Passaging capability of human corneal endothelial cells derived from old donors with and without accelerating cell attachment

Authors

Mohit Parekh[1,2], Gary Peh[3,4], Jodhbir S. Mehta[3,4,5,6], Tiago Ramos[1], Diego Ponzin[2], Sajjad Ahmad[1,7] and Stefano Ferrari[2]

Affiliations

[1]Institute of Ophthalmology, University College London, London, UK

[2]International Center for Ocular Physiopathology, The Veneto Eye Bank Foundation, Venice, Italy

[3]Tissue Engineering and Stem Cell group, Singapore Eye Research Institute, Singapore

[4]Duke-NUS Graduate Medical School, Singapore

[5]Singapore National Eye Centre, Singapore

[6]School of Material Science and Engineering and School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore

[7]Moorfields Eye Hospital NHS Foundation Trust, London, UK

Correspondence

Mohit Parekh, PhD

Institute of Ophthalmology, University College London,

11-43 Bath St, London, EC1V 9EL, UK

Tel: +44 7427652996

e-mail: m.parekh@ucl.ac.uk

Running title

Passaging human corneal endothelial cells

Abstract

In a recent report, we showed that it is possible to establish the culture of Human Corneal Endothelial Cells (HCEnCs) from older donor corneas (usually over 65 year olds) when left to attach in the presence of a viscoelastic solution, potentially increasing the donor pool for culturing HCEnCs. Therefore, we set out to evaluate the outcome of using a viscoelastic solution (Viscoat) to accelerate the attachment of passaged cultured human corneal endothelial cells (HCEnCs). The cells from 28 donor tissues were isolated using peel-and-digest method and evenly seeded into two wells of an 8-well chamber slide. The cells were left to attach after topical application of Viscoat. At confluence, one well was subjected to end-stage characterization, whereas the other well was passaged into another two wells. The cells at P1 were attached with and without the use of Viscoat. The growth rate was monitored; and at confluence, morphometric analysis, corneal endothelial specific (CD166-Tag1A3 & PRDX6-Tag2A12), mitochondrial and respiration assessment (Tom-20 and Seahorse); function-associated (Na^+/K^+ATPase & ZO-1); proliferative (Ki-67) marker analysis, and viability (Hoechst, Ethidium Homodimer and Calcein AM-HEC) studies were performed. Cells at P0 (with Viscoat) showed 100% confluence at day 9. Cells at P1 with and without Viscoat showed significant difference of confluence 67.0% v 18.8% respectively ($p<0.05$). Confluence rate, cell density, hexagonality, Ki-67 positivity and mitochondrial intensity was significantly higher ($p<0.05$), whereas cell-area and polymorphism was significantly lower ($p<0.05$) in the cells attached with Viscoat compared with the cells attached without Viscoat. There was no significant difference in oxygen consumption rate between the groups. In conclusion, we observed that acceleration in the attachment of passaged HCEnCs with the assistance of Viscoat, could be beneficial for the propagation of HCEnCs isolated from older donors, to increase their propensity to proliferate, without loss of the expression of vital proteins and heterogeneity in cellular morphology.

Keywords

Cornea; corneal endothelial cells; old donor; primary cell culture; passage; adherence

1. Introduction

Transparency of human cornea is important for clear vision. This is maintained by the posterior monolayer of corneal endothelial cells (CEnCs) through dynamic regulation of corneal hydration between a leaky cell barrier and the ionic pumps (Joyce 2003). It is believed that Human CEnCs (HCEnCs) do not have a regenerative capability in vivo (Bourne 2003). As such, excessive damage to these cells will diminish their functional capacity in regulating corneal hydration and in turn it may result in corneal oedema and eventual blindness if left untreated (Engelmann et al. 1999; Edelhauser 2006). Corneal transplantation using a procured healthy cadaveric donor cornea, is a widely used surgical technique as a treatment to replace the damaged endothelium to restore vision (Parekh et al. 2015). According to the 2016 Eye Bank Association of America report, nearly 60% of the total number of donated corneas from the US eye banks was utilized for treating corneal endothelial dysfunctions.

The availability of the donor corneas with good endothelium is a severe limitation in many countries (Gain et al. 2016). With the capacity to propagate and expand the number of primary HCEnCs, several plausible therapeutic alternatives have been suggested for the treatment of corneal endothelial disorders, potentially increasing the number of transplants from a single donor tissue. Indeed, through both cell/tissue engineering of cultured HCEnCs grown onto suitable replacement graft materials or through injection of cultivated HCEnCs, these approaches have been developed and validated by various research groups around the world (Koizumi et al. 2012; Choi et al. 2010; Peh et al. 2011a,b; Peh et al. 2013a,b). Different isolation techniques, media constituents, donor corneal characteristics and plating techniques have been adopted (Koizumi et al. 2012; Choi et al. 2010; Peh et al. 2011a,b; Peh et al. 2013a,b; Parekh et al. 2013; Parekh et al. 2016).

Earlier reports have shown that culturing HCEnCs from younger donor corneas may have advantages related to cellular expansion as they have a higher proliferative capacity compared with aged donor corneas (Peh et al. 2015). However, the availability of younger donors for the propagation of HCEnCs is much less frequent,

as they are generally not as easily obtainable compared to older donor corneas. We have recently shown that the culture of HCEnCs from older donor corneas (usually over 65 year olds) can be established when left to attach in the presence of a viscoelastic solution (Parekh et al. 2017), potentially increasing the donor pool for culturing HCEnCs. The aim of the present study was to evaluate the use of a viscoelastic solution on the culture and serial passage of HCEnCs isolated from older donor corneas, and the subsequent characterization of the expanded cells.

2. **Material and Methods**

2.1 Ethical Statement

All the corneal samples [n=28] were obtained by the Veneto Eye Bank Foundation (FBOV). A written consent was taken from the donor's next-of-kin for the tissues to be used for research purposes. The experiments followed the 2013 tenets of declaration of Helsinki. The tissues were deemed unsuitable for transplantation because of their poor endothelial cell count (<2200 cells/mm^2) and were used according to the Centro Nazionale di Trapianti (Rome, Italy) laws. No other indications or complications were registered in the donor corneas like Diabetes, HIV or HBV.

2.2 Endothelial cell count and donor characteristics

The tissues prior to the study were preserved in tissue culture medium (TCM), Cornea Max (Eurobio, Paris, France) at 31°C for not more than 35 days. Endothelial cell density (ECD) and mortality were checked by applying 100 µL of trypan blue (TB) stain (0.25%) (Thermo Fisher Scientific (Rochester, NY, USA), topically on the corneal endothelium for approximately 20 seconds followed by washing with phosphate buffered saline (1X PBS). Mortality was assessed as percentage of trypan blue positive cells (TBPCs). ECD and TBPCs of donor corneas were determined as previously described (Parekh et al. 2017), before primary cell isolation. The number of cells at confluence was checked using a 10X10 area reticule within the eyepiece. It was also used to check the rate of confluency by counting each box that was filled with cells every alternate day. For each cornea, 5 readings from random sites were counted and averaged. Finally, basic donor information (age, gender, post-mortem time, TBPCs and ECD) was obtained retrospectively from FBOV database.

