YIGSR sequence contained elastin like recombinamer for sustaining retinal pigment epithelium as a possible tool for retinal regenerative medicine

Girish K. Srivastava [1, 3], Laura Martin [2], David Rodríguez-Crespo [1], Amar K. Singh [1], Manuel J. Gayoso [1], José C. Rodríguez-Cabello [2], José C. Pastor [1,3]

1. Instituto Universitario de Oftalmobiología Aplicada, Universidad de Valladolid, 47011 Valladolid, Spain.
2. BIOFORGE Group, Universidad de Valladolid, CIBER-BBN, 47011 Valladolid, Spain.
3. Centro en Red de Medicina Regenerativa y Terapia Celular de Castilla y León, 47011 Valladolid, Spain.

Abstract

Retinal pigment epithelial (RPE) cell replacement therapy is promising for AMD patients. However, it needs a suitable surface to sustain RPE characteristics. Constructed elastin like recombinamer containing YIGSR sequence (ELR-YIGSR), using solvent-casting onto glass cover slips followed by cross-linking, was evaluated along with TCP polystyrene (control) for ARPE19 cells sustainability (cell adhesion, viability, proliferation, epithelial characteristics and cellular junctions) at 4, 24, 72 and 120 hours. Cell viability was over 90% at 72 and 120 hours. Numbers of cells adhered (42.29±24.24%) at 4 hours and grew at 4, 24, 72, and 120 hours on ELR-YIGSR surface was significantly lower (25±16, 63.5±25, 420±60 and 662±45) than that of control TCP polystyrene (49.5±25.21, 190.5±57.27, 768±67.81 and 810.3±145.2). Microscopic results confirmed epithelial characteristics (hexagonal morphology, Ecad and ZO-1protein expressions, and tight and gap junctions). Thus, it confirmed ELR-YIGSR surface sustained RPE cell characteristics. However, this needs subsequent studies with fresh human RPE cells to conclude its application in transplantation.

Keywords

ELR, YIGSR, RPE, AMD, Biomaterial

Corresponding Author

Girish K. Srivastava, Ph.D., Instituto de Oftalmobiología Aplicada (IOBA), Universidad de Valladolid, Campus Miguel Delibes, Paseo de Belén, 17, 47011 Valladolid, Spain. E-mail: girish@ioba.med.uva.es

Publication

Received: 20042018
Accepted: 30062018
Article in Press:01062018
Published Online:15062018

Citation
Allbiosolution (2018): A0100005
Allbios (2018): A0100005

Introduction

Age-related macular degeneration (AMD) is a multifactorial disease which produces, in a high percentage of cases, irreversible vision impairment and posterior legal blindness in old population over 65 years. Basically there are two pathological forms of this disease: wet and dry. Wet AMD has at least palliative treatments such as anti-vascular endothelial growth factors (anti-VEGF) intravitreal injections paradigm. However, dry AMD does not have any treatment up to date, even palliative although 60-80% cases of the AMD patients belong to this form. Pathogenesis of the AMD is not well understood yet due to complex nature of interactions of metabolic, functional, genetic and environment factors that produce this disease. Nevertheless there are many studies which have concluded that in initial stage of the AMD, alterations are limited to external layer of retina known as Retinal Pigment Epithelium (RPE), then in next stage neuroretina is affected (Bhutto and Lutty, 2012; Khan et al., 2014; Nowak, 2006; Yonekawa and Kim, 2014; Zarbin et al., 2014).

RPE, its basal membrane and Bruch's membrane (BrM) are crucial for sustaining retina normal functions. Among others, prominent is that they act as a regulatory barrier between blood vessels and retinal layer including photoreceptors for nutrient supply and metabolic waste elimination. Dysfunction of RPE could cause waste products deposits underneath of RPE forming drusen. Drusen formations are typical signs of the AMD. It probably contributes to BrM damage. BrM supports RPE to sustain its health, polarity and functionality having also an important role in so named blood-retinal external barrier (Bhutto and Lutty, 2012; Nita et al., 2014). Thus one of ideal approaches for treating AMD patients is to replace damaged RPE and BrM.

Several approaches have been explored up to now for repairing damage of RPE. Macular relocation, patch grafting, fresh RPE cells injection and many others have proved a concept that healthy RPE can rescue retina including photoreceptors and thus, can prevent visual loss (Alonso-Alonso and Srivastava, 2015; Binder et al., 2007). Nevertheless, RPE cells have been shown to gain adult cells to mesenchymal transition property if they lose their contact to BrM. They can migrate into vitreous cavity and differentiate other type of cells including fibroblast to produce proliferative vitreoretinopathy (PVR) (Chen et al., 2012). However it has been also demonstrated that ex vivo RPE cell proliferation leads to rejuvenate these cells from aging influence (Pfeffer and Philp, 2014). Hence, one of best approach could be to grow ex vivo proliferated RPE cells on an extracellular matrix (ECM). This ECM must be able to deliver physiological signals for proper cell adherence and proliferation, and sustaining adult RPE cell characteristics and functions. The ECM could act as RPE cell carrier minimizing surgical and immune complications and, if requires it could able to integrate at location of damaged BrM.

Elastin-like recombinamers (ELRs) are artificial protein sequences which have shown excellent results due to their specific properties including biocompatibility, mechanical properties and bioactivity (Arias et al., 2014; Girotti et al., 2011; Rodríguez-Cabello et al., 2011). ELR containing RGD (ELR-RGD) and REDV (ELR-REDV) sequences have been used in many tissue engineering and other biomaterial applications including drug delivery, culture surfaces, and in engineering of tissues like cartilage, bone, eye, liver, oral mucosa and corneal stroma substitute (Arias et al., 2014; Girotti et al., 2011; Rodríguez-Cabello et al., 2011). Even our previous reports have

shown suitability of ELR as a substratum to culture cells from ocular surface and ELR-RGD for ARPE19 cells and human RPE cell growth (Martínez-Osorio et al., 2009; Singh et al., 2014; Srivastava et al., 2011).

