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Epithelial basement membrane of human decellularized cornea as a suitable substrate for differentiation of embryonic stem cells into corneal epithelial-like cells

Thaís Maria da Mata Martins[a,*], Pricila da Silva Cunha[b], Michele Angela Rodrigues[c], Juliana Lott de Carvalho[d,e], Joyce Esposito de Souza[b], Junnia Alvarenga de Carvalho Oliveira[f], Dawidson Assis Gomes[b], Alfredo Miranda de Goes[c]

[a] Department of Morphology, Institute of Biological Sciences, Federal University of Minas Gerais, Avenida Presidente Antônio Carlos, 6627, Belo Horizonte 31270-901, Minas Gerais, Brazil
[b] Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais, Avenida Presidente Antônio Carlos, 6627, Belo Horizonte 31270-901, Minas Gerais, Brazil
[c] Department of Pathology, Institute of Biological Sciences, Federal University of Minas Gerais, Avenida Presidente Antônio Carlos, 6627, Belo Horizonte 31270-901, Minas Gerais, Brazil
[d] Department of Genomic Sciences and Biotechnology, Catholic University of Brasília, QS 07 – Lote 01, EPCT - Taguatinga, Brasília, Distrito Federal 71966-700, Brazil
[e] Faculty of Medicine, University of Brasília, Campus Universitário Darcy Ribeiro, Brasília, Distrito Federal 70910-900, Brazil
[f] Department of Microbiology, Federal University of Minas Gerais, Avenida Presidente Antônio Carlos, 6627, Belo Horizonte 31270-901, Minas Gerais, Brazil

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ABSTRACT

The ability to decellularize and recellularize the corneas deemed unsuitable for transplantation may increase the number of available grafts. Decellularized corneas (DCs) may provide a natural microenvironment for cell adhesion and differentiation. Despite this, no study to date has evaluated their efficacy as a substrate for the induction of stem cell differentiation into corneal cells. The present study aimed to compare the efficiency of NaCl and NaCl plus nucleases methods to decellularize whole human corneas, and to investigate the effect of epithelial basement membrane (EBM) of whole DCs on the ability of human embryonic stem cells (hESCs) to differentiate into corneal epithelial-like cells when cultured in animal serum-free differentiation medium. As laminin is the major component of EBM, we also investigated its effect on hESCs differentiation. The decellularization efficiency and integrity of the extracellular matrix (ECM) obtained were investigated by histology, electron microscopy, DNA quantification, immunofluorescence, and nuclear staining. The ability of hESCs to differentiate into corneal epithelial-like cells when seeded on the EBM of DCs or laminin-coated wells was evaluated by immunofluorescence and RT-qPCR analyses. NaCl treatment alone, without nucleases, was insufficient to remove cellular components, while NaCl plus nucleases treatment resulted in efficient decellularization and preservation of the ECM. Unlike cells induced to differentiate on laminin, hESCs differentiated on DCs expressed high levels of corneal epithelial-specific markers, keratin 3 and keratin 12. It was demonstrated for the first time that the decellularized matrices had a positive effect on the differentiation of hESCs towards corneal epithelial-like cells. Such a strategy supports the potential applications of human DCs and hESCs in corneal epithelium tissue engineering.

1. Introduction

The cornea is the transparent and outer protective layer of the eye. It consists of three cellular layers: the anterior epithelium, which is attached to the epithelial basement membrane (EBM) and is renewed by limbal stem cells (LSCs); the multilamellar collagenous stroma, and the

Abbreviations: CCs, control corneas; CECs, corneal epithelial cells; DCs, decellularized corneas; EBM, epithelial basement membrane; ECM, extracellular matrix; FBS, fetal bovine serum; GAG, glycosaminoglycans; hESCs, human embryonic stem cells; HS, pooled allogeneic human serum; LF, limbal fibroblast; LF-CM, limbal fibroblast-conditioned epithelial medium; LSCD, limbal stem cell deficiency; LSCs, limbal stem cells; PSCs, pluripotent stem cells; SEM, scanning electron microscopy; TEM, transmission electron microscopy

* Corresponding author.
E-mail address: thaismmmartins@gmail.com (T.M. da Mata Martins).

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inner endothelium [1]. Corneal diseases are the fourth leading cause of blindness globally [2]. Although transplantation has been the only effective treatment for most corneal illnesses, it presents drawbacks, such as the risk of immune-mediated rejection and the shortage of high-quality donor corneas [3,4]. A recent global survey estimated that 12.7 million patients are on waiting lists for keratoplasty [3].

The development of bioengineered corneas is essential to meet the growing demand for transplantation [3]. Different natural or synthetic materials have been used to engineer full or partial thickness corneas, including amniotic membrane, collagen, and poly (L-lactic acid) [5,6]. However, all of these materials have limitations and fail to mimic the compositional, microarchitectural, biomechanical, and optical properties of the corneal extracellular matrix (ECM), whose highly ordered microstructure is notoriously difficult to be tissue engineered [7,8]. Today, bottom-up strategies are emerging as alternatives to scaffold-based approaches for the biofabrication of corneal stromal equivalents with more native-like structure and composition [9,10]. But the main limitations of bottom-up approaches are the limited thickness of the stromal tissue produced, the long culture time required, and the inability to produce full-thickness corneas [11,12].

In this context, decellularized corneas (DCs) are emerging as one of the most promising materials since they replicate the complex corneal ECM structure and composition, which should support proper optical and biomechanical properties [13,14]. DCs also provide a natural microenvironment for the adhesion, growth, migration, and differentiation of cells [15,16]. Despite this, no study to date has evaluated their efficacy as a substrate for the induction of stem cell differentiation into corneal cells. On average, 41–47% of donated corneas are rejected for transplantation, mainly due to low endothelial cell count [17,18]. Endothelial cell density is the main criterion for the evaluation of the donor corneal tissue quality and has been one of the factors used to predict the outcome of penetrating and endothelial keratoplasty [19–21]. Most of the discarded tissues are structurally intact and could potentially be used to engineer new corneas by the decellularization process [22]. Still, relatively few studies have investigated the use of human DCs for such application, in contrast to xenogeneic tissues, which have been comprehensively studied [23–30]. Nevertheless, in the clinical context, human tissues are always preferred over xenogeneic materials to minimize the risks of zoonotic infections and xenogeneic immune response [16,31]. In most corneal decellularization studies, only thin stromal sheets have been decellularized [13,32–34]. However, the presence and integrity of the EBM is very important for corneal epithelium reconstruction, as it plays critical roles in many corneal epithelial cells (CECs) functions, including adhesion, proliferation, and differentiation [35].

A previous study indicated that the use of hypertonic sodium chloride (NaCl) plus nucleases is the best method for whole human corneal decellularization [15]. However, nucleases are difficult to be removed from the tissue and might be cytotoxic [36,37]. It was reported that only NaCl treatment is sufficient to decellularize porcine corneas [24,26,27,30], but there is no evidence that this protocol may be used to successfully decellularize human corneas.

