TESTING THE COMPATIBILITY OF MATRIGEL, PURAMATRIX AND PURIFIED BASEMENT MEMBRANE PROTEINS IN SUBCULTURING RPE CELLS DIFFERENTIATED FROM HUMAN EMBRYONIC STEM CELLS

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MUSTALAHTI RISTO: TESTING THE COMPATIBILITY OF MATRIGEL, PURAMATRIX AND PURIFIED BASEMENT MEMBRANE PROTEINS IN SUBCULTURING RPE CELLS DIFFERENTIATED FROM HUMAN EMBRYONIC STEM CELLS

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Vain osa ihmisen alkion kantasoluista voidaan erilaistaa retinan pigmenttiepiteelisoluiksi (RPE) nykyisillä erilaistusmenetelmillä. Tämän tutkimuksen tarkoituksena on isoloida erilaistuneet RPE solut ja saada ne kasvamaan kompaktina yksisolukerroksena. Tutkimuksessa testattiin eri kasvatusalustojen sopivuutta RPE solujen kasvun tukemiseksi ja erilaistumisen kontrolloimiseksi.

Tutkimukset suoritettiin kantasolututkimuskeskus Regean solulinjalla 023/08, joka on lähtöisin implantaatiovaihetta edeltävistä ylijäämä- ja huonolaatuisista alkioista. Solut on kasvatettu ilman seerumia, hyvän tuotantotavan (GMP) saavuttamiseksi. Tutkimuksessa testattiin kolme kasvatusalustaa. Ensimmäinen sisälsi seoksen solun tyvikalvon proteiineja; kollageeni neljää, laminiinia, fibronektiiniä ja nidogeeniä. Toinen ja kolmas alusta olivat kaupalliset Matrigel ja Puramatrix. Kasvua seurattiin valomikroskopian ja immunfluorenssimikroskopian keinoin. Monia solujen erottelutekniikoita kokeiltiin.

Kolmen alustan vertailussa Puramatrix oli selvästi kahta muuta heikompi. Matrigel vauhditti hyvin solujen kasvua, mutta erilaistumista tapahtui myös muunlaisten solujen suuntaan. Tyvikalvoproteiinien seoksella kasvu ei ollut aivan yhtä nopeaa, mutta erilasituminen RPE solujen kaltaisiksi soluiksi oli toivotumpaa tällä alustalla.

Suurimmaksi ongelmaksi koituivat vaikeudet solujen erottelussa riittävän hellässä ja tehokkaassa erottelussa, mikään tutkituista keinoista ei tuottanut tyydyttäviä tuloksia. Paras kasvatusalusta tässä tutkimuksessa oli tyvikalvoproteiinien seos. Immunofluoresenssimikroskopialla määritettynä tällä kasvatusalustalla kasvatetut solut olivat eniten RPE solujen kaltaisia. Kunhan vaikeudet solujen erottelussa ja kasvatusmaljojen pinnaoittamisessa saadaan ratkaistua, olen luottavainen, että RPE solujen kasvattaminen kompaktina yksisolukerroksena on mahdollista. Tämä tutkimus antaa arvokasta tietoa jatkotutkimuksille, joissa tähdätään kohti yksityiskohtaisempaa numerodataa.

ABSTRACT

Purpose: Only a part of the undifferentiated hESC population can be differentiated to RPE cells with existing differentiation methods. The goal of this study is to isolate the differentiated RPE cells, and to have the cell population as a compact monolayer. Different culture surfaces are tested to find a surface, which would support the growth and maturation of differentiated cells to functional RPE monolayer.

Methods: The experiments were carried out with hESC line Regea 023/08 which has been derived from pre-implantation stage surplus and low quality embryo. The hESC line was cultured on irradiated human foreskin feeder fibroblasts in the presence of human basic fibroblast growth factor (bFGF). The cells were cultured without serum to attain conditions closer to Good Manufacturing Procedure. Three cell culture surfaces were tested. A mixture of cell attachment proteins collagen IV, laminin, fibronectin and nidogen, second and third were commercial products Matrigel and Puramatrix. Growth and differentiation was monitored with light microscopy, Live/Dead kit and immunofluorescence. Various cell dissociation techniques were tested.

Results: The most distinguishable difference comparing the three surfaces was the inability of Puramatrix to support cell attachment and sustain cell survival in comparison with the two other surfaces. Matrigel was a good growth booster but there was also rapid growth to directions we did not desire. Growth with the protein mixture, growth was not as rapid but more purely RPE-like. Difficulties with cell dissociation complicated monitoring.

Conclusions: The biggest challenge encountered was how to gently and effectively dissociate the cells to one cell suspension. None of the methods used offered satisfactory results. The best cell culture surface examined in this study was the mix of attachment proteins. The growth was not as fast as with matrigel but the growth was more parallel towards RPE cells. The Live/Dead kit offered an easy way of monitoring cell vitality. This will be used in further studies also. Based on immunofluorescence analyses the results were promising. After more focus on cell dissociation techniques, and overcoming minor difficulties in plating phase, I am confident that growing RPE cells as a homogenous monolayer is possible. This study provides valuable information to lead us towards studies with more numerous data.

CONTENTS

1. INTRODUCTION

Retinal degenerative diseases, such as age-related macular degeneration (AMD), diabetic retinopathy and retinitis pigmentosa, affect millions of people worldwide. In the developed world, AMD is the leading cause of blindness affecting mainly elderly people [1]. Although various means to prevent, delay or repair retinal cell degeneration are under investigation, no effective treatment is currently available for advanced stages of retinal degenerations.

Retinal pigment epithelium (RPE) cells are important for the development of the eye. They also play a key role in photoreceptor function and survival in the adult by providing the photoreceptors nutrients, controlling regeneration of visual pigments, ion flow and oxidative damage in the retino-choroidal border, forming the blood-retinal barrier, absorbing stray light and phagocyting the outer segment of the photoreceptors, ensuring their renewal. The interplay between RPE and retina is bidirectional, and abnormalities in one tissue can lead to secondary degeneration of the other. In mammalian development, RPE and neural retina both derive from optic neuroepithelium and share the same progenitor [2].

Embryonic stem (ES) cells are derived from pluripotent cells of the early mammalian embryo and are capable of unlimited, undifferentiated proliferation *in vitro*. The term "ES cell" was introduced to distinguish these embryo-derived pluripotent cells from teratocarcinoma-derived pluripotent embryonal carcinoma (EC) cells. Essential characteristics of primate ES cells include derivation from the preimplantation or periimplantation embryo, prolonged undifferentiated proliferation, and stable developmental potential to form derivatives of all three embryonic germ layers even after prolonged culture. For ethical and practical reasons, in many primate species, including humans, the ability of ES cells to contribute to the germ line in chimeras is not a testable property. Nonhuman primate ES cell lines provide an accurate *in vitro* model for understanding the differentiation of human tissues [3].

