Expanded Polytetrafluoroethylene as a Substrate for Retinal Pigment Epithelial Cell Growth and Transplantation in Age-related Macular Degeneration.

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Abstract

Background: Retinal pigment epithelial (RPE) transplantation presents a potential treatment of Age-related Macular Degeneration (AMD). A suitable transplant membrane that can support an intact functioning RPE monolayer is required. Expanded polytetrafluoroethylene (ePTFE) possesses the physical properties required for a transplanting device, however, cells do not attach and spread on ePTFE. This study investigated the ability of surface modified ePTFE to optimise the growth and function of healthy RPE monolayers.

Methods: ePTFE discs were modified by ammonia gas plasma treatment. ARPE-19 cells were seeded on the membranes and maintained in media supplemented with retinoic acid and reduced serum. Cell number, morphology and proliferation were analysed. RPE monolayer function was investigated through formation of cell-cell junctions and phagocytosis of photoreceptor outer segments (POS).

Results: Ammonia gas plasma treatment resulted in enhanced cell growth, good monolayer formation with evidence of cell-cell junctional proteins. Furthermore, RPE monolayers were able to phagocytose POS in a time-dependent manner.

Conclusions: ePTFE can be surface modified to support an intact functional monolayer of healthy RPE cells with normal morphology and the ability to perform RPE-specific functions. With further investigation ePTFE may be considered for use in transplantation.

Introduction

Replacing retinal pigment epithelial cells under the failing macula represents a potential treatment for age-related macular degeneration.[1-4] For transplants to be successful they

will need to be introduced under the macula as an intact functioning monolayer as delivery of isolated cells can cause serious complications including PVR.[5] A range of biological substrates and degradable synthetic substrates have been investigated but are of limited use due to lack of reproducibility of the biological substrates or the potential to leave cells exposed to underlying damaged Bruch's membrane following degradation, as well as the unknown fate of the degradation products.[4;6-8]

The aim in this study was to identify if expanded polytetrafluoroethylene (ePTFE) is a suitable biostable substrate and address the options to enhance its properties required for transplantation. ePTFE is a fluoropolymer, which has been used in a range of surgical devices with well-established biocompatibility, biostability, porosity and flexibility.[9;10] ePTFE 'as received,' however, is hydrophobic and does not promote cell adhesion. Previous studies have shown that surface modification can enhance cell attachment.[9;11] The present study investigated how surface modification of ePFTE using an ammonia gas plasma treatment enhanced the growth of RPE cell monolayers in the presence of retinoic acid and reduced serum levels and furthermore preserved their differentiation and functional characteristics.

Materials and Methods

Substrate and plasma treatment

Samples 3mm in diameter were punched from a 100μm thick ePTFE sheet and cleaned in 100% ethanol. A scanning electron micrograph of the ePTFE is presented in Figure 1. Untreated samples were placed into sterile distilled water and stored until cell culture experiments. The remaining discs were surface modified by ammonia gas (100% purity; BOC Ltd, Surrey, UK) plasma treatment, following the procedure previously reported.[12;13] Following treatment, the samples were placed directly into sterile

distilled water for 24 hours prior to use in cell culture. No change in the surface topography was observed after plasma treated under scanning electron microscopy. All samples were UV sterilised (CL-1000 U.V. Crosslinker; UVP, Cambridge, UK) for 3 minutes immediately before use in cell culture.

Cell culture

ARPE-19 cells, an established but non-immortalised human RPE cell line (American Type Culture Collection, Manassas, VA, USA), were seeded on ammonia gas plasma treated ePTFE samples and controls (tissue culture polystyrene; TCPS) at a density of 1500 cells/mm^2. The samples were maintained in a 1:1 (vol/vol) mixture of DMEM/F12 (Sigma-Aldrich Ltd, Ayrshire, UK) containing 2mM L-glutamine (Gibco, Paisley, UK), 50U/ml penicillin G, 50µg/ml streptomycin (Gibco), 2.5ug/ml amphotericin B (Gibco), and supplemented with 10% FBS (Gibco) for the first 4 days and incubated at 37^oC with 5% CO_2. From day 4 cells were grown in culture media with 2.5% serum levels supplemented with 10µM all-trans retinoic acid (RA) (Sigma), as described in previous studies.[14-16] At 1, 4, 7, 10 and 15 day time points the samples were fixed in 10% neutral buffered formalin. Primary human RPE cells (hRPE) were isolated and expanded as described in Kent et al., 2003[17] and seeded at a density of 800 cells/mm^2 (cells were used from 2 donor eyes and were passage number 5-12). Samples were maintained F10 medium (Sigma) containing 2mM L-glutamine, 50U/ml penicillin G, 50µg/ml streptomycin, 2.5ug/ml amphotericin B, and supplemented with 20% FBS. At day 2, FBS was reduced to 5% and medium was supplemented with 10µM RA. Samples were fixed at 7, 14, 21 and 28d.