2.3 Peel and digest method

All the tissues were pre-washed in sterile PBS to remove any media remnants. The Descemet membrane-endothelial complex was peeled gently in various pieces ensuring quicker isolation of the cells. The peeled pieces were incubated in a solution of 2mg/mL Collagenase Type 1 (Thermo Fisher Scientific, Rochester, NY, USA) for 2-3 hours at 31°C and 5% CO_2. Subsequently, following the digestion of the

Descemet's membrane, the cell suspension was obtained and centrifuged for 5 minutes at 1000 rpm. After removing the supernatant, the cells were re-suspended in TrypLE Express (1X), phenol red (Life Technologies, Monza, Italy) for 5 minutes at 37^{o}C to obtain smaller clusters of cells and single cells. The cells were re-suspended in 200 µL of the cell culture media supplemented with ROCK inhibitor and counted with TB using a haemocytometer slide.

The cell culture medium was a 1:1 mixture of HamF12 and M199 (Sigma Aldrich, St. Louis, Missouri, USA) supplemented with 5% FBS (Sigma Aldrich, St. Louis, Missouri, USA), 1% ascorbic acid (Sigma Aldrich, St. Louis, Missouri, USA), 0.5% Insulin Transferrin Selenium (ITS) (Thermofisher Scientific, Waltham, Massachusetts, USA), Rec human FGF basic (10ng/mL) (Thermofisher Scientific, Waltham, Massachusetts, USA), 10 µM ROCK inhibitor (Y-27632) (Miltenyi Biotec, Bergisch Gladbach, Germany) and 1% PenStrep (Thermofisher Scientific, Waltham, Massachusetts, USA). The procedure was slightly modified from the previously published article (Peh et al. 2011b).

2.4 Plating cells

The plating density was recorded and the cells were plated in Lab-Tek II chamber slides (8 chambers, 25X75 mm, 0.7 cm^2 culture area; Thermo Fisher Scientific Rochester, NY, USA). All the chambers were pre-coated with FNC coating mix (50 µL) (BRFF AF-10, US Biological Life Sciences, Salem, Massachusetts, USA) at 37^{o}C, 5% CO_2 for approximately 30 minutes. 200 µL of the final cell suspension was prepared from each donor cornea and mixed well. The cells were plated equally in each chamber of the 8-well chamber slide with 100 µL of cell suspension in two wells from each donor. Immediately following cell seeding, approximately 50-100 µL viscoelastic [Viscoat (Alcon, Texas, USA) − 0.8 mL containing 3% sodium hyaluronate and 4% chondroitin sulphate] was gently applied onto the cell suspension of both the chambers. The viscosity of HA being very dense, forces the cells to attach quicker accelerating the process of cellular attachment onto the coated base (Parekh et al. 2017). Cell culture media were refreshed every alternate day and the cells were monitored till confluence.

2.5 Passaging cells

At confluence, the cells from one chamber were used for end-stage analysis and the cells from other chamber (from the same eye of the donor cornea) were dissociated for subsequent study. Dissociation of these cells were achieved with TrypLE Express (1X) treatment for approximately 5-10 minutes at 37°C, 5% CO_2. The cell suspension was centrifuged at 1000 rpm for 5 minutes. The cells were re-suspended with 200 µL of cell culture media. In order to assess the effect of the application of Viscoat for the continual propagation of HCEnCs at Passage 1 (P1), 100 µL of the obtained cell suspension was seeded in each of the two chambers, where only one of the chambers was treated with the application of Viscoat as described above.

2.6 Morphology of the cultured HCEnCs

HCEnCs were monitored every alternate day till they were confluent using an inverted microscope (Axiovert, Zeiss, Germany). The percentage confluency was recorded using a 10X10 reticule (0.1mm^2) attached to the eyepiece by counting the number of boxes filled with cells at every alternate day.

2.7 The following analysis was performed as end stage characterization.

Live and dead cell analysis using Hoechst, Ethidium Homodimer and Calcein AM (HEC) staining

Cultured HCEnCs in the wells [n=3] were washed with PBS. 5 µL, 4 µL and 2 µL of Hoescht 33342 (H) (Thermo Fisher Scientific, Rochester, NY, USA), Ethidium Homodimer EthD-1 (E) and Calcein AM (C) (Live/Dead viability/cytotoxicity kit, Thermo Fisher Scientific, Rochester, NY, USA) respectively, were mixed in 1 mL of PBS. 100 µL of this solution was added in each well with cultured cells and incubated in the dark for 30 minutes at room temperature. Cells were then viewed and imaged within 2 hours with Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad, Amsterdam) microscope using NIS Elements software (Nikon).

2.8 Immunostaining for cultured HCEnCs

HCEnCs [n=3 for each marker] were rinsed with PBS. 4% paraformaldehyde (PFA) was used as a fixative agent at room temperature (RT) for 20 minutes. With the

197 exception of 2A12 staining, all the remaining cells were permeabilized with 0.5%
198 Triton X-100 incubated at room temperature for 30 minutes. 5% goat serum was
199 used as blocking buffer for 1 hour at RT. The cells were incubated overnight at 4°C
200 with the following primary antibodies: anti-Ki-67, 1:200 (MIB-1, Milan, Italy); anti-
201 2A12, 1:100 (Tag-2A12) & anti-1A3 (Tag-1A3, 1:100 (Bioprocessing Technology
202 Institute, Singapore); anti-Na/K-ATPase, 1:50 (Na/K ATPase, Santacruz, Texas, USA)
203 and; anti-TOM-20, 1:100 (Tom-20, Santa Cruz Biotechnology, Texas, USA). The cells
204 were further incubated with goat anti-mouse fluorescein isothiocyanate (FITC)-
205 conjugated secondary antibody in 10% goat serum for 2 hours at RT. For anti-ZO-1
206 (1:200, ZO-1, Thermo Fisher Scientific Rochester, NY, USA), as it was directly
207 conjugated to Alexa Fluor 488, samples were analyzed following 3 hours of
208 incubation at room temperature. The cells were washed in regular intervals of 3
209 washes with 1X PBS. The wall of the Lab-Tek slide was removed carefully and the
210 cells were mounted using VectaShield with 4',6-diamidino-2-phenylindole (DAPI).
211 The cells were examined using Nikon Eclipse Ti-E (Nikon, Burgerweeshuispad,
212 Amsterdam) microscope with NIS Elements software (Nikon).
213
214 *2.9 Metabolic assay using Oxygen Consumption Rate (OCR) analysis*
215 Mitochondrial respiration [n=4] was performed using an extracellular flux analyzer
216 (XFw24; Seahorse Bioscience) at 37°C. The cells were plated as above following
217 manufacturer's protocol using XF Cell Mito Kit. 15 oxygen concentration
218 measurements, initiating with three basal readings in glucose-supplemented media,
219 three readings each after adding 1 µM oligomycin (ATP synthase inhibitor), 0.6 µM
220 and 1 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, ETC
221 uncoupler), and 1 µM Rotenone (Rot, ETC inhibitor) with 1 µM Antimycin A (AA, ETC
222 inhibitor). Quantification of the mitochondrial respiration was carried out in the
223 extracellular media by measuring the oxygen concentration change. The oxygen
224 consumption rate (OCR; pmole/min) was thus recorded.
225
226 *2.10 Measurements and statistical analysis*
227 All the measurements and data analysis were performed using ImageJ (FIJI) software.
228 Surface areas of cells were measured based on Calcein AM uptake, where each cell

was marked using a free-hand tool and measured with size limits of 150-10,000 µm^2. Ki-67 positivity was assessed using random areas, and analyzed with an outline option. Watershed function was applied when necessary. The percentage of Ki-67 positive cells was counted based on its positive immune-signals observed in the area compared with the number of cells counted using nuclei staining.