YIGSR (Y=L-tyrosin; I=L-isoleucine, G=glysin, S=L-serin and R=L-arginin) is a sequence that has been shown to enhance cell binding, proliferation and spreading of various cell types especially endothelial and epithelial cells as well as fibroblasts (Kilic et al., 2014). This study proposes to evaluate suitability of ELR containing YIGSR sequence (ELR-YIGSR) for ARPE19 cell growth and behaviours.

Materials and methods

ELR-YIGSR expression and purification

ELR-YIGSR was produced, purified and characterized according to previous published protocols (Girotti et al., 2011; Martínez-Osorio et al., 2009; Singh et al., 2014; Srivastava et al., 2011). Briefly a gene for expression of a sequence of amino acids ELR-YIGSR is constructed combining different corresponding gene sequences. Amino acids sequence of ELR-YIGSR is as depicted below.

ELR-YIGSR:

MESLLP-{[(VPGIG)2 VPGKG (VPGIG)2]2 (VPGIG)2 DPGYIGSR (VPGIG)2[(VPGIG)2 VPGKG (VPGIG)2]2}8-V
(Mw = 89,366 Da)

Escherichia coli is transformed with this gene construct and subjected to induction for producing ELR-YIGSR amino acids sequence. For this purpose synthetic oligonucleotides, and Taq DNA polimerase and restriction enzyme Eam 1104 I were purchased from IBA GmbH (Goettingen, Germany) and Stratagene. The restriction enzymes Xba I, Hind III, DpnI, EcoRI, and Sap I were purchased from Fermentas (Burlington, ON), and T4 DNA ligase, Shrim Alkaline Phosphatase (S.A.P), and Antartic alkaline phosphatase were purchased from New England Biolabs. PCR amplifications were performed in an Eppendorf AG 22331 thermocycler. Gel photographs were taken with Gel logic 100 Imaging System running Kodak 3.6 software. Escherichia coli BLR (DE3) strain and expression vector pET (25+) from Novagen (Madison WI), and Ampicillin from Apollo Scientific (Bredbury, UK), were employed during elastin-like recombinamer (ELR) production, which was performed with several cycles of temperature-dependent reversible precipitation.

SDS-PAGE gels and mass spectrometry (MALDI-TOF/MS) was used to determine purity and molecular weight of proteins. Correctness of sequence was checked by amino acid analysis and amino acid sequencing. Physical properties were characterized by differential scanning calorimetry (DSC) and turbidity measurements.

ELR-YIGSR film preparation

Solvent-casting onto glass and subsequent cross-linking with hexamethylene diisocyanate (HDI), a lysine-targeted homobifunctional crosslinker was used to prepare ELR-YIGSR films. Briefly aqueous solution (80 µl, 50 mg/mL) of ELR-YIGSR was deposited on circular glass cover slips (diameter: 12 mm; Thermo Scientific) and dried for 8 h at 60 °C. Subsequently dry films were cross-linked immersing them for overnight in 10% HDI/acetone solution. Then they were washed extensively with type I water. All of these reagents were purchased from Sigma Aldrich.

The ELR-YIGSR film coated glass cover slips (test surface) were inserted in a 24-well cell-culture plate, exposed to UV light for overnight and then with Dulbecco's Modified Eagle Medium (DMEM) with antibiotics for 2 hours for sterilization before cell seeding.

RPE cell seeding

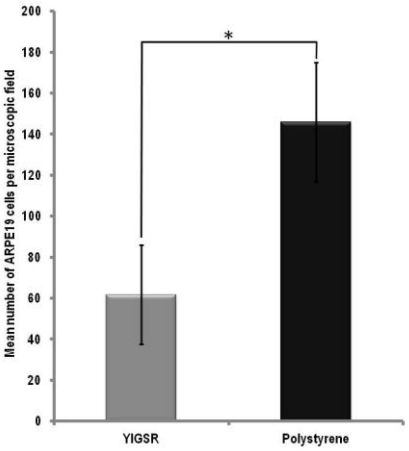


Figure 1. Adherence of ARPE19 cells to surface of ELR-YIGSR and TCP polystyrene. The graph bar shows mean number of adhered cells per microscopic field (x10) ± SD for each substrate at 4 hours of growth period. The data is generated by staining with DAPI the nuclei of attached cells on each surface, following fluorescence microscopic observations, photography of ten fields (x10) per surface at random and statistical analysis. The graph shows that surface of ELR-YIGSR is less favourable to support adhesion of ARPE19 cells in comparison to surface of TCP polystyrene. Asterisk (*) represents a significant p-value between mean numbers of ARPE19 cells adhered to surface of ELR-YIGSR and control TCP polystyrene at 4 hours as presented in the graph. Statistical significance is established as $p<0.05$ (*).

The ARPE19, a human RPE cell line, were purchased from American Type Culture Collection (Manassas, VA, USA). They were grown in a DMEM/F12 medium supplemented with 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin G, 50 mg/ml streptomycin and 2.5 mg/ml amphotericin B (Invitrogen-Gibco). The standard culture condition for these cells was humidified atmosphere at 37 °C in presence of 5% CO2. The medium was replaced every 3 days intervals. When ARPE19 cell culture became 90% confluent, cells were detached using 0.1% trypsin and 0.04% tetrasodium ethylenediaminetetraacetate (EDTA; Invitrogen-Gibco). Further it was incubated with medium supplemented with FBS to stop trypsin-EDTA effect. Then cells were washed and re-suspended in phosphate-buffered saline (PBS; Invitrogen-Gibco).

Trypan blue exclusion assay was used for viability and quantification of cell numbers. The 1x104 of ARPE19 cells were seeded on each ELR-YIGSR coated glass cover slip (test surface) and standard tissue culture plate (TCP) polystyrene surface (Control). Then they were incubated in standard culture conditions for time intervals of 4, 24, 72, 120 and 168 hours. Late passages of cell cultures were discarded and were not used for experimental purpose.