Quantification of cell number (nuclear counts)

ARPE-19 cells were nuclear stained with 10µg/ml propidium iodide (Molecular Probes, UK) and visualised using fluorescence microscopy. Five fields were photographed per

substrate (three replicate substrates per time period). The mean number of nuclei per field of view ($2.76x10^{-2}$ mm^2) was calculated for each time interval for each treatment and control.

Qualitative assessment of cellular cytoskeletal morphology

Fixed samples were stained with 5U/ml Alexa Fluor 488 phalloidin (Molecular Probes) F-actin stain and visualised by fluorescence microscopy.

Identification of proliferating cells

At Day 4, 7 and 15 ARPE-19 cells grown on treated ePTFE and control TCPS surfaces were incubated with a 10μM bromodeoxyuridine (BrdU; Sigma) solution in serum-free media for 2 hours at 37°C. Samples were fixed and stained with 10μg/ml murine monoclonal anti-BrdU Alexa Fluor® 488 conjugate antibody (Molecular Probes). Mouse IgG_1-(FITC) 10μg/ml was used as a negative control. Samples were counterstained with 10μg/ml propidium iodide and visualised using fluorescence microscopy. The nuclei of proliferating cells were counted and expressed as a percentage of the total number of cells on the substrate.

Immunohistochemistry for cell-cell junctions

Cell-cell contact and monolayer formation were evaluated by staining for protein components of tight junctions (ZO-1 and occludin), for adherens junctional proteins (cadherins), and for gap junctional protein connexin 43. Treated substrates and control TCPS were fixed and incubated with 10μg/ml murine monoclonal anti-ZO-1-(FITC) (Invitrogen, Paisley, UK). Mouse IgG-(FITC) (Dako) 10μg/ml was used as a negative control. Duplicate samples were incubated with 10 μg/ml rabbit anti-ZO-1, 10μg/ml

rabbit anti-pancadherin (Invitrogen), 2μg/ml rabbit anti-occludin (Invitrogen) or 2.5μg/ml rabbit anti-connexin 43 (Sigma) antibodies for 1 hour RT post blocking for 30 minutes with 10% goat serum (Dako). Rabbit IgG (Dako) was used as a negative control.. After 1 hour the samples were rinsed and incubated with 20μg/ml secondary antibody Alexa Fluor 488 or 594 goat anti-rabbit. All samples were visualised using fluorescence microscopy. At least 3 replicate experiments were performed with 6 samples in each experiment.

Phagocytosis of photoreceptor outer segments (POS)

The methods used to harvest and label the POS were adapted from procedures previously described.[18;19] ARPE-19 were seeded onto the treated ePTFE and control substrates and incubated as above. All samples were treated with 10μM RA in culture media with 5% FBS at Day 4. At Day 7, when cells were known to have reached confluence, each of the samples was rinsed with fresh media and challenged with 10μg/ml of POS and then incubated for 3 hours and 24 hours. At each time-point, samples were rinsed with PBS, mounted in glycerol at pH 9 and immediately visualised using fluorescence microscopy. Ten fields of view were photographed per substrate per time interval and the number of attached (which fluoresced red at the external pH 9) and internalised POS (which fluoresced green at an acidic pH inside phagosomes) were counted. The mean number of attached and internalised POS per field of view ($2.76x10^{-2}$ mm^2) were recorded for ePTFE and control for each time-point.

