Acta Ophthalmologica

Human corneal endothelial cells from older donors can be cultured and passaged on cell-derived extracellular matrix

Mohit Parekh,[1] Tiago Ramos,[1] Finbarr O'Sullivan,[2] Paula Meleady,[2] Stefano Ferrari,[3] Diego Ponzin[3] and Sajjad Ahmad[1,4]

[1]Institute of Ophthalmology, University College London, London, UK
[2]Dublin City University, Dublin, Ireland
[3]Fondazione Banca degli Occhi del Veneto Onlus, Venice, Italy
[4]Moorfields Eye Hospital NHS Foundation Trust, London, UK

ABSTRACT.

Purpose: **To investigate the effect of culturing human corneal endothelial cells (HCEnCs) from older donors on extracellular matrix (ECM) derived from human corneal endothelial cell line (HCEC-12).**

Methods: **HCEC-12 cells were cultured on lab-tek chamber slides for 9 days. Upon confluence, the cells were ruptured using ammonium hydroxide leaving the released ECM on the slide surface which was visualized using scanning electron microscope (SEM). HCEnCs from old aged donor tissues (n = 40) were isolated and cultured on either fibronectin-collagen (FNC) or HCEC-12 ECM at passage (P) 0. At subsequent passages (P1 and P2), cells were sub-cultured on FNC and ECM separately. Live/dead analysis and tight junction using ZO-1 staining were used to record percentage viability and morphological changes. The protein composition of HCEC-12 ECM was then analysed using liquid chromatography–mass spectrometry.**

Results: **SEM images showed long fibrillar-like structures and a fully laid ECM upon confluence. HCEnCs cultured from older donor tissues on this ECM showed significantly better proliferation and morphometric characteristics at subsequent passages. Out of 1307 proteins found from the HCEC-12 derived ECM, 93 proteins were evaluated to be matrix oriented out of which 20 proteins were exclusively found to be corneal endothelial specific.**

Conclusions: **ECM derived from HCEC-12 retains protein and growth factors that stimulate the growth of HCEnCs. As the current clinical trials are from younger donors that are not available routinely for cell culture, HCEnCs from older donors can be cultured on whole ECM and passaged successfully.**

Key words: cell culture – cell therapy – cornea – endothelial cells – extracellular matrix – proteins

Acta Ophthalmol.

doi: 10.1111/aos.14614

Introduction

Human corneal endothelial cells (HCEnCs) are the posterior hexagonal monolayer of neural crest-derived cells that maintain optimal corneal hydration (Joyce 2003). As a result, these cells are responsible for maintaining the transparency required for clear vision, and any damage or absence of endothelium can lead to corneal oedema (Joyce 2003). Relatively recent advances in the field of endothelial keratoplasty, techniques such as Descemet Stripping Automated Endothelial Keratoplasty (DSAEK) and Descemet Membrane Endothelial Keratoplasty (DMEK; Melles 2006), have efficiently replaced the damaged recipient endothelial layer with healthy donor endothelium (Parekh et al. 2013). However, these techniques rely heavily on donor material and are mostly limited to one donor per patient. A global survey has reported that the supply to demand ratio of corneal tissues is about 1:70, indicating a huge demand of corneal tissues worldwide (Gain et al. 2016). Therefore, to overcome this limitation, HCEnCs have been isolated from donor tissues and cultured *in vitro* using different techniques. Rho-kinase inhibitor (ROCK) supplemented cultures in particular have shown the most promise so far (Okumura et al. 2012). Although HCEnCs do not have regenerative capacity *in vivo*, HCEnCs can

proliferate *in vitro* provided optimal conditions are available for cell growth (Joyce 2005). Many studies have suggested techniques to isolate and propagate these cells *in vitro* (Peh et al. 2011a,b, 2013a,b, 2015; Parekh et al. 2016, 2017, 2019a,b,c) with only one successful clinical trial reported so far (Kinoshita et al. 2018).

Having a higher endothelial cell density (ECD) compared to old aged donor tissues (>60 years of age), young donors seem to be an ideal choice for culturing HCEnCs (Parekh et al. 2017; Kinoshita et al. 2018). However, due to higher endothelial cell counts, young donor tissues are often selected for transplantation, which is the principle aim of corneal donation. It is therefore difficult to source these tissues for cell culture optimization and clinical trials. Simultaneously, old aged donor tissues are easy to obtain due to endothelial cell counts that are usually lower than the threshold required for transplantation. As the old aged donor cells go into cell senescence quickly, it becomes challenging to culture these cells (Parekh et al. 2017). However, if these cells can be efficiently cultured *in vitro*, then we may utilize available donor material more effectively. In the studies outlined here, we therefore used old-age donor HCEnCs to overcome the challenge associated with young donor tissue availability.

Several studies have shown that corneal endothelial cells strongly attach and interact with Descemet's membrane *in vivo* and their extracellular matrix (ECM) *in vitro* (Underwood & Bennett 1993; Joyce 2003; Engler et al. 2009). Human corneal endothelial cells have been shown to produce an ECM that helps in promoting cellular adhesion, proliferation, migration, morphogenesis and differentiation (Hsieh & Baum, 1985; Underwood & Bennett, 1993). ECM proteins found in Descemet's membrane include fibronectin, laminin, collagen (IV, VI and VIII) and proteoglycans containing heparan sulphate, dermatan sulphate or keratan sulphate (Yue et al. 1978; Hassell et al. 1980; Lee & Davison 1984; Nakayasu et al. 1986; Zimmermann et al. 1986; Gordon 1988; Marshall et al. 1991; Choi et al. 2013). The ECM laid by corneal endothelial cells may have all the proteins and growth factors that are required to promote cell proliferation and maintenance of HCEnCs in culture. Thus, in the studies outlined here, our aims were to investigate the effect of corneal endothelial cell-derived ECM on HCEnC culture to optimize the culture of old aged donor cells and to evaluate the proteins in this ECM.

Materials and Methods

Ethical statement and donor characteristics

Human donor corneas were shipped from Fondazione Banca degli Occhi del Veneto Onlus (FBOV, Venice, Italy) to UCL Institute of Ophthalmology (London, UK) with written consent for research use as they were not suitable for transplantation due to poor endothelial cell count (<2200 cells/mm^2). The tissues were utilized and discarded as per standard Human Tissue Authority (HTA, UK) requirements. The experiments were approved by the UCL ethics committee (10/H0106/57-2011ETR10) and were performed in accordance with the Declaration of Helsinki.

Endothelial cell evaluation

Donor endothelium of all the tissues was stained with trypan blue (0.25% wt/vol), a vital dye that selectively stain dead cells (VisionBlue, D.O.R.C., Zuidland, The Netherlands), to detect the percentage of dead/necrotic cells. Approximately 100 µl of trypan blue was applied on the endothelial surface for 20 seconds and then washed with sterile phosphate-buffered saline (PBS). The endothelium was exposed to a hypotonic sucrose solution (1.8%) to swell the intercellular borders for counting the number of endothelial cells and to examine general morphology before processing. A reticule (10 × 10) was fixed to the eyepiece of an inverted microscope (Nikon Eclipse TS100, Nikon, Surrey, UK) to count the number of endothelial cells and trypan blue positive cells (TBPCs) manually. An average of five different counts was recorded (Parekh et al. 2019a).

Human corneal endothelial cell line culture and exposure of extracellular matrix (ECM)

Human corneal endothelial cells (HCEC) from a certified cell line (HCEC-12) were cultured on 75 cm^2 culture flasks (Nunc, Thermo Fisher Scientific, Rochester, NY, USA) until confluence using culture medium containing 1:1 Ham's F12 and Medium 199 (cat no: 31765027 and 41150020, respectively, Thermo Fisher Scientific) supplemented with 5% foetal bovine serum (FBS). The cells were trypsinized upon confluence and cultured on lab-tek II chamber slides (8 chambers, 25 × 75 mm, 0.7 cm^2 culture area, Thermo Fisher Scientific) to obtain the ECM required for primary endothelial cell culture. Upon confluence, medium was removed and the HCEC-12 cells were washed with sterile PBS. The cells were treated with 20 mM ammonium hydroxide (NH_4OH, Thermo Fisher Scientific) in sterile PBS for 5 min (Gospodarowicz 1984; Todorovic et al. 2010). The resulting ruptured cells were washed away several times with sterile PBS followed by a single wash of double distilled water (ddH_2O) to remove any remaining cell remnants. This resulted in the lab-tek II chambers coated with whole ECM proteins. Different time-points (days 1, 5 and 9) were evaluated for optimal conditions required for ECM production.

