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Change of Morphological and Functional Characteristics of Retinal Pigment Epithelium Cells during Cultivation of Retinal Pigment Epithelium-Choroid Perfusion Tissue Culture

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Key Words
Perfusion tissue culture · Retinal pigment epithelium · Apoptosis · Wound healing · Vascular endothelial growth factor

Abstract
Aims: To evaluate the changes of morphological and functional characteristics of the retinal pigment epithelium (RPE)-choroid perfusion culture during cultivation. ***Methods:*** Porcine RPE-choroid tissue was cultivated in a perfusion tissue culture system. After the indicated times, histology, immunolocalization of collagen IV and von Willebrand factor, RPE cell viability with calcein-AM, TUNEL assay and occludin immunolocalization of RPE cells were examined. The tissue was treated with selective RPE treatment laser after different time periods and the wound healing response was characterized. Vascular endothelial growth factor secretion was measured by enzyme-linked immunosorbent assay. ***Results:*** On day 8, prominent morphological degenerative changes of RPE cells were observed in histology. According to the immunohistochemistry for collagen IV, the Bruch's membrane did not display any obvious decomposition until day 8. Von Willebrand factor staining decreased during cultivation, especially at the choriocapillaris. Calcein-AM staining and TUNEL assay displayed the increase of apoptotic changes in only a minority of the cells on day 4, but in many cells on day 8. Occludin delocalization was observed on day 8. Selective RPE treatment laser-produced wounds were completely closed by monolayer RPE when wounded on fresh and 3-day-old cultures, but not when wounded on 6-day-old cultures. Vascular endothelial growth factor secretion was stable between days 2 and 5, but increased after that. ***Conclusion:*** Under the stated culture perfusion conditions, porcine RPE-choroid tissue was suitable for experimentation up to 5 days of maintenance.

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Introduction

Many chorioretinal diseases are retinal pigment epithelium (RPE)-related disorders, e.g. macular degeneration. Therefore, most of the treatments (e.g. laser treatments, intravitreal injections) try to influence RPE cells. For the further understanding of the healing mechanisms or for the establishment of novel treatments, a good experimental model with RPE would be helpful.

The perfusion tissue culture system of Minuth et al. [1] might be a suitable model for these kinds of studies. This system was introduced to cultivate tissues in an organotypical environment. The tissue is constantly perfused with medium without manual replacement, guaranteeing a consistent supply of nutrients and drainage of metabolites. It has already been used in ophthalmology, e.g. to examine tissue response after conventional continuous wave laser treatment or to test the safety of indocyanine green [2–5], as well as in several other fields [6, 7]. A re-

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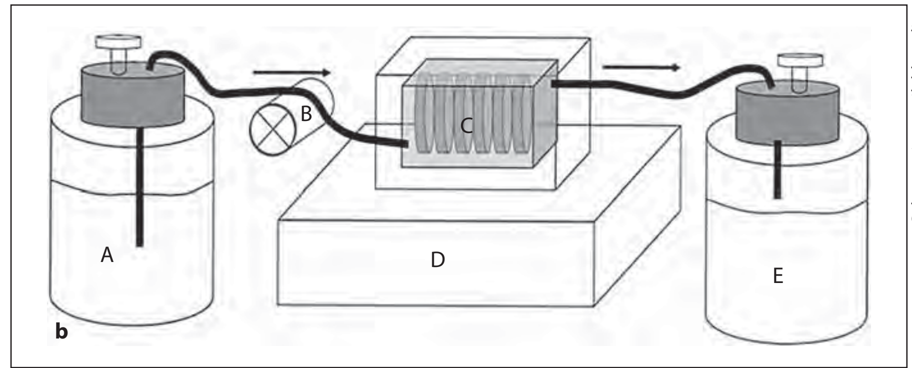


Fig. 1. Picture of the tissue culture container (**a**), and the schematic drawing of the whole system of the perfusion culture system (**b**). **a** The view from the upper side of the container with 6 tissue rings and the culture medium inside. **b** Fresh medium is stored in bottle A. The medium is pumped out from the bottle by the function of the pump B and flows in the direction of the arrow, with a flow speed 2.5 ml/h. The medium flows into the culture container C, which is on the heating plate D. After the medium perfuses the tissues, it runs out from the opposite side of the container and flows into the bottle E.

cent publication characterized RPE and retina in perfusion organ culture on a morphological level, using light and electron microscopy [4]. They compared the histological characteristics between perfusion and static cultures of retina-RPE-choroid, and proved the superiority of perfusion culture in morphological maintenance of the tissue. However, they mainly focused on retinal tissue, and the characteristics of RPE-choroid tissue have still not been investigated in-depth, even though the functional aspect of RPE is one of the major interests.

Organ culture resembles the in vivo tissue situation to a higher degree than cell culture models, but a major problem of the organ culture is a faster degeneration of the tissue. Although the perfusion system is superior to the static culture in preserving the tissue, this problem is still inevitable. Perfusion organ culture offers new options, but in order to obtain reliable results, the basic behavior of the tissue has to be evaluated. In this study, we focused on the morphological and functional characteristics of RPE cells in RPE-choroid tissue culture in the perfusion culture system, and evaluated the optimal time period of cultivation for experimental settings.

Materials and Methods

Organ Culture

For the preparation of RPE-choroid sheets, freshly slaughtered pig eyes which were kept under a cool condition until use were used 3–5 h after enucleation. Eyes were cleaned of adjacent tissue and immersed briefly in antiseptic solution. The anterior part of the eye was removed, as well as the lens and vitreous. Retina-RPE-choroid sheets were separated from the sclera using forceps and scissors. The retina was gently removed, leaving the RPE untouched. Prepared tissue was fixed between the lower and upper part of a fixation ring (diameter of the exposed tissue preparation is 9 mm), excess tissue was removed and the ring was placed in a culture container (Minucells and Minutissue, Bad Abbach, Germany). One eye per fixation ring was used. In the culture chamber (fig. 1a), the 6 tissue rings are set in parallel. The chamber was placed on a heating plate as previously described [2]. The medium was a mixture of equal amount of Dulbecco's modified Eagle's medium (DMEM; PAA, Cölbe, Germany) and Ham F12 medium (PAA) which was supplemented with penicillin/streptomycin (1%), L-glutamine, HEPES (25 mM), sodium pyruvate (110 mg/ml) and 10% porcine serum (PAA). The medium enters the container at its front end, passes between the tissue carriers and leaves the container at the rear side at a velocity of 2.5 ml/h. The schematic drawing of the perfusion culture system is described in figure 1b.