Results

Cell number and morphology

Both ARPE-19 and hRPE demonstrated well-defined circumferential actin staining (Figure 2) with homogenous cell coverage of the ePTFE surface and close cell packing. The number of ARPE-19 cells (Figure 3) attached to the treated ePTFE was lower than that attached to the control TCPS at each time point but the number was increasing on both surfaces. Very few ARPE-19 cells were counted on untreated ePTFE at all time points.

Identification of proliferating cells

At Day 4 the bright green BrdU staining clearly highlighted a high proportion of nuclei, from the total number of nuclei, on both ePTFE and control TCPS surfaces (68.4% and 71.6%, respectively) (Table 1). At Day 7, however, only a very small percentage of nuclei took up the BrdU stain from the total (0.7% and 1.3%, respectively). By Day 15 the mean percentage of BrdU labelled nuclei further dropped (0.5% and 0.6%, respectively). The staining, however, could only be demonstrated in a small area of cell overgrowth on both surfaces at Day 7 and 15 and was not present in all other areas of the substrate and control, where a homogenous coverage of the surfaces was observed. Overall, the number of proliferating cells noted on ePTFE was lower than that seen on control ($p=0.047$) (Table 1).

Immunohistochemistry for cell-cell junctions:

Presence of tight junctional protein components, ZO-1 and occludin, were demonstrated at Day 7 by ARPE-19, on ePTFE and control TCPS (Figures 4a and 4b) at cell-cell borders rather than perinuclear. Furthermore, their staining could only be shown in confluent monolayers and not in pre-confluent cells before Day 7. Circumferential pancadherin staining was evident on both surfaces (Figures 4c). The gap junctional protein, connexin 43, was also extensively observed by the bright punctate

immunofluorescent staining at cell-cell contacts over the whole surface area on both ePTFE and controls. hRPE exhibited similar trends, although junctional protein components were not observed until later time points. ZO-1 was observed only in small patches at Day 7, but was visible over the whole surface area at 14 and 28 days (Figure 4d). Similar results were obtained when cells were stained for occludin, pancadherin and connexin 43.

Phagocytosis of photoreceptor outer segments (POS)

Cells attached to the ePTFE were able to phagocytose the fluorescently labelled POS in a time-dependent fashion and in a similar manner to the cells on the control TCPS. At 3 hours a mean of 61 (±6.0) POS were attached to the cell membranes and a mean of 12.7 (±8.9) POS were internalised on the ePTFE whereas 65.4 (±9.2) were attached to the cells adhered to the control and 21.2 (±3.6) were internalised (Figure 5). By 24 hours the mean attached and internalised POS were very similar for both ePTFE and the control. There was a significant difference for both attached and internalised POS between 3h and 24h ($p<0.001$) for the 2 surfaces together. However, there was no statistically significant difference found between control and ePTFE.

Discussion

AMD is a major problem with between 20-25 million people affected worldwide and the figure is expected to triple in the next 40 years with the growing ageing population.[20] For RPE transplantation to be a clinical possibility it is essential to provide a membrane on which to grow the RPE cells that will provide a replacement for the natural Bruch's membrane and maintain the health and functionality of the transplanted cells for the patient's lifetime. The approach was to investigate ePTFE as a biostable synthetic membrane that would satisfy the prerequisites of a suitable

transplanting device, and support growth of a healthy intact functioning monolayer of RPE cells.

Previous studies have shown ammonia gas plasma treatments followed by a post-treatment storage in distilled water results in a defluorination of the fluoropolymer surface and the incorporation of surface polar groups.[12;13] This increase in hydrophilicity promotes cell attachment and growth.

Once a monolayer of cells has formed it is important to arrest cells in their differentiated state (G_0 phase). Retinoic acid (RA) performs this function in the eye and is known to promote RPE differentiation during development.[15] A differentiated monolayer was achieved on ePTFE in the presence of RA with homogeneous coverage of the substrate surface. Growth arrest and evidence that a differentiated monolayer will remain is vital, prior to implantation into the eye, in order to ensure there is less risk of developing complications such as PVR or neoplasia. This was further confirmed by the very low percentage uptake of BrdU staining, which was used as a marker of cell proliferation, by the RPE cells grown on ePTFE at the later time-points. Furthermore, culture of RPE cells in reduced serum levels would probably give a realistic indication of their survival and maintenance as a monolayer if implanted into the eye, especially in patients with geographic atrophy and associated atrophy of the choriocapillaris.