For ZO-1 analysis, the images were converted to overlay masks using pre-determined macroinstructions to define the parameters of both hexagonality and polymorphism within a particular area (Parekh et al. 2017, 2019). 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 6 borders per cell for hexagonal cells and less than 4 borders for severe polymorphic cells in the investigated area. For mitochondrial staining intensity, the image was converted, using ImageJ into gray scale image, followed by auto ROI to normalize all the readings. The intensity of the gray scale image per cell was recorded and analyzed with an average of 15 cells from each group (Greiner et al. 2015; Aldrich et al. 2017). All the data were recorded and expressed as mean ± SD.

SAS software for statistics was employed to check the statistical significance between P1 with and without Viscoat. Data with $p<0.05$ was significant using Non-parametric Wilcoxon test and Student's T-test for paired data. A post-hoc correction to the significance was ensured using Bonferroni.

3. Results

3.1 Donor characteristics and the number of cells plated

A total of 25 donor corneas (16 male and 9 female donors) were used for the final analysis in this study. Isolated HCEnCs from donors [n=3] that did not proliferate in both, the control and treatment groups were excluded from the study. Average donor age was 69.89 ± 10.13 (65-78) years. The mean recorded post-mortem time was 9.72 ± 5.43 (3.5-19.5) hours. Mean donor corneal ECD before isolation was 1972 ± 144.73 (1600-2100) cells/mm^2 and 1.56 ± 3.84 (0-5)% of TBPCs were found. 187,331 ± 13,747 cells were obtained in average from each cornea. No other pathologies were noted.

3.2 Confluence rate

Following isolation of HCEnCs, twenty-one tissues were seeded equally into two wells and observed every alternate days of culture (Figure 1) for morphology, growth pattern and confluency. At day 1, the state of confluence between the seeded P1 cells with and without Viscoat was found to be 21.2% and 5.0% respectively. By day 5, confluency increased to 43.0% and 10.6% respectively with statistical significance ($p<0.05$) between the two treatments. At day 9, P1 with and without Viscoat showed 67.0% and 18.8% confluence respectively, which was statistically significant ($p<0.05$; Figure 2a). At day 9, the endothelial cell densities (cells/mm^2) from the groups were found to be statistically significant ($p<0.05$; Table 1; Figure 2b).

3.3 Hoechst, Ethidium homodimer and Calcein AM (HEC) staining

Cultured HCEnCs at both P0 and P1, established with or without the use of Viscoat were found to be equally viable when evaluated at day 9 (last day) of the culture period. P0 cells were monitored as control (figure 3a). The difference in cell area (µm^2) was found to be statistically significant between P1 with and without Viscoat ($p<0.001$; Table 1). Morphological assessment showed that cell sizes of P1 HCEnCs that adhered in the presence of Viscoat (figure 3b) were significantly smaller than cells that had attached without Viscoat (figure 3c). The cell area is shown in Figure 3d.

282 *3.4 Expression of biomarkers on HCEnCs*

283 In the presence of Viscoat, expression of cell-surface markers CD166 (Tag1A3),
284 PRDX-6 (Tag2A12), function-associated markers Na^+/K^+-ATPase and ZO-1, as well as
285 proliferative marker Ki-67 were detected in P1 HCEnCs. Interestingly expression of
286 these markers were not detected for 2A12, Na^+/K^+-ATPase (Figure 4), and ZO-1
287 (Figure 5) for donor-matched P1 HCEnCs that were left to attach without the
288 presence of Viscoat. Based on ZO-1 expression (5a-5c), hexagonality (Figure 5d) and
289 related values of polymorphism (Figure 5e) in each group was evaluated and
290 tabulated in table 1. Significant differences were found in overall hexagonality
291 between both groups. Polymorphic cells ($p<0.001$) were observed in the group
292 without Viscoat. Although Ki-67 was detected in both groups (figure 6a-6c),
293 significantly more Ki-67 positive cells (%) were observed in HCEnCs that were left to
294 attach in the presence of Viscoat as compared to control (figure 6b-6d; table 1;
295 $p<0.001$).
296
297 *3.5 Oxygen Consumption Rate and mitochondrial intensity*

298 Oxygen Consumption Rate (OCR) (Rose et al. 2014) [n=4] was measured before and
299 after the addition of inhibitors to assess mitochondrial respiration. No statistical
300 difference was observed in terms of OCR between P1 cells with or without Viscoat
301 (Table 2) at basal level, after oligomycin, after increasing amounts (0.6 µM and 1
302 µM) of FCCP, or after antimycin A and rotenone as represented in Figure 7. TOM-20
303 [n=3] was expressed in all the conditions (figure 8a-8d), and the intensity values
304 were found to be significantly higher in P1 cells with Viscoat compared with P1 cells
305 without Viscoat ($p<0.05$) (figure 8d).
306

4. **Discussion**

Due to the limited supply of donor corneas, alternative replacement options such as cell-based therapeutics using propagated primary HCEnCs have been investigated (Koizumi et al. 2012; Choi et al. 2010; Peh et al. 2011a,b; Peh et al. 2013a,b; Parekh et al. 2013; Parekh et al. 2016). Most recently, a clinical report by Kinoshita et al. described a landmark breakthrough of a successful clinical trial where by 11 patients received a cellular injection of cultured HCEnCs grown in medium supplemented with ROCK inhibitor (Kinoshita et al. 2018). It should be noted that isolation and propagation of HCEnCs have been described earlier using different methods with vastly different media formulations (Shima et al. 2011; Kimoto et al. 2012; Zhu et al. 2012; Okumura et al. 2012; Okumura et al. 2013; Peh et al. 2013), and studies have indicated that primary HCEnCs established from younger donors are generally more proliferative and possesses a better expansion profile (Peh et al. 2013).

It has been observed that cultivated HCEnCs that are derived from old donor tissues have lower proliferative capability with a senescent cell phenotype and enlarged cellular morphology, which may in turn affect overall cell yield as well as its inherent functional ability (Joyce & Zhu 2004). However, older donor tissues i.e. above 65 years of age are more frequently available for research due to lower endothelial cell threshold that is required for transplantation (FBOV annual statistical report). Hence, expansion of HCEnCs from older donor tissues therefore could be advantageous, as it may reduce the waiting time for a suitable source of endothelial cells (Parekh et al. 2017).

All the tissues in this study were obtained from TCM, as it has been shown to have less mortality and hence higher plating density (Parekh et al. 2018) especially for the older donor corneas. We have previously shown that endothelial cells from old donor corneas cultured directly from TCM survived and showed confluence in presence or absence of Viscoat solution (Parekh et al. 2017). The current study aimed at assessing the outcome of using a viscoelastic solution during the attachment phase of the HCEnCs at passage 1 on the proliferative and propagative capacity of these cells.