Characterization by Histology

On days 1, 4 and 8, the RPE-choroid tissue was taken out of the chamber and fixed in 4% formaldehyde. Following the initial fixation, the samples were dehydrated in a series of alcohols, removed from the ring, embedded in paraffin, and precisely oriented cross sections were obtained. Semi-thin sections were stained with hematoxylin-eosin and examined with light microscope (Carl Zeiss, Jena, Germany).

Characterization by Collagen IV Immunohistochemistry

Immunohistochemistry for collagen IV was performed in order to characterize the morphological preservation of Bruch's membrane in the RPE-choroid culture on days 1, 4 and 8. The tissue was fixed and embedded as described above. Semi-thin sections were processed with anti-collagen IV antibody (1:100, Abcam, Cambridge, UK). Immunoenzymatic staining was performed by the streptavidin-biotin (LSAB) method using the Dako LSAB™ kit (Dako, Glostrup, Denmark). Counterstaining was performed with hematoxylin. The section was examined with a light microscope.

Characterization by von Willebrand Factor Immunohistochemistry

Immunohistochemistry for von Willebrand Factor (vWF), one of the endothelial cell markers, was performed to characterize the choroidal endothelial cells in the RPE-choroid culture on days 1, 4 and 8. The tissue was fixed and embedded as described above. Semi-thin sections were processed with anti-vWF antibody (Dako). Immunoenzymatic staining was performed by the streptavidin-biotin (LSAB) method using the Dako LSAB™ kit (Dako). Counterstaining was performed with hematoxylin. The section was examined with a light microscope.

Characterization by Calcein-AM Staining

On days 0, 2, 4 and 8, the tissue was incubated with calcein-AM (AnaSpec, Inc., San Jose, Calif., USA) for 30 min, washed with PBS, and the RPE cells were observed using a fluorescence microscope, with λex/λem = 497/517 nm (Carl Zeiss). Calcein-AM is widely used as a membrane permeability marker that readily passes through the cell membrane of living cells. After non-fluorescent calcein-AM permeates into the cytoplasm, it is hydrolyzed by endogenous esterase into the highly green fluorescent calcein, which retains in cytoplasm. Therefore, it can be utilized to distinguish live and dead cells through the cytoplasm green fluorescence intensity. Furthermore, calcein-AM provides morphological evidence of apoptotic changes such as chromatin condensation and segregation in blebs, together with functional information about plasma membrane integrity and intracellular esterase activity [8, 9].

Characterization of Tissue Apoptosis by TUNEL Assay

RPE-choroid tissue apoptosis was detected using the ApopTag Peroxidase in situ apoptosis detection kit (Millipore, Billerica, Mass., USA). This assay detects the apoptotic cells by labeling and detecting DNA strand breaks by the TUNEL method. The RPE-choroid culture on days 1, 4 and 8 was fixed and embedded as described above and semi-thin section was used for the assay. The apoptotic cells are identified by the brown-stained nucleus. In order to avoid the disturbance in the analysis by the pigment in RPE cells and choroid, the semi-thin section was bleached by bathing in 3% hydroxyperoxide before the assay. Counterstaining was performed with hematoxylin. The section was examined with a light microscope.

Characterization by Occludin Immunostaining in RPE Cells

On days 0, 4 and 8, the RPE cells in RPE-choroid tissue culture were assessed for occludin immunolocalization. The tissue was washed with cold PBS and fixed with 4% formaldehyde for 15 min on ice. After washing with PBS three times, cells were permeabilized with 5% Triton-X 100 for 10 min at room temperature. The tissue was blocked with 1% BSA in PBS for 20 min in room temperature and incubated with the primary antibody (rabbit anti-goat occludin antibody, diluted 1:100 in 2.5% Triton-X 100 and 1% BSA containing PBS; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at 4°C overnight. The tissue was washed with PBS three times and incubated with a second antibody conjugated with TRITC. After washing with PBS three times, the tissue was removed from the ring, mounted onto a glass slide, covered with mounting solution and observed with a fluorescence microscope (λex/λem = 554/570 nm).

Characterization by Wound-Healing Response

The wound-healing capacity of the RPE in RPE-choroid tissue in perfusion culture was characterized at various time points of the cultivation. The selective retina therapy (SRT) laser was used to make RPE cell defects of identical size, as described before [10, 11]. The SRT laser damages only the RPE while sparing the surrounding tissues [12]; it is an Nd:YLF laser with 527 nm wavelength, 1.7 μs of pulse duration, 100 Hz of repetition rate, 30 pulses per irradiation. The diameter of the spot is 200 μm. The laser is coupled to a slit lamp, and therefore the laser could be applied to the RPE cells of the tissue in medium through a mirror set on the slit lamp. On days 0, 3 and 6 after the beginning of the cultivation of the tissue culture, SRT laser beams (300 mJ/cm^2) were applied on the RPE cells in the tissue culture, to make a round wound (diameter = 200 μm). For the laser irradiation, the tissue rings were carefully removed from the perfusion culture chamber and each ring was replaced in a well of conventional 6-well normal culture plate, in which 2.5 ml of the phenol red-free medium (warmed to 37°C) was added beforehand. The control rings were also replaced in this plate and were carried as the treated cultures. Immediately following the laser irradiation, the rings were placed back to the perfusion culture chamber. After treatment, the tissue was cultured further for up to 4 days. The wounds were compared morphologically 2 and 4 days after wounding. To visualize the cell shape, F-actin was stained. F-actin is located directly inside the cell membrane and its staining pattern is intimately related to cell morphology. For F-actin staining, the cells were incubated with FITC-conjugated phalloidin (diluted 1:500 in PBS; Sigma, St. Louis, Mo., USA) after permeabilization by Triton-X 100. After washing with PBS three times, the tissues were removed from the ring and mounted on a glass slide, covered with mounting solution and cover glass. They were observed with a fluorescence microscope (λex/λem = 492/517 nm).

Characterization by Vascular Endothelial Growth Factor (VEGF) Secretion

For the detection of VEGF secretion from the tissue, the supernatant of the perfusion organ culture was collected from the output line for 1 h each at the time point of 2 h (day 0), up to day 8. The VEGF content was measured by a VEGF-ELISA kit (R&D Systems, Minneapolis, Minn., USA) following the manufacturer's instructions.