The ability of RPE cells to polarise and form a monolayer is dependent on the formation of junctional complexes typical for an epithelial cell phenotype. The formation of tight junctions (of which ZO-1 and occludin are protein components) depends on the integrity of the adherens junctions (which contain cadherins).[14;21] Good cell-cell

junctional staining was exhibited by both cell types for ZO-1, occludin, pancadherins and gap junctional protein connexin 43 on treated ePTFE. Formation of functional junctional complexes is important for effective barrier function, which is an essential function of RPE cells *in vivo*.

Failure of RPE to phagocytose POS has been well documented to cause retinal degenerations, including AMD where the macula is particularly predisposed because of its high photoreceptor density.[22-24]Hence many studies have targeted their investigation into factors controlling POS attachment and internalisation as a measurement of phagocytosis.[25;26] Cells grown on ePTFE substrates were able to phagocytose POS in time dependent manner similar to that of cells on the control TCPS. Findings from this study, however, were consistent with those reported in earlier studies showing large numbers of isolated POS phagocytosed by confluent RPE cell cultures *in vitro.*[22] Previous studies have used RPE cells grown on control TCPS as a comparator in phagocytosis assays [18;19] and thus were used as controls for the present study. Phagocytosis of POS has also been demonstrated by IPE cells, although at a lower rate to that of RPE cells.[19;27] This is important when considering which would be the appropriate cell type to eventually transplant. It would be essential to ascertain whether the candidate cell types such as genetically modified RPE cell-lines, primary RPE or IPE, or RPE derived from stem cells, would still be able to phagocytose POS and maintain visual function and how the subretinal environment may influence this *in vivo*.[4;28]

In summary, this study demonstrated that the surface properties of ePTFE could be manipulated by gas plasma treatment to support the growth of an intact functional monolayer of RPE cells. Furthermore, by controlling the cell culture conditions in

terms of addition of differentiating agents and serum reduction, it was possible to optimise and maintain a monolayer of well spread cells displaying evidence of intercellular tight junctions. The cells grown on gas plasma modified ePTFE were able to phagocytose POS in a time dependent manner, consistent with those reported in previous studies, demonstrating an essential function of RPE cells *in vivo*. The advantages of using a biostable membrane rather than a synthetic biodegradable or tissue-based material will be the long term support and an effective disease-free basement membrane for the transplanted cell monolayer. Thinner ePTFE membranes are available and will be required for future *in vivo* studies.

Although it is recognised that ARPE-19 cells in their present state would not be used for subretinal transplantation, results from this well-characterised cell-line and the observation of similar trends in terms of cell morphology and cell-cell junction formation using primary human RPE provide compelling evidence that ePTFE may be considered as the membrane of choice to proceed with future in vivo experiments.

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	Day 4	**Day 7**	**Day 15**
Control	71.6 ±3.3	1.3 ±1.6	0.6 ±1.1
Treated ePTFE	68.0 ±6.1	0.7 ±1.4	0.5 ±0.8

Table 1: The mean percentage of ARPE-19 cells undergoing cell division on control TCPS and ammonia gas plasma treated ePTFE surfaces. Proliferating cells were highlighted by the uptake of BrdU nuclear staining and were expressed as a percentage of the total nuclear counts per field of view on control and treated ePTFE. (n=6 for control and ePTFE). *Control vs ePTFE p=0.047; Friedman*.