RPE cell adhesion and proliferation

Cells were counted for growth periods of 4, 24, 72, 120 and 168 hours for evaluating cells adhesion and proliferation on ELR-YIGSR coated glass and TCP polystyrene surface. Following each growth period substrates surfaces were washed with PBS to remove non-adhered cells. Then adhered ARPE19 cells were incubated with DAPI (Invitrogen, Eugene, OR) for 1 minute at room temperature (RT) to stain cell nuclei. The nuclear stained ARPE19 cells were mounted using a fluorescencent mounting medium and visualized using a phase contrast and fluorescence microscope (Leica Microsystems, Mannheim, Germany). Ten fields (x10) were photographed per surface at random (from centre to peripheral area). The ARPE19 cells and their nuclei for each field of view were counted using Adobe Photoshop Elements software. The mean number of nuclei (cells) per field of view (x10) was calculated for each growth period and presented as a histogram. The histogram presents mean number of cells per field of view ± standard deviation (SD) versus growth period for evaluating cell adhesion and growth pattern.

The cell growth in terms of the cell culture doubling time on each surface was measured for growth periods of 20 h (between 4 to 24 hours), 68 hours

(between 4 to 72 hours), 116 hours (between 4 to 120 hours), 58 hours (between 24 to 72 hours), 96 hours (between 24 to 120 hours), 48 hours (between 72 to 120 hours). The formula used was; DoublingTime = duration∗log(2) / (log(FinalConcentration)−log(InitalConcentration), where "log" is the logarithm to base 10 or 2 or any other base. The software used was available online http://www.doubling-time.com/compute.php.

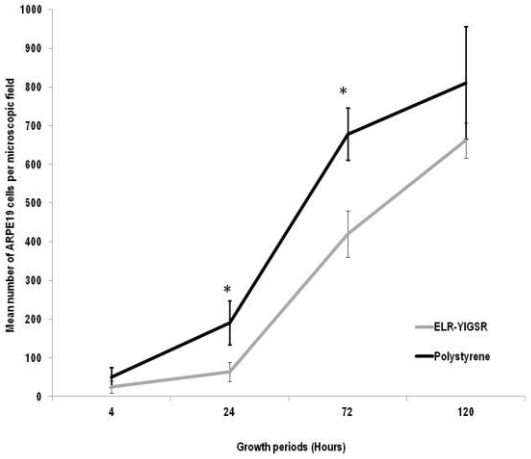


Figure 2. Growth of ARPE19 cells on surface of ELR-YIGSR and TCP polystyrene. The line bar shows mean number of cells per microscopic field (x10) ± SD for each substrate at growth periods of 4, 24, 72 and 120 hours. The data is generated by staining with DAPI the nuclei of cells found on each surface at growth periods of 4, 24, 72 and 120 hours, following fluorescence microscopic observations, photography of ten fields (x10) per surface at random and statistical analysis. The graph shows that surface of ELR-YIGSR is less favourable to support growth of ARPE19 cells in comparison to surface of TCP polystyrene. Asterisk (*) represents significant p-values between mean number of ARPE19 cells found on surface of ELR-YIGSR and control TCP polystyrene at each time period as presented in the graph. Statistical significance is established as p<0.05 (*).

ELR-YIGSR surface cytotoxicity for cell viability

Trypan blue exclusion assay was performed following cell seeding at 4 hours for analysing ELR-YIGSR and TCP polystyrene substrates cytotoxicity to ARPE19 cells. Furthermore, cell viability and cytotoxicity assay kit for live and dead cells® (Biotium Inc., Hayward, CA, USA) was used for 72 and 120 hours growth periods. The viable ARPE19 cells were seeded on surfaces of ELR-YIGSR and TCP polystyrene substrates and incubated in standard culture condition. Following each growth period substrate surface was washed; cells were stained with trypan blue or staining kit and visualized immediatly under an optical microscope for counting stained cells. Cell viability and cytotoxicity assay kit was used in accordance with previous published protocol (Singh et al., 2014). The kit contains two different colour florescent probes staining live ARPE19 cells in green (Calcein acetoxymethyl ester staining) and dead and damaged ARPE19 cells in red (Ethidium homodimer III staining). The ARPE19 cells were incubated in stain for 30 minutes at room temperature, and then they were washed with PBS and visualized using a fluorescence microscope. Ten fields (x10) were photographed per surface at random (from centre to peripheral area). The mean number of green and red stained cells per field of view (x10) was calculated for each growth period and presented as a histogram. The histogram presents percentage of stained cells per field of view ±1 standard deviation (SD) versus growth period for evaluating cell viability (substrate surface cytotoxicity to ARPE19 cells) on surface of each substrate. The formula used for calculation was; percentage of stained cells (dead or live) = number of dead or live cells * 100 / total number of cells (dead+live).

RPE cell morphology, Ecad and ZO-1 proteins expressions and intercellular junctions

RPE cell morphology, Ecad and ZO-1 epithelial proteins expressions and intercellular junctions were evaluated as described in previous article (Singh et al., 2014; Srivastava et al., 2011). Morphology of growing ARPE19 cells on each substrate surface was observed using phase contrast microscopy at different growth periods. Epithelial characteristic was evaluated by simple microscopic observation as well as using Rhodamine-Phalloidin (Molecular Probes-Invitrogen, Paisley, UK) staining for cytoskeleton actin filaments and immunostaining with primary antibodies against Ecad and ZO-1 specific epithelial protein markers (Invitrogen, Camarillo, CA). Electronic microscopy was performed for determining presence of intercellular tight junction and gap junctions for cells grown for 168 hours.

Briefly, growing ARPE19 cells were washed with PBS three times for 5 minutes (3x5 min), fixed with 4% paraformaldehyde for 10 minutes. Then they were permeabilized with PBT (0.2% TritonX-100 in PBS) for 10 minutes, again washed with PBS (3x5 min) and blocked for one hour in antibody blocking buffer (10% Normal goat serum, 1% Bovine serum albumin in PBT). The cells were incubated with a 1:50 dilution of primary antibody in PBT overnight at 4 °C, and then washed with PBT (3x5 min). Following they were incubated with a 1:100 dilution of anti-mouse IgG-FITC (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) in PBS for one hour at RT. The cells were also co-stained with a 1:40 dilution of Rhodamine-Phalloidin (200unit/ml) in PBS for 20 minutes. Then they were mounted and observed under a fluorescence microscope.