In tissues, extracellular matrices provide support and store growth factors, cytokines and other biological signals to promote and maintain cell differentiation. In cell cultures, adhesion of biomolecules to the cell culture surface can be used to manipulate cell differentiation, adhesion, proliferation and function [2].

Collagen IV, laminin, fibronectin and nidogen (entactin) are major components of the basement membrane of the RPE cells [4].Collagen IV is the most prominent protein in epithelial tissue of the basal lamina. Collagen IV molecule contains three polypeptide chains like other collagens. These

protomers of three polypeptide chains then form dimers and furthermore tetramers. Finally, the type IV collagen scaffold is formed when collagen tetramers interact end-to-end with each other. This scaffold forms the superstructure of the basal lamina [5].

So far, some studies have been published about the effects of different culture surfaces to RPE cells. On Matrigel contradictory results have been shown. In some studies Matrigel has significantly improved RPE growth, but on the other hand some studies report inhibition of the growth. Matrigel contains growth improving factors which enhance cell differentiation to other cell types as well [6,2]. Laminin which is a major component of Matrigel has independently been shown to increase RPE cell growth, so other components of Matrigel may induce the adverse effects [6]. Adhesion proteins of the normal RPE basement membrane, collagen I and IV, fibronectin and laminin, have slightly increased RPE growth when examined as a combination. In most cases the growth has not been as rapid as with Matrigel but the cells have differentiated more purely to the desired direction [6]. To evaluate the effect of cell culture surface composition on the growth and maturation of the hESC-derived RPE cells we tested Matrigel, a mixture of cell attachment proteins containing collagen IV, laminin, fibronectin and nidogen, and peptide hydrogel Puramatrix, which to our knowledge has not yet been tested with RPE cells.

BD™ PuraMatrix™ Peptide Hydrogel is a synthetic matrix that is used to create defined threedimensional (3D) micro-environments for a variety of cell culture experiments. To achieve optimal cell growth and differentiation, it is necessary to determine the appropriate mixture of this material and bioactive molecules (e.g., growth factors, extracellular matrix (ECM) proteins, and/or other molecules). BD PuraMatrix Peptide Hydrogel consists of standard amino acids (1% w/v) and 99% water. Under physiological conditions, the peptide component self-assembles into a 3D hydrogel that exhibits a nanometer scale fibrous structure [7].

BD Biosciences Matrigel (BD Biosciences, San Jose, CA) basement Membrane Matrix is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is laminin, followed by collagen IV, heparan sulfate proteoglycans, entactin/nidogen [8].

1.1 Objectives and hypothesis

Only a part of the undifferentiated hESC population can be differentiated to RPE cells with existing differentiation methods. The goal of this study is to isolate the differentiated RPE cells, and to have

the cell population as a compact monolayer. Different culture surfaces are tested to find a surface, which would support the growth and maturation of differentiated cells to functional RPE monolayer.

2.MATERIALS AND METHODS

2.1 hES cell culturing

The experiments were carried out with hESC line Regea 023/08 which has been derived from pre-implantation stage surplus and low quality embryo [9]. Regea holds the approval of the National Authority for Medicolegal Affairs Finland (TEO) to study human embryos (Dnro1426/32/300/05) and has the support of the Ethical Committee of the Pirkanmaa Hospital District to derive, culture, and differentiate hESC lines from surplus human embryos (R05116).

The hESC line was cultured on irradiated human foreskin feeder fibroblasts in the presence of human basic fibroblast growth factor (bFGF). The cells were cultured without serum to attain conditions closer to Good Manufacturing Procedure quality, needed for human trials in future.

Culture medium (RPE DM- medium) was changed six times a week and consisted of KnockOut Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 20% KnockOut serum replacement (SR) (Invitrogen), 2 mM Glutamax (Invitrogen), 0.1 mM □-mercaptoethanol (Invitrogen), 1% nonessential amino acids (NEAA) (Lonza, Walkersville, MD), 50 U/ml penicillin and streptomycin (p/s) (Lonza) and 8 ng/ml bFGF (R&D Systems Inc. Minneapolis, MN)

2.2 Cell differentiation

For RPE differentiation, the SR concentration was reduced to 15% and bFGF was removed from the medium. After one week culture on the feeders, cell clusters were cut to suspension and from this on the medium was changed three times a week. The floating aggregates were passaged mechanically with a scalpel to allow gas and nutrient exchange. Once black areas, assumed to consist of pigmented RPE cells, appeared to the cell clusters they were separated from the transparent ones with a scalpel for the experiments. The experiments were done in triplicate and repeated twice. In each experiment three cell clusters per 96-well plate well were plated. Schedule of the study is presented in tables 1 & 2. The cells had been on differentiation for approximately for two and a half months.

2.3 Cell culture surfaces

In the experiment, three plates of each culture surface were coated. Wells A1-A3 of each plate were coated with collagen IV (Sigma-Aldrich, St. Louis, MO), laminin (Sigma-Aldrich), fibronectin (R&D Systems Inc., Minneapolis, MN) and nidogen (R&D Systems Inc) mixture. Wells B1-B3 were coated matrigel (BD Biosciences, San Jose, CA). With Puramatrix (3DM Inc. Cambridge, MA) two consentrations were used, so also wells C1-C3 were coated.

1. A mixture of cell attachment proteins containing collagen IV, laminin, fibronectin and nidogen. With the cell attachment protein mix, the following consentrations were used.

- Collagen IV 5 µg/cm2
- Fibronectin 5 µg/cm2
- Laminin 5 µg/cm2
- Nidogen 1 µl/ml

The solution was incubated at 37 degrees for 24 hours and taken to room temperature 2.5 hours before coating. 100 µl of mixture was used for coating each well.

2. BD Matrigel hESC-Qualified Matrix Matrigel is a mixture of cell attachment proteins and growth factors.

Matrigel was used as instructed in the product specification sheet, taken from 4°C with cooled pipettes, and added to cooled well plates. Then put at 37°C for 30 minutes. A 280 µl aliquot of Matrigel stock solution was diluted to 25 ml of DMEM-F12. Matrigel is instructed to be used 1 ml per well for a 6-well plate. Considering the smaller surface area of a 96-well plate, 37.5 µl Matrigel was used per well. The well plates were taken to room temperature 2.5 hours before coating.

3. Puramatrix (3DM)

With Puramatrix concentrations of 0,15% and 0,25% were used. With Puramatrix 50 µl of solution per well was used. As advised in the product specification sheet, Puramatrix was put in incubation at 37°C in the afternoon, the day before the cell platings.

2.4 Cell dissociation and plating

Cell clusters from line 08/023 consisted of black pigmented and transparent areas. Black areas were assumed to contain the desired RPE cells. The goal was to separate the black areas from the transparent areas and be able to dissociate cell clusters to one-cell-suspension. With this kind of suspension controlling the number of plated cells would be easier and thus comparability would be better.

