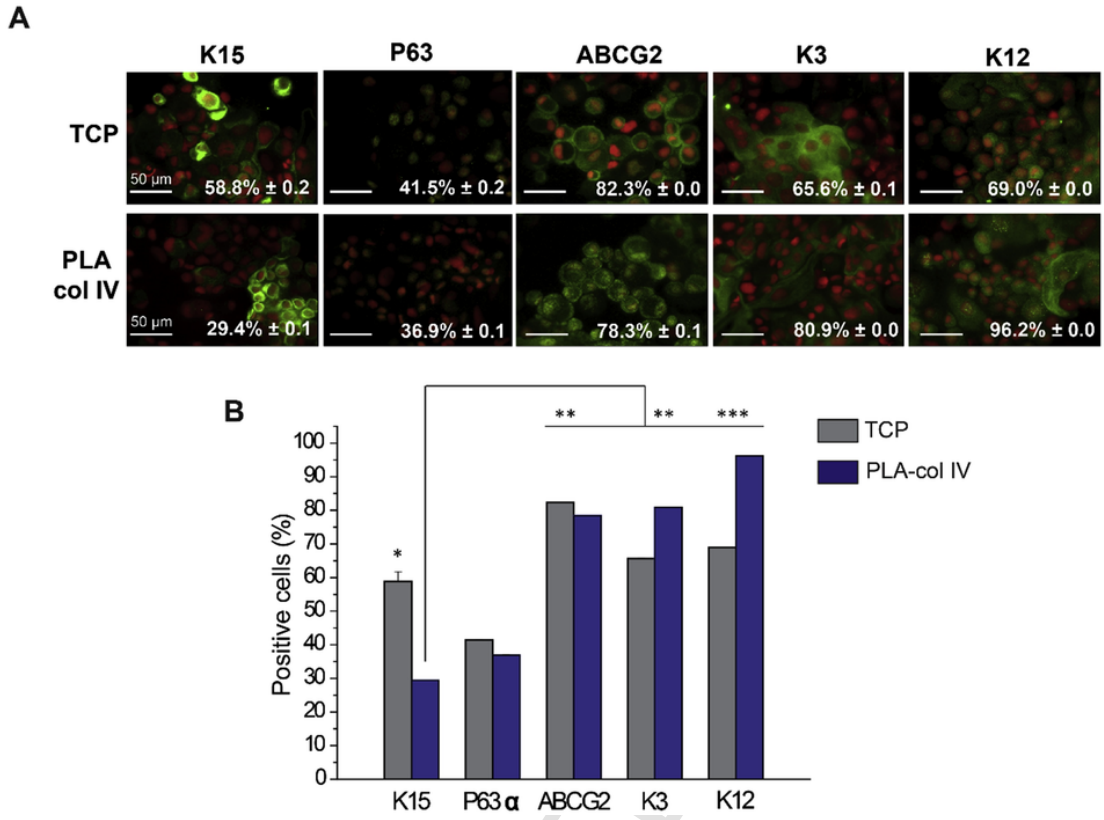

Fig. 5. Human LESCs cultivated on PLA-col IV films maintain the phenotype (at protein expression level) of the LESC population. (A) Immunofluorescence staining (green) for K15, P63α, ABCG2, K3 and K12. Red nuclear counterstain with propidium iodide; magnification ×40. Scale 50 µm. B) Histogram representing the percentage of positive cells ± SEM of each marker on TCP (gray, left bars) and PLA-col IV (blue, right bars). Micrographs are representative of four independent experiments (N = 4). *p < 0.05, **p < 0.01, ***p < 0.001. PLA-col IV: poly-L/DL-lactic acid 70:30 biopolymers functionalized with type IV collagen; TCP: tissue culture plastic; LESC: limbal epithelial stem cell.

markers, with decreased expression of corneal differentiated markers [52].

Considering all these findings, col IV was used in the present study to functionalize the PLA films, to improve the culture, attachment, and enrichment of LESCs. Col IV has been described in some studies not only as an adhesion protein, but also as a biomaterial that supports the culture of LESCs and stromal cells. Several types of collagen, such as collagen I (Vitrigel) [53], collagen III [16], or collagen together with chitosan [54] have been used for the *in vitro* reconstruction of the corneal epithelium [55]. Moreover, some studies have demonstrated the use of a compressed collagen matrix containing embedded fibroblasts, which simulate the corneal stroma, as substrates for LESCs [21,24,56]. In most of those studies, the authors tried to get a stratified and differentiated corneal epithelium using the air-lifting technique. However, in our case, this was not one of our aims because when the cells start to form more than one layer, they lose the stem cell phenotype [41]. Therefore, the aim of our work was to obtain cell monolayers in order to avoid cell differentiation and retain the stem cell-ness to further transplant a stem-cell-rich population onto the ocular surface. Furthermore, to elude non-human, animal components in the cell cultures, murine feeder cells were not used and the culture medium was free of animal supplements [18]. To our knowledge, there are no studies that have used PLA-col IV substrates with similar goals to our study, making it impossible to compare our results with any publication.