Based on confluency assessment of the HCEnCs from P1, the cells that were left to attach by gravity following passaging did not grow over 20% and showed larger cell area. The cells that were left to attach in the presence of Viscoat, showed 67% confluence by day 9, with smaller cell area and better morphology, hence increasing the chances for passaging old age donor corneas. However, compared with P0 cells, cell sizes of P1 cells were found to be significantly larger whether Viscoat was used, or not, to aid the adherence of cells. In our experience, we found that passaging of older donor corneas was extremely difficult as they lose their morphology. This is not surprising, as corneal endothelial cells from older donors, have been shown to express many different genes, compared to HCEnCs, that were isolated and expanded from younger donors (Chng Z et al. 2013). However, with accelerated attachment, the morphology can be improved with better growth rate.

As such without Viscoat, the lower adherence capacity at P1 may have resulted in weakly attached cells that were washed off during the initial media change following cellular passage, thus reducing the total number of attached cells, and lowering seeding density. This further leads to poor cellular morphology (Peh et al. 2013B), as well as the lack of formation of intercellular borders, and minimal expression of ZO-1 and Na^{+}/K^{+}-ATPase. Indeed, it has been previously shown, that seeding density plays an important role in the proliferative potential of HCEnCs (Peh et al., 2013b). Even though the study (Peh et al., 2013) described the cell-density associated proliferative propensity of donor-matched HCEnCs isolated from younger donors, it can be expected that such observation may hold true or even be more applicable to older donors. Indeed, we observed the expression of tight junction protein and better preservation of cellular hexagonality (>65% in the visual field) with <20% of severe polymorphism only in P0 cells attached with Viscoat. In the group without the application of Viscoat, the overall number of adhered cells was less and scattered, with minimal indication of intercellular connection in most cases. This was evident by the minimal expression of ZO-1 and Na^{+}/K^{+}-ATPase, which were not detected in the cells without Viscoat. Although this observation is not enough to conclude on the quality of the cells. Hexagonality could be achieved if the cells show complete confluence and once the monolayer has stabilized following long-term confluency.

The function-related proteins, such as Na^+/K^+-ATPase and ZO-1, are only expressed on confluent cells, explaining why it was not observed in the sub-confluent conditions especially in P1 cells without Viscoat.

CD166 and Prdx-6 (cell surface markers) have been monitored using TAG-1A3 and TAG-2A12, respectively. It has been shown that the expression of these markers has a good correlation with the current standard of morphological grading of the cultured HCEnCs (Ding et al. 2014). Low expression profile of these markers was observed in P1 cells without Viscoat.

As mentioned earlier, we have noticed that when the cells are attached with the help of Viscoat, almost all the plated cells are firmly attached to the base due to the viscosity of the Viscoat. This does not occur when the cells are attached without Viscoat and many cells get washed off during the first media change. This could be a reason for finding a significant difference in cell proliferation (Ki-67) between the groups at confluence. Also, it has been previously shown, that if the seeding density is too low, then corneal endothelial cells may not be able to proliferate even if there is a potential for proliferation (Peh et al. 2013b). The overall proliferative capability at P1 could have been low due to the low seeding density making it stressful for the corneal endothelial cells specially when they are obtained from the old donor tissues (Peh et al. 2013b).

Although mitochondrial density assay may reflect metabolic data, they cannot measure the total metabolic output that can be obtained using extracellular flux assays. In this study, oxygen consumption rate (OCR), as described earlier (Rose et al. 2014), was measured pre- and post- the addition of inhibitors to obtain multiple parameters of mitochondrial respiration. A report by Greiner MA et al. did not show any statistical difference in OCR readings between the central and peripheral corneal endothelium but the authors reported a significant change in the density using mitochondrial density measurements (Greiner et al. 2015). We did not find any significant difference in OCR readings at any phase of the study between P1 cells with or without Viscoat. The readings did not follow the usual trend of OCR. These

unusual findings led us to investigate further using mitochondrial specific markers and density quantification by recording its intensity values as an alternative to extracellular flux analysis. Mitochondrial density assay (Rose et al. 2014), i.e. intensity of the marker was found to be significantly higher in the P1 cells with Viscoat, therefore indicating better metabolic functionality compared with P1 cells without Viscoat. It has also been noted previously that the most important metabolic pathways for producing the required ATP are mitochondrial respiration and glycolysis. This is also crucial for proper human corneal endothelial cell function especially the pump function, and overall cellular health (Laing et al. 1992).

Viscoelastics have been widely used as a protective coat for corneal endothelial cells during cataract operations (Glasser et al. 1989; Probst & Nichols 1993; Hammer & Burch 1984; MacRae et al. 1983; Nguyen et al. 1992; Lane et al. 1991; Smith & Lindstrom 1991; Fry & Yee 1993). It has been noted that the high coat-ability i.e. viscoelastic's surface tension that determines its ability towards the tissue adherence, is because of chondroitin sulfate (Liesegang 1990; Soll et al. 1980). A fine layer of viscoelastic applied on the surface of endothelium is usually enough to protect the cells from mechanical stress (Shimmura et al. 1992; Artola et al. 1993). High viscosity, which is related to the molecular weight and concentration, and hence density offers the necessary protection against compressive and drag forces on the corneal endothelium (MacRae et al. 1983) caused after plating the cells. It has been shown that there are specific receptors on the corneal endothelium for hyaluronan (Madsen et al. 1989; Stenevi et al. 1993; Forsberg et al. 1994). The higher the molecular weight of hyaluronan, greater the affinity for receptors (Ravalico et al. 1997). However, it has been noted that cell surface heparan sulfate proteoglycan is required for adhesion to fibronectin-coated surfaces (Woods et al. 1986), which could be similar to our results, as we have used FNC coating in our studies both, in the experimental and control group. Proteoglycans have been associated with cell migration, which relates to constant breakage and reformation of cell adhesion sites (Fullwood et al. 1996). We believe that the components in Viscoat are linking with the surface proteoglycans further helping the cells to adhere

434 to the extracellular matrix, protecting the cells and increasing the focal points to
435 initiate cell migration, observed from the results in this study.
436
437 We have observed that although donor characteristics may have an influence on the
438 cell culture (Peh et al. 2011A; Peh et al. 2011B; Beck et al. 1999; Miyata et al. 2001),
439 it was difficult for us to correlate the outcome of cell culture with donor
440 characteristics, as every donor was different. We have also found that younger
441 donor corneas sometimes result in a culture failure, compared to old donors and we
442 speculate that this is dependent on the donor, but the exact cause is still unknown
443 (Parekh et al. 2017). In our series, we lost three tissues during the cell culture phase,
444 as they did not show any proliferation or migration although the cells were attached.
445
446 In conclusion, our findings indicate that HCEnCs established from older donor
447 corneas can be better cultured into passage 1 when cells were attached in an
448 accelerated manner using Viscoat. However, it should be noted that the number of
449 cells may still not be enough for transplantation, and hence, there may be a need to
450 pool tissues together to obtain a higher number of cells to passage and expand them
451 for transplantation purposes. It would be interesting to study the effect of such
452 accelerated attachment described in this study on younger donor cells as they
453 already have a better proliferation capacity. Future studies would be responsible for
454 looking into the expansion of passaged P1 cells established using older donors and
455 their functional capacity within an animal model via cell injection as described by
456 Kinoshita (Kinoshita et al. 2018) or via a tissue engineered cell carrier DSAEK
457 approach (Peh et al. 2017).