Various cell dissociation techniques were attempted.

TrypLE™ (Invitrogen)

TrypLE is a replacement for the human native proteolytic trypsin enzyme. TrypLE is free of animal- and human-derived components so it is ideal for this experiment. The solution used contained an instructed 1% of TrypLE. Solution was at at 37°C . When testing TrypLE the cells were exposed to the solution and results were checked at two minutes and five minutes.

We found TrypLE rather unefficent with our cells. Very little dissociation was attained when the cells were held in instructed 1% solution. At two minutes almost nothing was seen. Cells were then over the instructed two minutes at the suspension. This also had very little effect. With trituration (cell suspension sucked in to the pipette and bursted out) against well plate's surface pretty good dissociation was attained but apparently this handling with both TrypLE and trituration was too

harsh for the cells. When plating cells dissociated with this method nearly all cells were detached after a few days.

Collagenase IV

Collagenases are enzymes that break peptide bonds in collagen matrix. Collagen IV stock solution (10 mg/ml) was tested to dissociate cells. Collagenase IV was incubated at 37°C for half an hour.

Results were pretty much equal with TrypLE, not enough dissociation with gentle enough methods.

pH variation

A pH variation technique [10] was also tested. In this method, the cells were exposed to RPE DM- medium with different pH values in order to disengage cell junctions. pH values were set with a pH meter. Firstly some cell clusters were harvested with a pipette to an eppendorf tube with normal RPE DM- medium. This medium was aspirated and 0.2 ml of RPE DM- medium with pH value of 7.5 was added. Then 0.2 ml of alkaline RPE DM- medium, pH 11,6 (to create an overall pH of 10), was added and the solution was triturated two times after two and five minutes. Last step was to add 0.2ml of acidic medium, pH 1.7, to decrease overall pH to 8.1, and the solution was triturated five times. Then the cells were moved to normal RPE DM- medium.

Results of this technique were viewed on a 24-well plate under a microscope. One-cell-suspension was not possible to attain with this method either. The results were slightly better than with TrypLE and collagenase IV but also with this method the cons were bigger than the pros. The cells seemed to suffer too much in contrast to the dissociation. This method though proved to be the best of the non-mechanical methods tested.

Mechanical dissociation

Cell dissociation was also tested with merely cutting the cell clusters to smaller segments with a scalpel under a microscope followed by trituration,. With the final experiment the cell clusters were dissociated mechanically with a scalpel. Black areas, presumed to contain RPE cells were separated and collected. Three cell clusters were plated per each well. Under ocular inspection, one of them contained mostly very dark cells, one mostly lighter brown cells and one also some transparent areas.

2.5 Monitoring of cells

With both experiments all the culture surfaces were handled identically. Assays and immunostainings were done at the same time with all the culture surfaces. The only date that varied between experiments was the timepoint of second immunofluorescence analysis. Schedules are seen in tables 1 & 2.

In the experiments, day one was the day of cutting and plating. RPE DM- medium was changed twice a week. On day seven, Live/Dead assay was done on one of the plates/culture surface. This way we detected how many of the cells were alive at an early stage of the experiment. This helped to evaluate the timing and necessarity of the following immunofluorescence stainings.

On day nine, the first immunofluorescence stainings were done. One plate per each culture surface was stained. The used antibodies are listed in the cell characterization chapter. Second stainings took place on day 56 with experiment one, and on day 49 of the second experiment.

Growth and differentiation was also monitored throughout the experiment with a light microscope. Pictures were taken just after plating. In the beginning, pictures were taken daily or every other day to see how the cells were attached and how rapid the growth was when the cells started to spread from the cell clusters. As the experiment progressed the light microscopy frequency was reduced and focus was centralized on immunofluorescence microscopy.

Table 1. Schedule of experiment 1

Day 1	Day 7	Day 9	Day 56
Cutting and plating the cells	Live/Dead assay, 1 plate/culture surface	Fixing for immunofluorescence I, 1 plate/surface	Fixing for immunofluorescence II, 1 plate/surface

Table 2. Schedule of experiment 2

Day 1	Day 7	Day 9	Day 49
Cutting and plating the cells	Live/Dead assay, 1 plate/culture surface	Fixing for immunofluorescence I, 1 plate/surface	Fixing for immunofluorescence II, 1 plate/surface

2.6 Cell characterization

Live/Dead kit

LIVE/DEAD (Viability/Cytotoxicity Kit for mammalian cells, Molecular Probes, Inc, Invitrogen.) was used to monitor cell viability on the seventh day of the experiment. Also culture surface toxicicity can be seen with this method at an early phase. Stainings were performed according to the instructions of the manufacturer. Shortly, live cells were distinquished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually nonfluorescent cell-permeant celcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells. Dead cells were detected with EthD-1 (Ethidium homodimer-1 which enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing bright red fluorescence. EthD-1 is excluded by the intact plasma membrane of live cells Immunostainings.

Immunofluorescence I (after 9 days)

First immunofluorescence stainings were done after the cells had been growing for 9 days. All antibodies Alexa Fluor, Invitrogen.

well	primary 1	secondary 1	primary 2	secondary 2
A1	mouse anti-PAX-6, 1:75	Goat anti-mouse 488	rabbit anti-Bestrophin, 1:250	goat 568
A2	rabbit anti-MAP-2, 1:400	donkey anti-rabbit 488	mouse anti-CRALBP, 1:1000	goat 568
A3	both primaries	none	both primaries	none

Immunofluorescence II

well	primary 1	secondary 1
A1	rabbit anti-Bestrophin, 1:250	goat 568 anti-rabbit
A2	mouse anti-CRALBP, 1:500	donkey 568 anti-mouse
A3	no primaries	no primaries

The cellular markers

Bestrophin

Bestrophin is a 68-kDa basolateral plasma membrane protein expressed in RPE. It is encoded by the *VMD2* gene, which is mutated in Best macular dystrophy [10].

PAX-6

PAX-6 is a member of the *PAX* gene family. PAX functions in the developing eye and central nervous system of vertebrates [11].

MAP-2

Microtubule associated proteins (MAPs) stabilize microtubules during cell division. MAP-2 is present especially in dendrites and postmiotic neurons [12].

CRALBP

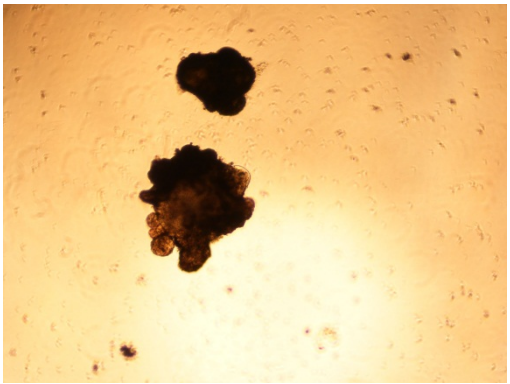
Cellular retinaldehyde-binding protein (CRALBP) functions in the RPE as an acceptor of 11-*cis*-retinol in the isomerization step of the rod visual cycle and as a substrate carrier for 11-*cis*-retinol dehydrogenase and is a specific RPE cell marker [13].