Scanning electron microscopy

Samples were prepared in a laminar flow hood, and extra care was taken to ensure the samples did not dry out prematurely. The samples were washed twice for 5 min in ddH_2O, and then, osmium tetroxide (1%, aqueous) was added to the samples, followed by further washes with distilled water three times for 10 min each. The samples were then washed with ethanol in increasing order of concentrations, that is 30%, 50%, 70%, 90% and 100% three times for 10 min each, and dried using methanol twice for 10 min each. The samples were dipped in hexamethyldisilazane (HMDS) in a small glass jar thrice for 3 min each and allowed to dry under a petri-dish in the hood for at least 1 hr. The samples were finally mounted into a labelled stub using a conductive carbon disc and sealed at the edges with silver paint. Samples were stored in a drying cabinet and coated with 1.5 nm platinum (Cressington sputter coated) before imaging using Zeiss Sigma VP SEM (Oberkochen, Germany).

Human corneal endothelial cell culture from old aged donor tissues

Peeling and plating the cells at passage 0
The Descemet membrane–endothelial complex of the tissues was peeled in several pieces to ensure a quicker isolation process. The excised pieces were incubated in 2 mg/ml collagenase Type 1 (Thermo Fisher Scientific) solution for approximately 2 hr at 37°C and 5% CO_2. The solution with cell clumps was then centrifuged for 5 min at 1000 rpm. The supernatant was removed, and the pellet was re-suspended with trypLE express (1×) phenol red (Life Technologies, Monza, Italy) for 5 min at 37°C to further dissociate the pellet into single cells. The supernatant was removed, and the cells were re-suspended in 200 µl of the cell culture medium, a formulation of 1:1 Ham's F12:M199 (Sigma-Aldrich), 5% FBS, 20 µg/ml ascorbic acid (Sigma-Aldrich), 1% insulin transferrin selenium (Gibco), 10 ng/ml recombinant human FGF basic (Gibco), 10 µM ROCK inhibitor (Y-27632; Miltenyi Biotech) and 1% penstrep (Sigma-Aldrich; Peh et al. 2011a,b, 2013a,b, 2015; Parekh et al. 2016, 2017, 2019a,b, c). The resulting cells were counted using a haemocytometer, and the number of plated cells was recorded for all the cultures. Control lab-tek II chamber slides were coated with 50 µl fibronectin-collagen (FNC) coating mix (US Biological Life Sciences, Salem, MA, USA) for at least 30–45 min at 37°C and 5% CO_2. The residual coating was removed before plating cells. 200 µl of the cell suspension from each cornea was divided into two equal halves and plated on each chamber of (i) FNC coated slide, and (ii) ECM coated slide derived from HCEC-12 cells. The medium was replaced, and the cells were monitored every alternate day until confluence followed by end-stage characterization or further passaging.

Passaging the cells
Upon confluence, primary cells were briefly washed with PBS and incubated at 37°C and 5% CO_2 for 5–10 min in trypsin-EDTA solution. The reaction was stopped using culture medium. The cell suspension was collected in a 15-ml tube and centrifuged at 1000 rpm for 5 min. The supernatant was removed, and the pellet was re-suspended using the medium. The lab-tek II slides were precoated with FNC and ECM (as mentioned above), and the cells were further divided into two groups at P1: cells obtained from the FNC group were sub-cultured on FNC and ECM coated slides separately, and cells from the ECM group were sub-cultured on FNC and ECM coated slides separately. Similarly, at P2, the cells from each group were further sub-cultured on both FNC or ECM coated slides separately. This resulted in the following different matrix formats of culture/sub-culture for P0-P1-P2, that is FNC-ECM-FNC; FNC-ECM-ECM; FNC-FNC-FNC; FNC-FNC-ECM; ECM-ECM-FNC; ECM-ECM-ECM; ECM-FNC-FNC; and ECM-FNC-ECM, respectively.

Hoechst, ethidium homodimer and calcein AM (HEC) staining to determine live/dead cells

Cells at confluence were washed with PBS after preservation prior to the assay. Five microlitre of hoechst 33342 (H) (Thermo Fisher Scientific), 4 µl of ethidium homodimer EthD-1 (E) and 2 µl calcein AM (C) (live/dead viability/cytotoxicity kit, Thermo Fisher Scientific) were mixed in 1 ml of PBS. 100 µl of the final solution was directly added to the cells and incubated at room temperature in dark for 45 min, followed by a single washing step. The walls of the lab-tek slides were detached, and the cells were mounted with cover slips without 4′,6-diamidino-2-phenylindole (DAPI) mounting media. The zeiss LSM 700 confocal microscope (Carl Zeiss, Cambridge, UK) was used to image the cells that were captured using in-built zen software. Triple labelling showed the presence of ethidium homodimer stained in red representing dead cells, hoechst in blue representing nuclei and calcein AM in green marking viable cells (Pipparelli et al. 2011).

Immunostaining of zonula occludens-1 (ZO-1) tight junction protein

Cells at confluence were washed with PBS and fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 20 min. The cells were permeabilized with 0.25% triton X-100 in PBS for 30 min. After blocking with 10% goat serum for 1 hr at RT, the cells were incubated overnight at 4°C with primary antibody anti-ZO-1, 1:200 (FITC-conjugated, Thermo Fisher Scientific). Hoechst (33342) was mixed (1:1000), and 100 µl of the solution was added to the cells to stain the nucleus. After each step, the cells were washed three times with PBS. After detaching the walls of the lab-tek slides, the cells were covered with mounting medium and cover slips. The expression of ZO-1 was examined using the LSM 700 confocal microscope (Carl Zeiss), and images were captured using in-built zen software.

Data analysis time-points

For SEM analysis, the images were obtained at days 1, 5 and 9. The data for HCEnCs cultured on both FNC and whole ECM coatings were obtained at days 1, 3, 5, 7 and 9 for P0 and at days 1, 5 and 9 for P1 and P2.

Cellular analysis

Confluence and cell doubling of the cultured HCEnCs
The percentage confluence was measured every alternate day using a 10 × 10 reticule (0.1 mm^2) attached to the eyepiece of an inverted microscope (Nikon Eclipse TS100; Nikon). The number of endothelial cells per mm^2 was counted using the same reticule to determine the cell numbers in the given area and the doubling time.

Determining viable/dead cells, hexagonality, polymorphism and cell area
All the measurements and data analysis were performed using ImageJ (FIJI) bundled with 64-bit Java 1.8.0 112. Viability of cells was measured as the number of calcein AM positive cells compared with number of Hoechst positivity. Similarly, dead cells (ethidium homodimer positive) were counted using ImageJ and converted to a percentage (Parekh et al. 2019a,b). For ZO-1 analysis, the images were converted to overlay masks using predetermined macroinstructions to define the parameters of both hexagonality and polymorphism within a particular area (Parekh et al. 2019a,b). The images were auto-converted, and the total number of cells in the investigated area was counted using the macros for ZO-1. The hexagonal and polymorphic cells were counted manually depending

on the cellular structure comprising six borders per cell for hexagonal cells and less than four borders for severely polymorphic cells in the investigated area. Cell area (μm^2) was measured by marking the borders of the cell using the free-hand tool and then using the area measurement tool.