Statistics

Every experiment was repeated at least three times and repeatability was confirmed. All values were tested for normal distribution and statistical evaluations were performed with paired Student's t test. $p < 0.05$ was considered significant.

Results

Morphology

In figure 2, the histological findings of RPE cells from days 1, 4 and 8 in RPE-choroid perfusion tissue culture can be seen. On day 1, RPE cells display their typical morphological polar organization with nuclei in the basal and

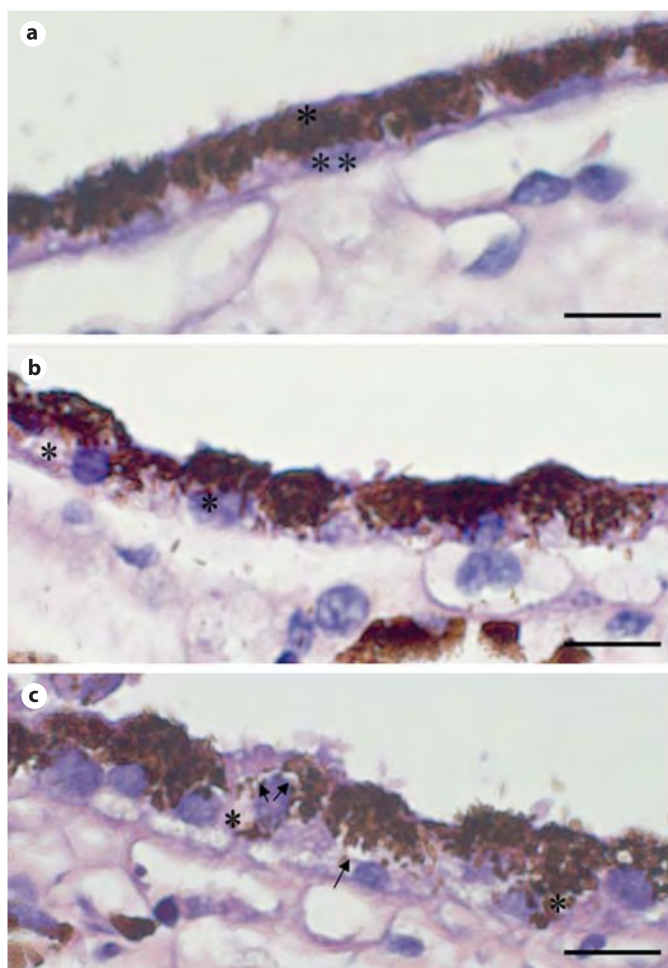


Fig. 2. Hematoxylin-eosin staining of RPE-choroid in tissue culture on day 1 (**a**), day 4 (**b**) and day 8 (**c**). In day-1 culture, RPE cells demonstrate a polar organization with melanosomes in the apical side (asterisk) and nuclei in the basal side (double asterisk) (**a**). In day-5 culture, this polarization is still preserved, although some cells show a slight dome-shaped change (asterisk) (**b**). In day-8 culture, the morphological polarization is significantly lost (pigment delocalization; asterisk) and the prominent changes such as dome-shaped change and vacuolization (arrows) could be observed (**c**). Bar = 10 μm.

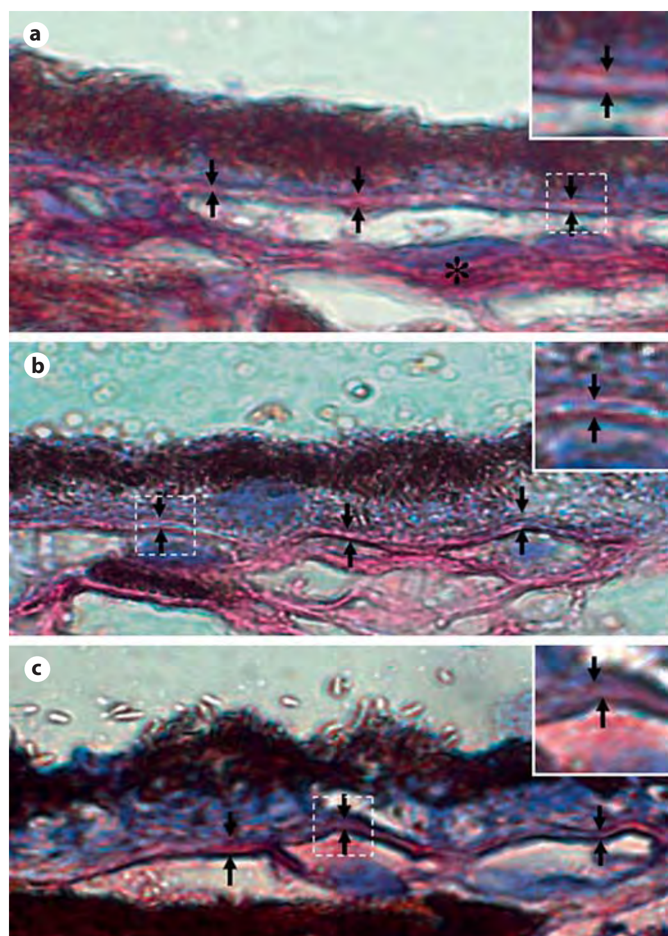


Fig. 3. Immunohistochemistry for collagen IV in RPE-choroid tissue culture on day 1 (**a**), day 4 (**b**) and day 8 (**c**). Collagen IV is stained red (see online version). Counterstaining (nucleus staining) was performed with hematoxylin. Collagen IV located in Bruch's membrane (**a–c**; arrows) and the basement membrane of choroidal vascular endothelium (**a**; asterisk). Throughout the cultivation time until day 8, collagen IV was well detected in both Bruch's membrane and the basement membrane of choroidal endothelium. In Bruch's membrane, two different layers of collagen IV are observed; one is at the RPE basement membrane layer, another is at the vascular endothelial cell basement membrane layer, which can be observed as double lines (**a–c**; arrows). The inset in each picture shows the magnification of the Bruch's membrane.

melanosomes in the apical cell compartment (fig. 2a). On day 5, this morphological polarity is still preserved, although the cell's shape appears slightly 'dome-shaped' in some cells (fig. 2b). On day 8, many of the RPE cells show obvious degenerative changes, e.g. loss of morphological polarity, dome-shape and vacuolization around the cell nucleus (fig. 2c).