Figure Legends:

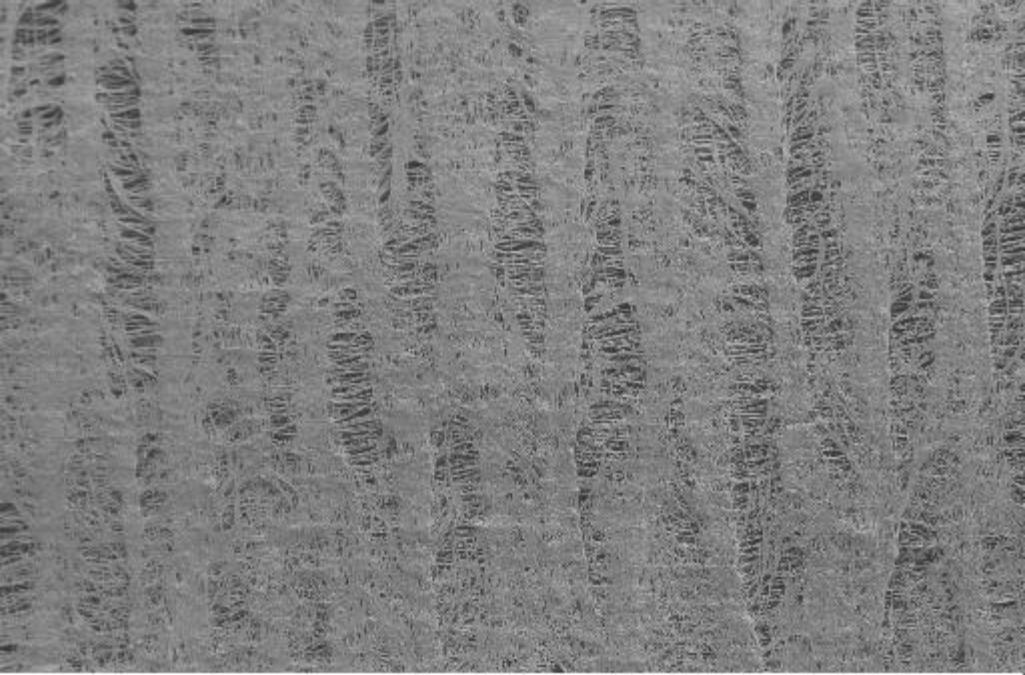
Fig 1: Representative scanning electron micrograph of ePFTE.

Fig 2: Representative photomicrographs displaying the cell cytoskeletal morphology of (a) ARPE-19 cells at Day 15 in the presence of 10μM RA and 2.5% FBS and (b) hRPE cells at Day 28 in the presence of 10μM RA and 5% FBS on the surface of ammonia gas plasma treated ePTFE showing good circumferential actin staining and a confluent cell monolayer . Scale bar: 20μm.

Fig 3: Histogram showing the mean cell number of ARPE-19 (nuclear counts) per field with increasing number of days on control TCPS and ammonia gas plasma treated ePTFE in the presence of 10mM RA and 2.5% serum. Bars: +1SD.

Fig 4: Cell-cell junctional staining of ARPE-19 cells at Day 7 grown on ammonia gas plasma treated ePTFE. Tight junctional protein components, ZO-1 (a) occludin (b), adherens junctional proteins (pancadherin) c) were clearly evident at cell borders. (d) hRPE cells exhibited circumferential ZO-1 staining at Day 28. Scale bar: 20μm.