3.RESULTS

3.1 Light microscopy

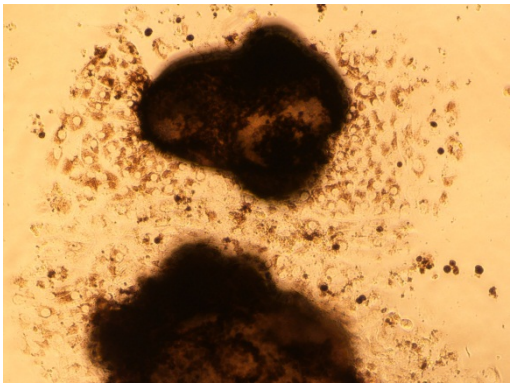
Cell culturing with collagen IV, laminin, fibronectin and nidogen mixture

Microscopy done with Nikon Eclipse TE2000-S phase contrast microscope (Nikon Instruments Europe B.V. Amstelveen, The Netherlands)



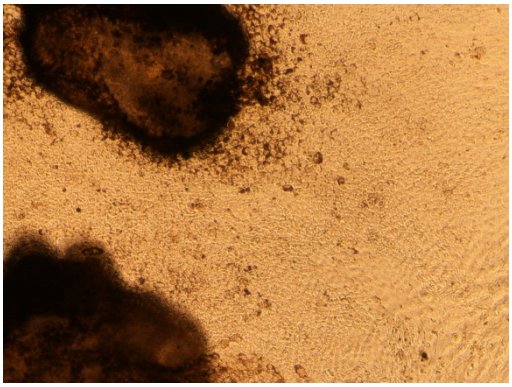
4x magnification, experiment day 0

The initial state after plating. Two highly pigmented cell clusters and smaller colonies were detected.



10x magnification experiment day 3

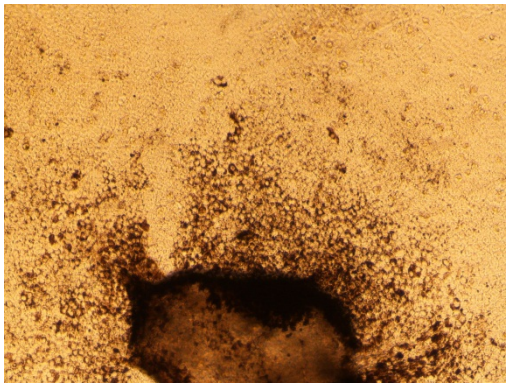
With the cell attachment protein mix, the cells were very well attached to the surface from the first days on. At first the most noticeable growth started from the bigger cell clusters. A layer of variedly pigmented cells started to spread from the clusters. On the first days the cell morphology was roundish, a bit plumper than with a highly differentiated RPE cell.



10x magnification, experiment day 8

After about a week from the plating, the cells outgrown from the big clusters started to resemble the morphology of native RPE cells. Differentiated RPE cells should have a cobblestone-like appearance, form a single monolayer, and contain dark brown pigment in their cytoplasm. An important finding was that when cells started to grow and divide from the clusters, the amount of cytoplasm pigment had great variation. In some sites the cell colonies started to spread with a transparent appearance, and in some the growth near the cluster was highly pigmented and started to fade towards more transparent appearance when progressing further away from the cluster.

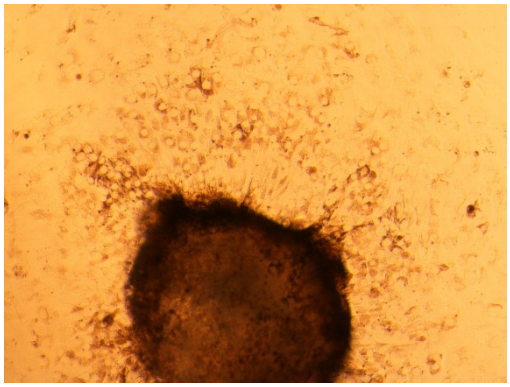
In the areas with smaller clusters and single cells, the growth was less uniform than near the clusters. In these areas cell morphology was more rotund or in some areas elongated, also no pigment was seen in these areas.



10x magnification, experiment day 13

In two weeks the cells near the clusters appeared to have the morphology of differentiated RPE cells with an ocular inspection. The area covered by cells had also increased to cover the majority of the well surface. The pigmented area

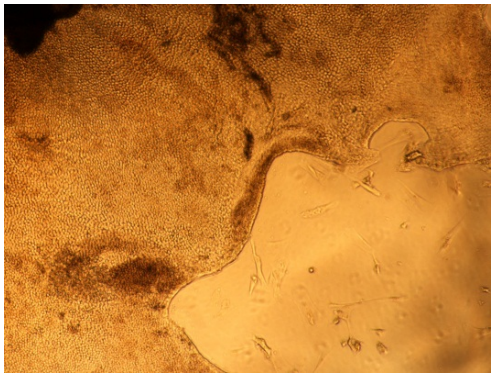
had not increased in the same phase with dividing cells in many sites it seemed to have stayed the same from day 8. Outside the areas of rapid growth near the big pigmented clusters a distinctive border could be seen. After the border behind the sprawling layer of RPE redolent cells, only minor colonies of miscellaneous cells could be seen. Morphology of the cells was similarly rotund or elongated as described on day 8.



10x magnification, day 55

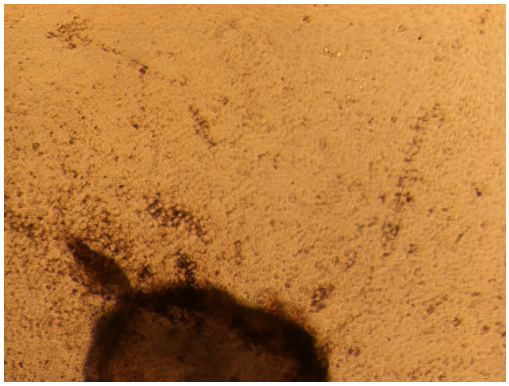
After the cells had been growing nearly two months in differentiation medium, their growth had slowed down. Rate of pigmentation of the RPE layer varied between different areas, some being dark brown and some seeming to have no pigmentation at all. At this point the growth was minimal and small bubble-like openings started to appear to the cell layer.