Expression of Collagen IV

In order to characterize the Bruch's membrane's morphological preservation, the immunolocalization of collagen IV was assessed by immunohistochemistry. Collagen IV is detected in Bruch's membrane and the basement of choroidal endothelium (fig. 3a–c). Collagen IV expression in Bruch's membrane showed no significant change throughout the whole culture period. In

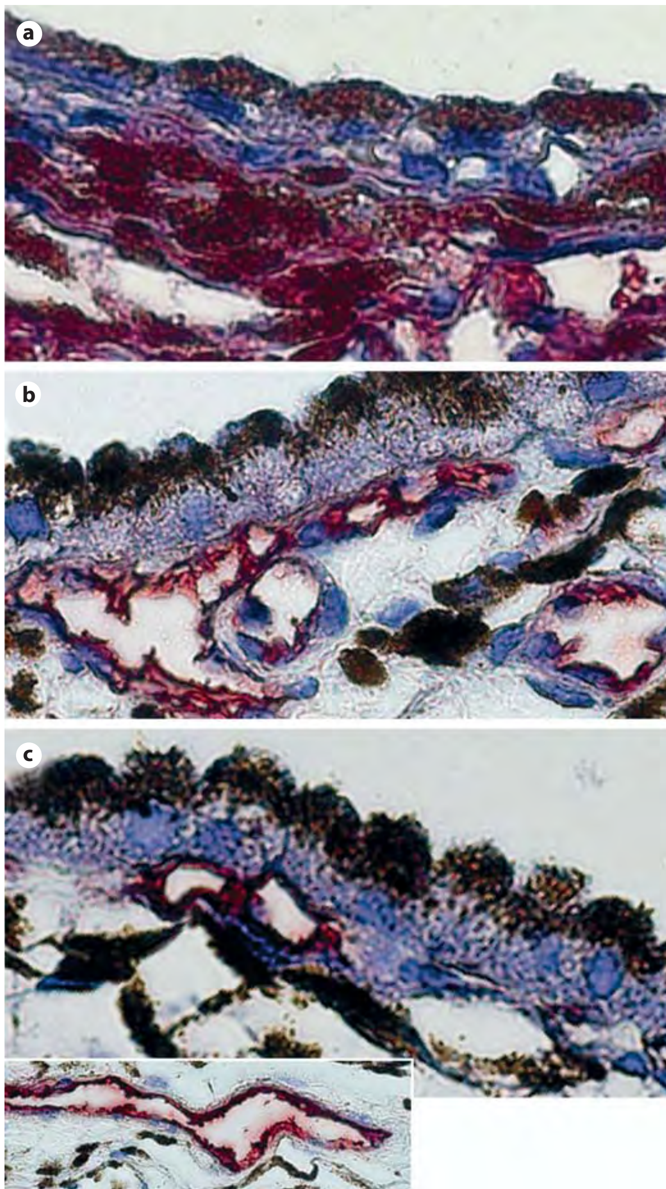


Fig. 4. Immunohistochemistry for vWF in RPE-choroid tissue culture on day 1 (**a**), day 4 (**b**) and day 8 (**c**). vWF is stained red (see online version). Counterstaining (nucleus staining) was performed with hematoxylin. On day 1, vWF was well stained in whole choroid (**a**). On day 4, the staining is less than on day 1, especially at the choriocapillaris (**b**). On day 8, the decrease of the staining at the choriocapillaris is prominent, and main vessels are still stained (inset) (**c**).

Bruch's membrane, the double lines staining can be observed (fig. 3a–c, arrows, insets), which is considered to be at the basement membrane of the RPE and at the basement membrane of the choriocapillaris, as known in vivo.

Expression of von Willebrand Factor

On day 1, vWF was detected in the vascular endothelial cells in whole choroid (fig. 4a). During the cultivation, this staining decreased especially at the choriocapillaris, with slightly less staining on day 4 (fig. 4b), and prominently reduced staining on day 8 (fig. 4c). The staining at the main vessels was maintained longer than at the choriocapillaris (fig. 4c, inset).

Calcein-AM Staining of RPE Cells

Figure 5 shows fluorescence microscopy pictures of RPE cells on RPE-choroid perfusion tissue culture treated with calcein-AM. On day 2, the RPE cells exhibit a typical green fluorescence staining indicating normal living cells (fig. 5a), which is similar to the freshly prepared cells (data not shown). The staining pattern complies with previous observation, with equally distributed cytoplasm intensity and a slightly higher intensity found in the nucleus [8]. On day 4, some cells begin to show an increased and sharp-edged signal from the nucleus (fig. 5b). This complies with the previous observation of chromatin condensation in apoptotic change [8]. On day 8, the cells show a variety of staining patterns. Some exhibit an equally high intensity staining of whole cell body accompanying a slight shrinkage of the cell (fig. 5c), which is one of the forms of apoptotic changes, and the others exhibit a high nucleus intensity with a slight decrease of cytoplasmic staining, causing dark areas in the culture (fig. 5d), indicating a slight permeability increase of the cell membrane. Calcein-AM negative, which is called 'blind points' as shown in figure 5e, is obtained as a typical finding for necrotic cells, and is an indication of a long postmortem time or unfavorable postmortem conditions (fig. 5e). Cultured in appropriate conditions, the tissue exhibits almost no 'blind points' during the first 8 days of cultivation.