For electronic microscopy growing cells were fixed for two hours in 0.5 % paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4), washed in same buffer and postfixed in 1 % OsO4 in phosphate buffer. After dehydration in alcohols pieces were embedded in low-viscosity epoxy resin (Spurr resin embedding kit. TAAB Laboratories Equipment Ltd, Berks, UK). Thin sections were examined in a JEOL 1200-E II electron microscope (TEM) after staining with uranyl acetate and lead citrate.

Statistical Analysis

The results obtained for each substrate were expressed as mean number of cells per field of view (x10) ± standard deviation (SD), as calculated using MS excel 2007. Data were tested for normality and investigated for statistical significance using Student's t-test. Statistical significance was set at p<0.05.

Results

RPE cell adhesion and growth

For each substrate, ELR-YIGSR as well as TCP polystyrene, surface the numbers of ARPE19 cell were counted to perform statistical analysing for determining cell adherence and growth. The mean numbers of adhered ARPE19 cells to ELR-YIGSR and TCP polystyrene surfaces were 61.72±24.24 and 145.96±29.06 per microscopic field respectively after 4 hours (figure 1). Statistical calculation showed that only 42.29±24.24% of total ARPE19 cells (100%) which were adhered to TCP polystyrene, was adhered to ELR-YIGSR substrate surface. ARPE19 cell adherence to TCP polystyrene surface was significantly higher (p<0.05) that that of ELR-YIGSR surface.

The ARPE19 cell growth was analysed for each substrate surface after growth periods of 4, 24, 72 and 120 hours by counting numbers of cells per microscopic field. The data was presented in figure 2. The results showed that there were 25±16, 63.5±25, 420±60 and 662±45 ARPE19 cells on ELR-YIGSR surface and 49.5±25.21, 190.5±57.27, 768±67.81 and 810.3±145.2 ARPE19 cells on TCP polystyrene surface in each microscopic field. The comparisons of data showed that there were differences between numbers of cells growing on surface of both substrates at each growth period and these differences increased along with growth periods of 4, 24, 72 and 120 hours. These differences were significant (p<0.05) at growth periods of 24 and 72 hours. The surface of TCP polystyrene was always found to contain numbers of ARPE19 cells higher than that of ELR-YIGSR. Further analysis of growth rate (= cell culture doubling time) for growth periods of 20 h (between 4 to 24 hours), 68 hours (between 4 to 72 hours), 116 hours (between 4 to 120

hours), 58 hours (between 24 to 72 hours), 96 hours (between 24 to 120 hours), 48 hours (between 72 to 120 hours) was performed. The results showed 14.87, 16.71, 24.54, 21.28, 28.39 and 73.12 cell doubling times for ELR-YIGSR surface and 10.29, 17.19, 28.76, 28.84, 45.96, 620.3 for TCP polystyrene surface. Hence, surface of ELR-YIGSR was less suitable for cell adherence but growth rate was higher than that of TCP polystyrene to support growth of ARPE19 cells.

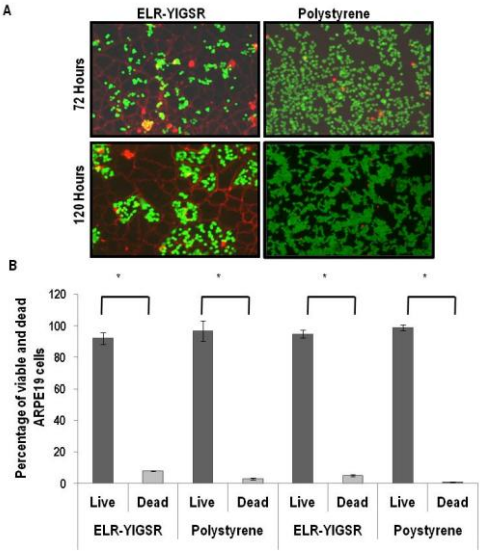


Figure 3. Viability of ARPE19 cells on surface of ELR-YIGSR and TCP polystyrene. A) At random selected images of x10 microscopic fields confirming detection of live (green) and dead cells (red) revealed on each substrate at growth periods of 72 and 120 hours after staining with cell viability and cytotoxicity assay kit for live and dead cells®. The kit has also stained the ELR-YIGSR surface, showing red fluorescent chicken-wire structure, however it has been not seen on TCP polystyrene surface. B) The line bar shows mean number of live and dead cells per microscopic field (x10) ± SD for each substrate at growth periods of 72 and 120 hours. The data is generated by staining with cell viability and cytotoxicity assay kit for live and dead cells® to ARPE19 cells found on each surface at growth periods of 72 and 120 hours, following fluorescence microscopic observations, photography of ten fields (x10) per surface at random and statistical analysis. The graph shows that surface of ELR-YIGSR is favourable to support viability of ARPE19 cells as observed for surface of TCP polystyrene. Asterisk (*) represents significant p-values between mean numbers of live and dead ARPE19 cells found on surface of ELR-YIGSR and control TCP polystyrene at different growth periods as presented in graph. Statistical significance is established as $p<0.05$ (*).