Matrigel



10x magnification, experiment day 3

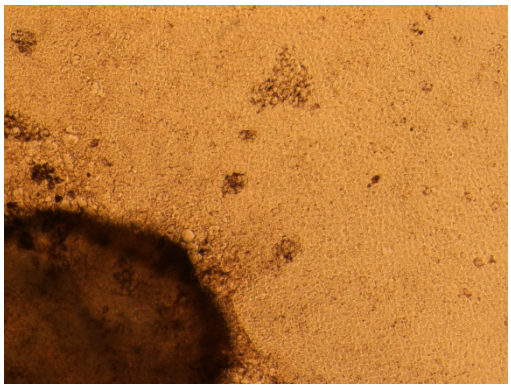
With Matrigel the growth also started from the bigger cell clusters. Cells were well attached to the surface from start on. As with the protein mixture, morphology of the cells was rounded and pigmented in the beginning of the experiment.



10x magnification, experiment day 8

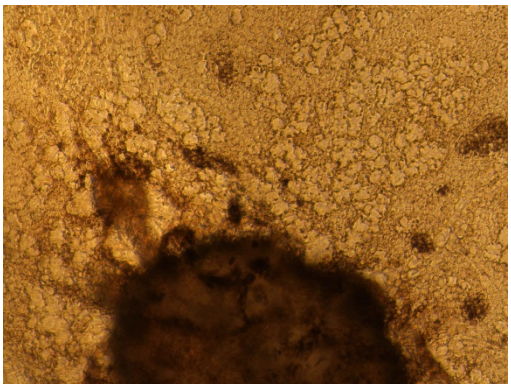
On the eighth day the cells had attained more RPE cell-like morphology. Pigmentation was also seen in some sites, mostly near the big pigmented clusters. With Matrigel the growth was more rapid but also more

multiform than with the cell attachment protein mixture. With Matrigel we could see large colonies of more rotund cells outside the RPE-like cell colonies. The border between cells growing from big pigmented clusters and other areas was not so strict. Rather, the cell type changed litlle by little form RPE-like to something else. With Matrigel we saw cell colonies more rapidly taking over the whole surface of the well.



10x magnification, experiment day 15

After two weeks, the morphology of the cells had transformed more and more towards a fully differentiated RPE cell. With pigmentation much was not happened from last time point. Small bubble-like holes started to emerge to the cell layer, especially near the big pigmented cell clusters, probably because the beginning of cell death on these sites.



10x magnification experiment day 39

With Matrigel cell death was seen earlier than with the cell attachment protein mixture. At this stage, good pigment development and a cobblestone like morphology was detected. All the white bubbly areas in this picture are dead cells.

Puramatrix

Cells plated with Puramatrix did not attach properly to the surface, thus no growth was seen. This matter is discussed more thoroughly further in the study.

