Title: Plasma polymer surface modified expanded polytetrafluoroethylene (ePTFE) promotes epithelial monolayer formation in vitro and can be transplanted into the dystrophic rat subretinal space.

Short title: epithelial monolayer formation and transplantation of modified ePTFE in rat retina

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Summary

The aim of this study was to evaluate whether the surface modification of ePTFE using an *n*-heptylamine (HA) plasma polymer would allow for functional epithelial monolayer formation suitable for subretinal transplant into a non-dystrophic rat model. Freshly isolated iris pigment epithelial (IPE) cells from two rat strains [Long Evans (LE) and Dark Agouti (DA)] were seeded onto HA, fibronectin-coated *n*-heptylamine modified (F-HA) and unmodified ePFTE and fibronectin-coated tissue culture (F-TCPS) substrates. Both F-HA ePTFE and F-TCPS substrates enabled functional monolayer formation with both strains of rat. Without fibronectin coating, only LE IPE formed a monolayer on HA-treated ePTFE. Functional assessment of both IPE strains on F-HA ePTFE demonstrated uptake of POS that increased significantly with time that was greater than control F-TCPS. Surgical optimization using Healon GV and mixtures of Healon GV:PBS to induce retinal detachment demonstrated that only Healon GV:PBS allowed F-HA ePTFE substrates to be successfully transplanted into the subretinal space of RCS rats, where they remained flat beneath the neural retina for up to 4 weeks. No apparent substrate-induced inflammatory response was observed by fundus microscopy or immunohistochemical analysis, indicating the potential of this substrate for future clinical applications.

Keywords (n=8)
cell transplantation, rats, plasma polymer, pigment epithelium of the eye/transplantation, retinal detachment, retinal degeneration, tissue engineering.

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Introduction

Retinal degenerative diseases, such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP), can severely affect vision and have limited effective treatments available to the majority of patients (Jones, Lu, Girman, & Wang, 2017; Lu, Liu, & Adelman, 2017; Tang et al., 2017). It is well recognized that defects in retinal pigment epithelial (RPE) cells and its underlying Bruch's membrane have a crucial role in the development of these diseases (Lund et al., 2001). Therefore, transplantation strategies to replace degenerative or missing RPE cells with a functioning RPE monolayer represent a promising therapeutic strategy. A number of cell sources for transplantation continue to be researched and have varied in approach using ocular (ciliary-, retinal- and iris- pigmented epithelial cells) or non-ocular sources ocular (embryonic-, induced pluripotent-, mesenchymal- and bone

marrow- derived stem cells)(Mason, Stewart, Kearns, Williams, & Sheridan, 2011; Pennington & Clegg, 2016).

Immunogenic responses to all non-autologous cells transplanted cells have been reported irrespective of their derivation and, therefore, autologous transplant of cells offers an attractive strategy as adjunct therapies to limit innate immune (adaptive and innate) responses are not well tolerated by aged patients (Kennelly, Holmes, Wallace, O'Farrelly, & Keegan, 2017). Iris pigment epithelial (IPE) cells have been studied as a substitute of RPE cells for transplantation as autologous IPE cells can be more readily obtained and have similar characteristics to RPE cells as they are both derived from the neural ectoderm. The shared similarities include heavy pigmentation, apical/basolateral polarity, tight junctions, retinol metabolism and capacity of outer segment phagocytosis (Abe et al., 2007; K. A. Rezai, L. Kohen, P. Wiedemann, & K. Heimann, 1997; K. A. Rezai, A. Lappas, L. Farrokh-siar, et al., 1997; Schraermeyer, Kociok, & Heimann, 1999). However, previous studies have shown that IPE cells transplanted as a cell suspension did not attach to the Bruch’s membrane in AMD patients, and formed cell clumps at the transplanted site instead of an organized cell monolayer (Aisenbrey et al., 2006; Lappas et al., 2000; Thumann et al., 2000). Indeed, irrespective of the cell type chosen, it has become clear that cell suspensions do not fully integrate as a functioning monolayer in both animal models and in human studies (Petrus-Reurer et al., 2017). Moreover, the inability to settle and function is compounded in a more hostile/diseased environment. Thus, transplantation of a well-differentiated cell monolayer on a substrate is necessary to maintain the appropriate orientation of transplanted cells and their contact with host photoreceptors.

An underlying substrate must satisfy a number of prerequisites in that it should support a functioning monolayer whilst being biocompatible, as well as being amenable to surgical manipulation. A number of substrates have been studied as a supportive membrane for cells to grow on, including biological substrates as well as

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synthetic degradable and non-degradable substrates which fit many of the required criteria (Da Silva et al., 2013; Goncalves et al., 2016; Krishna, Sheridan, Kent, Grierson, & Williams, 2007; Sorkio et al., 2015; Stanzel et al., 2014; Thomas et al., 2016; Thumann et al., 2009; Warnke et al., 2013; Williams et al., 2005; Xiang et al., 2014). One substrate showing promise is expanded polytetrafluoroethylene (ePTFE), a non-degradable synthetic polymer that has been widely used in many medical applications, in particular vascular graft transplantation, facial and orthopedic, due to its good biocompatibility, flexibility and biostability (Chen et al., 2019; Joo & Jang, 2016; Kannan, Salacinski, Butler, Hamilton, & Seifalian, 2005). However, unmodified ePTFE is hydrophobic and does not support cell attachment. We have demonstrated previously that gas plasma and plasma polymer surface modification to introduce amine functionality to ePTFE and other surfaces can produce a substrate that can support RPE growth and function *in vitro* (V. Kearns et al., 2012; V. R. Kearns et al., 2017; Krishna et al., 2011).