Besides the decellularization method, another critical issue in the context of corneal tissue engineering is the source of the cells used for recellularization. Given their pluripotency and unlimited self-renewal, human embryonic stem cells (hESCs) can serve as a source of clinically relevant cells, including CECs, and hold great potential for epithelium reconstruction in severe ocular surface diseases such as LSCs deficiency (LSCD) [28,38–40]. Currently, transplantation of *ex vivo* expanded LSCs or oral mucosal epithelial cells, commonly using amniotic membrane or fibrin as a carrier, are considered the most effective treatments for LSCD [41]. However, clinical trials have demonstrated that, despite favorable early results, the long-term outcomes are less satisfactory [42,43]. These may be due to inadequate properties of the transplanted cells and the lack of an appropriate substrate for them [44,45].

Given the importance of the structural, biochemical, and biomechanical support of the EBM and stroma for the function of the CECs, we hypothesized that the EBM of whole DCs might provide a proper and instructive substrate for the induction of stem cell differentiation to contribute to corneal epithelium reconstruction. Therefore, the present study aimed to compare the efficiency of NaCl and NaCl plus nucleases methods to decellularize whole human corneas, and to investigate the effect of EBM of DCs on the ability of hESCs to differentiate into corneal epithelial-like cells when cultured in animal serum-free differentiation medium. As laminin is the major component of EBM, we also investigated its effect on hESCs differentiation.

2. Materials and methods

All reagents were purchased from Thermo Fisher Scientific (Waltham, MA) unless otherwise stated.

2.1. Decellularization of human corneas

Sixty-two human corneoscleral buttons that had been stored in Optisol-GS media (Bausch & Lomb, CA) were obtained from the MG Transplantes Eye Bank/FHEMIG, BR. They were provided for research after being deemed unsuitable for transplantation due to low cell count or expiration date. The donor age ranged from 27 to 63 years old, and the female-male donor ratio was 2:3. The Ethics Committee in Research from the Universidade Federal de Minas Gerais (ETIC-UFMG no. 49967715.0.0000.5149) approved the use of human tissue, in accordance with the Helsinki Declaration, following informed consent from the donors' relatives. The excess of the sclera was removed, and the following two decellularization methods were performed to remove cells from the whole corneas (with central, paracentral, and peripheral limbal regions):

1-NaCl: the procedure was carried out at room temperature (RT) as described previously with some modifications [24]. Briefly, whole corneas were incubated in 1.5 M sterile NaCl (Sigma-Aldrich, St Louis, MI) dissolved in Milli-Q water for 48 h, followed by three washes with phosphate-buffered saline (PBS) for 24 h each.
2-NaCl plus nucleases: after incubation in 1.5 M NaCl, the corneas were treated with DNase 5 U/mL and RNase 5 U/mL at 37 °C for 48 h, and then washed with PBS for 72 h [15].

Untreated corneas were used as controls (CCs).

2.2. Evaluation of the optical properties

The corneas were imaged and the light transmittance percentage at 300–800 nm was measured using the Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) before and after deturgescence with glycerol for 10 minutes (min). The transparency was estimated from the light transmittance at an average of 450–600 nm [23].

2.3. Evaluation of the decellularization efficiency and the integrity of the ECM

Histological analyses: DCs and CCs were fixed in 4% paraformaldehyde, incubated in sucrose, and frozen in optimal temperature compound (Sakura, Torrance, CA). Cryosections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Alcian blue 8GX.
Scanning electron microscopy (SEM): the samples were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde, postfixed in 1% osmium tetroxide, and dehydrated in a graded series of ethanol. Then, they were critical point-dried, coated with gold, and examined by SEM at 15 kV (DSM 950 Zeiss, Jena, Germany).
Transmission electron microscopy (TEM): after fixation and

dehydration, the samples were embedded in Epon resin. Ultrathin sections were contrasted with 2% uranyl acetate followed by Reynold's lead citrate and observed by TEM (Tecnai G2-12-SpiritBiotwin FEI-120 kV).

Immunofluorescence and nuclear staining with Hoechst 33258: cryosections of DCs and CCs were permeabilized with 0.3% Triton X-100, blocked with 1% bovine serum albumin, and incubated with the following antibodies: rabbit monoclonal anti-laminin, mouse monoclonal anti-collagen type I (both 1:200, Abcam, Cambridge, MA), mouse monoclonal anti-collagen type IV (1:100, Santa Cruz Biotechnology, Dallas, TX), and rabbit polyclonal anti-fibronectin (1:100, Rockland, Limerick, PA). They were then incubated with Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG secondary antibodies (1:500). To detect nuclei, they were stained with 1 μg/mL Hoechst 33258 pentahydrate. The sections were analyzed with a Zeiss 5 Live confocal microscope (Jena, Germany).

Genomic DNA quantification and PCR: DNA was extracted from 25 mg of DCs and CCs using QIAamp DNA Mini Kit (Qiagen, Germantown, MD) according to manufacturer's instructions. The isolated DNA was quantified and used as a template for PCR. The primers used to amplify *SOX17* were: F: 5′-GTGAATCTCCCCGACAGC-3′ and R: 5′-TGTTTTGGGACACATTCAAAGC-3′.

2.4. Cell culture

The hESCs line H1 (WA01, WiCell Research Institute) [46] was maintained on Matrigel (Corning, Corning, NY) coated 6-well plates in mTeSR™1 medium (Stem Cell Technologies, Vancouver, BC) with medium changes daily. Cells were passaged at 1:6–1:8 ratio using Versene solution every 4–5 days.

Limbal fibroblasts (LF) were isolated from twelve human corneoscleral buttons (six donors aged 35–56 years old; female-male donor ratio of 1:1) not suitable for transplantation. The limbal stromal tissue was digested with 3 mg/mL collagenase type I for 3 h. The LF were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% antibiotic/antimycotic solution (both from Sigma-Aldrich, St Louis, MI) and 10% pooled allogeneic human serum (HS). When the cells reached 80–90% confluence, they were detached using 0.05% trypsin–EDTA and replated at a 1:4 ratio. HS was obtained from the whole blood of distinct blood-group-typed donors, as previously described by our group [47]. All donors provided written informed consent according to protocols approved by the ETIC-UFMG (no 49967715.0.0000.5149).

2.5. Conditioning of corneal epithelial medium by the LF

Confluent LF at passage 3 were mitotically inactivated with 10 μg/mL mitomycin C (Sigma-Aldrich, St Louis, MI) and on the next day 200 μL/cm^2 corneal epithelial medium was added. This medium consisted of DMEM/F12 supplemented with human epidermal growth factor, insulin, transferrin, hydrocortisone, tri-iodothyronine, cholera toxin, adenine, antibiotic/antimycotic solution (all from Sigma-Aldrich, St Louis, MI), and 10% HS. The LF-conditioned epithelial medium (LF-CM) was collected daily for 7 days. It was pooled, centrifuged, and stored at −80 °C. For all experiments, LF-CM was mixed with fresh corneal epithelial medium at a ratio of 3:1 [38].

2.6. Cell seeding and in vitro biocompatibility evaluation of DCs

One decellularized matrix (whole DC with central, paracentral, and peripheral limbal regions) per well was placed onto the bottom of a 24-well plate with the EBM side facing up. DCs were incubated in DMEM:F12 medium for 24 h at 37 °C in a CO_2 incubator before cell seeding. When hESCs reached approximately 80% confluence, they were dissociated into small colonies and split at a ratio of 1:10 (one well of 6-well plates:ten DCs). This corresponded to a density of approximately 5–6 colonies/DC. The hESCs colonies in 1 mL of mTeSR™1 medium were seeded on the EBM side of each DC. On the next day, the medium was replaced with a fresh mTeSR™1 medium or LF-CM. The recellularized matrices were maintained in mTeSR™1 medium for 7 days or in LF-CM for 14 days. Then, they were incubated with 5 μM Calcein-AM, and visualized through confocal microscopy (Zeiss LSM 880, Jena, Germany).