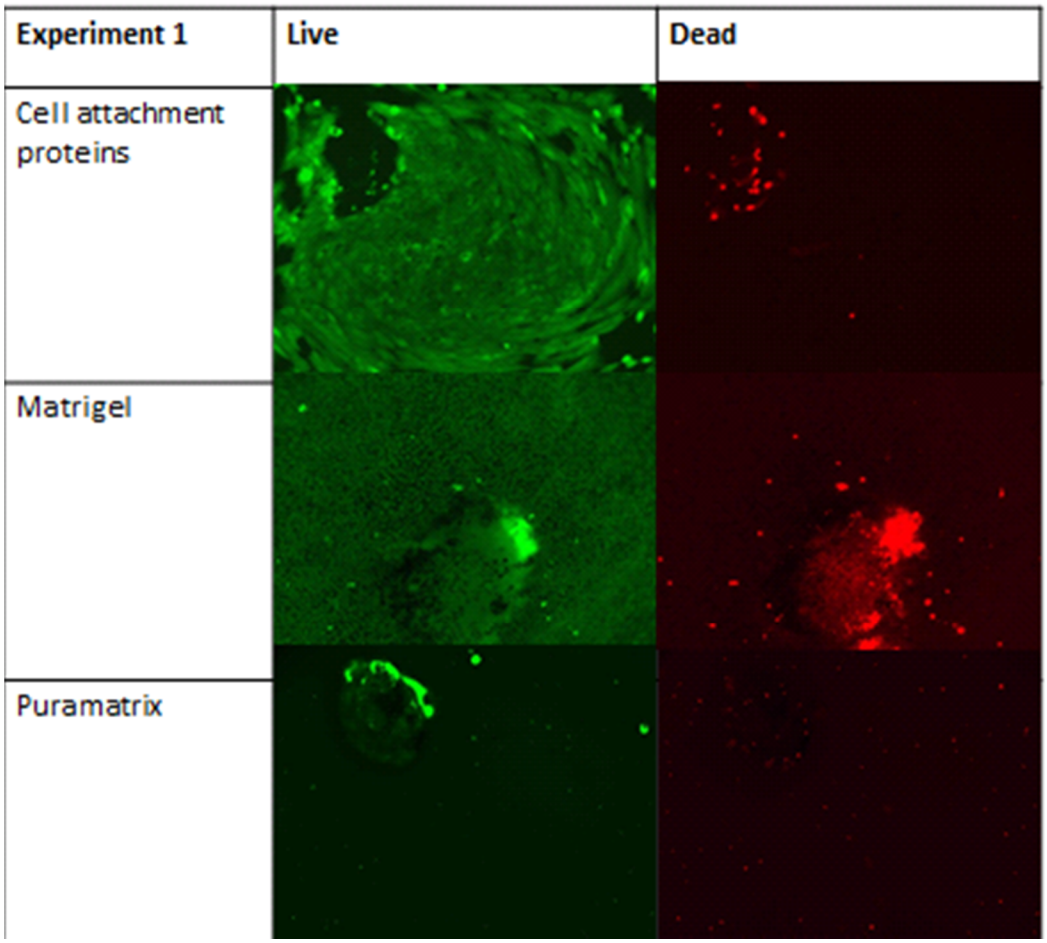
3.2Live/Dead cytotoxicity kit

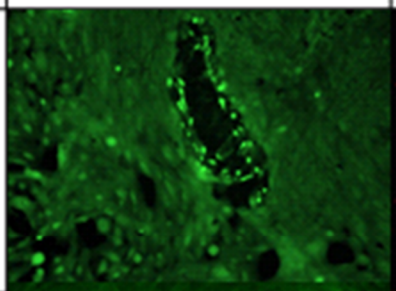
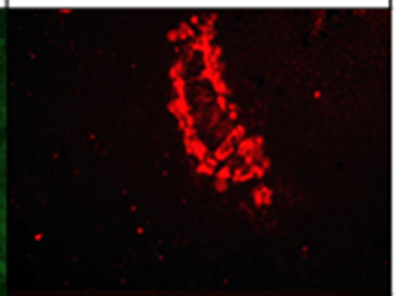
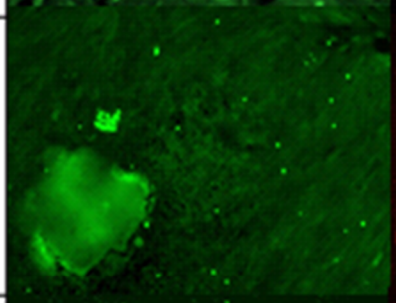
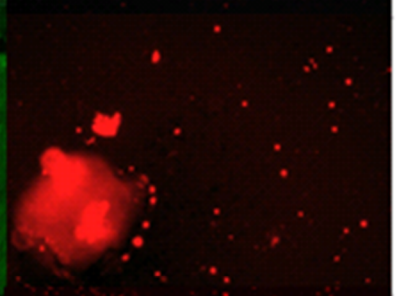
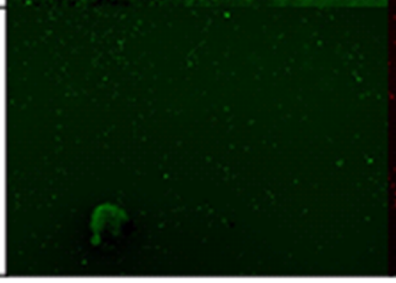
Microscopy done with Olympus IX51 phase contrast microscope with fluorescence optics and with Olympus DP30BW camera.

Experiment with Live/Dead cytotoxicity kit, experiment day 7

With the cell attachment protein mixture a favorable live/dead cell ratio was achieved. With both the protein mixture and Matrigel, almost all cells that had attached to the surface and started growing seemed to be alive. Most of the dead cell signal could be seen in the big clusters. Some small colonies around the well also emitted the dead cell signal.

With Puramatrix we saw about equally dead and live signal. As with the light microscope no cell growth was seen.



Experiment 2	Live	Dead
Cell attachment proteins		
Matrigel		
Puramatrix		

3.3Immunofluorescence

Microscopy done with Olympus IX51 phase contrast microscope with fluorescence optics and with Olympus DP30BW camera.

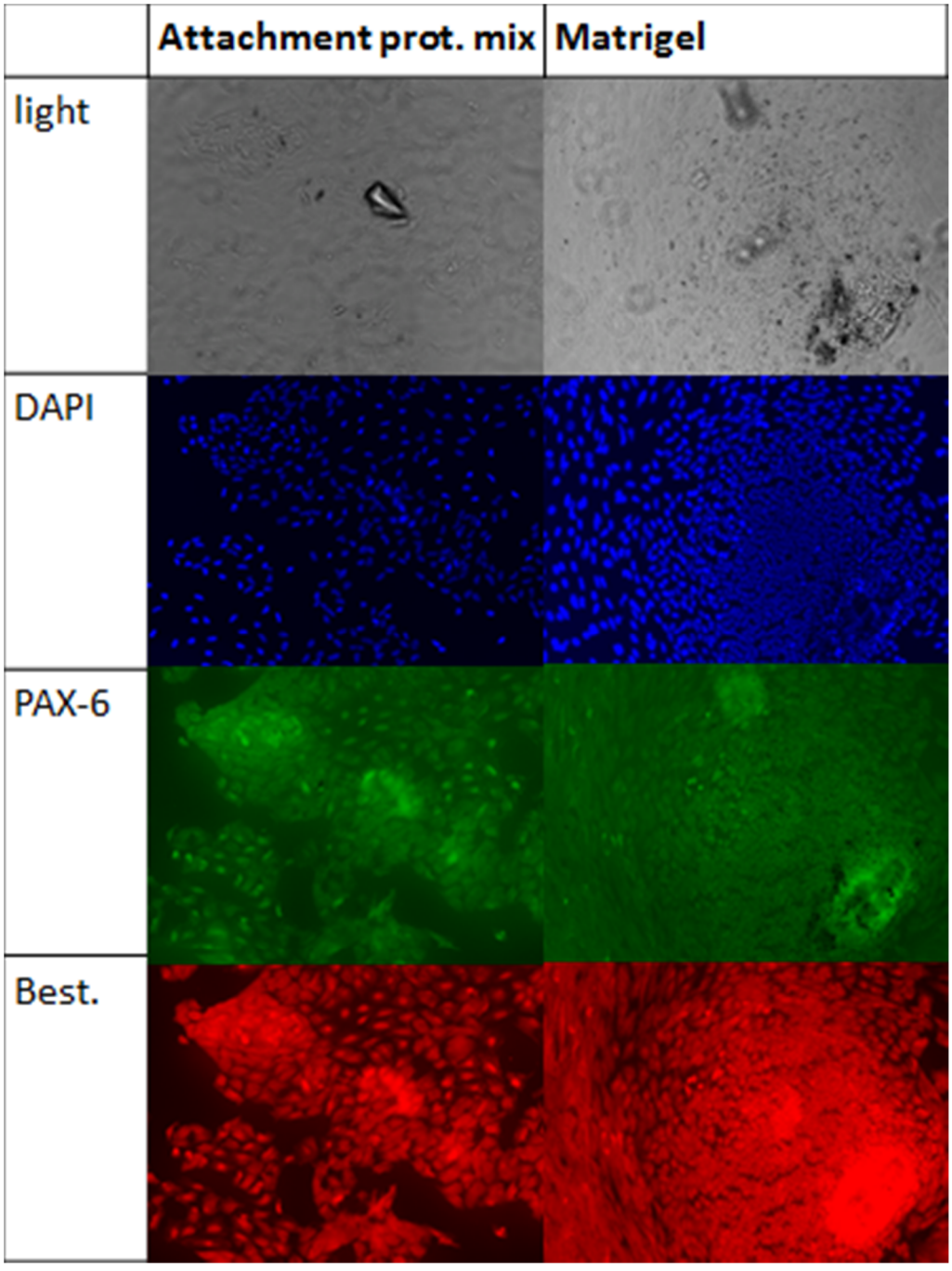
Immunofluorescent stainings were performed with all culture surfaces. Results with Puramatrix in plating and growth were so unsatisfactory that it will not be dealt with in this chapter.
The first stainings were problematic because the well plates chosen to the first experiment were too small to be imaged properly.

Immunofluorescence I after 9 days
In this staining the location of the nuclei is confirmed with DAPI. As seen with light microscopy, the cell layer was thicker and growth was more rapid at this point with matrigel.than with the protein mix.