The surgical manipulation of substrates into the subretinal space is key to transplant success. The RCS rat is a classic, well-studied animal model of inherited retinal degeneration, first reported in 1938 (Bourne, Campbell, & Tansley, 1938). In this model, RPE cells are to phagocytose shed photoreceptor outer segments, resulting in the death of rod and cone photoreceptors. The RCS rat has used for studies of RPE and IPE transplantation (Coffey et al., 2002; Idelson et al., 2018; Inoue et al., 2007; Kamao et al., 2014; Schraermeyer et al., 2000) and other studies of retinal therapy (Francisca Siu Yin Wong et al., 2019; Francisca S. Y. Wong, Wong, Chan, & Lo, 2016). Transplantation of foetal neural retina, with or without its underlying RPE, into the subretinal space of RCS rats has been reported to partially restore the functions of the degenerated retina (Aramant & Seiler, 2004; Woch, et al., 2001). The procedure for transplanting artificial substrates into the subretinal space is challenging.

This study aimed to compare the *in vitro* characteristics of two strains of primary rat IPE cells cultured on surface modified ePTFE substrates and evaluate their ability to

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form a functioning monolayer. In addition we have sought to examine whether surgical approaches using Healon GV and mixtures of Healon GV:PBS to induce retinal detachment could facilitate surgical success of a subretinal transplantation of an ePTFE substrate into the RCS rat model of retinal degeneration.

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Methods

Substrate Preparation

Expanded-polytetrafluorethylene (ePTFE) substrate was commercially available as a porous (0.4µm pore size) 40 µm thick membrane sealing the bottom of a polystyrene cylinder (Millicell® culture plate insert, PICM 01250, Millipore, UK). These membranes have been subjected to a proprietary surface modification which results in a lower water contact angle than virgin ePTFE and the incorporation of oxygen functionalities onto the surface (V. R. Kearns et al., 2017). ePTFE substrates were surface modified with the pure *n*-heptylamine plasma polymer in a reactor using a frequency of 13.56MHz and pressure of 0.2 Torr, forming a 20nm thick layer to enhance the attachment of cells (Vasilev, et al., 2009). The *n*-heptylamine modified ePTFE substrate inserts were UV sterilized within 24-well tissue culture plates for 40 minutes to prevent any contamination. Some were coated with bovine fibronectin (5.31µg/ml, 1030-FN; R&D Systems, Abingdon, UK) overnight at 4°C for the further enhancement of cell attachment. The bases of the wells of the 24-well plates were treated with bovine fibronectin in the same way.

Animals

Long Evans (LE) rats were obtained from Harlan Laboratories Inc., USA. Dark Agouti (DA) rats were obtained from the Laboratory Animal Unit, LKS Faculty of Medicine, The University of Hong Kong. RCS/lav rats (described hereafter as "RCS rats") were obtained from Dr. Matthew LaVail (UCSF School of Medicine). All the animals were maintained and manipulated in accordance with the ARVO Statement for the Use of Animal in Ophthalmic and Vision Research. The use of animals conformed to the Cap. 340 Animals (Control of Experiments) Ordinance and Regulations in Hong Kong, and was approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong (CULATR 1726-08, 2222-10 and 2423-11).

3 to 4-week old LE and DA rats were used as donors of IPE cells in this experiment. 4-

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week old, dystrophic RCS rats were the recipients of subretinal transplantation of F-HA ePTFE substrates with cells.

Isolation of rat IPE cells

Isolation of IPE cells was carried out according to previous studies (Kourous A. Rezai, Leon Kohen, Peter Wiedemann, & Klaus Heimann, 1997). In brief, the eyes were immediately enucleated after rats were sacrificed and immersed in CO_2 independent medium (18045, Life Technologies, UK) before dissection. A circumferential 360-degree excision, just anterior to the ora serrata and 2mm posterior to the limbus, was performed. The iris was then excised along its root to avoid the contamination of the ciliary body. The isolated iris was immersed in 0.25% trypsin solution containing EDTA (25200, Gibco) for 15 min at 37℃. To deactivate trypsin effect, Ham's F12 Nutrient Mixture (N6760, Sigma) containing 20% fetal bovine serum (FBS, Hyclone), 1% L-glutamine, 1% antibiotics (penicillin and streptomycin, Life Technologies) and 1% Amphotericin B (Life Technologies) was added to the iris. The IPE cells were carefully and gently separated from the stroma of central iris regions by pipetting iris up and down. The isolated IPE cell suspension was centrifuged, resuspended in culture medium, and cells counted using a hemocytometer.