Tissue Apoptosis

We performed the detection of apoptotic cells in RPE-choroid tissue sections with the Apoptag test, which utilizes the TUNEL assay method. With this method, the nucleus of the apoptotic cells is stained brown. Day-1 culture has almost no apoptotic RPE cells (fig. 6a), occasionally on day 5 (fig. 6b), and prominently on day 8 (fig. 6c). This finding is consistent with the tendency observed in the calcein assay. The TUNEL-positive RPE cells tend to detach from Bruch's membrane during the process of making the semi-thin section. In choroid, the number of the apoptotic cells increase over time (fig. 6a–c). Choroidal apoptosis seems to begin earlier than RPE, since while

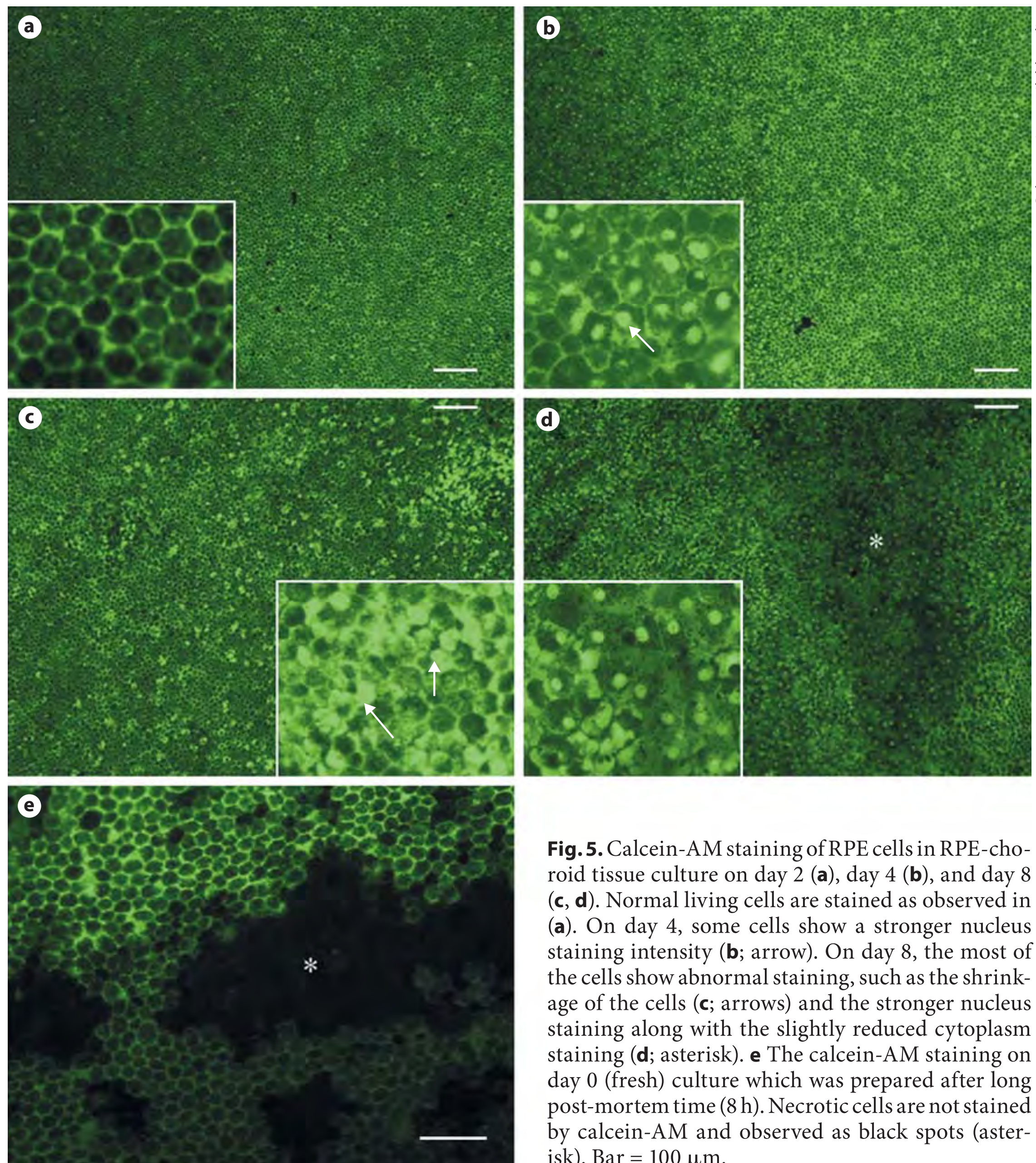


Fig. 5. Calcein-AM staining of RPE cells in RPE-choroid tissue culture on day 2 (**a**), day 4 (**b**), and day 8 (**c**, **d**). Normal living cells are stained as observed in (**a**). On day 4, some cells show a stronger nucleus staining intensity (**b**; arrow). On day 8, the most of the cells show abnormal staining, such as the shrinkage of the cells (**c**; arrows) and the stronger nucleus staining along with the slightly reduced cytoplasm staining (**d**; asterisk). **e** The calcein-AM staining on day 0 (fresh) culture which was prepared after long post-mortem time (8 h). Necrotic cells are not stained by calcein-AM and observed as black spots (asterisk). Bar = 100 μm.

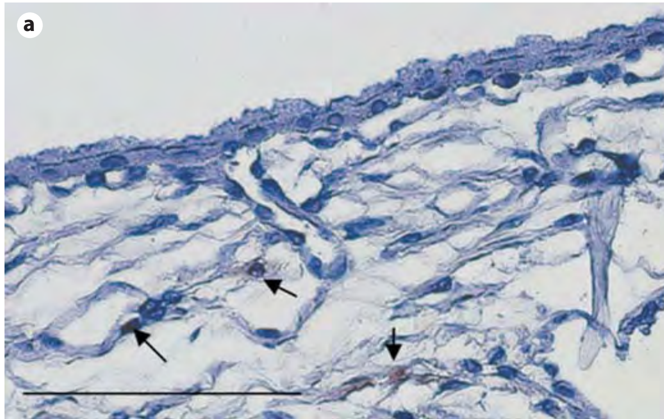
no RPE cells are positive, a few cells are already positive in choroid in day-1 culture (fig. 6a).

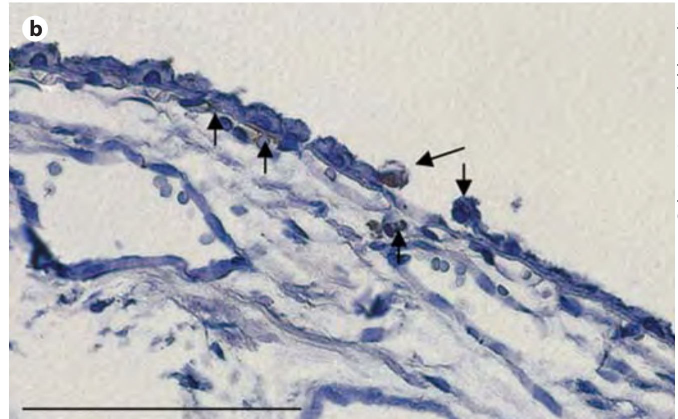
Occludin Localization

The localization of occludin in RPE cells on day 0 and day 4 are similar in that occludin creates linear staining near the cell border (fig. 7a, b). However, on day 8, less clear linear staining at the cell border and more staining in the cytoplasm can be observed (fig. 7c, d).