RPE cell viability

Trypan blue exclusion assay was performed to check ARPE19 cell viability at cell seeding time as well as at end of each cell growth period for each substrate surface. The results showed that ARPE19 cells were >90 % viable at cell seeding time as well as at end of experiment (120 hours). However, at end of each cell growth period for making more visual the observations, ARPE19 cells were also stained with a viability and cytotoxicity assay kit for live and dead cells® (Biotium Inc., Hayward, CA, USA). This assay allowed live ARPE19 cells to stain in green while damaged and dead cells to red. The microscopic observations and cell quantifications results (figure 3 A and B) showed that over 90% of ARPE19 cells, which were growing on surface of ELR-YIGSR and TCP polystyrene, were viable (green). Less than 8% of ARPE19 cells were found dead on surface of both substrates. Nevertheless, within this 8% dead cell population, percentage of died cells was higher for surface of ELR-YIGSR than TCP polystyrene (figure 3 A and B). The ARPE19 cells growing on surface of TCP polystyrene were more confluent than that found on surface of ELR-YIGSR (figure 3 A). There were more ARPE19 cells found at 120 than 72 hours on surface of each substrate (figure 3 A). Hence, this again demonstrated previous results that surface of TCP polystyrene contained numbers of cells higher than that of surface of ELR-YIGSR, and this number of cells increased on each surface along with growth period. Fig 3A also showed that kit stained the ELR-YIGSR surface, showing red fluorescent chicken-wire structure, which was not seen on TCP polystyrene surface.

Epithelial morphology, protein expression and intracellular junctions

The images of phase-contrast microscopy taken at 4 hours showed that surface of ELR-YIGSR substrate favoured adherence of ARPE19 cells and posterior observation showed that these cells grew as cell patches of different sizes (figure 4 A and 3 A). Within adhered cell population at 4 hours, few were still rounded with nucleus and pigmentation at centre, and few of them had acquired elongated morphology retaining nucleus and pigmentation at one end (figure 4 A). The images taken of the cells growing on both surfaces at 24-72 hours showed variations in cell size. Images 4 C and D of full microscopic fields with scales in µm showed circular, elongated to hexagonal morphology, and small to big sized cells growing on both surfaces (indicated by arrows). Images 4 E showed the cell area, circularity, and aspect ratio of cells growing on ELR-YIGSR surface calculated and analyzed by Image J software. Nevertheless it has to be taken into account the cells are growing forming cell monolayers over ELR-YIGSR and control surfaces. At 120 hours ARPE19 cell culture became confluent towards centre of cell culture area at surface of ELR-YIGSR substrate (figure 4 B). Images showed that they had also acquired hexagonal morphology. However, cell density was not as high as it was seen on surface of TCP polystyrene. The ARPE19 cells found less dense on surface of ELR-YIGSR. This cell density was found containing increasing tendency from periphery to center at both substrates surfaces. The cells growing in center of surface of ELR-YIGSR were more packed and homogenous demonstrating mosaic structure, and which was observed losing along with center to peripheral direction. Similar was the case for TCP polystyrene, however, this mosaic pattern was observed more homogenous on surface of TCP polystyrene due to increased cell density (Srivastava et al., 2011). This observation was further confirmed by staining of ARPE19 cells with rhodamin-phalloidin stains (figure 5 A). The rhodamin-phalloidin stains circumferential actin fibers of a cell. Immunofluorescence microscopy observations showed good packed hexagonal cells growing in patches on surface of ELR-YIGSR (figure 5 A). Further cells were immunostained for Ecad-1 and ZO-1 proteins expressions at 72 and 120 hours respectively for evaluating cell to cell contacts, thus confirming epithelial nature of these cell patches. The images of immunofluorescence microscopy showed that ARPE19 cells growing on surface of ELR-YIGSR expressed Ecad-1 protein at 72 hours and ZO-1 protein at 120 hours (figure 5 B and C). Image 5 E showed a representative image of all negative controls for immunofluorescence stainings. In this study, different times were used to detect Ecad-1, ZO-1 and actin filaments; however, in further extensive study with RPE primary cell cultures, all protein markers will be detected at a same time to localize them inside a cell. In all images, DAPI staining showed that cell nuclei were rounded (figure 5 B and C). The surface of ELR-YIGSR sustained epithelial morphology of ARPE19 cells were further strengthened by electronic microscopy. The images of electronic microscopy showed tight and gap junctions (indicated using red arrows) between ARPE19 cells growing on surface of ELR-YIGSR (figure 5 D). The tight junctions (zonula occludens) were characterized by groups of union points where plasma membranes of two adjacent cells were connected and gap junctions (nexus) were observed as zones of membrane apposition with a dense line between cell membranes. Chicken-wire structure could be seen in figure 4 a and b. It seemed neurite like structures; however these are knots of chicken-wire structure.

Discussion

Bruch membrane (BrM) supports to sustain RPE phenotype and functionality. The healthy RPE executing all functions properly is essential for neuroretina which is main spot for translating lights to vision signals (Bhutto and Lutty, 2012; Nita et al., 2014; Zarbin et al., 2014). Any type of disruption in this signal translation leads to vision impairment or sometime complete loss of vision. Hence, sustaining proper RPE can lead to solve many pathological issues, most of them related to genetic alterations (dystrophies) or ageing process (AMD). The advance stage of some of these retinal diseases requires RPE cell replacement therapy.

Nature is offering big sources of ECM that consist different characteristics including biocompatible and biodegradable. Besides it also provides opportunity to identify and decode genetic sequence of a particular

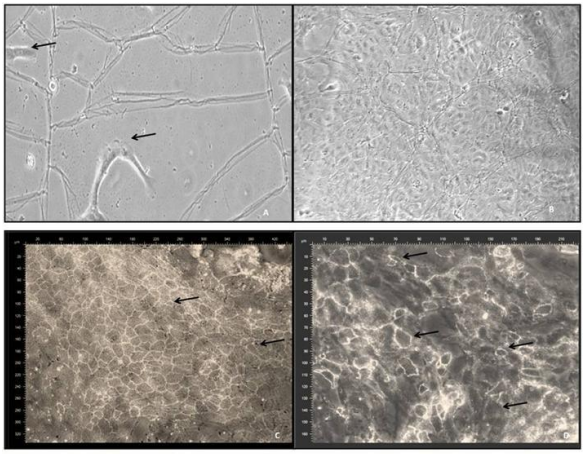


Figure 4. Morphology of ARPE19 cells growing on surface of ELR-YIGSR and TCP polystyrene. Representative images of x10 microscopic field showing ARPE19 cell morphology A) at 4 hours B) at 120 hours in the centre of culture plate. The ARPE cells are rounded or elongated with pigmentation at 4 hours but at 120 hours, they have acquired hexagonal morphology showing packed and homogenous mosaic structure. These figures have also demonstrated the ELR-YIGSR surface showing a chicken-wire structure. It has appeared neurite like structures; however, there are knots of chicken-wire structure. C) cell size on TCP polystyrene surface. D) cell size on ELR-YIGSR surface during 24 to 72 hours. E) cell area, circularity, and aspect ratio on ELR-YIGSR surface are calculated and analyzed by Image J software. Each arrow indicates an observation; circular, elongated to hexagonal and small to big size, and pigmentation. White marks around cells are due to detection of E-cad protein expression.