Cell Culture

Prior to cell seeding, the fibronectin was discarded and substrates were further incubated with 2% BSA for 60 minutes at 37°C. After that, 400µl Ham's F12 Nutrient Mixture supplemented with 20% FBS was placed inside the inserts and 600µl culture medium was placed outside the inserts. 1ml culture medium was added to the F-TCPS. Freshly isolated IPE cells from both Long Evans and Dark Agouti rats were seeded onto 4 different substrates at the optimal density of 4.2×10^4 cells/cm^2, including the bovine fibronectin coated *n*-heptylamine modified (F-HA) ePTFE substrates, *n*-heptylamine modified (HA) ePTFE substrates, unmodified ePTFE substrates and bovine fibronectin coated 24-well tissue culture plates (F-TCPS) as the

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control. Plates were then incubated at 37°C with 5% CO_2 and 95% air. For assessment of IPE cell proliferation, Ham's F12 Nutrient Mixture supplemented with 20% FBS was used as the culture medium; for other evaluations, this was replaced with a 1:1 mixture of Ham's F12 Nutrient Mixture and Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% FBS when the IPE cells became approximately 90% confluent.

Immunocytochemistry

Immunocytochemical staining for pan-cytokeratin was performed to ensure the isolated cells were pure IPE cells without contamination of other cell types. Only these cells (passage 1) were used in further experiments. Cell morphology and tight junction formation was also assessed. Details of all antibodies and concentrations are found in Table 1. The cells were fixed in 4% formaldehyde solution (PFA) for 15 min. For antibody staining, samples were permeabilized in 1% Triton X-100 (Sigma) for 5 min then blocked with 10% goat serum (Vector Laboratories Inc.) and incubated overnight at 4°C with the relevant primary antibody. Samples were were rinsed with PBS three times then incubated with the appropriate secondary antibody for 60 min at 37°C, counterstained with DAPI and observed with the inverted confocal laser scanning microscope under 20X magnification.

Assessment of cell number

At 1, 2, 4, 7, 10, 14 and 28 days after cell seeding, IPE cells were nuclear stained with 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI 1:1000; D9564; Sigma) and visualized with the fluorescent microscope under 20X magnification (TE 2000-S, Nikon, Japan). Six fields (5.7×10^5 μm^2 per field) of each substrate were chosen to photograph by a digital camera and imaging software SPOT Advanced™. Nuclei were counted in each field at different time points (18 fields were counted per substrate per time point in total). The average number of nuclei counts per field of view was calculated for different time points for different substrates and exhibited as a bar

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chart demonstrating the average number of nuclei counts per field of view + standard deviation versus time.

Evaluation of cell morphology

Prior to fixation, the morphology of rat IPE cells was observed under an inverted phase-contrast microscope (Nikon, Japan). Photomicrographs were then captured under 200X magnification by a digital camera and imaging software SPOT Advanced^{TM}. Qualitative evaluation of cell morphology was performed by cellular cytoskeletal F-actin staining (Fig. S1). Briefly, after fixation with 4% PFA, IPE cells were stained with Alexa Fluor 488 Phalloidin (5 units/ml, Invitrogen) for 30 min at 37°C followed by the nuclear staining with DAPI. The F-actin staining was visualized by inverted confocal laser scanning microscope under 20X magnification.

Transmission electron microscopy (TEM)

IPE cells cultured on F-HA ePTFE substrate were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences) in cacodylate buffer overnight at 4°C, and post-fixed with 1% osmium tetroxide (OsO_4, Electron Microscopy Sciences) in 0.1M cacodylate buffer for 30 min at room temperature. Samples were then dehydrated in a series of graded ethanol (50%, 70%, 90% and 100%) and propylene oxide, which in turn were infiltrated with 1:1 mixture of propylene oxide/resin and pure resin for 90 min respectively. The IPE cells with underlying substrates were embedded in pure resin pre-filled in the flat embedding mold. Ultrathin (90nm) sections were mounted onto copper grids, stained with 2% uranyl acetate and lead citrate subsequently and observed under an electron microscope (HT: 80kV, Philips EM208S).

Phagocytosis of photoreceptor outer segments (POS)

The following POS isolation procedures, following the protocol described in (Higgins, Wang, Dockery, Cleary, & Redmond, 2003), were carried out under dim far-red light, since the rhodopsin is light sensitive and could convert to opsin when exposing under

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the visible light. Freshly isolated bovine neural retinas were gently homogenized in 0.73M sucrose solution by mechanical agitation sonicator. After agitation for 30 seconds, the mixtures were filtered through 100µm nylon mesh and layered carefully onto the surface of a discontinuous sucrose density gradient layers containing 1.2M, 1.0M and 0.8M sucrose in phosphate buffer from bottom to top. Then they were centrifuged in P40ST swing-out rotor (Hitachi, Japan) at 60,000g (21,700rpm) at 4℃ for 60 min. The POS sample was slowly collected at the interface of 1.0M and 1.2M sucrose solution and suspended in the phosphate buffer, which in turn were spun at 27,000g (12,000rpm) at 4℃ for 20 min, 3 times to completely remove the sucrose solution. The final pellets were resuspended in 1ml phosphate buffer and stored at -80℃**.** The protein concentration of POS was assessed using Biorad DC protein assay (Bio-Rad, Hercules, CA).