Fig 5: Histogram demonstrating mean number of POS per field of view attached and internalised by ARPE-19 cells grown on ePTFE and control TCPS at Day 7 over 3h and 24h post POS challenge. Bars: 1SD. *Attached on ePTFE vs attached on control $p=0.47$; internalised on ePTFE vs internalised on control $p=0.42$; total attached at 3h vs total attached at 24h $p<0.001$; total internalised at 3h vs total internalised at 24h $p<0.001$; Multivariate 2-way ANOVA with univariate comparisons which included Bonferroni correction*. (n=4 for both ePTFE and control).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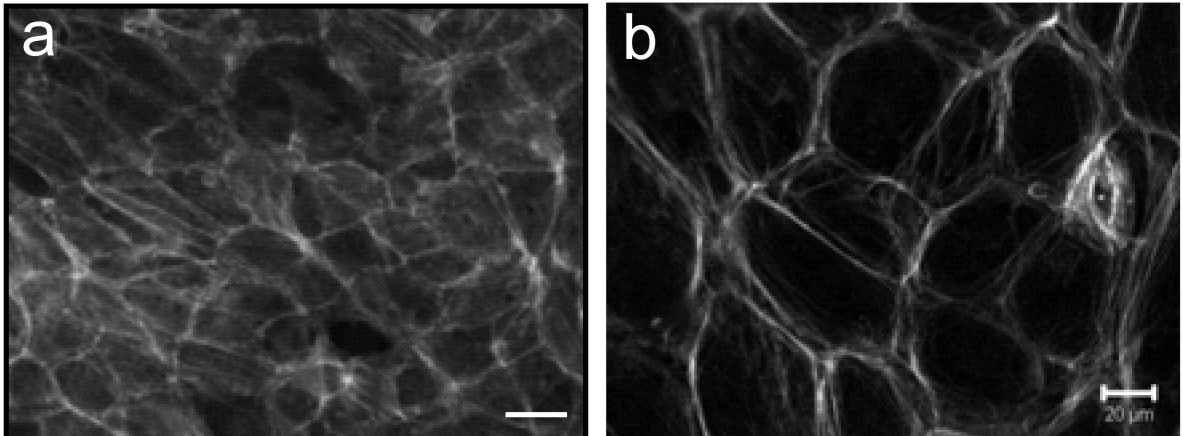
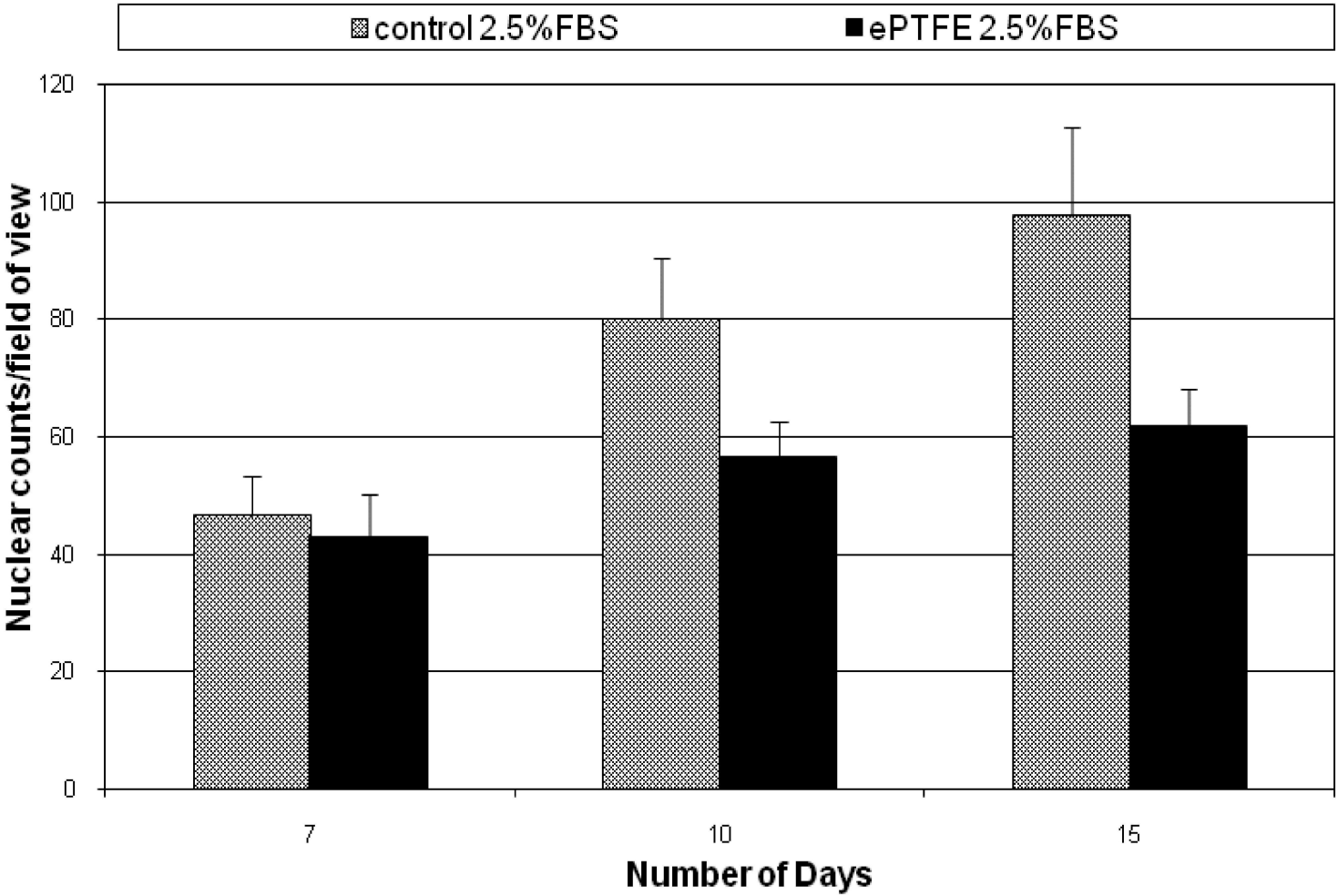