Liquid chromatography–mass spectrometry

ECM analysis by mass spectrometry

Cell lines from human conjunctival epithelial cells (HCjE-Gi; Gipson et al. 2003) and human telomerase-immortalized corneal epithelial cells (hTCEpi; Robertson et al. 2005) were cultured as controls for comparative protein analysis and were treated the same way as HCEC-12 to obtain the ECM (as described previously) for mass spectrometry. HCjE-Gi cells were cultured using keratinocyte serum-free media (KSFM; Gibco, Thermo Fisher Scientific), supplemented with 0.2% (V/V) bovine pituitary extract (BPE), 0.2 ng/ml of epithelial growth factor (EGF) and 1% (V/V) penstrep (Sigma-Aldrich, Haverhill, UK) and 0.4 mM of $CaCl_2$ (Sigma-Aldrich). The hTCEpi cells were cultured in KSFM with $CaCl_2$ (Gibco), supplemented with 0.2% (V/V) BPE, 0.23 ng/ml EGF (all supplied with the medium) and $CaCl_2$ to a final concentration of 0.13mM. Basal cell protein proteolysis and extraction were performed on HCjE-Gi, hTCEpi and HCEC-12 cells according to the method described by Gospodarowicz (1984) and Todorovic et al. (2010) with minor modifications (50 mM of NH_4HCO_3 instead of 100 mM, 0.33% of trypsin gold instead of 0.5 μg and instead of storing the samples the protocol for Mass Spec was continued immediately). A solution of 50 mM NH_4HCO_3 (Sigma-Aldrich) in ddH_2O, pH 8.0, containing: 0.02% (V/V) ProteaseMax surfactant (Promega, Southampton, UK), 0.33% (W/V) trypsin gold (Mass Spec grade; Promega) was added to the exposed ECM, and the samples were digested for 1 hr at 37°C in a humidified chamber. The peptides (resulting solution) were transferred into low-binding tubes (Eppendorf LoBind, Sigma-Aldrich). The enzymatic reaction was stopped by adding acetic acid (Sigma-Aldrich) setting the pH to 3–4; 1,4-dithiothreitol (DTT, Sigma-Aldrich; 1% (V/V) of 0.5M) was added for 20 min at 56°C; and 2.5% (V/V) of 0.55 M iodoacetamide (Sigma-Aldrich) was added for 15 min in the dark at room temperature. 0.3125% (W/V) of trypsin gold and 0.00625% (V/V) ProteaseMax surfactant were added, and the solution was heated at 37°C for 3 hr for protein digestion. Trifluoroacetic acid (TFA, Sigma-Aldrich) was added to a final concentration of 0.5% (V/V).

LC-MS analysis was performed at Dublin City University, Ireland, using an ultimate 3000 RSLCnano system (Dionex, Thermo Fisher Scientific) coupled to a hybrid linear ion trap/Orbitrap mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific). The digested samples were sonicated, and 1 μg of digested proteins was loaded onto a C18 trap column (C18 PepMap, 300μm i.d. × 5mm, 5 μm particle size, 100 μm pore size; Dionex) and desalted for 3 min at a flow rate of 25 μl/min using 2% acetonitrile containing 0.1 M TFA. The trap column was then switched online with the analytical column (PepMap C18, 75 μm i.d. × 500 mm, 3 μm particle, and 100 μm pore size; Dionex), and peptides were eluted in a 180-min gradient at a flow rate of 300 nl/min using 2% acetonitrile with 0.1% formic acid (FA) to 50% acetonitrile containing 0.08% FA. Mass spectrometry data was acquired with Xcalibur software, version 2.0.7 (Thermo Fisher Scientific). The mass spectrometer was operated in data-dependent mode and externally calibrated. MS1 survey scan (m/z 400–1200) was set at a resolution of 30 000 in the Orbitrap, followed by ten MS2 scans using collision-induced dissociation (CID) activation mode in the ion trap. Dynamic exclusion was enabled with the following settings: repeat count, 1; repeat duration, 30 seconds; exclusion list size, 500; and exclusion duration, 40 seconds. The activation time was 30 mseconds, with an isolation width of 2 Da for ion trap mobility spectrometry (ITMS); the normalized activation energy was 32%; and the activation (q) was 0.25 (Henry & Meleady 2017).

ECM protein data analysis

Proteome Discoverer (PD) version 2.1.0.81 (Thermo Scientific) was used to perform the database search against the human sequences in the UniProt Swiss-Prot protein database (version January 2016 with 20 151 entries) for the mass spectrometry raw data files. The search engines SEQUEST-HT and Mascot (version 2.4.0) were utilized in PD. The search parameters used were as follows: 20ppm tolerance for precursor ion masses, 0.6 Da for fragment ion masses analysed by ion trap. A total of two missed cleavages were permitted for fully tryptic peptides. Carbamidomethylation of cysteines (+57.0215 Da) was set as a fixed modification, and variable modifications of methionine oxidation (+15.9949 Da) and N-terminal acetylation (+42.0106 Da) were allowed. The false discovery rate (FDR) was determined by using a target–decoy search strategy using Percolator in PD2.1. The sequence database contains each sequence in both forward and reverse orientations, enabling FDR estimation. The FDR was set to 0.01 at both the peptide and the protein levels. A higher percentage coverage was considered better, which was calculated by dividing the number of amino acids in all found peptides by the total number of amino acids in the entire protein sequence of the samples (Henry & Meleady 2017).

Statistical analysis

A two-tailed non-parametric Wilcoxon signed rank for paired test with 95% confidence interval was used to check the statistical difference between the cells cultured on ECM and FNC, respectively, at passage 0. Non-parametric Kruskal–Wallis test without Gaussian approximation with *post hoc* Dunn's test with a significance level of Alpha = 0.05 (95% confidence intervals) was used to compare the data at passage 1 and 2 using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA) software. A probability value of < 0.05 was deemed statistically significantly different.

Results

Donor characteristics

Corneal tissues ($n = 40$) used in these studies were unsuitable for transplantation due to their low endothelial cell counts. Average endothelial cell density (ECD) before the isolation of cells was found to be 1885 (±203) cells/mm^2 with 1.4 (±0.9) % TBPCs. No other complications or indications were noted from the donor database. The mean age of the

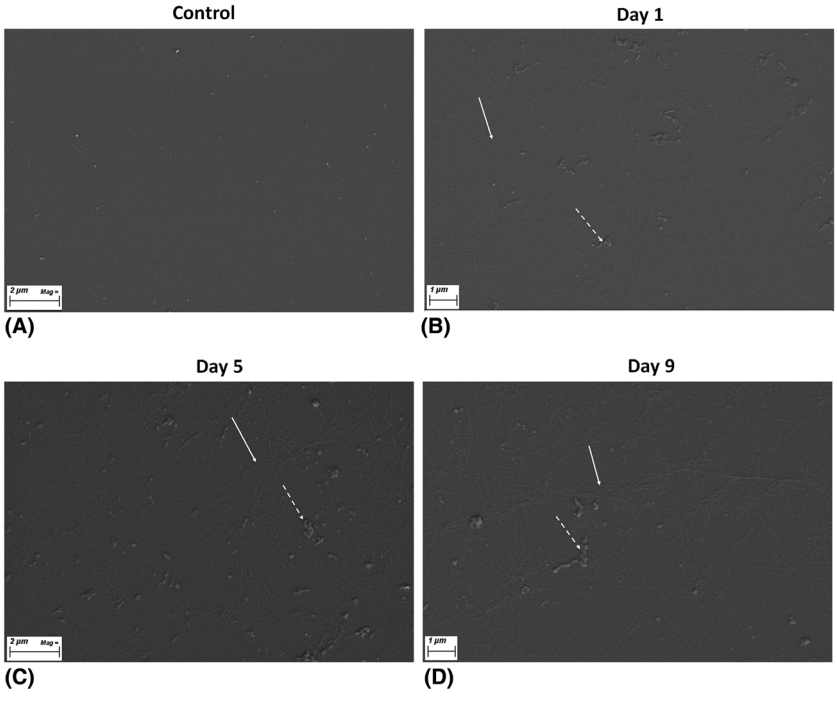

Fig. 1. Scanning electron microscopy (SEM) images of (A) control group without cells or extracellular matrix (ECM). (B) SEM image showing the ECM at day 1 with minimum ECM generated. (C) Long collagen-like fibrillary structures started appearing by day 5. (D) The entire base was coated with fibrillary structures by day 9. Cellular debris was observed at all time-points even after rigorous washing steps marked with dotted white arrows. The fibrils of the generated ECM are marked with white arrows.

donors was found to be 70.9 (±7.6) years comprising 22 males and 18 females and post-mortem time of 11.3 (±7.2) hours. Tissues were stored in tissue culture medium at 37°C for 25 (±4.4) days in the eye bank before use.