458 **5. Author contributions statement**

459 MP – Design of experiments, lab work, analysis, writing and approval of the draft

460 GP – Design of experiments, analysis, writing and approval of the draft

461 JM – Design of experiments, analysis and approval of the draft

462 TR – Writing, critical revision and approval of the draft

463 DP – Design of experiments, analysis, writing and approval of the draft

464 SA – Design of experiments, analysis, writing and approval of the draft

465 SF – Design of experiments, analysis, writing and approval of the draft

466

467 **6. Financial interest**

468 No authors have any financial or potential conflict of interest

469

470 **7. Acknowledgements**

471 The authors thank -

472 1. Bioprocessing Technology Institute, Singapore for kindly providing Tag-2A12 and

473 Tag-1A3 monoclonal antibodies.

474 2. Prof. Luca Scorrano and Dr. Stephanie Herkenne from University of Padova,

475 Padova, Italy for their help on mitochondrial staining procedures.

476 **8. References**

477 Aldrich, B.T., Schlötzer-Schrehardt. U., Skeie. J.M., et al., 2017. Mitochondrial and
478 Morphologic Alterations in Native Human Corneal Endothelial Cells Associated With
479 Diabetes Mellitus. Invest Ophthalmol Vis Sci. 58, 2130-2138.
480
481 Artola, A., Alio, J.L., Bellot, J.L., Ruiz, J.M., 1993. Protective properties of viscoelastic
482 substances (sodium hyaluronate and 2% hydroxymethylcellulose) against
483 experimental free radical damage to the corneal endothelium. Cornea. 12, 109-114.
484
485 Beck, R.W., Gal, R.L., Mannis, M.J., et al., 1999. Is donor age an important
486 determinant of graft survival? Cornea. 18, 503–510.
487
488 Bourne, W.M., 2003. Biology of the corneal endothelium in health and disease. Eye.
489 17, 912–918.
490
491 Chng, Z., Peh, G.S., Herath, W.B., et al., 2013. High throughput gene expression
492 analysis identifies reliable expression markers of human corneal endothelial cells.
493 PLoS One. 8, e67546.
494
495 Choi, J.S., Williams, J.K., Greven, M., et al., 2010. Bioengineering endothelialized neo-
496 corneas using donor-derived corneal endothelial
497 cells and decellularized corneal stroma. Biomaterials. 31, 6738-6745.
498
499 Ding, V., Chin, A., Peh, G., et al., 2014. Generation of novel monoclonal antibodies
500 for the enrichment and characterization of human corneal endothelial cells (hCENC)
501 necessary for the treatment of corneal endothelial blindness. MAbs. 6, 1439-1452.
502
503 Engelmann, K., Bednarz, J., B¨ohnke, M., 1999 Endothelial cell transplantation and
504 growth behavior of the human corneal endothelium. Ophthalmologe. 96, 555–562.
505
506 Edelhauser HF., 2006. The balance between corneal transparency and edema: the
507 Proctor Lecture. Invest Ophthalmol Vis Sci. 47, 1754–1767.

508 Forsberg, N., Von Malmborg, A., Madsen, K., et al., 1994. Receptors for hyaluronan
509 on corneal endothelial cells. Exp Eye Res. 59, 689-696.
510
511 Fry, L.L., Yee, R.W., 1993. Healon GV in extracapsular cataract extraction with
512 intraocular lens implantation. J Cataract Refract Surg. 19, 409-412.
513
514 Fullwood, N.J., Davies, Y., Nieduszynski, I.A., Marcyniuk, B., Ridgway, A.E., Quantock,
515 A.J., 1996. Cell surface-associated keratan sulfate on normal and migrating corneal
516 endothelium. Invest Ophthalmol Vis Sci. 37, 1256-1270.
517
518 Gain, P., Jullienne, R., He, Z., et al., 2016. Global Survey of Corneal Transplantation
519 and Eye Banking. JAMA Ophthalmol. 134, 167-173.
520
521 Glasser, D.B., Katz, H.R., Boyd, J.E., et al., 1989. Protective effects of viscous solutions
522 in phacoemulsification and traumatic lens implantation. Arch Ophthalmol. 107,
523 1047-1051

524 Greiner, M.A., Burckart, K.A., Wagoner, M.D., et al., 2015.
525 Regional assessment of energy-producing metabolic activity in
526 the endothelium of donor corneas. Invest Ophthalmol Vis Sci. 56, 2803-2810.
527
528 Hammer, M.E., Burch, T.G., 1984. Viscous corneal protection by sodium hyaluronate,
529 chondroitin sulfate, and methylcellulose. Invest Ophthalmol Vis Sci. 25, 1329-1332.

530 Joyce, N.C., 2003. Proliferative capacity of the corneal endothelium. Prog Retin Eye
531 Res. 22, 359–389.
532
533 Joyce, N.C., and Zhu, C.C., 2004. Human corneal endothelial cell proliferation:
534 potential for use in regenerative medicine. Cornea. 23, S8–S19.
535
536 Kimoto, M., Shima, N., Yamaguchi, M., et al. 2012. Role of hepatocyte growth factor
537 in promoting the growth of human corneal endothelial cells stimulated by L-ascorbic
538 acid 2-phosphate. Invest Ophthalmol Vis Sci. 53, 7583–7589.

539 Kinoshita, S., Koizumi, N., Ueno, M., et al., 2018. Injection of Cultured Cells with
540 a ROCK Inhibitor for Bullous Keratopathy. N Engl J Med. 378, 995-1003.
541
542 Koizumi, N., Okumura, N., Kinoshita, S., 2012. Development of new therapeutic
543 modalities for corneal endothelial disease focused on the proliferation of corneal
544 endothelial cells using animal models. Exp Eye Res. 95, 60–67.
545
546 Okumura, N., Koizumi, N., Ueno, M., et al., 2012. ROCK Inhibitor Converts Corneal
547 Endothelial Cells into a Phenotype Capable of Regenerating In Vivo Endothelial
548 Tissue. Am J Pathol. 181, 268–277.
549
550 Okumura, N., Kay, E.P., Nakahara, M., et al., 2013. Inhibition of TGF-beta signaling
551 enables human corneal endothelial cell expansion in vitro for use in regenerative
552 medicine. PLoS One. 8, e58000.
553
554 Laing, R.A., Chiba, K., Tsubota, K., Oak, S.S., 1992. Metabolic and morphologic
555 changes in the corneal endothelium. The effects of potassium cyanide,
556 iodoacetamide, and ouabain. Invest Ophthalmol Vis Sci. 33, 3315–3324.
557
558 Lane, S.S., Naylor, O.W., Kullerstrand, L.J., et al., 1991. Prospective comparison of the
559 effects of Occucoat, Viscoat, and Healon on intraocular pressure and endothelial cell
560 loss. J Cataract Refract Surg. 17, 21-26.
561
562 Liesegang, T.J., 1990. Viscoelastic substances in ophthalmology. Surv Ophthalmol. 34,
563 268-293.

564 MacRae, S.M., Edelhauser, H.F., Hyndiuk, R.A., et al., 1983. The effects of sodium
565 hyaluronate, chondroitin sulfate, and methylcellulose on the corneal endothelium
566 and intraocular pressure. Am J Ophthalmol. 95, 332-341.