2.7. Corneal epithelial differentiation

Five to six hESCs colonies were seeded on the EBM side of the DCs as described above or plated in a 24-well plate coated with 2 μg/cm^2 laminin (Sigma-Aldrich, St Louis, MI) and cultured in LF-CM for up to 21 days. hESCs cultured on Matrigel in mTeSR™1 medium were used as controls (undifferentiated control cells).

2.8. Reverse transcription-qPCR (RT-qPCR)

Total RNA was extracted from control cells and differentiating hESCs seeded on the DCs or laminin-coated wells at days 3, 6, 9, 14, and 21 using Trizol reagent as described by the manufacturer. One μg of total RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit. The primers used are described in Table 1. RT-qPCR was carried out in Applied Biosystems 7500 Real-Time PCR System using Sybr Green PCR Master Mix, according to manufacturer's instructions. Relative gene expression was calculated using the mathematical model proposed by Pfaffl [48] and the relative expression software tool (REST, http://rest.gene-quantification.info) [49]. *GAPDH* was used as a reference gene and undifferentiated hESCs (cells cultured on Matrigel in mTeSR™1 medium) were used as calibrator. The undifferentiated control was assigned a value of 1, and all other values for differentiation time points were calculated relative to this.

2.9. Immunofluorescence

The hESCs maintained on the DCs or laminin-coated wells in LF-CM for 9 days were processed for immunofluorescence. The primary antibodies used were the mouse monoclonal anti-cytokeratin 3/2p and the affinity-purified goat polyclonal anti-cytokeratin 12 (both 1:100, from Santa Cruz Biotechnology, Dallas, TX). The secondary antibodies used were the Alexa Fluor 488 goat anti-mouse IgG and the Alexa Fluor 555

Table 1
Sequences of forward and reverse primers used in RT-qPCR assays.

Gene and accession number (GenBank)	Sequence (5′-3′)	Amplicon size (bp)
PAX6	F: TCACAAACACCTACAGCGCT	143
NM_000280.4	R: ATAACTCCGCCCATTCACCG	
TP63	F: ACGAAGATCCCCAGATGATG	141
NM_001114982.1	R: TGCTGTTGCCTGTACGTTTC	
KRT3	F: GAGAGTGTCCGAGTGCTGTC	86
NM_057088.2	R: GCCGTAACCTCCTCCATAGC	
KRT12	F: GTTATGGGGGAAGTGCCTTTGG	83
NM_000223.3	R: GCCGGAACTAGAACCAAACATG	
POU5F1	F: ATGTGGTCCGAGTGTGGTTC	94
NM_002701.5	R: GACCCAGCAGCCTCAAAATCC	
NANOG	F: CAGAAGGCCTCAGCACCTAC	111
NM_024865.3	R: ATTGTTCCAGGTCTGGTTGC	
SOX2	F: TGGGTTCGGTGGTCAAGTCC	67
NM_003106.3	R:CTGGAGTGGGAGGAAGAGGTAAC	
GAPDH	F: ACATCGCTCAGACACCATG	143
NM_002046.5	R: TGTAGTTGAGGTCAATGAAGGG	

PAX6 - paired box 6; *TP63* - tumor protein 63; *KRT3* - keratin 3; *KRT12* - keratin 12; *POU5F1* - POU class 5 homeobox 1; *NANOG* - Nanog homeobox; *SOX2* - SRY-box transcription factor 2; *GAPDH* - glyceraldehyde-3-phosphate dehydrogenase.

donkey anti-goat IgG. The images were captured using a Zeiss LSM 880 confocal microscope (Carl Zeiss, Jena, Germany). Images of cells labeled with anti-cytokeratin 12 were analyzed by ImageJ software (National Institute of Health, Bethesda, MD) to measure cell size.

2.10. Statistical analyses

All experiments were repeated three or more times with triplicate samples. Statistical analyses of DNA quantification and transmittance were performed using Student's *t*-test and one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, respectively, and the values are presented as the mean ± standard deviation (GraphPad Prism 6.0). Relative gene expression (and standard error of the mean) between differentiated and undifferentiated control cells was calculated using REST, and the pair-wise fixed reallocation randomization test was used to determine statistical significance. Significance was considered for $p < 0.05$.

3. Results

3.1. Decellularization efficiency

The removal of cellular components from the corneas exposed to two different decellularization treatments was investigated by H&E and nuclear staining, TEM, and DNA content analysis (Fig. 1). Corneas processed using NaCl treatment were only partially decellularized as evidenced by the presence of cellular and nuclear remnants in the stroma (Fig. 1A.2, B.2, C.2). In contrast, complete removal of the epithelial, stromal, and endothelial cells, including cell debris and nuclei, was observed in corneas treated with NaCl plus nucleases (Fig. 1A.3, B.3, C.3). Quantitative analysis of DNA content showed a significant average reduction of 43.7% and 99.6% after decellularization with NaCl and NaCl plus nucleases, respectively, compared to CCs ($p < 0.05$) (Fig. 1D). PCR analysis confirmed the presence of DNA remnants in NaCl treated corneas, whereas no amplification occurred with DNA from NaCl plus nucleases decellularized corneas (Fig. 1D.2, .4).

As NaCl treatment resulted in incomplete removal of cellular components, the complete characterization and all subsequent experiments were performed only with NaCl plus nucleases treated corneas.

3.2. Evaluation of the optical properties and the integrity of the ECM

The light transmittance percentage of DCs before treatment with glycerol was about 59%, whereas the transmittance of the glycerol-treated DCs was about 73%, similar to that of CCs (Fig. 2A). The macroscopic evaluation also demonstrated that the NaCl plus nucleases treatment caused opacity of the corneas; however, their transparency was recovered after deturgescence with glycerol (Fig. 2B).

Alcian blue (Fig. 2C), PAS staining (Fig. 2D), and immunofluorescence (Fig. 2E) demonstrated that glycosaminoglycans (GAG), glycoproteins, and collagen type I were retained after decellularization process. TEM analysis revealed that the ultrastructure of the DCs was well preserved (Fig. 2F).

SEM (Fig. 3A) and TEM (Fig. 3B) micrographs demonstrated the preservation of the ultrastructural integrity of the corneal EBM and Bowman's layer after decellularization. The immunostaining (Fig. 3C) showed the regular distribution of the major components of the EBM and Descemet's membrane, laminin, collagen type IV, and fibronectin, in DCs.