characteristic and thus to develop a new artificial ECM frame incorporating gene sequences of interest. In recent years, there are many reports describing success in stem cells differentiation to RPE cells. Similarly there are many papers describing about natural, synthetic, biodegradable, non-biodegradable, compatible ECMs which could serve as a BrM prosthesis. They showed sustaining RPE cell growth and characteristics, and subsequently graft behavior in transplantation. Unluckily most of them are failed or achieved partial success in transplantation study (Hynes and Lavik, 2010). This confirms that somewhere they failed to present characteristics of natural BrM.

Hence besides fresh adult RPE cells, an appropriate ECM is vital for damaged RPE replacement. An ideal ECM is one that has property to deliver appropriate physiological signals for proper RPE cell adherence and proliferation, and sustain adult RPE characteristics and functions. From surgical point of view it also requires that this ideal ECM should be flexible, thin and could be handled easily during transplantation procedure. Genetic engineering provides opportunity to framing structural and functional related genetic sequences in particular order. Thus it offers to fabricate recombinant polymeric films containing properties of interest. It also facilitates to combine sequences in complex order for properties which are not observed in living organism. Hence, a bioactive domain can be inserted to deliver a particular physiological signal to the cells.

An ideal BrM prosthesis must contain all property of natural BrM. Few of them which should be considered from beginning are: it should be porous for metabolic transportation between blood vessels and RPE, able to deliver appropriate signals to sustain RPE characteristics and unable to produce adverse effect in in vivo post-transplantation. Besides these characteristics, from surgical point of view, it should be flexible, easy to handle and adjustable to in situ of retina. If it is biodegradable, it must not produce adverse effects to other organs via systemic routes.

Elastin is a well know protein which is a part of composition of many ECMs including BrM. It normally provides backbone and flexibility to an ECM and could be biodegradable. The ELR film of this study contained repeated sequence of elastin i.e. a synthetic elastin frame, thus, it secures flexible and biodegradable nature of film. The genetic engineering technologies had been used to synthesize this recombinamer, hence it was possible to include bioactive domains where one or multiple sequences could be inserted. Since a sequence presents a single property, therefore, ELRs containing different properties could be synthesized. The ELR containing RGD sequence had been already tested with ARPE19 cells (a RPE cell line) and fresh human RPE cells, and these studied were already reported including the data with ELR-IK (control) surface (Singh et al., 2014; Srivastava et al., 2011). Since ELR-IK surface was already discussed in previous publications, therefore, in this study it would be discussed for comparison referring previous published data. Nevertheless, elastin does not contact the RPE. It may transmit signals to the RPE that counter normal development. Studies from the Del Priore lab shows that RPE adheres poorly to the elastin layer of RPE. However, this study describes modified ELR that contains bioactive domains. The previous published data for ELR-RGD (Singh et al., 2014; Srivastava et al., 2011) showed modified ELR surface was favorable for RPE growth. The ELR can be used to prepare porous hydrogels using a salt-leaching technique (Martín et al., 2009), thus, confirming its suitability to prepare porous films. The BrM is a porous ECM which facilitates metabolic transportation between blood vessels and RPE. Additionally, it was described that this ELR could degrade into natural amino acids that could eliminate via normal routes (Arias et al., 2014; Girotti et al., 2011; Rodríguez-Cabello et al., 2011). Thus, this provides sufficient period for RPE to synthesis its own ECM, to integrate into retina by establishing connections with nearby RPE cells and neuro-retina.

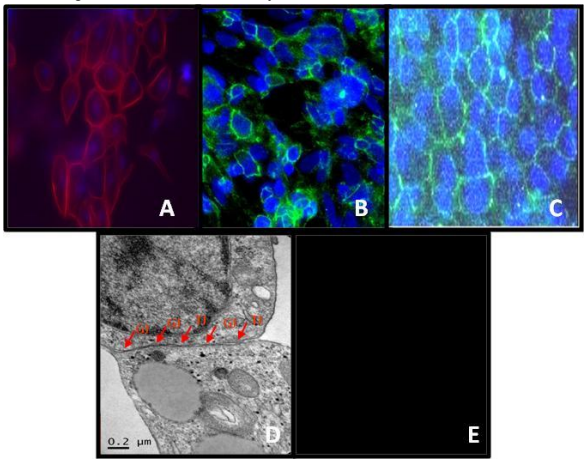


Figure 5. Morphology, epithelial proteins (Ecad and ZO1) expressions, and tight and gap junctions detection of ARPE19 cells growing on surface of ELR-YIGSR. Representative images of x10 microscopic field showing A) circumferential actin fibers of ARPE19 cells as detected by staining with rhodamin-phalloidin stains at 120 hours of growth periods, B) Ecad proteins expressions at 72 hours of growth periods, C) ZO1 proteins expressions at 120 hours of growth periods, D) a representative image of electronic microscopy showing tight and gap junctions (each arrow indicates a junction) and E) a representative image for all negative controls of immunofluorescence stainings (secondary antibody). Scale bars are not included because figures are made to fit to display the proteins expressions.