Figure 2


control 2.5%FBS
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100
80
60
40
20
0
Nuclear counts/field of view
7
10
15
Number of Days

Figure 3

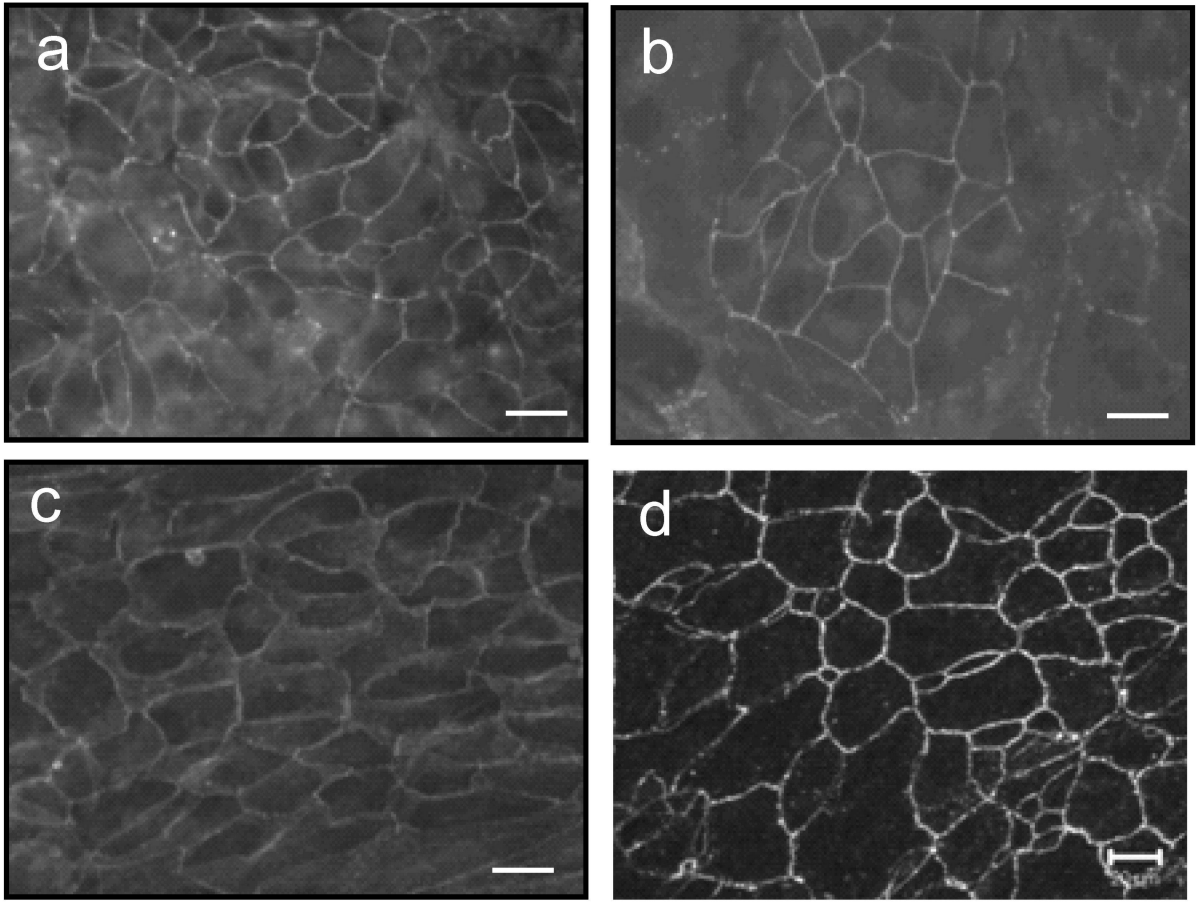