Scanning electron microscopy of the ECM

SEM images of the culture flasks were obtained without any cells as the control group (Fig. 1A) and of the laid ECM after days 1, 5 and 9 (n = 3). Collagen-like fibrillary structures were noticed in the ECM after day 1 of culture (Fig. 1B). At day 5, when cells were approximately 90% confluent, slightly longer fibres were observed (Fig. 1C). At day 9, most of the cellular debris was washed off without compromising the long fibrillary structures and maintaining the core ECM proteins (Fig. 1D). HCEC-12 cells were therefore cultured for 9 days to obtain ECM for the subsequent experiments.

Morphological analysis of primary HCEnCs on FNC and ECM coated base

Human corneal endothelial cells cultured on both FNC and whole ECM coatings at P0 showed compactly arranged hexagonal cells by day 9 (n = 40; Fig. 2A). Interestingly, the cells started showing hexagonal morphology and sharp intercellular junctions when cultured on whole ECM (Fig. 2B – marked with dotted circle) compared to those cultured on FNC by day 5. This indicates that ECM proteins may be providing an optimum environment facilitating cell growth and supporting the development of tight junction proteins as also shown later by immunostaining. At P1 (Fig. 2C; n = 24), the cells were compactly organized and showed limited polymorphism when they were switched from ECM to a FNC coated base. However, morphology deteriorated at passage 2 (n = 12), but the cultures still showed confluence by day 9 (Fig. 2D) due to polymegathism.

Confluence

At P0 (n = 40), confluence was found to be statistically higher ($p < 0.05$) at days 1, 5 and 7 for ECM compared to FNC cultures (Fig. 3A). At P1 (n = 24), FNC cultured cells which were sub-cultured from P0 ECM cultures yielded statistically significant higher confluence at days 1, 5 and 9 of the culture period (Fig. 3B). At P2 (n = 12), all sub-cultures which had been grown from ECM cultures at P0 (as opposed to FNC) showed significantly higher confluence (Fig. 3C).

Cell doubling rate and time

Exponential growth in cell numbers was observed at P0 in both sets of cultures, for ECM and FNC coatings when 90 866 (±8230) cells were plated in FNC group and 90 756 (±6980) cells were plated in the ECM group at P0 (Fig. 3D). Significantly higher cell numbers from ECM cultures were observed than those grown on FNC at P0. Indeed, in subsequent passages, cultures which had been grown on ECM at P0 maintained significantly higher cell numbers at P1 (Fig. 3E) and P2 (Fig. 3F). By the end of the experiment at P2, collectively, cell numbers were found to be 563 457 (±52 000) cells in FNC group and 639 047 (±52 516) cells in the ECM group ($p = 0.0355$).

Cell doubling time was found to be significantly less in the ECM group compared with the FNC group at P0 (Fig. 3G; n = 40). Cell doubling time was not found to be significantly different at P1 (n = 12; Fig. 3H). However, at P2 (n = 12) the doubling time was significantly less between cells that were cultured on ECM-ECM-FNC at P0-P1-P2 compared with FNC-ECM-FNC (Fig. 3I). It was observed that in general the cell doubling times at P0 were higher compared to those at P1 and P2).

Live/dead staining

HEC staining showed good viability of cells at P0 (n = 8; Fig. 4A), P1 (n = 6; Fig. 4B) or P2 (n = 6; Fig. 4C) without any significant difference between FNC and ECM groups at P0 (Fig. 4D), P1 (Fig. 4E) or P2 (Fig. 4F). However, a significant reduction ($p = 0.0014$) in the percentage of viable cells was observed between P0 and P2 in general. Simultaneously, the number of dead cells (ethidium homodimer positive) significantly increased with subsequent passaging from P0 to P2 ($p = 0.0005$; Fig. 4) with no significant difference observed between the FNC and ECM groups at either passages P0 (Fig. 4G), P1 (Fig. 4H) or P2 (Fig. 4I).

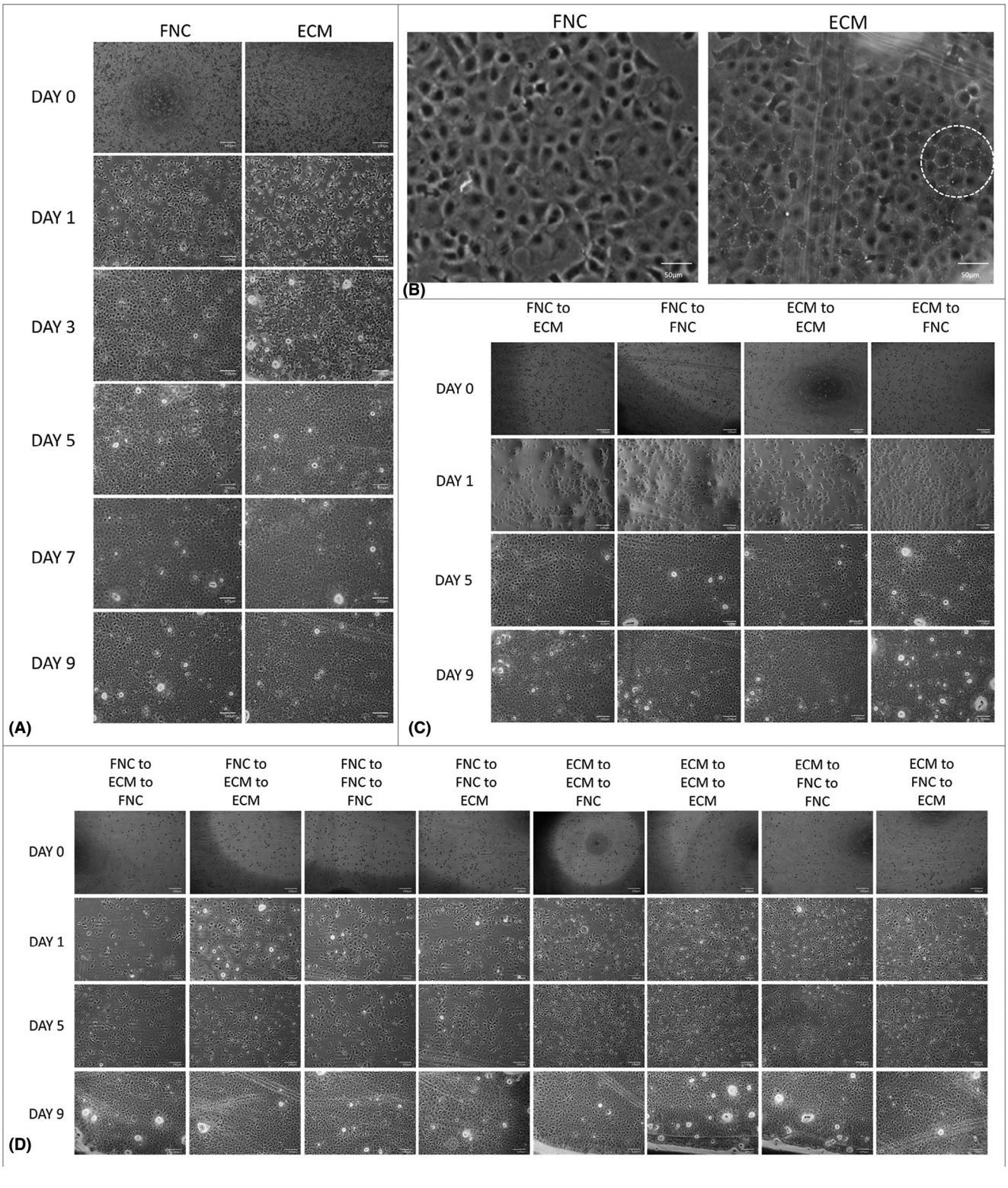

Fig. 2. Cell morphology at (A) passage 0 when plated on FNC and extracellular matrix (ECM), respectively. (B) Sharp intercellular junctions and perfectly arranged hexagonal cells were observed on the cells cultured on ECM at day 5 compared with the cells cultured on FNC (marked in white dotted circle). (C) Morphology of cells at P1 and (D) P2, from the FNC and ECM groups Scale: A, C, D—100 µm and; B—50 µm.