At Day 28, IPE cells cultured on F-HA ePTFE substrates and control F-TCPS were challenged with 10µg/ml POS. After 3 hours and 24 hours, cells were fixed in 4% PFA and incubated with mouse monoclonal anti-rhodopsin and Alexa Fluor® 488 goat anti-mouse as described previously. Cells were incubated with trypan blue to eliminate signal from attached but not internalized POS, counterstained with DAPI and observed with the inverted confocal laser scanning microscope under 20X and 40X magnification. Five fields (1.024 x10^4 µm^2 per field) of each substrate were chosen to photograph. The fluorescent regions showing internalized POS was measured using Image J. The results were calculated as a percentage of total fluorescence after 24h phagocytosis of IPE cells grown on F-HA ePTFE substrates, which was taken as 100% and exhibited in a histogram with bars + SD at each time point.

Preparation of IPE cell sheets on F-HA ePTFE substrates for the transplantation

Primary IPE cells isolated from Long Evans rats were seeded onto F-HA ePTFE substrates and allowed to reach to a confluent cell layer for approximately 28 days. These samples were then cut into 1x2 mm size rectangles and stored in culture

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medium without FBS just before transplantation.

Subretinal transplantation

RCS rats were injected intraperitoneally with Xylazine (7mg/kg) and Ketamine (70mg/kg) for general anesthesia before surgery. The eye was further treated with 0.5% proparacaine hydrochloride (Alcaine 0.5%, Alcon) for tropical analgesia and pupil was dilated with topical 1% Mydriacyl (Alcon). Then the cornea was punctured using a 30 Gauge needle to avoid an increase of intraocular pressure in subsequent procedures. A local retinal detachment was created by either subretinal injection of a mixture of Healon GV and PBS (1:1) or PBS alone using a 30 Gauge needle in the superior temporal portion of the eye. The conjunctiva and subconjunctival tissue were then dissected to expose the surface of the sclera. A small incision was made just behind the ora serrata by penetrating the sclera, choroid and Bruch's membrane with a 20G V-Lance™ knife (Alcon). The substrate with cells was inserted into the subretinal space with fine forceps. The incision and conjunctiva were then sutured using 10-0 thread respectively. After surgery, antibiotic ointment (Tobramycin) was applied tropically and rats were kept in a humidified chamber at 37°C for recovering from anesthesia. RCS rats in sham surgery groups underwent the same surgical procedures without inserting the F-HA ePTFE substrates into the subretinal space.

Histology

One week after surgery, fundus of each RCS rat was photographed, and then RCS rats were sacrificed. The eyes were immediately enucleated, fixed in 4% PFA, embedded in paraffin, sectioned into 5-µm thick sections. Sequential sections with F-HA ePTFE substrates and corresponding sequential sections from sham-treated and non-treated RCS rats were employed for the following experiments. Four sagittal sections of each eye were randomly selected. Unstained sections were studied for the presence of the implants and sections were stained with hematoxylin and eosin (H&E) or using immunohistochemical methods.

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Immunohistochemistry

Samples were stained for pan-cytokeratin, CD 11b, and glial fibrillary acidic protein GFAP (to evaluate position, cultured cell integrity and induced gliotic inflammation of the implants. Deparaffinization and dehydration treated sections were incubated with proteinase K in 1X PBS (20μg/ml) for antigen retrieval at room temperature for 4 minutes. Samples were then stained with the relevant antibodies (Table 1) and counterstained with DAPI as described previously. They were then mounted in the fluorescent mounting medium. The immunoreactivity was assessed using a semi-quantitative immunohistochemistry scoring method, in which level 1 demonstrated the lowest immunoreactivity while level 5 represented the strongest immunoreactivity (Eclipse 80i, Nikon, Japan).

Statistical analysis

Each individual experiment was performed at least three times and in each experiment, a minimum of three technical replicates were performed. All results were exhibited as mean +/- SD. One-way analysis of variance (ANOVA) followed by Bonferroni post-tests, unpaired student's t-test or a Kruskal-Wallis test followed by Dunn's post-hoc test was applied to assess statistical significance using GraphPad Prism5 software (Graphpad Software, Inc; San Diego) where appropriate. P values <0.05 were considered as statistically significant between compared groups.

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Results

IPE culture

IPE cells grown on each substrate presented an increasing trend of number with time except on native ePTFE substrate, on which only a few cells (1 to 2 cells per field of view) attached during the experimental period (Fig. 1). The number of cells cultured

on F-HA ePTFE substrates and control F-TCPS increased dramatically and then began to reach a plateau at Day 7 (Fig. 1). The number of cells was significantly higher on F-HA ePTFE substrates than on F-TCPS for both LE and DA IPE at the time points studied (days 1-14; $p<0.001$).