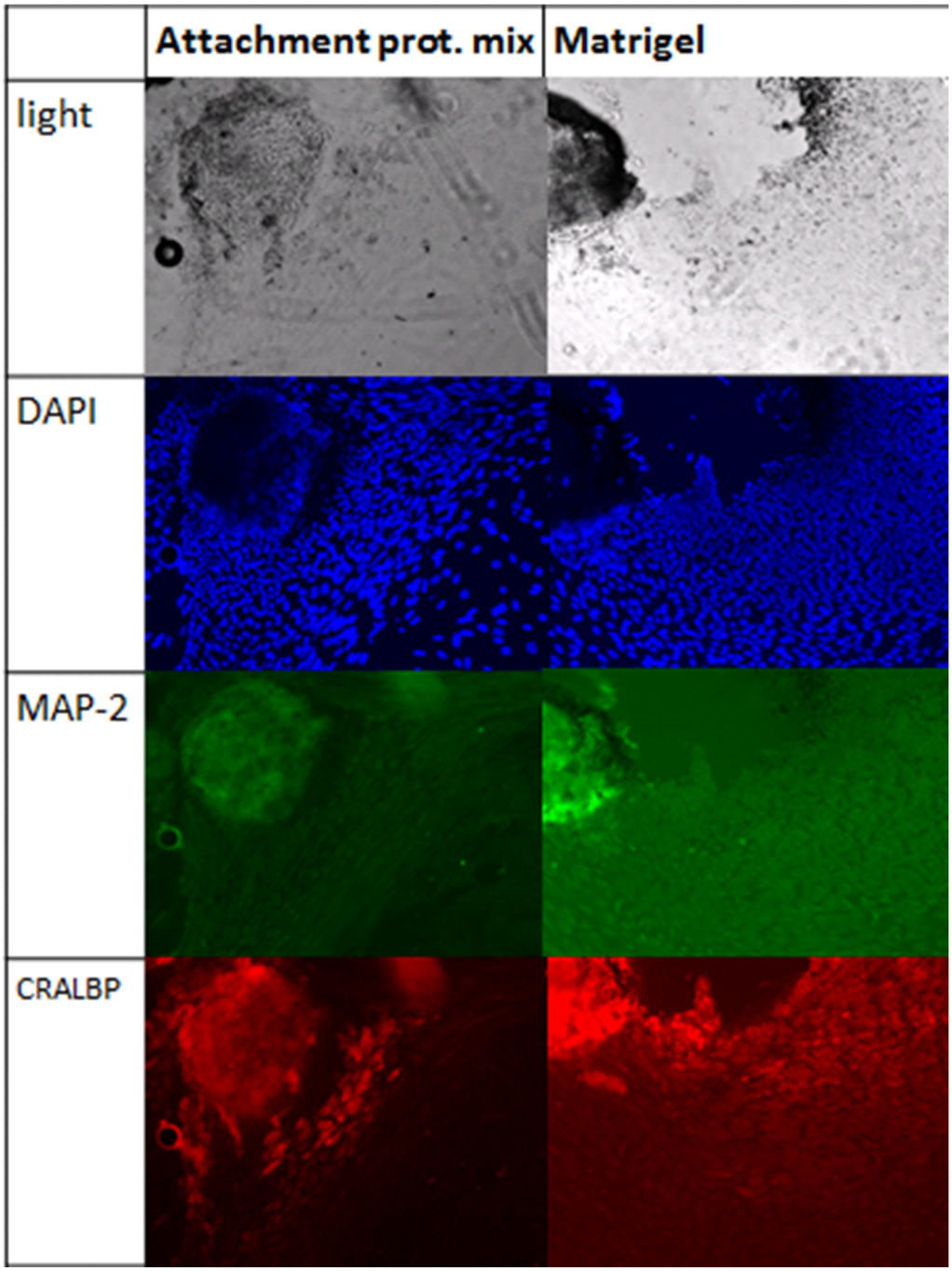
With PAX-6 we saw a good signal with both surfaces. With the cell attachment protein mixture the signal is more in the nuclei where it should be with this gene marker.

Bestrophin is expressed in the basolateral membrane of RPE cells, as cited before. With cell attachment protein mixture we saw a signal throughout the membrane of the cobblestone like cells. In cells with more of an oval morphology the signal is also in the nuclei. With matrigel, the growth had been more rapid and morphology was more like desired RPE like. Also signal with Bestrophin was more in the membranes where it should be.