3.3. In vitro biocompatibility of DCs

In vitro recellularization showed the ability of DCs to support hESCs culture (Fig. 4). The cells were seeded on the EBM of DCs and cultured in mTeSR™1 medium for 7 days or in LF-CM for 14 days. Calcein-AM staining demonstrated that hESCs remained viable and were uniformly distributed throughout the anterior surface of the DCs in both culture conditions. The cells maintained in mTeSR™1 medium formed large compact multicellular colonies (Fig. 4A). In contrast, the cells cultured

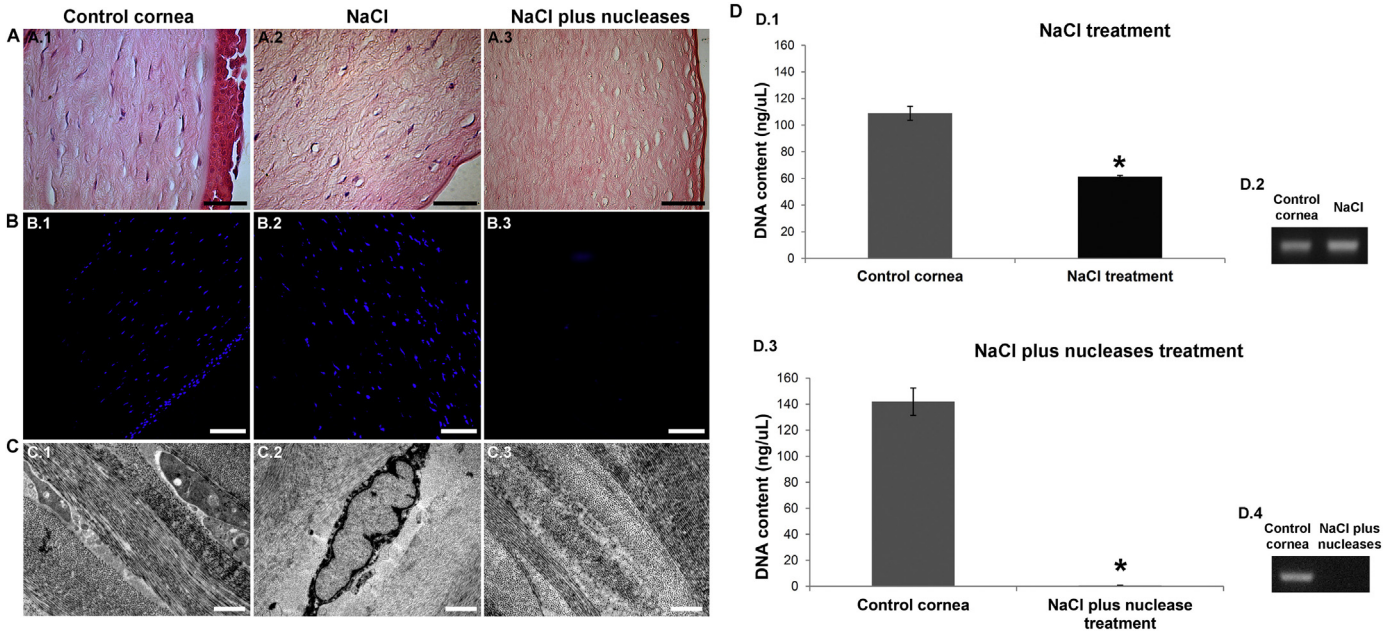

Fig. 1. Evaluation of the decellularization efficiency. H&E (A) and nuclear Hoechst 33258 (blue, B) staining, transmission electron micrographs (C), and DNA content analysis (D) of control corneas (A.1, B.1, C.1) and corneas exposed to the NaCl (A.2, B.2, C.2, D.1, D.2) or NaCl plus nucleases decellularization (A.3, B.3, C.3, D.3, D.4) methods. Representative images (A, B, C), genomic DNA quantification (D.1, D.3) and PCR analysis (D.2, D.4) showed the presence of cellular and nuclear remnants in the corneas processed using NaCl treatment whereas total removal of the cellular components was achieved using NaCl plus nucleases decellularization procedure. Data are presented as the mean ± standard deviation. Experiments were performed four times independently with triplicate samples. *Compared to control corneas ($p < 0.05$, Student's *t*-test). Scale bars: 50 μm (A), 100 μm (B), 1 μm (C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

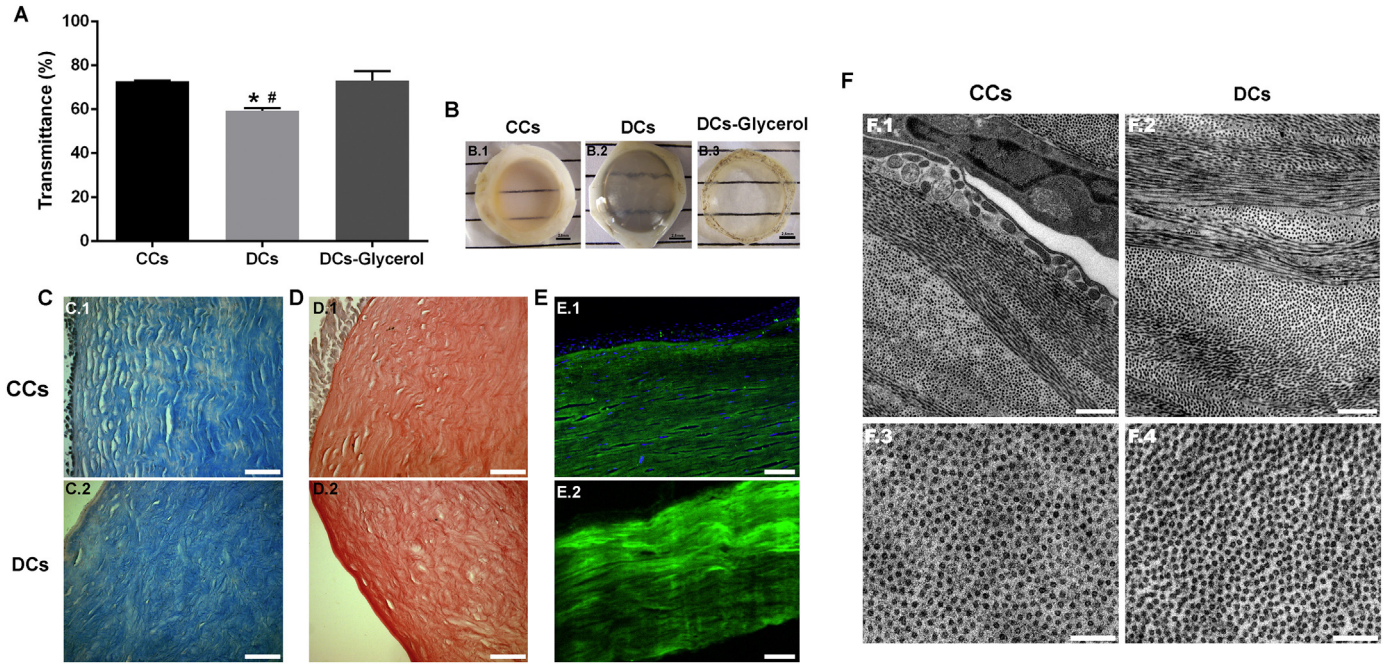

Fig. 2. Analyses of the corneal transparency and the integrity of the stromal matrix after decellularization with NaCl plus nucleases. (A) The light transmittance percentage of the CCs, DCs and glycerol-treated DCs. Data are presented as the mean ± standard deviation. This experiment was performed three times independently with triplicate samples. One-way ANOVA and Tukey's multiple comparison test was performed. $^{*}p < 0.05$ *versus* CCs; $^{\#}p < 0.05$ *versus* glycerol-treated DCs. (B) Macroscopic evaluation of decellularized corneal tissue transparency before (B.2) and after (B.3) deturgescence with glycerol. Scale bars: 2.6 mm. (C) Alcian blue staining indicated the presence of glycosaminoglycans and proteoglycans in the decellularized corneal stromal. Scale bar: 80 μm. (D) PAS staining demonstrating the retention of glycoproteins after the decellularization process. Scale bar: 80 μm. (E) Immunofluorescence image showing the preservation of collagen type I (green) in decellularized matrix. Scale bar: 80 μm. (F) Transmission electron micrographs showing the similarity of ultrastructure between control (F.1, F.3) and decellularized (F.2, F.4) corneas and the presence of keratocytes only in control corneas (F.1). Scale bars: 500 nm (F.1, F.2), 200 nm (F.3, F.4). Panels show representative images of three independent experiments. CCs, control corneas; DCs, decellularized corneas. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