Laminins are a family of large, trimeric basal proteins, present as a component of ECM. They influence largely differentiation, migration and adhesion as well as phenotype and survival of a cell. Interaction of integrin proteins of a cell and laminin proteins of ECM is well known because this interaction supports a cell to anchor on surface of an ECM. Additionally it generate appropriate stimulus to sustain cells on ECM surface without losing characteristics. Some of previous reports had shown that intermittent cell cultivation rejuvenates RPE cells and also increase expression of integrin proteins. Thus, growing these RPE cells on ELR surface which contains counterpart laminin proteins to sustain RPE layer seems suitable to proceed for RPE cell replacement therapy development (Berg et al., 2004; Gullapalli et al., 2008; Zarbin, 2003).

The laminins, type IV and V collagen and heparin sulfate are reported as components of basement membrane of RPE. The concomitant expression of these ECM components, therefore, plays a key role in stabilizing adhesion and metabolism for RPE/BrM complex (Aisenbrey et al., 2006). Thereby, interactions between integrins from RPE and laminins from BrM are vital for RPE stability. The laminin is a multidomain glycoprotein with different structure and functions. Therefore, understanding completely function of each domain or sequence present in laminins of BrM and their interactions with RPE cell expressed integrins is critical to develop an ELR-RPE interface. A suitable interface development increases probability of success to ELR-RPE scaffold transplantation in future studies. Hence, ELR must contain sequences for supporting RPE quiescence, adherence, polarity and health whilst also promoting proliferation after damage and migration into a wound. The functional role of each laminin sequence present in RPE basement membrane is not yet known (Aisenbrey et al., 2006). Therefore a recombinant ELR containing LEb domain of laminin β1 chain tryptophan-isoleucine-glycine-serine-arginine (YIGSR) was studied to obtain information regarding stability of RPE cells on the substrate ELR-YIGSR.

The ARPE19 cells were seeded on surface of ELR-YIGSR and TCP polystyrene and grown for 4, 24, 72 and 120 hours in standard culture conditions. The results at 4 hours showed that only 42.29±24.24% of total ARPE19 cells which were found at TCP polystyrene surface were adhered to ELR-YIGSR surface. The significant difference between cell adherence ($p<0.05$) to both surface confirmed that ELR-YIGSR substrate was less suitable than TCP polystyrene for ARPE19 adherence at initial period of growth. Nevertheless it has been performed a comparison between ELR-YIGSR and another ELR substrate ELR-RGD and a control ELR substrate ELR-IK which were used in our previous study (Srivastava et al., 2011). It showed that YIGSR sequence insertion in bioactive domain of ELR substrate improved its property to adhere ARPE19 cells over ELR-IK substrate surface which does not contain any bioactive sequence. But this improvement was not such as observed for ELR-RGD substrate which showed over 90% adherence. Further growth study showed that ARPE19 cells grew well on surface of both substrates; ELR-YIGSR and TCP polystyrene for 24, 72 and 120 hours. However, ARPE19 cell growth was higher on surface of TCP polystyrene than ELR-YIGSR. The difference between mean numbers of cells found on each surface was increasing along growth period and at 24 and 72 hours there was clear difference ($p<0.05$). This confirmed that YIGSR sequence insertion does not inhibit cell growth. Thus, YIGSR sequence is suitable to improve the ELR property for ARPE19 cell adherence and growth. However, a further comparison with ELR-RGD substrate showed that ELR-YIGSR surface is poor than ELR-RGD substrate to stimulate RPE cell growth (Srivastava et al., 2011). The cell viability study confirmed that ELR-YIGSR and TCP polystyrene substrates maintained over to 90% growing ARPE19 cells viable. Less than 8% of ARPE19 cells were dead and ELR-YIGSR surface contained more number of dead cells than TCP polystyrene. Considering a cell population containing up to 90% viable cells is suitable for performing any transplantation, ELR-YIGSR can be considered appropriate for RPE transplantation. Further studied showed that ARPE19 cells grew in patches, showed pigmentation and later acquired hexagonal morphology showing packed and homogenous mosaic structure on surface of ELR-YIGSR, which is the real situation of RPE in in vivo condition. The ZO-1, Ecad-1 and F-actin localization are present in various cells not only in epithelial cells, however, detecting their expressions provides first clue that cells are maintaining few characteristics which are essential part of RPE cells characterization (extensive study). The immunfluorescence and electronic microscopy study detected the expressions of Ecad-1 and ZO-1 proteins and presence of tight and gap junctions between growing ARPE19 cells. This further confirmed that surface of ELR-YIGSR substrate is suitable to sustain epithelial nature of ARPE19 cells. However, strengthnesses of these proteins expressions and junctions of cells growing on surface of ELR-YIGSR substrate are similar to that of TCP polystyrene substrate or the BrM surface is a question of the study. There are many other issues which should be solved before considering this ELR-RPE scaffold for transplantation. There are few differences between ARPE19 cells and primary human RPE cells (Alge et al., 2006), therefore, this ELR-YIGSR substrate must be tested with primary human RPE cells to detect and confirm similar response. This study did not test functional property; therefore, it must be tested. The TCP polystyrene surface responded well to sustain RPE, however its suitability for retinal transplantation is in question. Nevertheless, it is necessary to perform further study for how to bring more similarity.

Thus, identifying and sequencing of characteristics of natural ECM, and posterior their insertions into ELR constructs using genetic engineering technologies supports to produce a recombinamer containing particular properties such as ELR-YIGSR. The YIGSR sequence is known to enhance cell binding, proliferation and spreading of various cell types. Results of this study have showed that surface of ELR-YIGSR substrate is suitable to grow RPE cells It sustains RPE cells adherence, growth, maintain their viability over 90%, cells hexagonal morphology and expressions of epithelial and junctional proteins. However, few further studies are needed before considering an ELR-RPE scaffold for retinal transplantation.

Acknowledgments

This research work is supported by a grant from the Centro en Red de Medicina Regenerativa y Terapia Celular de Castilla y León, 47011 Valladolid, Spain. A.K. Singh was supported with a pre-doctoral scholarship from the Agencia Español de Cooperacion Internacional. The authors also acknowledge thanks to visiting students of practical trainings for their supports.