ZO-1 expression, hexagonality, polymorphism and cell area

Although ZO-1 was expressed at the tight junctions of cells at P0 ($n = 8$; Fig. 5A), P1 ($n = 6$; Fig. 5B) or P2 ($n = 6$; Fig. 5C), it was less strongly expressed at P1 and P2 compared with P0. Cultures from the ECM group showed significantly higher numbers of hexagonal cells compared with FNC at P0 (Fig. 5D). At P1, the ECM-FNC (P0-P1) group showed a significantly higher percentage of hexagonal cells compared with both P1 groups that were subcultured from P0 FNC cultures (FNC-

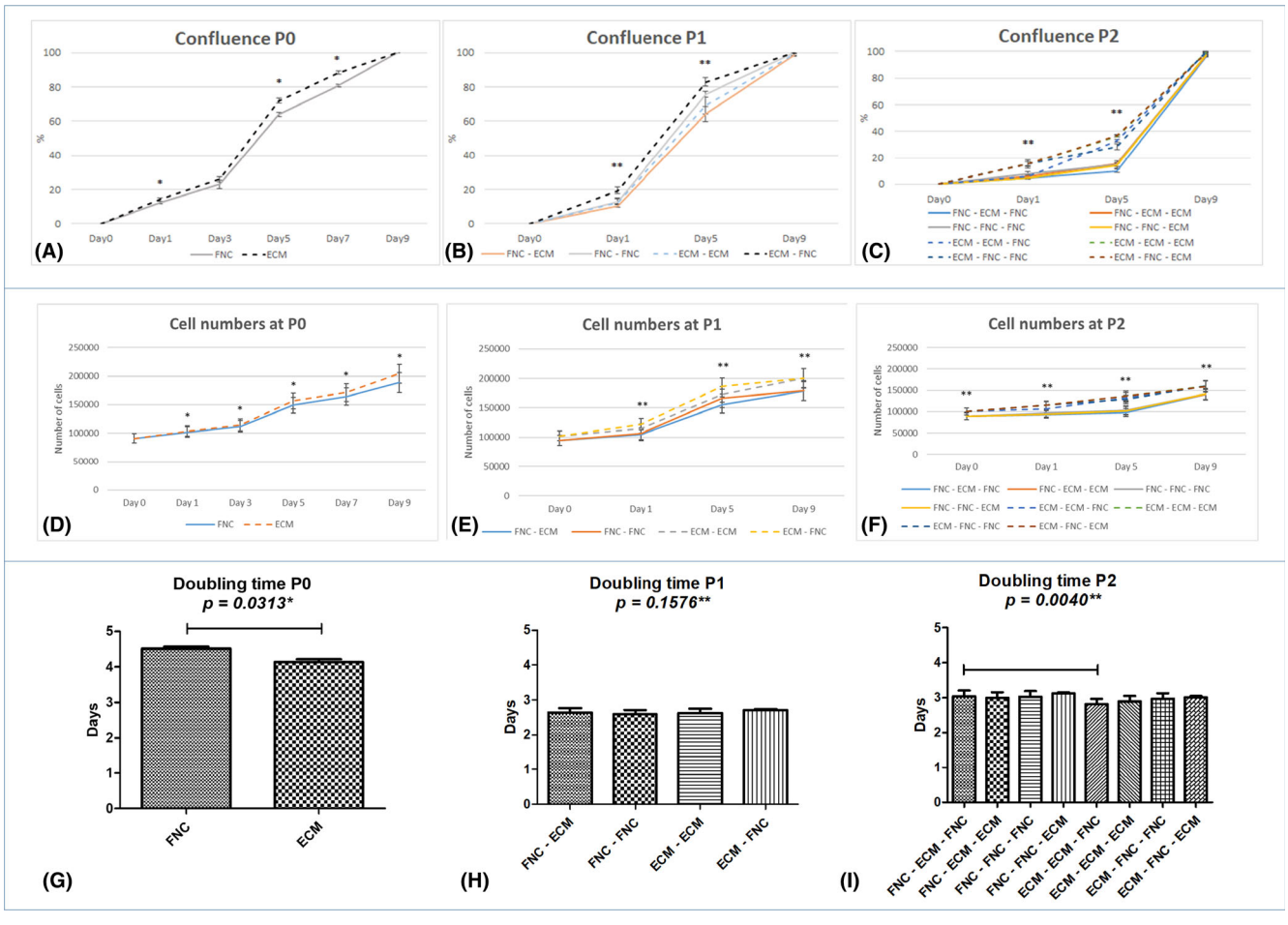

Fig. 3. Cells cultured on FNC and extracellular matrix showing confluence at (A) P0, (B) P1 and (C) P2; cell number at (D) P0, (E) P1, (F) P2 and; cell doubling time at (G) P0, (H) P1 and (I) P2. The data are presented as mean with SD. Statistical significance: * = Wilcoxon test and ** = Kruskal–Wallis test.

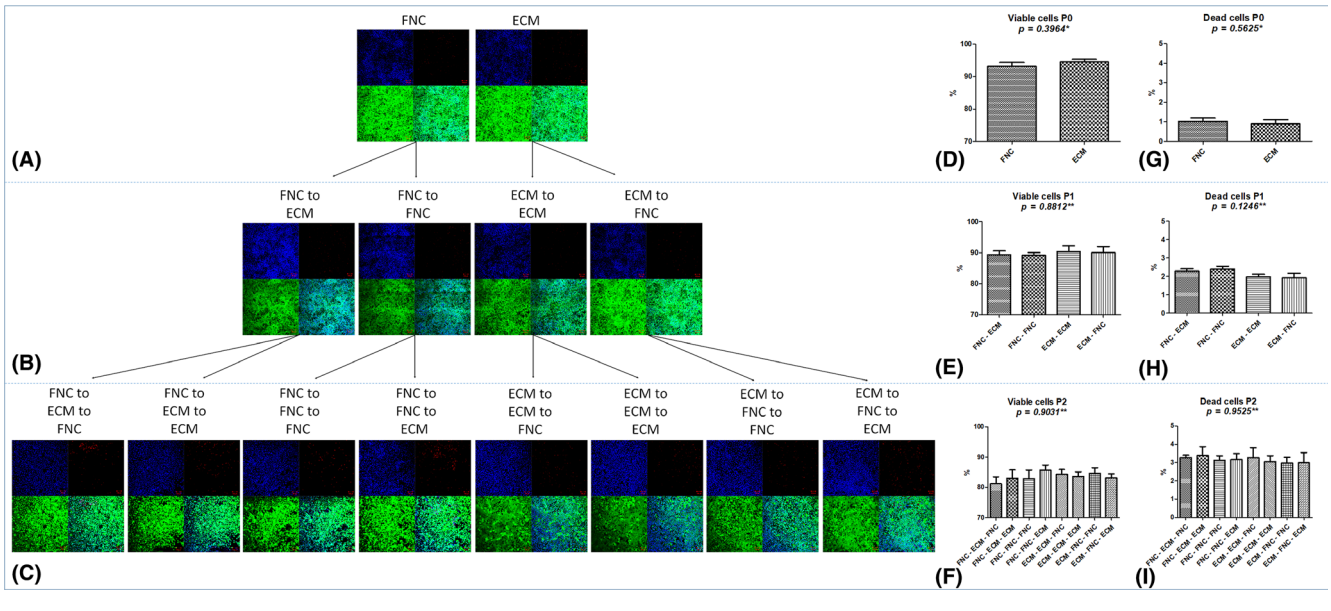

Fig. 4. Hoechst (blue), ethidium homodimer (red) and calcein AM (green; HEC) staining used for live/dead analysis showed viable and dead cells from FNC and ECM groups at (A) P0 (B) P1 and (C) P2. The data did not show any significant difference in terms of percentage viability at (D) P0, (E) P1 and (F) P2 or the percentage of dead cells at (G) P0, (H) P1 and (I) P2. Scale: 100 μm. The data are presented as mean with SD. Statistical significance: * = Wilcoxon test and ** = Kruskal–Wallis test.