IPE cultures exhibited heavy pigmentation surrounding the nuclei and cells were positively labeled with an antibody against pan-cytokeratin indicating pure IPE cells populations were obtained (Fig. S1). For both LE and DA IPE, IPE cells formed an intact cell monolayer on the F-HA ePTFE substrates and control F-TCPS at Day 28, whereas very few IPE cells attached to untreated ePTFE substrates (Fig. 1). LE IPE formed a monolayer on HA only treated ePTFE, but DA IPE did not. Attached cells on all substrates were highly pigmented with rather even distribution of round nuclei. Staining with phalloidin revealed the polygonal circumferential cytoskeleton fibers of actin in cells cultured on F-HA ePTFE substrates whilst control F-TCPS exhibited extensive noncircumferential positive staining throughout the cell. Cells cultured on native ePTFE exhibited a rounder shape when attached (Fig. 1).

At Day 28, analysis of actin distribution showed a circumferential pattern in both HA and F-HA treated substrates whilst control F-TCPS gave a more diffuse cytoplasmic appearance (Fig. 1). Immunostaining of occludin, one component of tight junction protein, was presented at cell-cell contact areas continuously over the cell monolayer on F-HA ePTFE substrates and control F-TCPS, indicating the formation of tight junction along cell borders (Fig. 2). The presence of occludin staining could only be viewed when cells became confluent but not pre-confluent and western blotting revealed no difference in the quantity of occludin between confluent samples (Fig. 2ii). To further reveal the ultrastructure of cell monolayer on different substrates and tight junctions between adjacent cells, TEM was performed on the cells cultured on F-HA ePTFE substrates. Fusion of plasma membranes of neighboring IPE cells were observed as high electron density at the apical-lateral area, suggesting the presence of tight junctions between adjacent cells (Fig. 2). Western blots analysis of CRALBP

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protein expression in IPE cells showed no statistical significant difference of CRALBP protein expression level was observed in IPE cells grown on either HA, F-HA ePTFE substrates and control F-TCPS for both strains of rat (Fig. S2)

Phagocytosis of POS

IPE cells cultured on F-HA ePTFE substrates and control F-TCPS were able to internalize POS in a time-dependent manner, increasing the number ingested significantly from 3h to 24 h ($p<0.001$; Fig. 3). LE and DA IPE significantly ingested more POS when confluent on F-HA ePTFE than on F-TCPS over 24 hours ($p<0.001$). POS internalized by cells cultured on control F-TCPS only reached 64.08%±3.6% (LE) and 67.16%±11.3% (DA) at 24h compared to those cultured on F-HA ($p<0.001$; Fig. 3).

In vivo study

Surgical procedure

Prior to the surgery, the cornea was punctured to avoid the damage of increased IOP when retinal detachment was induced. In addition, the pupil was dilated to enable more retinal areas to be clearly viewed during the surgical operation. During the surgery, a regional retinal detachment was induced using either the 100% Healon GV (Group 1) or a 1:1 mixture of Healon GV and 1X PBS (Group 2) to minimize the relevant trauma. The substrate could be transplanted into the subretinal space using fine forceps and refined glass capillary and the orientation of the substrate at transplanted site was controllabl.. After transplantation of the substrate, the incision was sutured to ensure that the substrate remained in the subretinal space.

Immediately after the surgery, retinal detachment was observed with fundus microcopy in both Group 1 and Group 2 following subretinal injection to create retinal detachment (Fig 4. A&E). However, retinal detachment was not observed after

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the first day after surgery in either group by fundus microscopy (Fig. 4B-D, F-H). During the whole experimental period, the rat eyes demonstrated clear corneas and no signs of cataract, intraocular hemorrhage or infection in either group. A drop in IOP was observed in both groups immediately following surgery which then recovered and remained relatively constant for the remaining time points studied (Fig 4.)

Post-operative histological analysis

Histological analysis of both surgical procedures showed a marked difference in retinal attachment following processing, with Group 1 treated eyes showing extensive retinal detachment in both sham and F-HA ePTFE surgery whilst Group 2 maintained retinal attachment (Fig. 5i). Similarly, histology sections from both groups revealed that the substrate was always located between RPE and neural retina. Punctures through Bruch's membrane into the choroidal space were never observed. Histological analysis showed the outer nuclear layer (ONL) thickness to be decreased at 4 weeks compared to one week ($p<0.05$) in all rats studied. No significant changes ($p>0.05$) to the thickness of ONL or the inner nuclear layer (INL) and ganglion cell layer (GCL) of the neural retina between the transplanted groups was observed at any time point (Fig. 5ii). In addition, the choroid structure also appeared normal in the transplantation region with patent capillary vessels readily observed (Fig. 5i). Further analysis of retinal response to substrate transplant by immunohistochemical staining of GFAP demonstrated activation of Müller cells and astrocytes in retinae of Group 1 treated eyes. GFAP immunoreactivity was mainly present in the ILM at 1week (Fig 6iA -C), whilst increased GFAP immunoreactivity was observed across the whole retina (D-F). Statistical analysis demonstrated a significant increase of GFAP immune-reactivity for non-treated and sham control eyes but not for F-HA ePTFE transplanted eyes at 4 weeks. The cells cultured on F-HA ePTFE substrates remained as an intact cell monolayer underneath the neural retina (Fig 6ii4D, E) and were

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further confirmed as the IPE cells using pan-cytokeratin staining (Fig 6iiE). CD11b staining, a marker of macrophages, was not found in either transplanted eyes or sham control eyes (Fig 6ii, G).