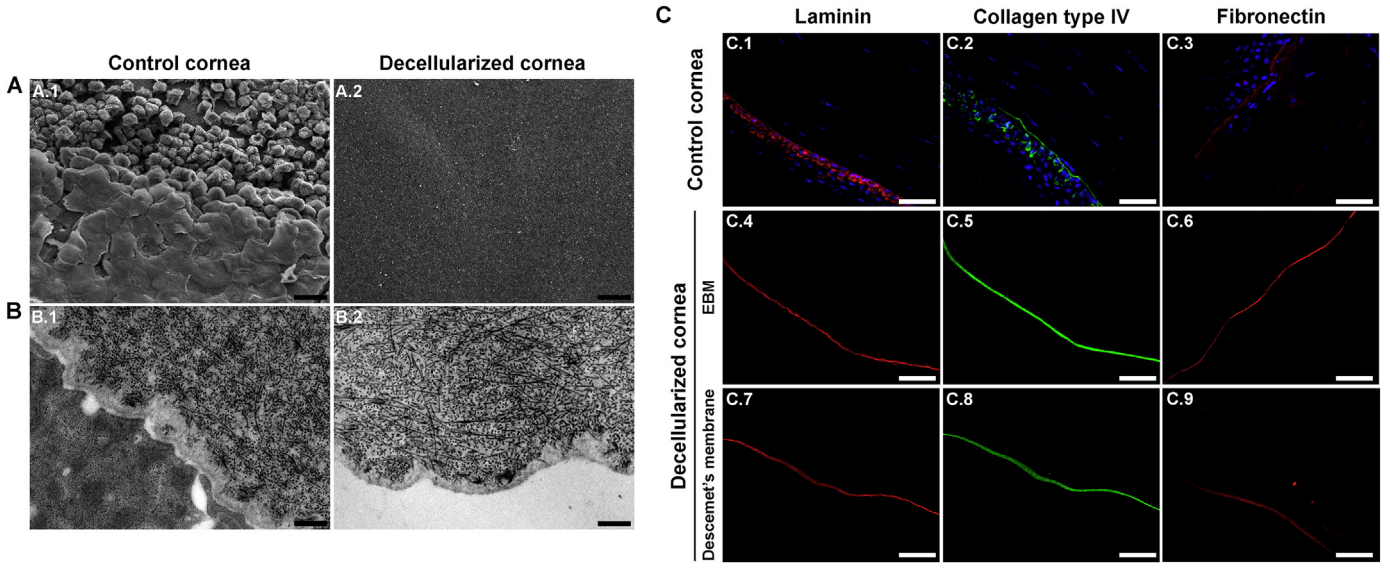

Fig. 3. Analysis of the integrity of the corneal epithelial basement membrane, Bowman's layer, and Descemet's membrane after decellularization with NaCl plus nucleases. Scanning (A) and transmission (B) electron micrographs of control (A.1, B.1) and decellularized (A.2, B.2) corneas showing absence of epithelial cells and preservation of the basement membrane and Bowman's layer ultrastructure upon decellularization. Scale bars: 20 μm (A), 500 nm (B). Immunofluorescence images of control (C.1–C.3) and decellularized (C.4–C.9) corneas showing positive staining for the epithelial basement membrane (C.4–C.6) and Descemet's membrane (C.7–C9) proteins, laminin (red, C.1, C.4, C.7), collagen type IV (green, C.2, C.5, C.8), and fibronectin (red, C.3, C.6, C.9). Scale bars: 50 μm. Panels show representative images of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

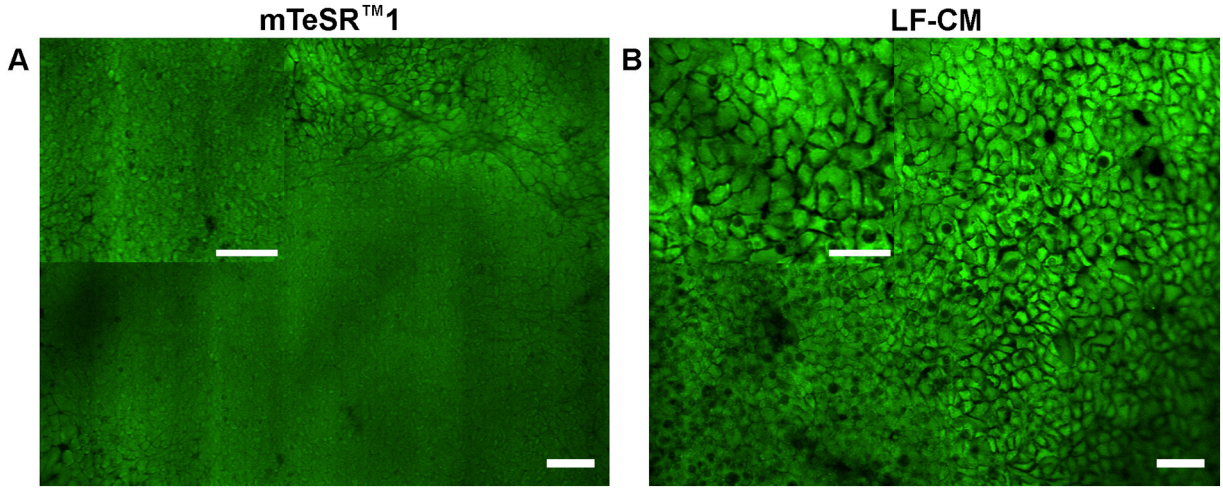

Fig. 4. Cell seeding and *in vitro* biocompatibility evaluation of decellularized corneas. Confocal images showing the viability, adhesion and morphology of the hESCs seeded on decellularized corneas and cultured in mTeSR™1 medium for 7 days (A) or in LF-CM for 14 days (B). Central regions of the recellularized corneas were represented. Magnified views are shown in the insets. Panels show representative images of three independent experiments. Scale bars: 100 μm.

in LF-CM lost the colony morphology and appeared predominantly polygonal (Fig. 4B).

3.4. Corneal epithelial differentiation

The effects of DCs and laminin on the ability of hESCs to differentiate into CECs were investigated. The loss of hESCs pluripotency during the differentiation process was assessed by RT-qPCR (Fig. 5). For cells differentiated on DCs, the expression of *POU5F1*, *SOX2*, and *NANOG* declined significantly by the third, sixth and ninth day of differentiation when compared to undifferentiated control hESCs, respectively, reaching nearly negligible levels by day 14. On the contrary, *POU5F1* and *NANOG* expression increased at days 6 and 9 for cells induced to differentiate on laminin, and the expression levels of these genes as well as of *SOX2* decreased significantly only by day 14.