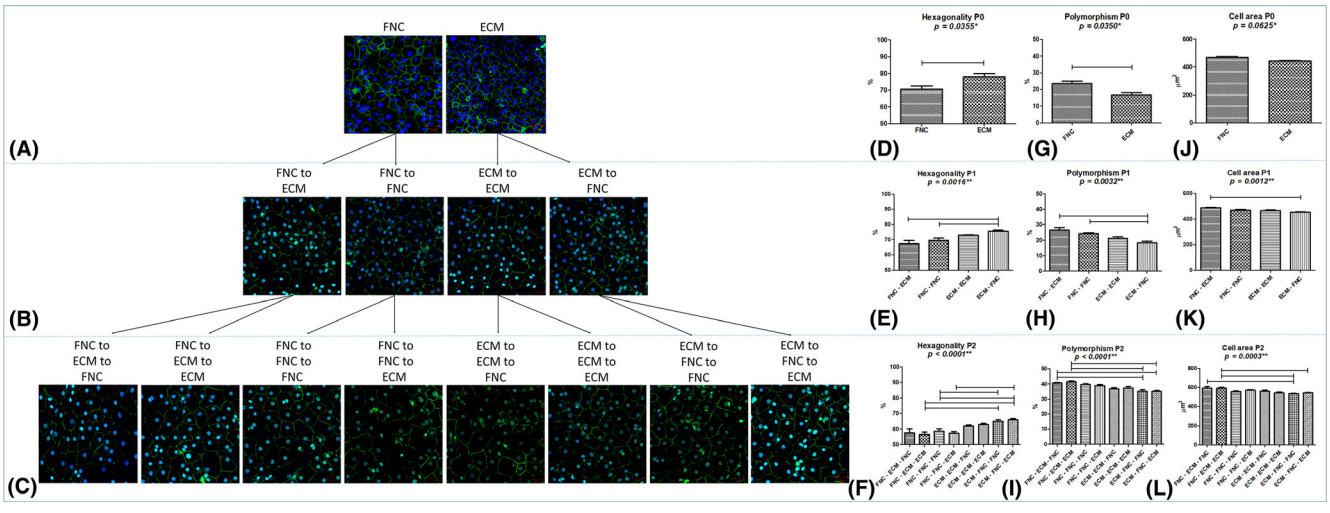

Fig. 5. Zonula Occludens-1 (ZO-1) protein for measuring the tight junctions was expressed at (A) P0, (B) P1 and (C) P2 in all the cultures. Hexagonality was found to be significantly better in the cells cultured on the ECM at (D) P0, (E) P1 and (F) P2. However, polymorphism was found to be significantly higher in the cells cultured on FNC coatings at (G) P0, (H) P1 and (I) P2. Cell area found between the cells cultured on FNC and ECM groups at (J) P0, (K) P1 and (L) P2. Scale: 50μm. The data are presented as mean with SD. Statistical significance: * = Wilcoxon test and ** = Kruskal–Wallis test.

FNC and FNC-ECM; Fig. 5E). P2 cultures that were grown on ECM at P0 showed a significantly higher number of hexagonal cells compared to those P2 cultures grown on FNC at P0 (Fig. 5F). The results for polymorphism were inversely proportional to those for hexagonality at all passages as would be expected, with those cultures grown on ECM at P0 showing significantly less polymorphism (Fig. 5G–I). In terms of cell area, no significant difference was observed at P0 between the groups (Fig. 5J). However, P1 cultures grown from the P0 ECM cultures had significantly smaller cell areas (Fig. 5K). A similar trend was found in P2 cultures derived from P0 ECM cultures compared to those from P0 FNC cultures (Fig. 5L).

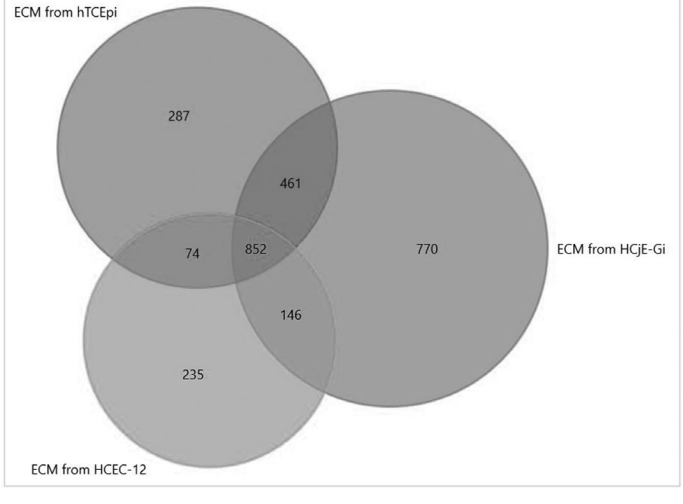

Fig. 6. A Venn diagram showing the total number of proteins extracted from the corneal epithelial, conjunctival epithelial and corneal endothelial extracellular matrix (ECM). Out of these, 235 proteins were exclusively found from the corneal endothelial ECM and 20 of these were found to be ECM oriented.

Mass spectrometry

A total of 1307 proteins were found in the corneal endothelial ECM ($n = 3$) using modified LC-mass spectrometry, out of which 92 proteins were found to be matrix oriented. 72 of these matrix oriented proteins overlapped with HCjE-Gi ($n = 3$) and hTCEpi ($n = 3$) derived ECM proteins leaving 20 specific to corneal endothelial ECM (Fig. 6; Table 1).

Discussion

Out of all corneal transplants carried out in the UK, 40% are due to endothelial disease (NHSBT data). Although corneal transplantation is the mainstay of treating symptomatic corneal endothelial dysfunction, it is restricted due to the limited availability of donor tissues around the globe. Although alternative solution to endothelial keratoplasty has been identified, cell culture techniques are further constrained as healthy and high number of endothelial cells are difficult to obtain (Shima et al. 2011; Kimoto et al. 2012; Zhu et al. 2012; Okumura et al. 2013; Peh et al. 2013a). Therefore, optimizing proliferation from older donor tissue-derived corneal endothelial cells (Khaireddin et al. 2003; Joyce & Zhu 2004; Coster & Williams 2005), especially those that are discarded due to poor endothelial cell counts, would be ideal considering the availability of these tissues.

Previous studies have used denuded human amniotic membrane, collagen type I matrices, silk fibroin, fish scale scaffolds etc. as potential substrates for the propagation of HCEnCs (Ishino et al. 2004; Gruschwitz et al. 2010; Madden et al. 2011; Palchesko et al. 2015; Parekh et al. 2018). Several

Table 1. List of 20 extracellular matrix oriented/supported proteins found in the endothelial extracellular matrix.

Description	Coverage (%)
Fibronectin type III domain-containing protein 1	1
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A containing DEAD/H box 1	1
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1	3
Insulin-like growth factor-binding protein 5	11
Vitronectin	3
Fibrillin-1	1
Fibrillin-2	1
Tensin-3	2
Protein Wnt-5b	6
Citron Rho-interacting kinase	1
Chondroitin sulphate proteoglycan 4	3
Cyclin-dependent kinase 1	19
Cyclin-dependent kinase 4	8
Periostin	5
Thrombospondin-4	1
Tubulin alpha chain-like 3	7
Tubulin alpha-1B chain	43
Tubulin beta-1 chain	10
Tubulin beta-4A chain	46
Versican core protein	1

specific ECM coatings including collagen I/IV, FNC, laminin and chondroitin sulphate have been shown to influence cellular adhesion, proliferation, morphology, molecular function, and the development and expression of markers for HCEnCs (Choi et al. 2013). Although several ECM proteins have already been investigated for the culture of HCEnCs *in vitro*, the culture of HCEnCs on HCEnC derived whole ECM has not been studied previously. Using SEM, we have shown that long collagen-like fibrillary structures can be seen in the whole ECM deposited by corneal endothelial cells. These appear at day 5 of culture and at day 9 there is wide coverage of the plasticware with these fibrils. FNC has been used commonly for HCEnC cultures (Peh et al. 2011a,b, 2013a,b, 2015; Parekh et al. 2016, 2017, 2019a,b,c) previously, and this was therefore used as a control to whole ECM we investigated here. Moreover, in the studies described here, we have shown that HCEnCs from older donor tissues that have suboptimal endothelial cell counts for transplantation, can be cultured with success on whole ECM. Morphologically, we have observed from our previous studies that HCEnCs cultured on FNC coated plastic at P0 show confluence by day 9. This trend was also observed when cells were cultured on whole ECM. However, systematic arrangement of hexagonal morphology and the development of intercellular tight junctions was observed in HCEnCs on whole ECM (compared to FNC) by day 5, further indicating that the ECM proteins and growth factors may provide an optimum environment facilitating the development of tight junction proteins at an earlier stage of culture.