Wound Healing Response

The SRT laser produces a round-shaped, equally sized defect specifically on the RPE (fig. 8a). The size corresponds to the size of the laser spot. We compared the response of RPE cells after wounding by SRT laser applied at different days of cultivation. In order to visualize the RPE wound healing process, F-actin staining was conducted. When the wound was produced on fresh (day 0) or 3-day-old cultures, active RPE migration can be ob-





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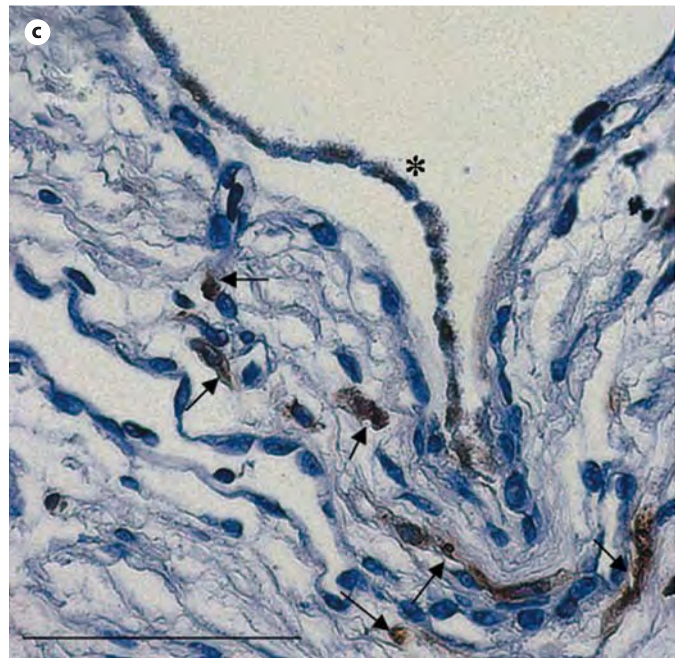
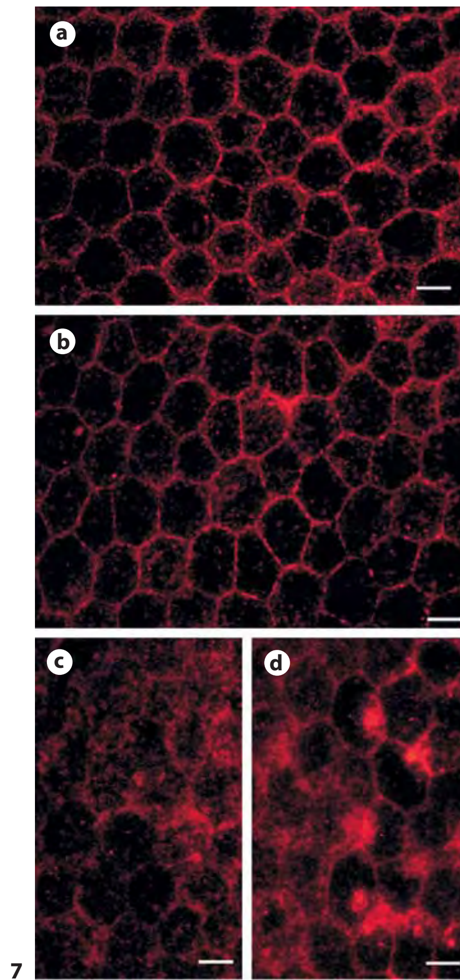


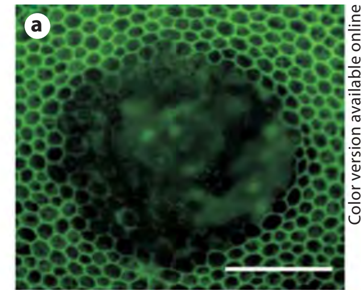
Fig. 6. Apoptosis detection using the Apoptag test. The RPE-choroid tissue cultures on day 1 (**a**), day 4 (**b**) and day 8 (**c**) were examined. The nucleus of apoptotic cell is stained brown (see online version). Counterstaining (nucleus staining) was performed with hematoxylin. On day 1, no positive staining is detected in RPE cell, while a few choroidal cells are positive (arrows) (**a**). On day 5, a few RPE cells and some choroidal cells are positive (arrows) (**b**). On day 8, marked increase of the positive cells in RPE (asterisk) and choroid (arrows) (**c**). Bar = 100 μm.

served 2 days after the wounding (fig. 8b, d, asterisks); that is, cells from the edge of the wound exhibit an elongated shape, directed toward the center of the defect. At most of the wounds produced on 6-day-old cultures, on the other hand, no active RPE cell migration can be observed, in which the edge of the cells at the wound front displays a clear linear staining without any extension, although these cells are slightly enlarged (fig. 8f). In a few cultures, fibroblastic change is observed (fig. 8h). Four days after wounding, the wounds produced on fresh and 3-day-old cultures are completely closed by monolayer RPE (fig. 8c, e). Comparing the healing response between fresh (fig. 8c) and 3-day-old cultures (fig. 8e), the wound produced on 3-day-old cultures was covered by bigger cells and diminished number of cells. The wound produced on 6-day-old cultures displayed no normal wound closure by a monolayer RPE cells, either with the lack of wound closure (fig. 8g), or covered with the over-layered fibroblastic tissue (fig. 8i).