For the hESCs differentiated on DCs, the downregulation of pluripotency markers was accompanied by an increase in the expression of corneal epithelial markers (Fig. 5). The gene expression of *PAX6* peaked at day 14 of differentiation on DCs, showing almost 80-fold increase when compared to undifferentiated control cells. In the case of the hESCs induced to differentiate on laminin, the peak in *PAX6* expression was much less pronounced, showing almost 7-fold increase in expression at day 3, and a significant decrease in *PAX6* expression was detected at days 14 and 21. The expression level of the putative LSCs marker, *TP63*, reached more than 50- and 230-fold increase at day 21 of differentiation on DCs and laminin, respectively. An increase in the expression of corneal epithelial cell-specific keratins, *KRT12* and *KRT3*, was only observed in cells differentiated on DCs, in which the expression of these markers reached more than 193- and 32-fold increase at days 6 and 21, respectively. In contrast, no change was observed in the expression levels of *KRT12* and *KRT3* in cells induced to differentiate on laminin in all the time points studied.

Cell differentiation was also assessed by immunofluorescence (Fig. 6), which confirmed high expression of KRT12 and KRT3 in hESCs

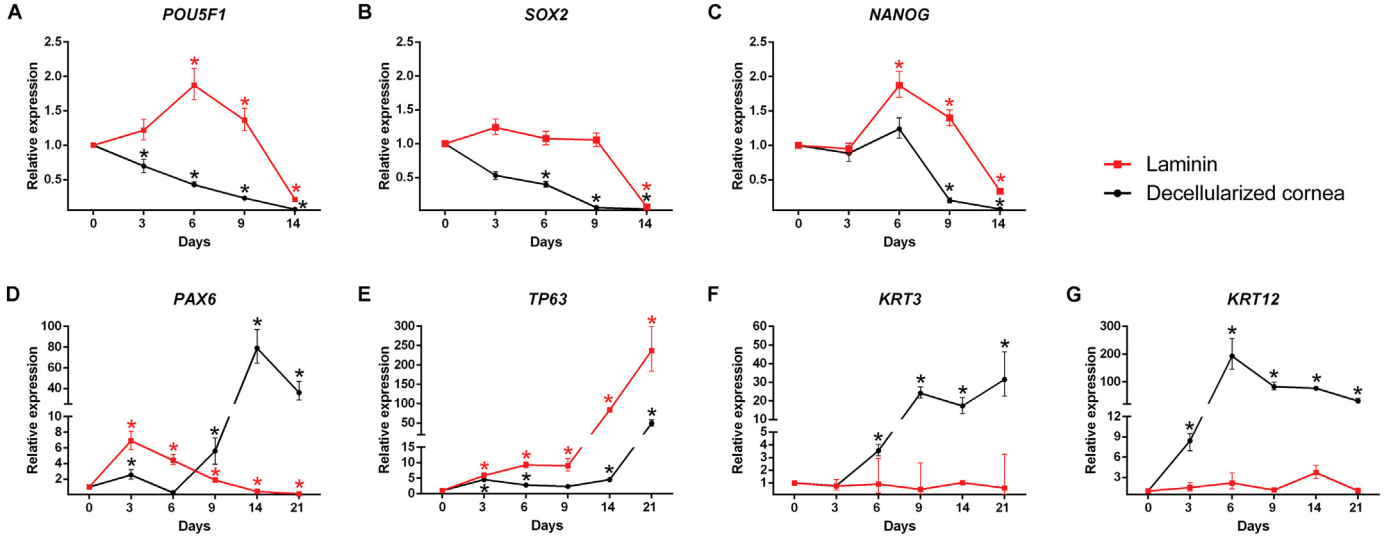

Fig. 5. Relative gene expression of pluripotency-associated transcription factors and corneal epithelial markers during the differentiation process. Graphical representation of RT-qPCR analysis of *POU5F1* (A), *SOX2* (B), *NANOG* (C), *PAX6* (D), *TP63* (E), KRT3 (F), and *KRT12* (G) expression during the differentiation of hESCs cultured on decellularized corneas (black line) or on laminin (red line). The relative gene expression ratios between differentiated and undifferentiated cells normalized to the reference gene (*GAPDH*) were calculated using REST. Statistical significance was determined using the pair-wise fixed reallocation randomization test. Data are presented as the mean ± standard error of the mean. Experiments were performed three times independently with triplicate samples. *Compared to undifferentiated cells ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