Discussion

We have demonstrated the ability of rat IPE cells to form a functioning monolayer on a surface-modified ePTFE substrate that can be successfully transplanted subretinally into a dysfunctional rat retina model. Both LE and DA IPE cultures demonstrated intact monolayer formation as evidenced by morphological and tight junction observations, as well as a functional phagocytic capacity at higher and similar levels than TCPS.

Our cell attachment and proliferation studies demonstrated that both LE and DA IPE could both be successfully isolated and cultured on TCPS whilst not adhering to untreated ePTFE. This was in alignment with previous human RPE culture studies (Krishna et al., 2011) in which the relatively hydrophobic surface chemistry of ePTFE required surface modification in order to allow cellular attachment.(V. R. Kearns et al., 2017; Krishna et al., 2011). Whilst LE IPE rat cultures have previously been cultured on TCPS and shown to demonstrate the phagocytotic ability (K. A. Rezai, A. Lappas, L. Farrokh-siar, et al., 1997) no such evidence in the literature regarding DA IPE cells has been reported previously. Interestingly, the two strains of rat IPE (LE and DA) differed considerably in our experiments in that only LE IPE could adhere sufficiently to form a functioning monolayer on HA-ePTFE substrates. DA IPE did not form a functioning monolayer on HA-ePTFE and, whilst both strains have been extensively studied in ocular research, this is the first report of any known *in vitro* functional differences with the same cell type. The unreported difference may be due to the paucity of such comparable studies. Indeed, we were only able to compare our rat IPE data to LE and not DA rats in previously published studies of IPE

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cell isolation and function *in vitro* (Jordan et al., 2002; K. A. Rezai, A. Lappas, L. Farrokh-siar, et al., 1997; Rezai, Lappas, Kohen, Wiedemann, & Heimann, 1997). The difference in strain attachment was absent with the combination of a surface of HA coated with FN, which resulted in both strains of IPE attaching and forming a monolayer within a similar time frame. This discrepancy in cellular response also demonstrates that caution must be taken when extrapolating data from similar studies utilizing differing strains of the same species. The different biological responsiveness/function of the same cell type in different strains of rat is becoming more apparent (Claassen, 1994) and, whilst not part of this investigation, it is known that sex (male or female) of the donor and/or recipient can impact on transplantation success in both ocular (Hopkinson et al., 2017) and non-ocular scenarios (Hsieh, Vaickus, & Remick, 2018).

Whilst ePTFE has previously been proposed to offer the required physical properties (rigidity, porosity) for human RPE cell monolayer formation *in vitro* (V. R. Kearns et al., 2017; Krishna et al., 2011), its ability to be transplanted into a diseased animal model was not known. We subsequently chose the RCS rat for transplantation studies as it is the most widely used rodent strain due to its defective phagocytosis of isolated rod outer segments and subsequent retinal degeneration (Edwards & Szamier, 1977). It has been extensively used as a model to assess RPE transplant (patch) (Coffey et al., 2002; Whiteley, Litchfield, Coffey, & Lund, 1996). Whilst there is no gold-standard model of subretinal transplantation method and transplantation of a substrate, the use of the rat model here presented additional challenges due to the size and anatomy of the rat globe. We utilized a transscleral approach by making a small incision just behind the ora serrata by penetrating the sclera, choroid and Bruch's membrane as far removed from the transplantation site as possible with, and without Healon. This approach was employed in the hope that minimal repair responses would occur distant from the placed substrate site. Ophthalmic viscoelastic devices, of which the first commercially available was Healon, are often used to create subretinal blebs to allow placement of cells and other devices (Ho et

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al., 2017; Jackson et al., 2003; Woo, Li, Lai, Wong, & Lo, 2013). Using a mixture of Healon GV and PBS (1:1) to induce retinal detachment, F-HA ePTFE substrates were successfully transplanted into the subretinal space of RCS rats, where the substrates remained flat beneath the neural retina up to 4 weeks. However, the procedures were not without complications, with surgical insult trauma and retinal tears frequently observed. In addition, the retinal detachment was observed pre- and post-histological processing in both surgical approaches. Additional analysis of activation of Müller cells and astrocytes in response to the surgery by GFAP expression demonstrated a significant increase of GFAP immunoreactivity in non-treated control eyes at 4 weeks. However, this is not entirely unexpected, as the RCS rat undergoes progressive photoreceptor degeneration and thus the upregulation of GFAP expression in eyes undergoing surgery may be due to the induced retinal detachment which is known to increase GFAP expression in long-standing detachments (Pastor et al., 2016). This was observed in Group 1 eyes where H&E staining revealed that the neural retina did not reattach up to 4 weeks after the surgery. However, it appears from the GFAP immunoreactivity data that the F-HA ePTFE substrates alone did not induce activation of Müller cells and astrocytes as GFAP immunoreactivity was similar to non-treated and sham control eyes at 4 weeks in both Group 1 and Group 2. In addition to the lack of significant increase of GFAP expression for F-HA ePTFE transplanted eyes at 4 weeks (compared to 1 week) also suggests no difference in activation of Müller cells and astrocytes.