Figure 4

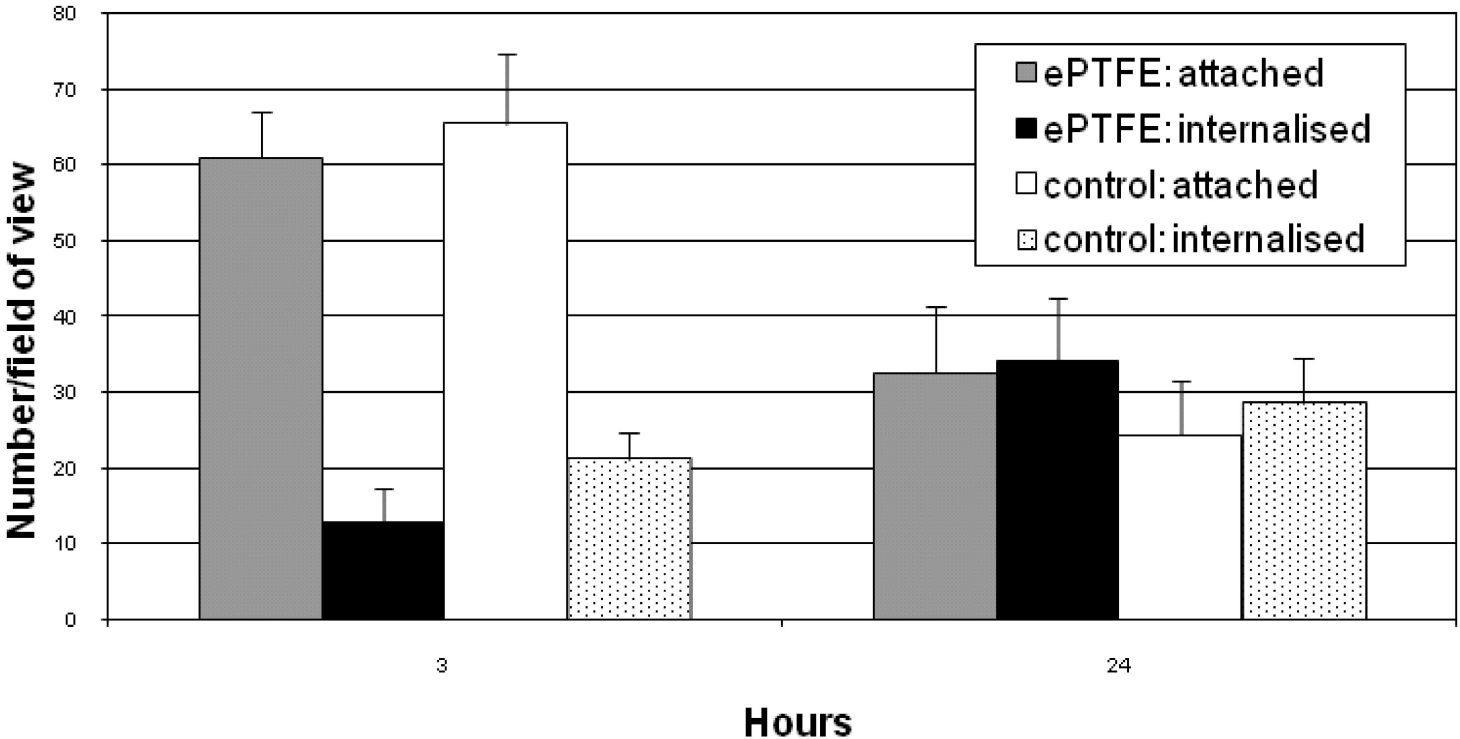
80
70
60
50
40
30
20
10
0
Number/field of view
ePTFE: attached
ePTFE: internalised
control: attached
control: internalised
3
24
Hours

Figure 5