VEGF Secretion

The secretion of VEGF into the supernatant of the perfusion tissue culture is shown in figure 9. In the second hour after onset of cultivation, no VEGF can be detected in the supernatant. After 1 day, high concentrations of VEGF (617.3 ± 123.8 pg/ml) can be detected. On day 2,





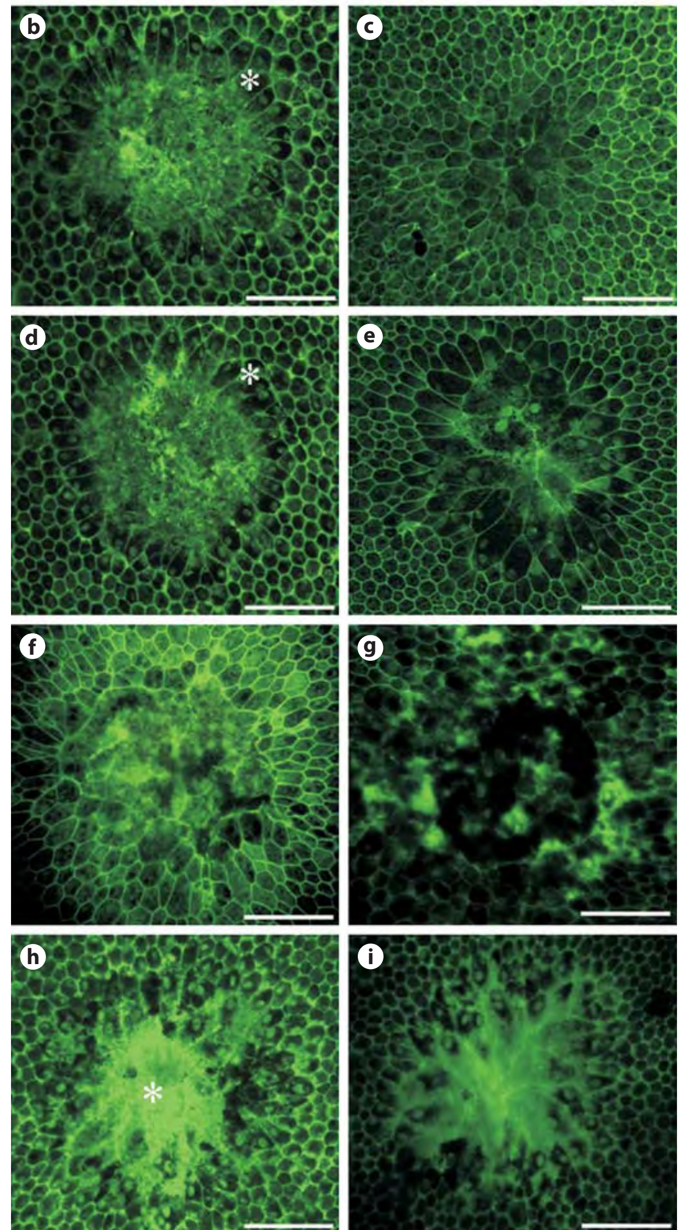


Fig. 7. Occludin immunostaining of RPE cells in RPE-choroid tissue culture on day 0 (freshly prepared) (**a**), day 4 (**b**) and day 8 (**c**, **d**). On day 0 and day 4, the clear linear staining at the cell border can be observed (**a**, **b**). On day 8, on the other hand, this linear staining becomes unclear (**c**, **d**). Bar = 10 μm.

Fig. 8. Wound healing response of RPE cells in RPE-choroid perfusion tissue culture after SRT laser treatment. SRT laser produces the round shaped wound of RPE. Culture immediately after wounding (**a**). The wound was produced either on day 0 (fresh) (**b**, **c**), 3 (**d**, **e**) or 6 (**f–i**) of cultivation. Actin staining using FITC-phalloidin was performed either 2 days (**b**, **d**, **f**, **h**) or 4 days (**c**, **e**, **g**, **i**) after wounding. At the wounds produced on fresh (day 0) and 3-day-old cultures, the RPE cells show an active migration 2 days after the wounding (**b**, **d**; asterisk), while at the most of the wounds produced on 6-day-old cultures, no active RPE cell migration can be observed 2 days after wounding (**f**). In a few cultures around the wounds produced on 6-day-old cultures, a multilayered fibroblastic tissue is observed in 2 days (**h**; asterisk). Four days after wounding, the wounds produced on fresh (day 0) and 3-day-old cultures are covered by monolayer RPE cells (**c**, **e**). The wounds produced on day 6 show no normal wound closure by monolayer RPE cells, either with incomplete closure (**g**) or covered by overlayered fibroblastic cells (**i**). Bar = 100 μm.

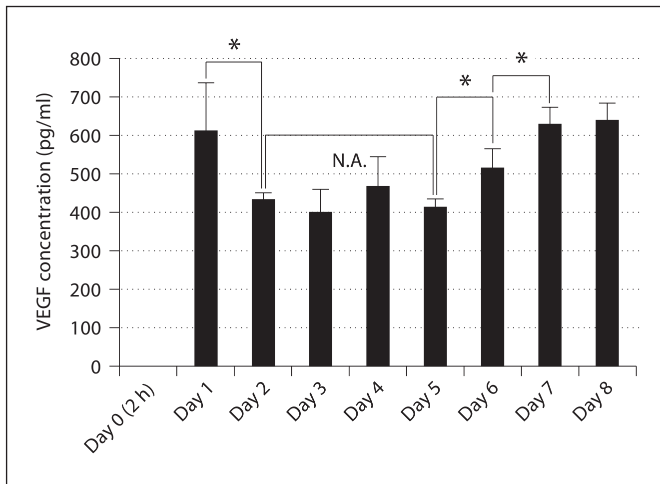


Fig. 9. VEGF secretion from RPE-choroid tissue in perfusion culture. The supernatant of perfusion tissue culture was collected for 1 h from day 0 to day 8 of cultivation, and VEGF concentration was measured with a VEGF ELISA-kit. VEGF secretion is observed from day 1. The secretion decreases on day 2 and this level is preserved until day 5. The VEGF level begins to increase from day 6 (* $p < 0.05$).

the level decreases (437.9 ± 16.9 pg/ml), and this level is stable until day 5. On day 6, the level starts to increase (520 ± 48.2 pg/ml, $p < 0.05$), and the further increase after day 7 was seen (day 7 and day 8; 665.3 ± 40.5 and 677.3 ± 48.0 pg/ml, respectively; $p < 0.05$).

Discussion

Studies on RPE-choroid static tissue culture were done mainly before 1990 [13–15], focusing on the morphological maintenance of the RPE cell monolayer using light or electron microscopy. Apart from some information on retinal histological aspects, not much data has been obtained about perfusion tissue culture in ophthalmologic research so far [2, 4]. Here, we assessed viability, morphology and functionality of RPE cells in perfusion RPE-choroid culture and discuss the ideal time period for its use as an experimental model.