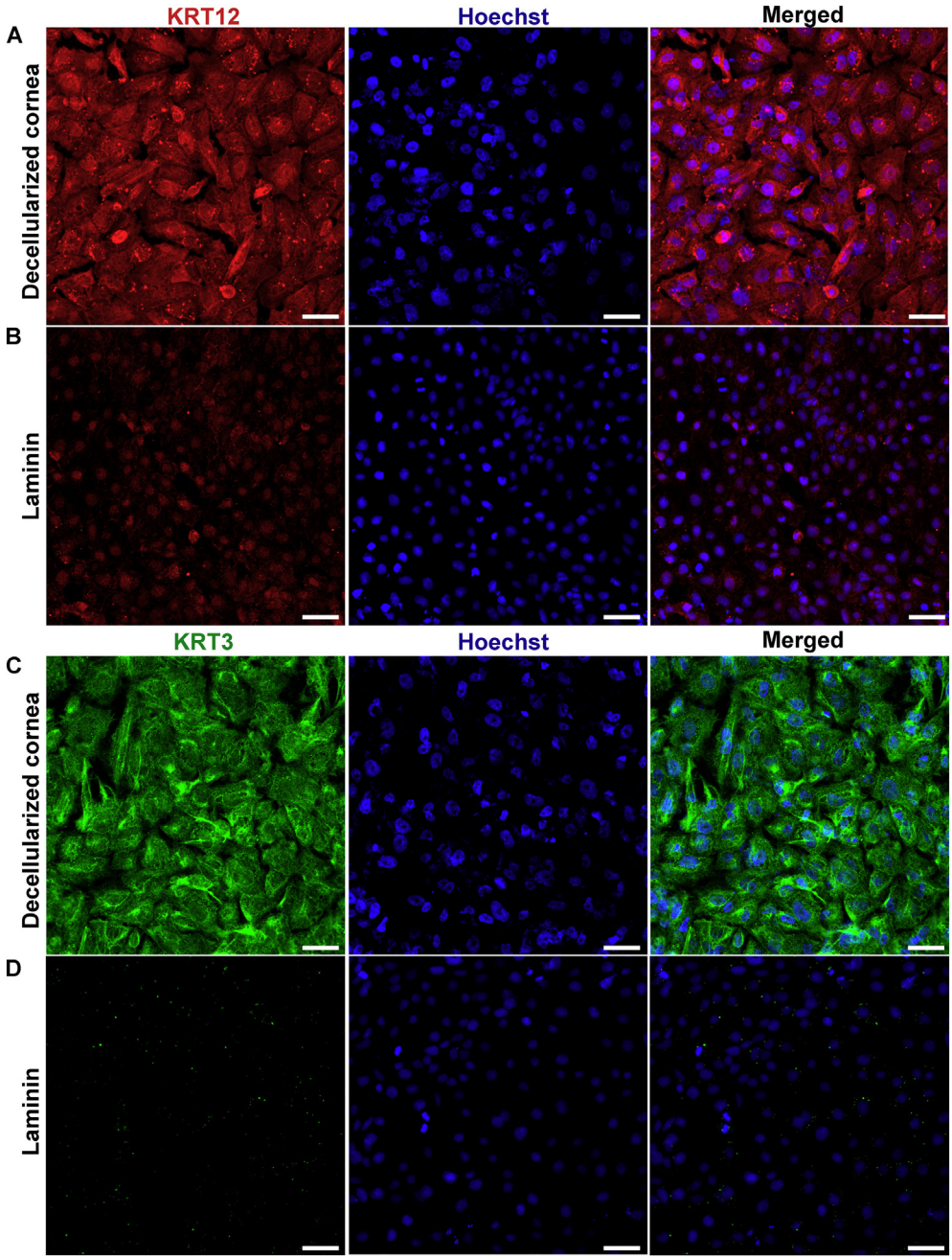

Fig. 6. KRT12 and KRT3 protein expression in hESCs after 9 days of differentiation. Confocal images showing the expression of KRT12 (red, A and B) and KRT3 (green, C and D) in cells cultivated on decellularized corneas (A and C) and on laminin (B and D). Nuclei were stained with Hoechst (blue). Panels show representative images of three independent experiments. Scale bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

seeded on DCs and cultured in LF-CM for 9 days. The average size of these cells was 54.5 ± 21 μm. Although cells induced to differentiate on laminin also expressed KRT12 and KRT3, a weak expression of these corneal epithelial-specific markers was observed in cells cultured on this substrate, in which average cell size was 41.4 ± 9.7 μm.

4. Discussion

One factor contributing to the limited supply of corneas for transplantation is that up to 40% of donor corneas are discarded [17,18,22]. In most cases, the corneal ECM is intact, so the ability to decellularize and recellularize these discarded tissues may increase the number of available corneal grafts [22]. However, few studies investigated the use of human corneas [29]. Most of the decellularization approaches proposed were tested in thin slices of animal corneas and did not meet the clinical transplantation requirements [26–28,50,51]. It has been shown that methods using Triton-X, liquid nitrogen, and poly(ethylene glycol) resulted in incomplete decellularization, whereas sodium dodecyl sulfate (SDS), trypsin, dispase, and NaOH altered protein and GAG content of the ECM, damaged the EBM and reduced the transparency of the cornea [15,22,24,30]. Supercritical carbon dioxide and high hydrostatic pressure are expensive and require specialized equipments [52,53]. For future clinical application of DCs, it is desirable to establish a cost-effective decellularization method that requires fewer steps and reagents [16]. A previous study indicated that a simple protocol based on NaCl and nucleases is effective at decellularizing whole

human corneas [15]. However, nucleases are difficult to be washed out and might be cytotoxic [36,37]. So, the first goal of this study was to compare two methods based on hypertonic NaCl, with and without nucleases treatment, to generate a full-thickness human DCs.

Our findings showed that only NaCl treatment resulted in incomplete decellularization, and contrast with other reports describing the efficient decellularization of porcine corneas after NaCl treatment [24,26,27,30]. These discrepant results may be due to species differences and may suggest that protocols used to decellularize xenogeneic tissues are not reproducible and do not work efficiently when applied to human corneas. Other reports also indicate that decellularization efficiency depends on the species of tissue origin [54,55].

Our results showed that NaCl plus nucleases method effectively removed cellular components and support the finding of Shafiq et al. [15], but contrast with data reported by Wilson et al. and Yam et al. which demonstrated that this procedure resulted in incomplete decellularization [22,56]. Such differing results may be due to the differences in the protocols and the source of the tissue (full cornea *vs* thin stromal lenticules). Wilson et al. incubated whole corneas in 1.5 M NaCl dissolved in PBS containing protease inhibitors only for 24 h [22], while in our study and in that of Shafiq et al. the incubation time was 48 h [15]. Yam et al. incubated thin corneal stromal lenticules in 1.5 M NaCl dissolved in PBS for 24 h [56]. In the present study the NaCl was dissolved in Milli-Q water. Recently, Islam et al. also have shown that changing the solvent of the solution from PBS to water optimized the decellularization effectiveness [53].

Here, we performed for the first time the analyses of DNA content, light transmittance, and Descemet's membrane integrity of whole human corneas decellularized by NaCl plus nucleases process. After this treatment, the residual DNA was effectively diminished to approximately 0,02 ng per mg tissue, with an average reduction of 99,6%, fulfilling the standard requirement of less than 50 ng DNA per mg ECM dry weight [36]. Genomic DNA was not detected even after PCR amplification.

Light transmittance and macroscopic evaluation revealed that although the NaCl plus nucleases method caused opacity of the corneas, their transparency was completely recovered after deturgescence with glycerol, suggesting an absence of gross change in the stromal lamellar organization, which was confirmed by TEM. The maintenance of the ultrastructural integrity and the protein content of the EBM, Bowman's layer, stroma, and Descemet's membrane were also demonstrated here. On the contrary, Yam et al. showed that the treatment of the corneal lenticules with hypertonic 1.5 M NaCl and/or nucleases negatively affects the stromal architecture and composition [56]. They suggested that the decellularization of whole cornea with basement membrane preserved on both sides may restrict fibril distortion. This may explain the retention of collagen, glycoprotein and GAG content and the normal fibrillar stromal architecture achieved in our study.

The integrity of the EBM is particularly important for recellularization and epithelialization. It was shown that the use of detergents damaged the EBM, rendering the DCs unsuitable for limbal epithelial cells attachment, whereas NaCl plus nucleases-treated corneas maintained an intact EBM and successfully supported the adhesion and differentiation of these cells [15]. In addition, the evaluation of expression of LSCs markers, TP63 and ABCG2, demonstrated that NaCl plus nucleases-treated corneas also maintained LSCs in an undifferentiated state, which, together with cell differentiation and migration processes, is important for corneal epithelial homeostasis and maintenance [15].

Our biocompatibility results indicated that decellularization agents were washed out thoroughly and the resulting DCs were not cytotoxic. Even though nucleases are considered difficult to be removed, they have been used and washed out successfully in corneal decellularization protocols, including those used in preclinical and clinical studies [32,57], supporting our results. Here, we showed for the first time the capacity of human DCs to support attachment and survival of undifferentiated hESCs as well as cells under corneal epithelial differentiation conditions. An increase in cell size and morphological changes were observed in cells induced to differentiate on DCs. They lost the colony morphology that are typical of undifferentiated hESCs and appeared predominantly polygonal, with average cell size of 54.5 ± 21 μm. The undifferentiated H1 cells grow as compact, multicellular colonies, and are heterogeneous populations that show distinct characteristics in size, with the presence of large and small cell subpopulations, with approximately 16 and 6.5 μm in size, respectively [58].