Expansion of HCEnCs by passaging them in culture from P0 to P2 was performed to understand the proliferative capacity of these cells on whole ECM. From our previous experience and that of others in the field, it is difficult to sub-culture HCEnCs as cells often become senescent (Parekh et al. 2017). Therefore, our aim was to investigate the effect of whole ECM on the proliferative capacity and subculturing capability of HCEnCs. This was investigated in comparison with FNC and by switching between whole ECM and FNC at each passage to determine the effects of these different matrices on cell morphology and proliferation. Interestingly, we observed that cells showed better morphology and cell proliferation when passaged and switched from one base coat to the other provided they were all cultured on whole ECM at P0. Thus, in general at P1, we observed that proliferation was best for ECM-FNC (P0-P1 matrix) compared to ECM-ECM or FNC-FNC. We noted limited polymorphism when cells were transferred from ECM to FNC coated base, indicating better morphology. In the same way, cells cultured on whole ECM at P0 showed significantly better results at P2. This shows HCEnCs must be provided with optimum conditions at P0 for them to survive subsequent passaging. We also found that higher passages lead to more dead cells, but this is without compromising cell shape, size and area of the viable cells. It is already known that tight junction proteins lose their ability to form continuously (Joyce 2005) leading to lower expression of ZO-1 at every subsequent passage (Kundu et al. 2013). We observed a similar trend in the expression of ZO-1 at different passages.

We also investigated the protein content exclusive to the whole ECM laid down by HCEC-12 cells but not found in the ECM of HCjE-Gi and hTCEpi cells. These proteins showed specificity towards cell adherence, migration, proliferation and cell cycle control and many of which have not been used previously as coatings for HCEnC culture. These include the following:

1 *SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1* plays an important role in cellular proliferation, differentiation and cell cycle control that causes cell cycle arrest in G0/G1 (Kalimuthu & Chetty 2016). Interaction with this protein could be one of the indirect causes of corneal endothelial cell cycle arrest and limited proliferative capacity *in vivo*.

2 *Insulin-like growth factor-binding protein 5* has been shown to modulate the growth-promoting effects of insulin-like growth factors (IGFs) on cell culture by altering the interaction of IGFs with their cell surface receptors (Jones et al. 1993).

3 *Vitronectin* is a glycoprotein found in serum, ECM and bone and binds to integrin alpha-V beta-3 further promoting cell adhesion and migration (Boron Walter & Boulpaep Emilie 2012) and therefore may play an integral role in the development of focal adhesions of HCEnCs.

4 *Chondroitin sulphate proteoglycan 4* (CSPG4) has a role in cell proliferation

and migration. It promotes retraction fibre formation and cell polarization through Rho GTPase activation. We have used Rho-kinase inhibitor for our cells and therefore believe that CSPG4 could be one of the important proteins involved in the migration of cells on the ECM coated base (Michelacci 2003) in combination with Rho-kinase inhibitor.

5 *Thrombospondin-4* is an adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions and is therefore an important protein required for HCEnCs culture (Lawler et al. 1995).

From our investigations, we believe that it is important to provide an environment close to physiologic conditions for culturing HCEnCs *in vitro*. We observed that for successful culture and passaging, maintenance of cellular morphology and expression of core proteins, the normal whole ECM plays a key role. It is not only that the cells must adhere and proliferate, but also that the cells show appropriate phenotypic characteristics and expression of core proteins in order for them to be considered suitable for transplantation. The 'de-roofed' culture system investigated here in the form of whole ECM not only provides a surface structure that enables optimal cell–matrix interactions (adhesion and migration), but also provides a pool of soluble growth factors trapped within the ECM. These cues have been shown to regulate central cell processes (Palchesko et al. 2015), including cell differentiation. We thus investigated a culture system, that mimics the cellular microenvironment, to be able to induce favourable HCEnC proliferation with good morphological and phenotypical behaviours for use in tissue engineering of the corneal endothelium (Koo et al. 2014).

In summary, we show in the studies outlined here that HCEnCs from older donors that are not suitable for conventional endothelial transplantation can be cultured on whole ECM and passaged successfully. Moreover, the HCEnCs cultured initially on whole ECM at P0 show better morphology and growth, than those grown on FNC at P0. In addition, analysis of this ECM by mass spectrometry identifies ECM proteins specific to HCEnCs that are of interest and warrant further study.

References

Boron Walter F & Boulpaep Emilie L (2012): Medical physiology. Philadelphia, PA: Saunders (Elsevier): 1097.

Choi JS, Kim EY, Kim MJ, Giegengack, M, Khan, FA, Khang, G & Soker, S. (2013): In vitro evaluation of the interactions between human corneal endothelial cells and extracellular matrix proteins. Biomed Mater **8**: 014108.

Coster DJ & Williams KA (2005): The impact of corneal allograft rejection on the long-term outcome of corneal transplantation. Am J Ophthalmol **140**: 1112–1122.

Engler C, Kelliher C, Speck CL & Jun AS (2009): Assessment of attachment factors for primary cultured human corneal endothelial cells. Cornea **28**: 1050–1054.

Gain P, Jullienne R, He Z et al. (2016): Global survey of corneal transplantation and eye banking. JAMA Ophthalmol **134**: 167–173.

Gipson IK, Spurr-Michaud S, Argueso P, Tisdale A, Ng TF & Russo CL (2003): Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. Invest Ophthalmol Vis Sci **44**: 2496–2506.

Gordon SR (1988): Changes in distribution of extracellular matrix proteins during wound repair in corneal endothelium. J Histochem Cytochem **36**: 409–416.

Gospodarowicz D (1984): Preparation of extracellular matrices produced by cultured bovine corneal endothelial cells and PF-HR-9 endodermal cells: their use in cell culture. In: Banes DW, Sirbasu DA, Sato GH, Liss AR (eds.). Methods for preparation of media, supplements and substrata for serum free animal cell culture. New York, NY: AR Liss Inc. 275–293.

Gruschwitz R, Friedrichs J, Valtink M et al. (2010): Alignment and cell–matrix interactions of human corneal endothelial cells on nanostructured collagen type I matrices. Invest Ophthalmol Vis Sci **51**: 6303–6310.

Hassell JR, Robey PG, Barrach HJ, Wilczek J, Rennard SI & Martin GR (1980): Isolation of a heparan sulfate-containing proteoglycan from basement membrane. Proc Natl Acad Sci USA **77**: 4494–4498.

Henry M & Meleady P (2017): Clinical proteomics: liquid chromatography-mass spectrometry (LC-MS) purification systems. Methods Mol Biol **1485**: 375–388.

Hsieh P & Baum J (1985): Effects of fibroblastic and endothelial extracellular matrices on corneal endothelial cells. Invest Ophthalmol Vis Sci **26**: 457–463.

Ishino Y, Sano Y, Nakamura T et al. (2004): Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. Invest Ophthalmol Vis Sci **45**: 800–806.

Jones JI, Gockerman A, Busby WH Jr, Camacho-Hubner C & Clemmons DR (1993): Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I. J Cell Biol **121**: 679–687.

Joyce NC (2003): Proliferative capacity of the corneal endothelium. Prog Retin Eye Res **22**: 359–389.

Joyce NC (2005): Cell cycle status in human corneal endothelium. Exp Eye Res **81**: 629–638.

Joyce NC & Zhu CC (2004): Human corneal endothelial cell proliferation: potential for use in regenerative medicine. Cornea **23**: S8–S19.