Picture 1, PAX-6 and Bestrophin, day 9



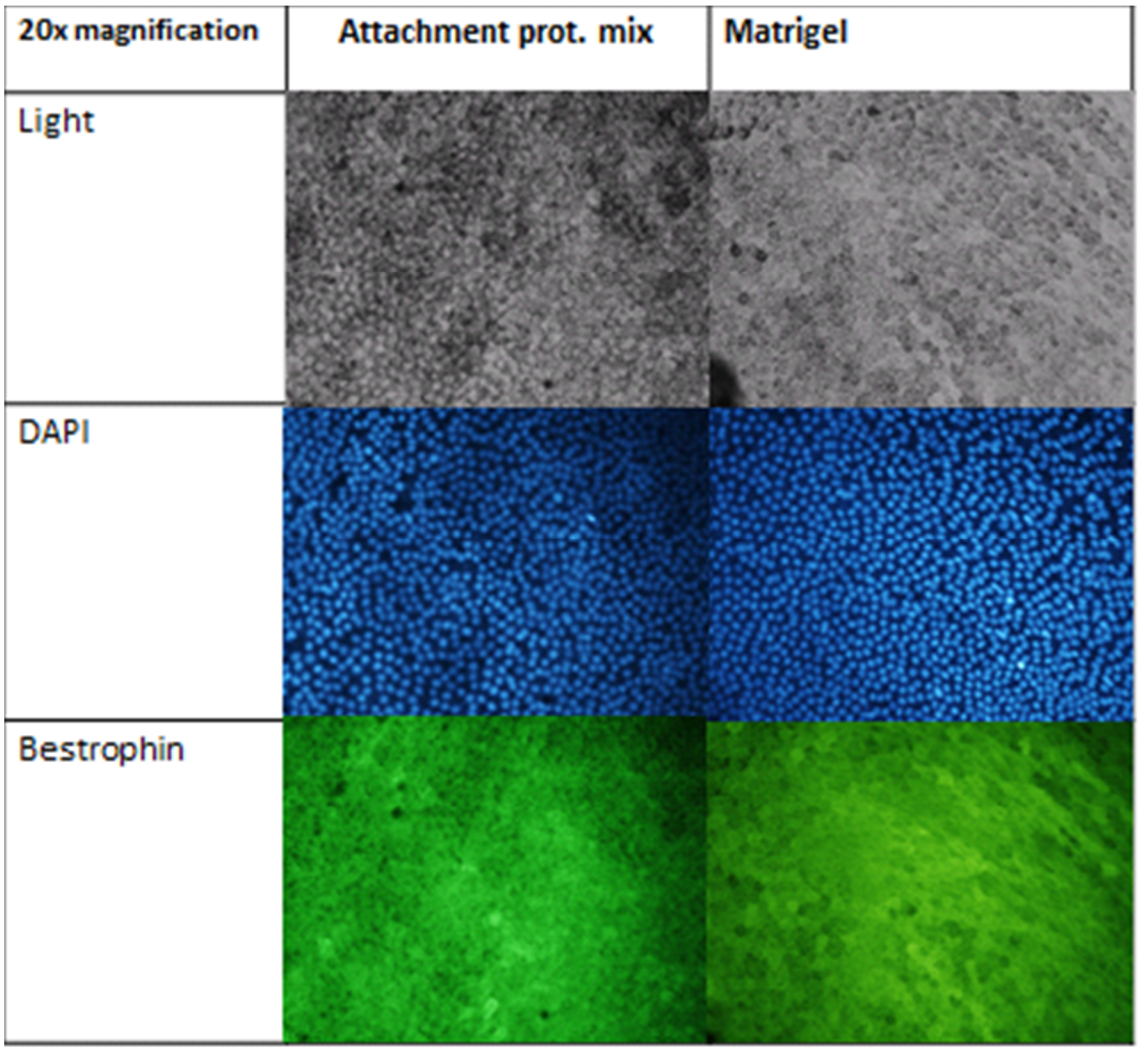
Picture 2, MAP-2 and CRALBP, day 9



Again as in the previous staining, nuclei are identified with DAPI. With MAP-2 antibody, some signal from the more oval cells was seen with cell attachment protein mixture. Overall, the signal was weak and did not strengthen in any part of the cells in particular. With matrigel, no clear signal was seen.

With CRALBP antibody, staining was stronger in cells that already had a resemblance to mature RPE cells. The signal faded towards the edges of the cell layer.

Immunofluorescence 2, day 56



A good signal from the cell membranes with Bestrophin was detected equally with both surfaces. At this point of culturing, the cells had a good amount of pigment and RPE cell like morphology. The signal was also a lot stronger from the membranes compared to the staining done in earlier timepoints.

CRALB was negative throughout the layer. This is contradictory to the results in the earlier stage. No explanation was found to this matter.

4.DISCUSSION

The most distinguishable difference comparing the three surfaces was the inability of Puramatrix to support cell attachment and sustain cell survival in comparison with the two other surfaces. Matrigel was a good growth booster but there was also rapid growth to directions we did not desire. Growth with the protein mixture, growth was not as rapid but more purely RPE-like. Difficulties with cell dissociation complicated monitoring.

4.1 hESC culturing

hES cell culturing was done with regea's standardized protocol. Culturing medium used in this protocol was always the same. The cells used in the experiments were from the same cell line. Spontaneous differentiation at Regea before our experiments causes some variation in cell age and type. This is not a factor that could not be controlled in this study.

4.2 Cell dissociation

Problems with dissociation might have been due to the differentiation rate of the original cell material. The cells from Regea might have been too differentiated and thus extracellular matrices already developed too firm for our techniques.

Cutting the cells

After testing various non-mechanical techniques, we evaluated that the best way is to cut the black areas of the cell clusters and collect them with a pipette. With this method an one cell suspension was not attained, but the least harm was done to the cells. After cutting, three dark cell clusters were plated per well. Each well contained ocularly the same type of clusters. This method is not a very precise one considering the amount of cells in each well. Also because the percentage of dark area was estimated ocularly, the results from this experiment will be more of an approximation of the cell culture surfaces.

4.3 Coating

With Matrigel and cell attachment protein mixture the platings were successful. The culture surface lay on the bottom of the surface. The amount of attached coating material or quality in the beginning of the experiments was only validated ocularly. This has caused some variation between experiments. Puramatrix did not form an even surface on the bottom of the well. Instead, it created an uneven three-dimensional matrix. This caused difficulties in imaging. The goal was to grow cells as a compact monolayer, this of course made it impossible.

4.4 Plating

Numbers of cells plated were evaluated ocularly. This was in consequence of the difficulties encountered with cell dissociation and could have an effect to the results.

4.5 Monitoring of the cells

Light microscopy

The most distinguishable finding comparing the three surfaces was the inability of Puramatrix to support cell attachment and sustain cell survival in comparison with the two other surfaces. With Puramatrix, there was almost no attachment or growth at all. Possible reasons for this was the three dimensional matrix that Puramatrix formed, this of course this cannot be the only reason for poor growth. The main problem in this case is clearly the issue with attachment. With no attachment, there is no layer to provide needed assistance.

The experiment displays well the significance of the right culture surface. With Matrigel the growth was rapid but Matrigel being a powerful booster for growth, there was also rapid growth to directions we did not desire. With the protein mixture, assumed to contain the correct proteins for RPE growth, the growth was slightly slower, but the result seemed more pure than with other surfaces.

With Live/Dead kit the results were as expected on ground of the light microscopy. Matrigel and the cell attachment protein mixture were equally good to sustain RPE cell survival based on our study.

4.6 Immunofluorescence

There were difficulties with the first immunofluorescence staining. This was because the well plates chosen were too small to be imaged properly with the microscope used. Due to this the experiment will not be discussed here.

With anti-PAX-6 staining we had good signals overall with Matrigel and protein mixture. The cells that had been growing on matrigel were closer to the desired cobblestone like morphology. This is understandable due to PAX-6's role in many phases of eye and RPE development [11].

Stainings with anti-Bestrophin also gave good results. Bestrophin is a rather specific marker for RPE cells, and good expression was detected with both surfaces. The signal appeared stronger in the cell attachment protein mixture under ocular inspection.

MAP-2 was chosen to differentiate cells of neurodermal origin from RPE cells. Some signal was seen in cells that had more of an oval morphology with both the protein mixture and Matrigel. Thus, it could be concluded that the cell material was not purely RPE cells. Biggest causes to this were difficulties in cell dissociation, and impurity of original material.

With CRALBP antibody a stronger signal was seen in the cells with RPE cell like morphology and good pigmentation. Overall, in this study it was seen that pigmentation started to spread from the clusters towards the outer regions.

5.CONCLUSION

The biggest challenge encountered was how to gently and effectively dissociate the cells to one cell suspension. None of the methods used offered satisfactory results. In order to achieve pure RPE cell masses, a lot of focus has to be designated to this step.

The best cell culture surface examined in this study was the mix of attachment proteins. The growth was not as fast as with matrigel but the growth was more parallel towards RPE cells. Matrigel might be better in conditions with a more pure cell colony to begin with.
This needs more research. Puramatrix has three dimensional state when plated. This was a big problem when plating the cells to well plates. Puramatrix might offer a more *in vivo* like conditions due to the three dimensional gel composition. A method worth trying for would be coating Puramatrix with basement membrane proteins. This could help cells to attach in an early stage of growth. This needs more research.

The Live/Dead kit offered an easy way of monitoring cell vitality. This will be used in further studies also.
Based on immunofluorescence analyses the results were promising. After more focus on cell dissociation techniques, and overcoming minor difficulties in plating phase, I am confident that growing RPE cells as a homogenous monolayer is possible. The main aim of this study was to test the above variables in culturing RPE cells. Although there were difficulties, valuable information was achieved to head us towards studies with more specific and numerous data.

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