Whilst the lack of CD11 expression (macrophage) indicates the substrates are well tolerated in the subretinal space of RCS rats, the technical challenge of optimization of injection vehicles for cell-substrate transplantation still needs to be addressed in both small and large-eyed globes (Tian et al., 2019). In keeping with the 3Rs principle involving animal research, we decided additional further animal experimentation in the small eye rodent model would not be performed. Future improvements in surgical techniques and instrumentation are required in larger globed rabbit and

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porcine models to develop more effective transplant procedures more akin to the human scenario (Brant Fernandes et al., 2016; Kamao et al., 2017).

In conclusion, different biological responses were observed between different inbred strains of rat but ultimately both could form a functioning monolayer on a HA-FN modified substrate. In addition, this substrate was shown to be amenable to subretinal transplantation into a retinal degenerative model and has the potential for use in RPE replacement surgery.

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Statement for the work contribution of each author

CS and AL are the principal investigators of the project involved in project design, data analysis and, manuscript writing. NS was involved in all cell culture and data acquisition. DW and WL were involved in in vivo surgery planning and execution. VK provided support in cell cultures, histological assessment, data analysis and manuscript preparation. RW provided support in data analysis and alongside AB and KV were involved in substrate modification and preparation. All authors contributed to critical revision and approval of the manuscript.

Conflict of Interests

None

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Ethical approval

All animals were treated according to the national and international rules of animal welfare, including the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The use of animals conformed to the Cap. 340 Animals (Control of Experiments) Ordinance and Regulations in Hong Kong, and was approved by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong (CULATR 1726-08, 2222-10 and 2423-11).

Availability of Datasets

The datasets generated and/or analyzed during the current study will be made available in University of Liverpool open access data repository.

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Table 1 - Details and dilutions of antibodies used in this study

Antibody	**Antibody details; supplier**	**Dilution**
pan-cytokeratin, Clone C-11	C2931; Sigma	1:100
anti-occludin	71-1500; Invitrogen	1:100
anti-rhodopsin	ab5417; Abcam	1:500
Alexa Fluor® 488 goat anti-mouse	A-11001; Invitrogen	1:200
Alexa Fluor® 568 goat anti-rabbit	A-11011; Invitrogen	1:200
CD 11b	ab1211; Abcam	1:100
Anti- Glial fibrillary acidic protein (GFAP)	Z0334; Dako	1:500

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Figure 1. Phase contrast and fluorescent micrographs demonstrating the morphology of IPE cells cultured on different substrates at Day 28. Under phase-contrast microscope, cells cultured on control F-TCPS and F-HA substrates exhibited a monolayer of cells with heavy pigmentation distributed around nuclei, while cells were barely observed on unmodified ePTFE substrates. LE IPE but not DA IPE formed a monolayer on HA only treated ePTFE. Cytoskeleton F-actin staining (green) revealed the polygonal or cuboidal morphology of cells cultured on control F-TCPS and F-HA substrates, however, cells grown on ePTFE substrates were round and small. Cells were counterstained with DAPI (blue). Scale bars, 50µm. Histograms demonstrating the mean number of IPE cells per field of view ($5.7x10^5 µm^2$) at different time points on control F-TCPS, F-HA ePTFE, HA ePTFE and unmodified ePTFE substrates (G). *** P<0.001, one-way ANOVA followed by Bonferroni's Multiple Comparison. Error bars: +1 SD, n=3.

Figure 2. (i) Photomicrographs of occludin staining of LE and DA IPE cells cultured on control F-TCPS (A) and F-HA ePTFE (B) substrates at Day 28. Occludin staining was apparent along the cell borders, forming the mosaic appearance. Cells were counter stained with DAPI (blue). Ultrastructural SEM morphology of IPE cells cultured on F-HA ePTFE (C) substrates at Day 28. The tight junction was observed as the merge of cell membranes (arrows) between the adjacent cells cultured on F-HA ePTFE substrates. Scale bar, 8µm IF; 100nm TEM;) **(ii)** Western blots of occludin protein expression in IPE cells with their corresponding densitometry analysis directly below. In Long Evans (LE) rats, no significant difference of occludin protein expression level was observed in IPE cells grown on HA, F-HA ePTFE substrates and control F-TCPS. Dark Agouti (DA) rats, expression of occludin protein in IPE cells cultured on F-HA ePTFE substrates was not significantly different from that on control F-TCPS. One-way ANOVA followed by Bonferroni's Multiple Comparison Test (Long Evans rats), unpaired student's t-test (Dark Agouti rats). n=6. Error bars +/- 1 SD.