567 Madsen, K., Schenholm, M., Jahnke, G., Tengblad A., 1989. Hyaluronate binding to
568 intact corneas and cultured endothelial cells. Invest Ophthalmol Vis Sci. 30, 2132-
569 2137.

570 Miyata, K., Drake, J., Osakabe, Y., et al., 2001. Effect of donor age on morphologic
571 variation of cultured human corneal endothelial cells. Cornea. 20, 59–63.
572
573 Nguyen, L.K., Yee, R.W., Sigler, S.C., Ye, H.S., 1992. Use of in vitro models of bovine
574 corneal endothelial cells to determine the relative toxicity of viscoelastic agents. J
575 Cataract Refract Surg. 18, 7-13.
576
577 Parekh, M., Graceffa, V., Bertolin, M., et al., 2013. Reconstruction and Regeneration
578 of Corneal Endothelium: A Review on Current Methods and Future Aspects. J Cell Sci
579 Ther. 4, 146.
580
581 Parekh, M., Ferrari, S., Ponzin, D., 2015. Eye Banking: an overview. In: Parekh M,
582 Ferrari S and Ponzin D. Eye Banking: Changing face of corneal transplantation. New
583 York: Nova Publications. 1-18.
584
585 Parekh, M., Ferrari, S., Sheridan, C., et al., 2016. Concise Review: An Update on the
586 Culture of Human Corneal Endothelial Cells for Transplantation. Stem Cells Transl
587 Med. 5, 258–264.
588
589 Parekh, M., Ahmad, S., Ruzza, A., Ferrari, S., 2017. Human Corneal Endothelial Cell
590 Cultivation From Old Donor Corneas With Forced Attachment. Sci Rep. 7, 142.
591
592 Parekh, M., Peh, G.S, Mehta, J.S., et al., 2018. Effects of corneal preservation
593 conditions on human corneal endothelial cell culture. Exp Eye Res. 179, 93-101.
594
595 Parekh, M., Romano, V., Ruzza, A., et al., 2019. Culturing discarded peripheral human
596 corneal endothelial cells from the tissues deemed for preloaded DMEK transplants.
597 Cornea. 38, 1175-1181.
598
599 Peh, G.S., Beuerman, R.W., Colman, A., et al., 2011A. Human corneal endothelial cell
600 expansion for corneal endothelium transplantation: an overview. Transplantation. 91,
601 811–819.

602 Peh, G.S., Toh, K.P., Wu, F.Y., et al., 2011B.
603 Cultivation of human corneal endothelial cells isolated from paired donor corneas.
604 PLOS One. 6, e28310.
605
606 Peh, G.S., Chng, Z., Ang, H.P., et al., 2013A. Propagation of human corneal
607 endothelial cells: a novel dual media approach. Cell Transplantation. 24, 287–304.
608
609 Peh, G.S., Toh, K.P., Ang, H.P., et al., 2013B. Optimization of human corneal
610 endothelial cell culture: density dependency of successful cultures in vitro. BMC Res
611 Notes. 6, 176.
612
613 Peh, G.S., Adnan, K., George, B.L., et al., 2015. The effects of Rho-associated kinase
614 inhibitor Y-27632 on primary human corneal endothelial cells propagated using a
615 dual media approach. Sci Rep. 5, 9167.
616
617 Peh, G.S.L., Ang, H.P., Lwin, C.N., et al., 2017. Regulatory compliant tissue-
618 engineered human corneal endothelial grafts restore corneal function of rabbits with
619 bullous keratopathy. Sci Rep. 7, 14149.
620
621 Probst, L.E., Nichols, B.D., 1993. Corneal endothelial and intraocular pressure
622 changes after phacoemulsification with Amvisc Plus and Viscoat. J Cataract Refract
623 Surg. 19, 725-730.

624 Ravalico, G., Tognetto, D., Baccara, F., Lovisato, A., 1997. Corneal endothelial
625 protection by different viscoelastics during phacoemulsification. J Cataract Refract
626 Surg. 23, 433-439.
627
628 Rose, S., Frye, R.E., Slattery, J., et al., 2014.
629 Oxidative stress induces mitochondrial dysfunction in a subset of autism
630 lymphoblastoid cell lines in a well-matched case control cohort. PLoS One. 9, e85436.
631

632 Shima, N., Kimoto, M., Yamaguchi, M., & Yamagami, S., 2011. Increased proliferation
633 and replicative lifespan of isolated human corneal endothelial cells with Lascorbic
634 acid 2-phosphate. Invest Ophthalmol Vis Sci. 52, 8711–8717.
635
636 Shimmura, S., Tsubota, K., Oguchi, Y., et al., 1992. Oxiradicaldependent
637 photoemission induced by a phacoemulsification probe. Invest Ophthalmol Vis Sci.
638 33, 2904-2907.
639
640 Smith, S.G., Lindstrom, R.L., 1991. 2% hydroxypropyl methylcellulose as a viscous
641 surgical adjunct. A multicenter prospective randomized trial. J Cataract Refract Surg.
642 17, 839-842.

643 Soll, D.B., Harrison, S.E., Arturi, F.C., Clinch, T., 1980. Evaluation and protection of
644 corneal endothelium. Am Intra-Ocular Implant Soc J. 6, 239 -242.
645
646 Stenevi, U., Gwin, T., Harfstrand, A., Apple, D., 1993. Demonstration of hyaluronic
647 acid binding to corneal endothelial cells in human eye-bank eyes. Eur J Implant
648 Refract Surg. 5, 228-232.
649
650 Woods, A., Couchman, J.R., Johnson, S., Hook, M., 1986. Adhesion and cytoskeleton
651 organization of fibroblasts in response to fibronectin fragments. EMBO J. 5, 665-670.
652
653 Zhu, Y.T., Chen, H.C., Chen, S.Y., & Tseng, S.C., 2012. Nuclear p120 catenin unlocks
654 mitotic block of contact-inhibited human corneal endothelial monolayers without
655 disrupting adherent junctions. J Cell Sci. 125, 3636–3648.