Kalimuthu SN & Chetty R (2016): Gene of the month: SMARCB1. J Clin Pathol **69**: 484–489.

Khaireddin R, Wachtlin J, Hopfenmuller W & Hoffmann F (2003): HLA-A. HLA-B and HLA-DR matching reduces the rate of corneal allograft rejection. Graefes Arch Clin Exp Ophthalmol **241**: 1020–1028.

Kimoto M, Shima N, Yamaguchi M, Amano S & Yamagami S (2012): Role of hepatocyte growth factor in promoting the growth of human corneal endothelial cells stimulated by L-ascorbic acid 2-phosphate. Invest Ophthalmol Vis Sci **53**: 7583–7589.

Kinoshita S, Koizumi N, Ueno M et al. (2018): Injection of cultured cells with a ROCK inhibitor for bullous keratopathy. N Engl J Med **378**: 995–1003.

Koo S, Muhammad R, Peh GS, Mehta JS & Yim EK (2014): Micro- and nanotopography with extracellular matrix coating modulate human corneal endothelial cell behavior. Acta Biomater **10**: 1975–1984.

Kundu B, Rajkhowa R, Kundu SC & Wang X (2013): Silk fibroin biomaterials for tissue regenerations. Adv Drug Deliv Rev **65**: 457–470.

Lawler J, McHenry K, Duquette M & Derick L (1995): Characterization of human thrombospondin-4. J Biol Chem **270**: 2809–2814.

Lee RE & Davison PF (1984): The collagens of the developing bovine cornea. Exp Eye Res **39**: 639–652.

Madden PW, Lai JNX, George KA, Giovenco T, Harkin DG & Chirila TV (2011): Human corneal endothelial cell growth on a silk fibroin membrane. Biomaterials **32**: 4076–4084.

Marshall GE, Konstas AG & Lee WR (1991): Immunogold fine structural localization of extracellular matrix components in aged human cornea: II. Collagen types V and VI. Graefes Arch Clin Exp Ophthalmol **229**: 164–171.

Melles GR (2006): Posterior lamellar keratoplasty: DLEK to DSEK to DMEK. Cornea **25**: 879–881.

Michelacci YM (2003): Collagens and proteoglycans of the corneal extracellular matrix. Braz J Med Biol Res **36**: 1037–1046.

Nakayasu K, Tanaka M, Konomi H & Hayashi T (1986): Distribution of types I, II, III, IV and V collagen in normal and keratoconus corneas. Ophthalmic Res **18**: 1–10.

Okumura N, Kay EP, Nakahara M, Hamuro J & Kinoshita S (2013): Inhibition of TGF-beta signaling enables human corneal endothelial cell expansion in vitro for use

in regenerative medicine. PLoS One **8**: e58000.
Okumura N, Koizumi N, Ueno M et al. (2012): ROCK inhibitor converts corneal endothelial cells into a phenotype capable of regenerating in vivo endothelial tissue. Am J Pathol **181**: 268–277.
Palchesko RN, Lathrop KL, Funderburgh JL & Feinberg AW (2015): In vitro expansion of corneal endothelial cells on biomimetic substrates. Sci Rep **5**: 7955.
Parekh M, Ahmad S, Ruzza A & Ferrari S (2017): Human corneal endothelial cell cultivation from old donor corneas with forced attachment. Sci Rep **7**: 142.
Parekh M, Ferrari S, Sheridan C, Kaye S & Ahmad S (2016): Concise Review: An update on the culture of human corneal endothelial cells for transplantation. Stem Cells Transl Med **5**: 258–264.
Parekh M, Peh G, Mehta JS et al. (2019a): Passaging capability of human corneal endothelial cells derived from old donors with and without accelerating cell attachment. Exp Eye Res **189**: 107814.
Parekh M, Peh G, Mehta JS, Ahmad S, Ponzin D & Ferrari S (2019b): Effects of corneal preservation conditions on human corneal endothelial cell culture. Exp Eye Res **179**: 93–101.
Parekh M, Romano V, Ruzza A et al. (2019c): Culturing discarded peripheral human corneal endothelial cells from the tissues deemed for preloaded DMEK transplants. Cornea **38**: 1175–1181.
Parekh M, Salvalaio G, Ruzza A et al. (2013): Posterior lamellar graft preparation: a prospective review from an eye bank on current and future aspects. J Ophthalmol **2013**: 769860.
Parekh M, Van den Bogerd B, Zakaria N, Ponzin D & Ferrari S (2018): Fish scale-derived scaffolds for culturing human corneal endothelial cells. Stem Cells Int **2018**: 1–11.
Peh GS, Adnan K, George BL et al. (2015): The effects of Rho-associated kinase inhibitor Y-27632 on primary human corneal endothelial cells propagated using a dual media approach. Sci Rep **5**: 9167.
Peh GS, Beuerman RW, Colman A, Tan DT & Mehta JS (2011a): Human corneal endothelial cell expansion for corneal endothelium transplantation: an overview. Transplantation **91**: 811–819.
Peh GS, Chng Z, Ang HP et al. (2013a): Propagation of human corneal endothelial cells: a novel dual media approach. Cell Transplant **24**: 287–304.
Peh GS, Toh KP, Ang HP, Seah XY, George BL & Mehta JS (2013b): Optimization of human corneal endothelial cell culture: density dependency of successful cultures in vitro. BMC Res Notes **6**: 176.
Peh GS, Toh KP, Wu FY, Tan DT & Mehta JS (2011b): Cultivation of human corneal endothelial cells isolated from paired donor corneas. PLoS One **6**: e28310.
Pipparelli A, Thuret G, Toubeau D et al. (2011): Pan-corneal endothelial viability assessment: application to endothelial grafts predissected by eye banks. Invest Ophthalmol Vis Sci **52**: 6018–6025.
Robertson DM, Li L, Fisher S et al. (2005): Characterization of growth and differentiation in a telomerase-immortalized human corneal epithelial cell line. Invest Ophthalmol Vis Sci **46**: 470–478.
Shima N, Kimoto M, Yamaguchi M & Yamagami S (2011): Increased proliferation and replicative lifespan of isolated human corneal endothelial cells with Lascorbic acid 2-phosphate. Invest Ophthalmol Vis Sci **52**: 8711–8717.
Todorovic V, Desai BV, Eigenheer RA et al. (2010): Detection of differentially expressed basal cell proteins by mass spectrometry. Mol Cell Proteomics **9**: 351–361.
Underwood PA & Bennett FA (1993): The effect of extracellular matrix molecules on the in vitro behavior of bovine endothelial cells. Exp Cell Res **205**: 311–319.
Yue BY, Baum JL & Silbert JE (1978): Synthesis of glycosaminoglycans by cultures of normal human corneal endothelial and stromal cells. Invest Ophthalmol Vis Sci **17**: 523–527.
Zhu YT, Chen HC, Chen SY & Tseng SC (2012): Nuclear p120 catenin unlocks mitotic block of contact-inhibited human corneal endothelial monolayers without disrupting adherent junctions. J Cell Sci **125**: 3636–3648.
Zimmermann DR, Trueb B, Winterhalter KH, Witmer R & Fischer RW (1986): Type VI collagen is a major component of the human cornea. FEBS Lett **197**: 55.

Received on May 27th, 2020.
Accepted on August 9th, 2020.

Correspondence:
Sajjad Ahmad MB BS, PhD, FRCOphth
Institute of Ophthalmology
University College London
11-43 Bath Street
London EC1V 9EL
UK
Tel: +44 7834 853610
Fax: +44 207 566 2320
Email: sajjad.ahmad@ucl.ac.uk

The study was fully supported by Moorfields Eye Charity grant titled 'The culture of human corneal endothelial cells for transplantation' MEC ref – GR000015 / R170041A to Mr. Sajjad Ahmad.

The authors thank Dr. Alice Davidson (UCL Institute of Ophthalmology, London, UK) for providing the human corneal endothelial cell line (HCEC-12), Professor Ilene Gipson (Schepens Eye Research Institute, USA) for providing the conjunctival cell line (HCjE-Gi) and Dr. James Jester (University of Southern California, USA) for providing the corneal epithelial cell line (hTCEpi).