Due to the difficulty of obtaining human limbal epithelial primary cultures, a human corneal epithelial (HCE) cell line was used for a viability-cytotoxicity test to determine the biocompatibility of the PLA-col IV substrates. The PLA-col IV substrates were not toxic, and even after observing a lower cell density at the final time point of cells cultured on the polymer (compared to the ones cultured on TCP), the cell density on the PLA-col IV substrates increased by approximately four times over 8 days, making this substrate a potential carrier for human corneal epithelial cells. Despite the lack of studies found on the specific interaction of this polymer with corneal epithelial cells, the successful biocompatibility results obtained with the HCE cells are in line with other studies that used analog biomaterials, and with other investigations related to collagen use for corneal epithelial cell culture [16,53,54]. Additionally, the increase in cell proliferation of HCE cells grown on PLA-col IV also coincides with other studies performed with similar materials combined with col IV.

Once biocompatibility of the PLA-col IV was demonstrated with HCEs, we wanted to try its suitability as a carrier substrate for human LESCs. To study cell adhesion from isolated cells, limbal epithelial cells were obtained by enzymatic dissociation of cadaveric human corneoscleral buttons instead of from small biopsies. The enzymatic action of dispase increased the purified isolation of corneal epithelial cells and decreased the stromal cell percentage [7,44,57]. Another advantage of using the presented technique is that it allows the initial plating cell density to be the same, making it possible to compare different experimental conditions easily.

A total of 4×10^4 human limbal epithelial cells/cm^2 were plated on each substrate and it was observed that the cells adhered significantly faster to the PLA-col IV films (2 h) compared to TCP (24–48 h). As mentioned previously, some authors have described the capacity of col IV to be used as a method to select LESCs. Considering this, the biopolymer used in the present work could not only improve the attachment capacity of LESCs, but also the selection and enrichment of a stem cell population. In order to test the maintenance of the stem cell population, the expression of LESC and differentiated corneal epithelial cell markers was analyzed. At the mRNA level, the expression of the

LESC specific marker, K15, was significantly higher in limbal epithelial cells grown on PLA-col IV compared to the expression in the same cells cultured on TCP. Moreover, K15 expression was also greater than some differentiated corneal epithelial cell markers (K3 and K12), and the relative expression of P63α (another specific marker of LESCs) was also higher than the expression of K3 in limbal epithelial cells cultured on the PLA-col IV substrates. Considering all this data together, our results suggest that the PLA-col IV substrate, apart from improving the LESC attachment to the surface, can maintain the undifferentiated stem cell phenotype of these cells.

At the protein level, the percentage of cells expressing p63α and ABCG2 was similar in limbal epithelial cells cultured on PLA-col IV and TCP. However, the percentage of cells expressing the LESC marker, K15, was lower than the percentage of cells that expressed ABCG2, K3, or K12. The mRNA and protein expression results might seem contradictory, but the parameters analyzed were different in each technique. Relative mRNA quantification was completed using real time PCR, while the percentage of cells expressing each protein was calculated by immunofluorescence, where a quantitative expression level for each protein was not determined. Previous reports have shown that only around 5–10% of cells present in the limbal niche are undifferentiated stem cells [2,58], so as expected, the percentage of cells grown on PLA-col IV expressing LESC markers (K15 and P63α) was lower than the percentage of cells expressing K3 and K12 as corneal epithelial cell markers. Nevertheless, due to the fact that only 5–10% of the limbal cells are stem cells [](58) [2,58], the percentage of cells found on PLA-col IV films expressing K15, P63α, and ABCG2 should be considered as adequate.

5. Conclusion

Our results demonstrate that PLA-col IV films are suitable for the attachment, proliferation, and maintenance of a stem-cell-containing population of limbal epithelial cells, and support the use of synthetic biopolymers as carrier substrata for the transplantation of limbal epithelial cells onto the ocular surface.

Conflict of interest

No competing financial interests exist.

Acknowledgments

This work was supported by the Carlos III National Institute of Health, Spain (CIBER-BBN and Spanish Network on Cell Therapy, (TerCel RD12/0019/0036), MINECO/FEDER, EU), and the Castilla y León Regional Government, Spain (Regional Center for Regenerative Medicine and Cell Therapy, SAN673/VA/28/08 and SAN126/VA11/09). M. López-Paniagua and S. Galindo were supported by scholarships co-financed by the Castilla y Leon Regional Government and the European-Social-Fund. We thank M. F. de la Paz, MD, (Barraquer Eye Bank of Barcelona, Spain) for providing human corneoscleral tissues. We also thank J.M. Herreras, MD, for his clinical advice, V. Sáez for her initial technical support and I. Fernández (Statistics Unit, IOBA, University of Valladolid, Spain) for statistical assistance.

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