9. Figure legends

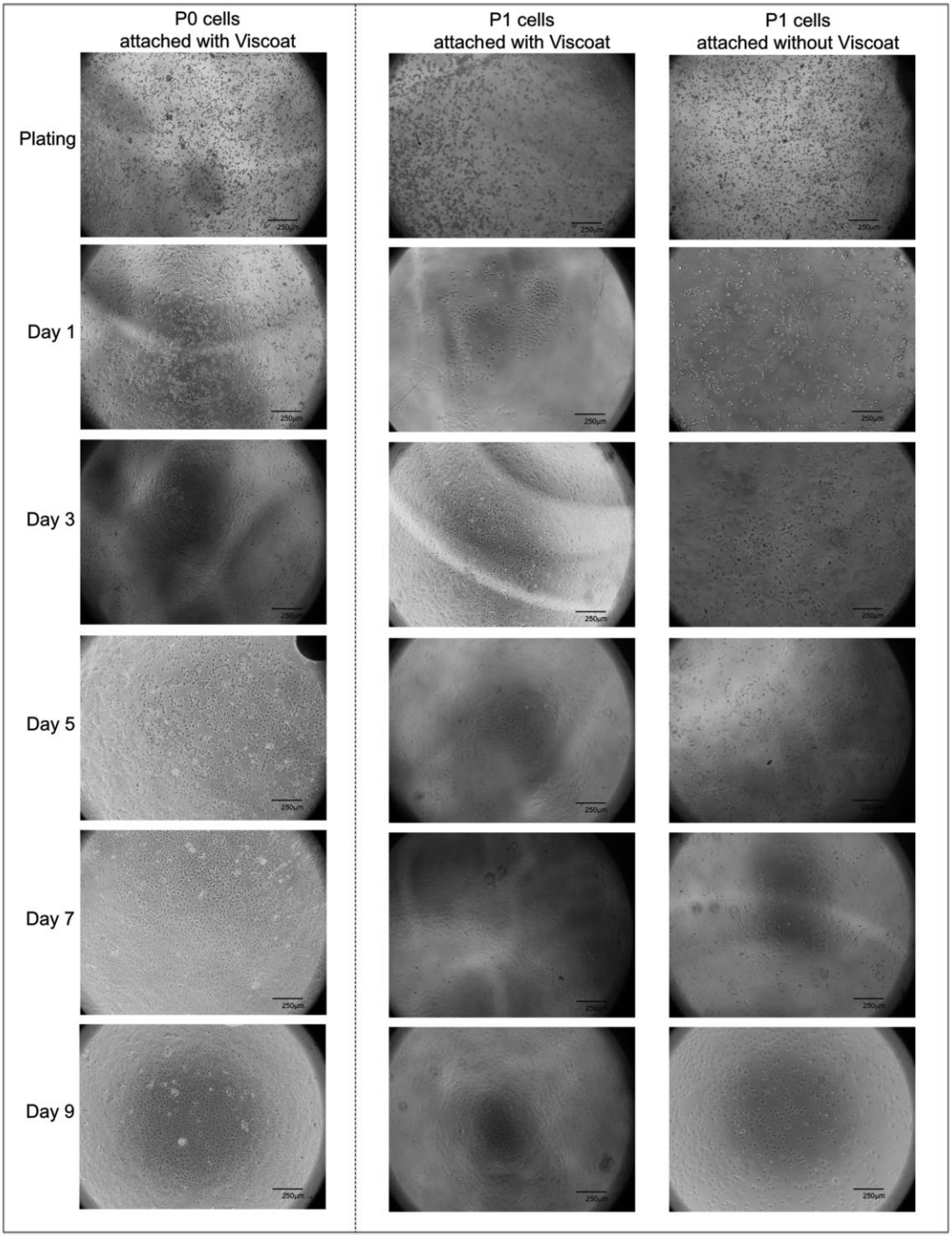

Figure 1: Morphological analysis of HCEnCs at alternate days of culture in primary cells (P0) and passaged cells (P1) with and without Viscoat. Morphology in terms of hexagonal cells was found to be significantly better in P0 cells compared to P1 cells with Viscoat. However, P1 cells without Viscoat showed poor morphology when compared to P1 cells with Viscoat.

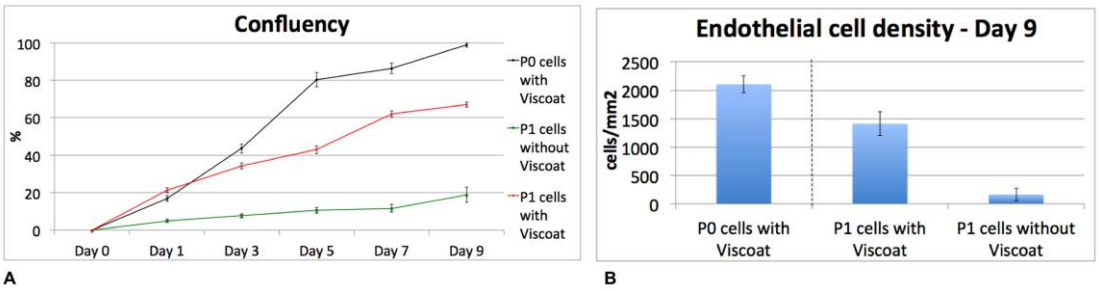

Figure 2: Confluence rate observed at every alternate day in A) P0 cells with Viscoat, P1 cells with and without Viscoat. Only P0 cells reached confluence. P1 cells did not reach confluence at day 9. B) Endothelial cell density (ECD) recorded at day 9 both, in P0 cells and at P1 with and without Viscoat. Significant difference was found in ECD data.

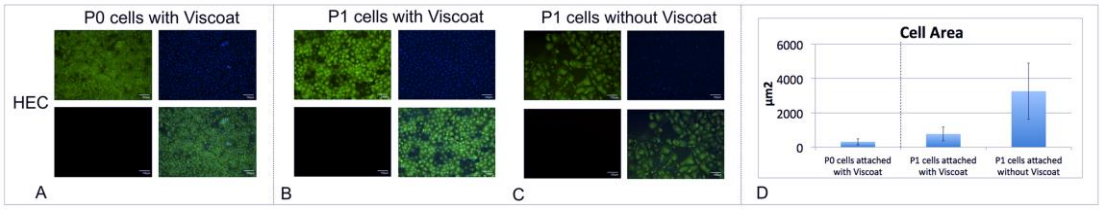

Figure 3: Triple endothelial labelling with Hoechst 33342 (H), Ethidium Homodimer (E), and Calcein-AM (C) showed expression of 'E' in red representing the dead cells, blue represents the nuclei 'H' and green marked the living cells 'C' determining live/dead in A) P0 cells, B) P1 with Viscoat, C) P1 without Viscoat and D) measurement of cell area at P1. Fully confluent areas were observed at day 9 in P0 cells with Viscoat. Cell area was significantly higher at P1 without Viscoat.