References

Aisenbrey, S., Zhang, M., Bacher, D., Yee, J., Brunken, W.J., and Hunter, D.D. (2006). Retinal pigment epithelial cells synthesize laminins, including laminin 5, and adhere to them through alpha3- and alpha6-containing integrins. Invest. Ophthalmol. Vis. Sci. 47, 5537–5544.

Alge, C.S., Hauck, S.M., Priglinger, S.G., Kampik, A., and Ueffing, M. (2006). Differential protein profiling of primary versus immortalized human RPE cells identifies expression patterns associated with cytoskeletal remodeling and cell survival. J. Proteome Res. 5, 862–878.

Alonso-Alonso, M.L., and Srivastava, G.K. (2015). Current focus of stem cell application in retinal repair. World J. Stem Cells 7, 641–648.

Arias, F.J., Santos, M., Fernández-Colino, A., Pinedo, G., and Girotti, A. (2014). Recent contributions of elastin-like recombinamers to biomedicine and nanotechnology. Curr. Top. Med. Chem. 14, 819–836.

Berg, M.C., Yang, S.Y., Hammond, P.T., and Rubner, M.F. (2004). Controlling mammalian cell interactions on patterned polyelectrolyte multilayer surfaces. Langmuir ACS J. Surf. Colloids 20, 1362–1368.

Bhutto, I., and Lutty, G. (2012). Understanding age-related macular degeneration (AMD): relationships between the photoreceptor/retinal pigment epithelium/Bruch's membrane/choriocapillaris complex. Mol. Aspects Med. 33, 295–317.

Binder, S., Stanzel, B.V., Krebs, I., and Glittenberg, C. (2007). Transplantation of the RPE in AMD. Prog. Retin. Eye Res. 26, 516–554.

Chen, H.-C., Zhu, Y.-T., Chen, S.-Y., and Tseng, S.C.G. (2012). Wnt signaling induces epithelial-mesenchymal transition with proliferation in ARPE-19 cells upon loss of contact inhibition. Lab. Investig. J. Tech. Methods Pathol. 92, 676–687.

Girotti, A., Fernández-Colino, A., López, I.M., Rodríguez-Cabello, J.C., and Arias, F.J. (2011). Elastin-like recombinamers: biosynthetic strategies and biotechnological applications. Biotechnol. J. 6, 1174–1186.

Gullapalli, V.K., Sugino, I.K., and Zarbin, M.A. (2008). Culture-induced increase in alpha integrin subunit expression in retinal pigment epithelium is important for improved resurfacing of aged human Bruch's membrane. Exp. Eye Res. 86, 189–200.

Hynes, S.R., and Lavik, E.B. (2010). A tissue-engineered approach towards retinal repair: scaffolds for cell transplantation to the subretinal space. Graefes Arch. Clin. Exp. Ophthalmol. Albrecht Von Graefes Arch. Klin. Exp. Ophthalmol. 248, 763–778.

Khan, M., Agarwal, K., Loutfi, M., and Kamal, A. (2014). Present and possible therapies for age-related macular degeneration. ISRN Ophthalmol. 2014, 608390.

Kilic, C., Girotti, A., Rodriguez-Cabello, J.C., and Hasirci, V. (2014). A collagen-based corneal stroma substitute with micro-designed architecture. Biomater. Sci. 2, 318–329.

Martín, L., Alonso, M., Girotti, A., Arias, F.J., and Rodríguez-Cabello, J.C. (2009). Synthesis and characterization of macroporous thermosensitive hydrogels from recombinant elastin-like polymers. Biomacromolecules 10, 3015–3022.

Martínez-Osorio, H., Juárez-Campo, M., Diebold, Y., Girotti, A., Alonso, M., Arias, F.J., Rodríguez-Cabello, J.C., García-Vázquez, C., and Calonge, M. (2009). Genetically engineered elastin-like polymer as a substratum to culture cells from the ocular surface. Curr. Eye Res. 34, 48–56.

Nita, M., Strzałka-Mrozik, B., Grzybowski, A., Mazurek, U., and Romaniuk, W. (2014). Age-related macular degeneration and changes in the extracellular matrix. Med. Sci. Monit. Int. Med. J. Exp. Clin. Res. 20, 1003–1016.

Nowak, J.Z. (2006). Age-related macular degeneration (AMD): pathogenesis and therapy. Pharmacol. Rep. PR 58, 353–363.

Pfeffer, B.A., and Philp, N.J. (2014). Cell culture of retinal pigment epithelium: Special Issue. Exp. Eye Res. 126, 1–4.

Rodríguez-Cabello, J.C., Martín, L., Girotti, A., García-Arévalo, C., Arias, F.J., and Alonso, M. (2011). Emerging applications of multifunctional elastin-like recombinamers. Nanomed. 6, 111–122.

Singh, A.K., Srivastava, G.K., Martín, L., Alonso, M., and Pastor, J.C. (2014). Bioactive substrates for human retinal pigment epithelial cell growth from elastin-like recombinamers. J. Biomed. Mater. Res. A 102, 639–646.

Srivastava, G.K., Martín, L., Singh, A.K., Fernandez-Bueno, I., Gayoso, M.J., Garcia-Gutierrez, M.T., Girotti, A., Alonso, M., Rodríguez-Cabello, J.C., and Pastor, J.C. (2011). Elastin-like recombinamers as substrates for retinal pigment epithelial cell growth. J. Biomed. Mater. Res. A 97, 243–250.

Yonekawa, Y., and Kim, I.K. (2014). Clinical characteristics and current treatment of age-related macular degeneration. Cold Spring Harb. Perspect. Med. 5, a017178.

Zarbin, M.A. (2003). Analysis of retinal pigment epithelium integrin expression and adhesion to aged submacular human Bruch's membrane. Trans. Am. Ophthalmol. Soc. 101, 499–520.

Zarbin, M.A., Casaroli-Marano, R.P., and Rosenfeld, P.J. (2014). Age-related macular degeneration: clinical findings, histopathology and imaging techniques. Dev. Ophthalmol. 53, 1–32.