Figure 3. Phagocytosis capacity of IPE cells harvested from LE and DA rats on control F-TCPS, F-HA ePTFE substrates and HA ePTFE substrates. Immunocytochemical staining with rhodopsin (green) represented the internalized POS after (A-C; G-H) 3h and (D-F; I-K) 24h incubation with cells. At higher magnification, the distribution of internalized POS was around nuclei stained with DAPI (blue). Histograms demonstrated the relative phagocytosis capacity, which was presented as the ratio to total fluorescence after 24h phagocytosis of IPE cells grown on F-HA and HA ePTFE substrates compared to TCPS. *P<0.05, *** P<0.001, one-way ANOVA followed by Bonferroni's Multiple Comparison Test. n=4. Error bars: + 1 SD. All figures sized to reflect to scale bars (F 20µm, inset 3µm).

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Figure 4. Representative fundus photos of rat eyes immediately after surgery, and 1, 7, 28 days after surgery. Retinal detachment (arrows) was observed in both group 1 (100% Healon GV; A-D) and 2 (50% Healon and 50% PBS; E-H) immediately after surgery (A,E). Rat eyes demonstrated clear cornea with retinal detachment, cataract, inflammation, intraocular hemorrhage was present after 1, 7 and 28 days (B-D, F-H). Line graphs demonstrate the IOP value in both groups over the same time course. IOP of non-treated control eyes were maintained at approximately 10 mmHg in the period studied. IOP of sham control and F-HA ePTFE transplanted eyes also remained at around 10 mmHg except the decrease to 6-7 mmHg immediately after the surgery. n=6. Error bars: + 1 SD.

Figure 5 (i). Photomicrographs of H&E stained retinal sections at 7 and 28 days post surgery following Sham or HA-implant surgery with 100% Healon (Group 1) and 50% Healon (Group 2) compared to un-operated controls (A,B, G,H). Retinal detachments were histologically observed in the eyes when retinal detachment was induced using 100% Healon (Group 1 E, F, K, L) at 7 and 28 days, whilst the 50% Healon/PBS injected eyes (group 2E, F, K, L) demonstrated neural retina adherence to the underlying RPE at 7 days. In both groups, F-HA ePTFE substrates (asterisk) were found flat in the subretinal space and in some cases cells (arrows) were attached to the surface of the F-HA substrate. *labels substrate, scale bars: 50µm.
(ii). Bar graphs representing retinal structure histological measurements of GCL, INL and ONL at 1 and 4 weeks for control, sham and F-HA operated RCS rat eyes. No significant difference was observed for any retinal measurements between the groups. ONL was shown to decrease from week one to four significantly ($p<0.05$) across all three groups but that difference was not significantly different between these groups ($p>0.05$). Data mean =1 SD.

Figure 6(i). Immunohistochemical staining of GFAP illustrating activation of Müller cells and astrocytes in retinae of group 1&2 (A-F). (A -C) GFAP immunoreactivity was mainly present in the ILM at 1 week. (D-F) While increased GFAP immunoreactivity was observed across the whole retina for both groups. (Group I and II G,H) Statistical analysis demonstrated a significant increase of GFAP immunoreactivity for non-treated and sham control eyes but not for F-HA ePTFE transplanted eyes at 4 weeks for both groups. **P<0.05, ** P<0.01*, Kruskal-Wallis test followed by Dunn's post-test. n=4-6. Error bars: SD. Scale bar: 50µm. **(ii).** Representative fundus photo of rat eyes 7 days after surgery, presenting the pigmented transplanted IPE cells (arrows, A). F-HA ePTFE substrates with cultured cells (arrowheads) after subretinal transplantation (B-D). In unstained sections, the substrate (*) was exhibited as a brown, flat membrane without folding (B). In H&E stained sections, substrates were found flat underneath the neural retina (C, D). No obvious reduction of ONL, INL and GCL thickness was observed. The cells cultured on F-HA ePTFE substrates remained as a cell monolayer (D, E) and further were identified as the IPE cells by pan-

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cytokeration staining (E). Immunohistochemical staining of CD11b illustrating host response (F, G). No immune response or inflammation was observed at the transplanted site in both transplanted eyes (G) and sham control eyes (F). (ONL- Outer nuclear layer; INL- Inner nuclear layer; GCL- Ganglion cell layer) Scale bar A:500µm; B, C, D: 50µm; E, F, G: 20µm.

Figure S1. Phase contrast and Immunoflourecence (IF) images demonstrating the morphology of cultured rat IPE cells and cytokeratin positive primary cultures. IPE cells isolated from DA and LE rats became confluent and dense pigmentation was observed in the cells. Cytokeratin staining of cultured rat IPE cells. The cells were positively stained with pan-cytokeratin (green), indicating they were IPE cells. The cells were counterstained with DAPI (blue). Scale bars, 50µm.

Figure S2: Western blots analysis of CRALBP protein expression in IPE cells. The higher panel are the western blots whilst the lower panel are the corresponding densitometry values. (A) In Long Evans rats, no significant difference of CRALBP protein expression level was observed in IPE cells grown on HA, F-HA ePTFE substrates and control F-TCPS. (B) In Dark Agouti rats, expression of CRALBP protein in IPE cells cultured on F-HA ePTFE substrates was not significantly different from that on control F-TCPS. One-way ANOVA followed by Bonferroni's Multiple Comparison Test (Long Evans rats), unpaired student's t-test (Dark Agouti rats). n=6. Error bars: SD

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