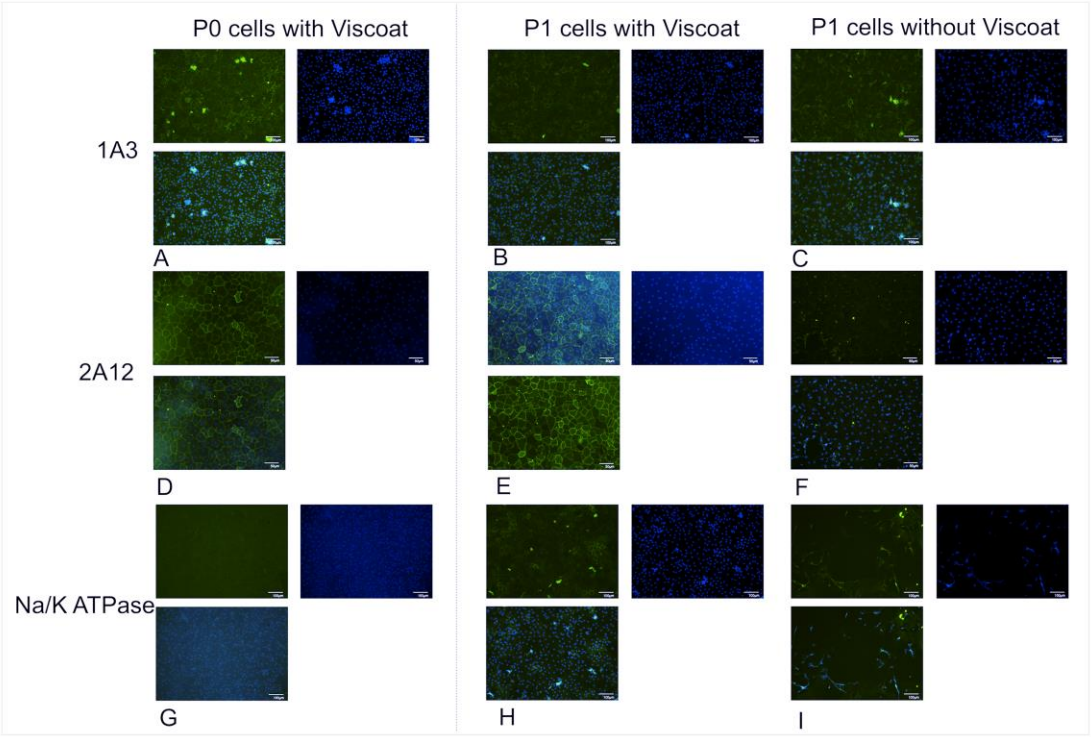

Figure 4: Expression pattern of 1A3 in A) P0 cells, B) P1 cells treated with Viscoat and C) P1 without Viscoat. Expression of cell surface marker 2A12 in D) P0 cells, E) P1 cells with and F) without Viscoat. Expression of Na^+/K^+ATPase marker in G) P0 cells, H) P1 cells with and I) without Viscoat. The cells showed expression mostly in primary cells and in the group with Viscoat and were limited to the group without forced attachment.

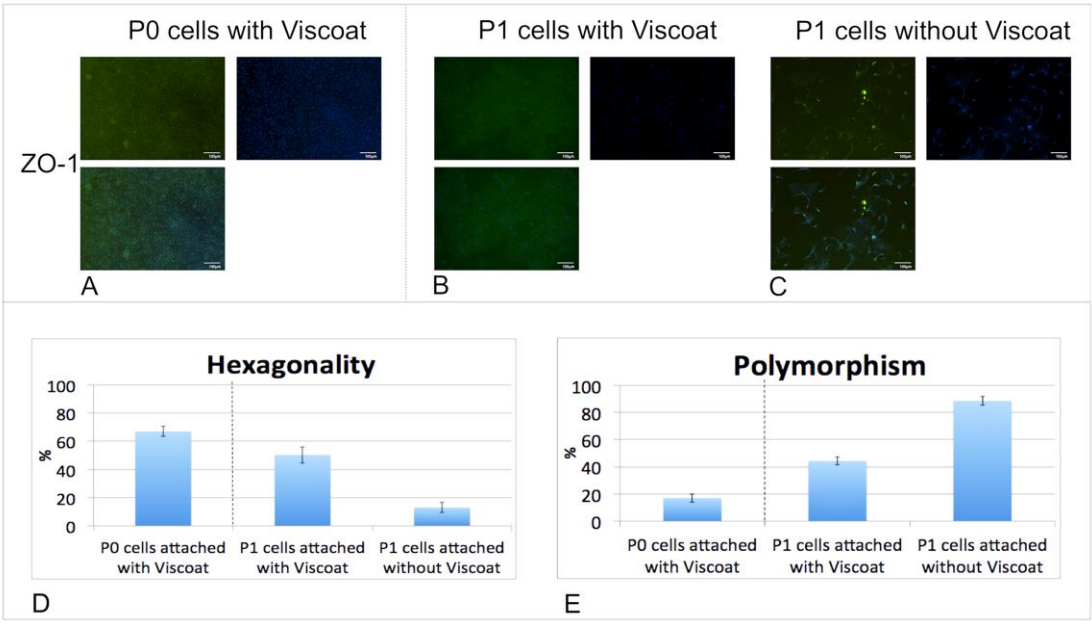

Figure 5: Expression of Zonula Occludens-1 (ZO-1) tight junction marker in A) P0 cells with Viscoat, B) cells at P1 with Viscoat and C) without Viscoat. ZO-1 was not expressed in the group without Viscoat. D) Hexagonality was found significantly higher in P1 cells with and without Viscoat whereas, E) polymorphism was found to be significantly lower in cells from P1 with and without Viscoat.

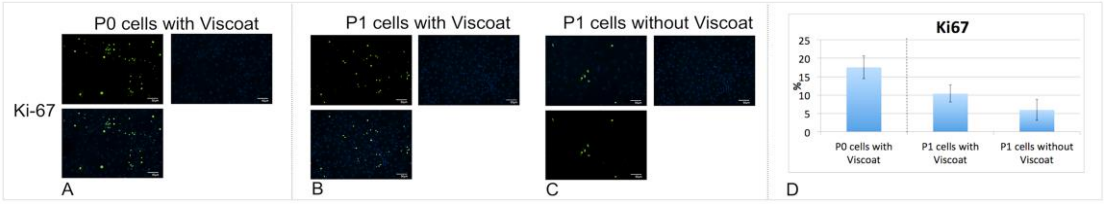

Figure 6: Expression of Ki-67 proliferative marker in A) P0 cells with Viscoat, B) cells from P1 with and C) without Viscoat. The amount of proliferative cells was found to be significantly higher in P1 with Viscoat compared to the group without Viscoat.

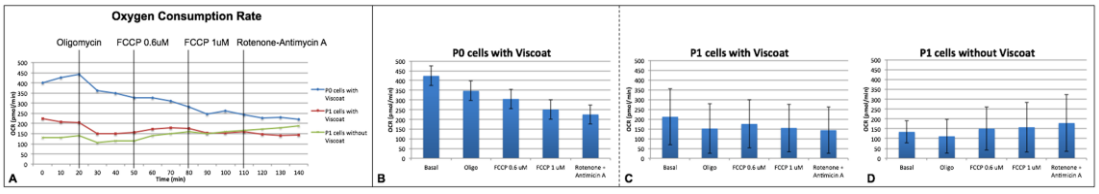

Figure 7: Metabolic assays deriving oxygen consumption rate analysis. OCR a) at different time points after each enzymatic reaction, b) on P0 cells with Viscoat, c) on P1 cells with and d) without Viscoat.

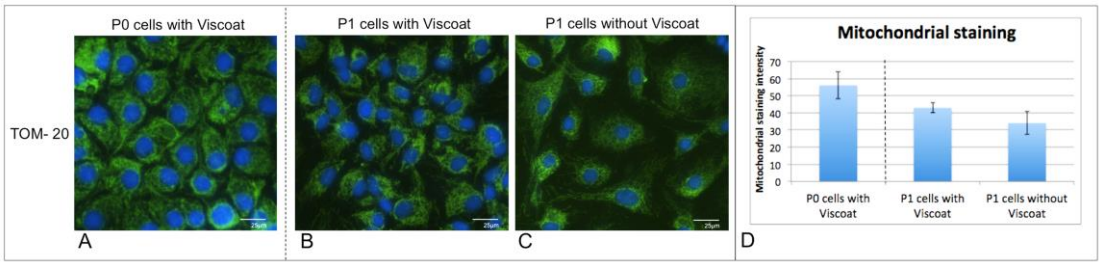

Figure 8: Expression of TOM-20 marker in A) P0 cells with Viscoat, B) cells from P1 with and C) without Viscoat. D) The amount mitochondrial intensity from P1 was significantly higher when the cells were attached with Viscoat compared with those cells at P1 that were not attached with Viscoat.