Different protocols have been proposed for the differentiation of pluripotent stem cells (PSCs) towards the corneal epithelial lineage, but fast and cost-effective methods have not been developed to date [59]. Most of the differentiation protocols have attempted to mimic the *in vivo* niche environment by coating the cell surfaces with commercially available EBM proteins, such as laminin and collagen type IV, by using murine PA6 feeder layer cells and/or conditioned medium harvested from limbal or corneal fibroblasts [38,59–63]. However, no study has evaluated the efficacy of human DCs as a substrate for the induction of PSCs differentiation into CECs, although the whole DCs better mimic the *in vivo* corneal microenvironment and may provide important biological, structural, and mechanical signals for cellular functions [15,24]. Previous studies involving DCs and PSCs used xenogeneic tissues and recellularized them with differentiated cells derived from hESCs, so they did not evaluate the DCs effect throughout the entire differentiation process [28,39,40,64,65]. The present study, on the other hand, explored the effect of human DCs on the ability of hESCs to differentiate into corneal epithelial-like cells since the beginning of the differentiation protocol. A study on liver decellularization suggested that the introduction of tissue-specific ECM signals may aid cell differentiation since very early stages of differentiation [66].

The interactions between the epithelium and its underlying EBM have been found to play a critical role in regulating CECs function during homeostasis and disease [35,67]. So, we hypothesized that the preserved EBM of the human DCs might provide a proper and instructive microenvironment for the induction of hESCs differentiation into corneal epithelial-like cells. As laminin is the major component of EBM [68,69], we also investigated its effect on hESCs differentiation.

Most CECs differentiation protocols and methods for the production of the limbal or corneal fibroblast-conditioned medium have used fetal bovine serum (FBS)-containing media [38,39,70–73]. Fibroblasts derived from limbal or corneal keratocytes have been commonly used to condition corneal epithelial medium for subsequent use in stem cell differentiation because they produce cytokines and growth factors that promote corneal epithelial wound healing and are crucial for the corneal epithelial fate [38,74]. Different studies have shown that serum-free media were able to maintain the quiescent phenotype of corneal stromal keratocytes, but did not support their proliferation, making the expansion of large numbers of cells difficult [75–77]. Although some reports have shown that serum-free media supplemented with factors such as retinoic acid or ascorbic acid enhanced keratocyte proliferation without encouraging fibroblastic differentiation, FBS-containing media were used for the isolation and initial expansion of cells in these studies [75–78]. However, the use of FBS in the clinical context raises safety concerns regarding the potential introduction of xenogeneic proteins and the transmission of zoonotic infections [79,80]. Therefore, we and others have attempted to replace FBS with pooled allogeneic HS due to its availability and the possibility of testing for human pathogens before use [47,81,82]. In this study, we were able to successfully expand LF, produce the LF-CM and induce the differentiation of hESCs using medium supplemented with HS.

Cell differentiation was assessed by RT-qPCR and immunofluorescence analyses. Surprisingly, although a weak protein expression of KRT12 and KRT3 was observed in cells induced to differentiate on laminin, no change was observed in the mRNA expression levels of these specific markers of terminally differentiated CECs compared to

undifferentiated cells. In contrast, a strong expression of these markers was observed in hESCs differentiated on DCs as early as 9 days of culture. Moreover, the upregulation of *TP63* in cells cultured on DCs after 21 days of differentiation suggests that, in addition to supporting differentiation, DCs also maintained cells with LSCs characteristics, which are important to renew the differentiated cell population. Besides TP63, the transcription factor PAX6 is essential for the maintenance of LSCs characteristics and their commitment to the corneal epithelial lineage [83,84]. Notably, our RT-qPCR data also showed the upregulation of *PAX6* in cells differentiated on DCs, especially at days 14 and 21, whereas a downregulation of this marker was observed in cells induced to differentiate on laminin at these times. Previous studies on liver, lung, and heart decellularization also showed improvement and acceleration of stem cell differentiation in the presence of decellularized matrices compared to commercially available ECM proteins [66,85,86]. These studies and ours indicate the advantage of using native and complex ECM niche, rather than coated plates or non-specific ECM, as a potent factor facilitating tissue-specific differentiation by providing specific morphogenic, biochemical, and biomechanical signals [66,85,86].

Mechanobiology studies have shown that corneal epithelial differentiation is controlled by changes in underlying matrix stiffness, *via* yes-associated protein (YAP), β-catenin, and TP63 signaling pathways, with stiffer substrates promoting differentiation and softer substrates promoting the maintenance of undifferentiated LSCs [87–89]. In our study, although laminin-coated wells are probably stiffer than the DCs, only a weak expression of the differentiated corneal epithelial markers was observed in cells cultured on them. In contrast, the DCs appear to support a more differentiated corneal epithelial cell phenotype as well as the maintenance of cells with LSCs characteristics. According previous reports, the limbus has lower bulk modulus compared to the central cornea, and the softer mechanical properties of the limbus maintain LSCs in an undifferentiated state, whereas stiffer central cornea is associated with increased cell activation, migration, and differentiation [88–90]. These studies demonstrated the importance of tissue biomechanics for maintaining corneal epithelial homeostasis and highlighted the significance of the intimate interaction between the epithelium and its underlying supporting matrix in dictating cell fate [87–89]. Thus, an ideal substrate for corneal epithelium tissue engineering should mimic the distinct biomechanical properties of the corneal ECM [91,92]. Corneal stiffness is determined by the amount and density of proteins present in the ECM, such as collagens, fibronectin, and proteoglycans, their degree of crosslinking, and their spatial orientation, which are very difficult to be recreated in a laboratory setting [8,93,94]. Therefore, decellularization methods provide unique advantages and whole human DCs with EBM and stroma preserved may provide the complex cornea-specific biochemical and biomechanical cues for reconstruction and maintenance of the corneal epithelium [11,15]. Future studies aiming to evaluate the preservation of the regional biomechanical differences in the DCs and the involvement of YAP, β-catenin, and TP63 signaling pathways in the regulation of cell phenotype across their surface may aid in the understanding of the factors involved in the epithelial differentiation and homeostasis with implications for future corneal epithelium tissue engineering strategies.

Here, we show for the first time that the EBM of DCs can serve as a suitable substrate to facilitate the differentiation process of hESCs into CECs. Although these cells hold great potential for reconstruction of the corneal epithelium in LSCD cases, the development of therapeutic approaches based on hESCs is hampered by immunological barriers and ethical issues [95]. The derivation of a universal hESCs line with a blocked HLA class I expression, the creation of HLA-typed hESCs banks or the use of patient-specific induced PSCs may circumvent these issues in the future [64,96].

5. Conclusion

Together, our data strongly support that decellularization of whole human corneas with NaCl plus nucleases treatment represents a simple and effective method for the development of decellularized matrices with therapeutic potential. Unlike laminin, DCs had a positive effect on the differentiation of hESCs towards corneal epithelial-like cells. Such a strategy supports the potential applications of human DCs and hESCs in corneal tissue engineering.

CRediT authorship contribution statement

Thaís Maria da Mata Martins: Conceptualization, Validation, Formal analysis, Investigation, Writing - original draft. **Pricila da Silva Cunha:** Investigation, Validation, Formal analysis. **Michele Angela Rodrigues:** Investigation, Validation. **Juliana Lott de Carvalho:** Conceptualization, Resources. **Joyce Esposito de Souza:** Investigation, Validation. **Junnia Alvarenga de Carvalho Oliveira:** Investigation, Validation. **Dawidson Assis Gomes:** Formal analysis, Writing - review & editing, Resources. **Alfredo Miranda de Goes:** Resources, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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