In the current study, the retina is absent. We have to mention that most retinas detach from the RPE layer during the cultivation. The detachment can be explained by the fact that the RPE and retina are not connected by tissue [16] and that postmortem metabolic changes in the subretinal space cause a loss of hydrostatic and osmotic pressure difference [17–20]. There is also a loss of adhesion between the RPE and photoreceptor outer segments mediated by interphotoreceptor matrix (IPM), which is considered to be responsible for most of the retinal adhesiveness [21–23]. As described in these reports, retinal adhesion is a multifactorial process and difficult to be maintained after enucleation. This lack of adhesion is a point for improvement of the present organ culture system in the future. Despite the loss of attachment of the retina, co-cultivation with retina might be beneficial due to its secretion that could affect the RPE. On the other hand, the retina makes the entire tissue thicker and more difficult for nutrients to reach the RPE. Through the thickened and detached retina, not only medication but also some physical treatment, such as laser treatment, could be impossible. Moreover, since the retina is known to degenerate earlier than RPE [4], RPE has to bathe in the secretion mix from the degenerating retina. Due to these reasons, we proceeded with the study without retina. The preservation of RPE in perfusion tissue culture was morphologically evaluated before, assessed according to a subjective grading score [4]. This evaluation concluded that the RPE can be preserved at least for 10 days in the perfusion system, with a gradual degenerative change. In our study, we were able to confirm that RPE cells demonstrate a gradual degenerative change. On day 8, most of the cells showed some major morphological alterations. One of the typical findings is the polarity change, in which the pigments are no longer localized in apical side and the nucleus in basal side only. The cells become dome-shaped, and some cells exhibit vacuolization in the cytoplasm.

In order to obtain a well-preserved RPE cell monolayer, Bruch's membrane has to be well preserved as a RPE substrate. Collagen IV is one of the major components of Bruch's membrane, existing at the most inner and outer layers, that is, the basement membrane of RPE and the basement membrane of choriocapillaris. According to the immunohistochemistry for collagen IV, Bruch's membrane did not display obvious signs of decomposition throughout the whole cultivation period.

vWF is produced by the endothelial cells and is known as an endothelial cell marker. Immunohistochemistry revealed a decreased expression of vWF at the choriocapillaris. Very little staining at the choriocapillaris on day 8 (fig. 4c) suggests that the choroidal vascular endothelial cells of the choriocapillaris have little functionality on day 8 and the RPE-choroid interaction is significantly decreased at this time point.

As a marker of plasma membrane integrity, calcein-AM is widely used in order to distinguish living and dead

cells. This neutral vital dye is loaded and rapidly hydrolyzed by endogenous esterase into high negatively charged green fluorescent analogue. The nucleus-cytoplasm signal intensity ratio is approximately 3:1 [8]. Calcein-AM staining of the RPE cells demonstrated different staining patterns during the time course: an increase in the intensity of nuclear fluorescence and a decrease of the intensity of cytoplasm, and some equally stained small-cell staining. The stronger nuclear staining was observed occasionally already in the culture at day 4, and was obvious in day-8 culture. The equally stained small cells, which were observed in day-8 culture, seem to be a product of cell shrinkage.

Calcein-AM proved to be both specific and sensitive for the detection and tracking of apoptosis in attached cells by confocal laser microscopy [24]. The calcein-loaded cells undergoing apoptosis demonstrate a high-intensity and sharp-edged nuclear fluorescence due to chromatin condensation. The persistence of intracellular calcein is one of the most significant features of apoptosis. This is in contrast to necrosis, where defects of membrane integrity lead to calcein leakage out of the cell and the signal vanishes even in the presence of residual esterase activity [25]. Therefore, we consider that some apoptotic changes may occur in the RPE cells on day 4 of cultivation, and the rate of this change increases up to day 8, in which some cells have already shrunk without losing membrane integrity.

The TUNEL assay findings demonstrate an increase of the apoptotic changes over time and prominent apoptotic changes in day-8 RPE-choroid tissue culture. It seems that apoptotic change in choroidal cell begins earlier than in RPE cells. Concerning RPE cells, day-4 culture had a few positive (apoptotic) RPE cells and many cells in day-8 cultures. Compared to the calcein-AM assay, the number of the cells considered to be undergoing apoptosis is smaller. An explanation for the discrepancy could be that the TUNEL assay using the Apoptag test detects advanced apoptosis, and the positive RPE cells are attached weaker than the normal cells and therefore taken off easily during the process of section preparation. As Gatti et al. [24] suggested, calcein-AM might detect the early stages of apoptosis.

The tight junction of RPE creates an outer blood retinal barrier. Occludin is one of the tight junction proteins which localizes mainly at the cell-cell border in healthy cells, and plays an important role in preserving cellular morphology and functions, as well as barrier function. Stress to the cells, such as an oxidative stress, can cause the barrier dysfunction of RPE correlated with the delocalization of tight junction proteins [26]. Immunostaining of occludin exhibits well-ordered occludin localization in fresh and day-4 cultures, while on day-8 culture, occludin is less ordered, and only partially remaining at the cell border. This indicates that the barrier function might be disturbed in day-8 culture compared to the cultures younger than 4 days, even though the tight junction still exists at the cell-cell border [4]. To our knowledge, perfusion culture system for RPE-choroid tissue, in which the transepithelial resistance (TER) is generated, has not been well established. Although the same manufacturer provides a gradient type container, in which the tissue is placed horizontally and the medium flows over the apical and the basal side of the tissue, it is still questionable in terms of TER preservation. The Ussing-type chamber is a well-known tissue chamber which allows the tissues to create TER and is suitable for the investigation of ion transport [27]. This is, however, used for short-circuit experiments and is not suitable for long-term cultivation. Establishment of the perfusion tissue culture system which can provide a stable TER environment to the tissue culture with the possibility of long-term cultivation is desired.

Wound healing is one of the basic biological responses in many pathological conditions. The SRT laser was first used clinically in 1999 [28], and further clinical studies have recently been published [10, 11]. This laser is able to specifically destroy RPE cells with a bubble formation around the melanosomes, without increasing the cell temperature, thus sparing the surrounding tissues [29]. This makes it an interesting tool to investigate wound healing processes of RPE cells without damaging the surrounding tissue. For an appropriate wound healing, both proliferation and migration are imperative, with the appropriate substrate for the migrating cells. To stop the wound healing, contact inhibition is necessary in order to stop the proliferation and/or the spreading of the cells as soon as the wound is closed. Otherwise, a multilayered, less-functional RPE is created. We observed that if the wound is made on 6-day-old cultures, the cells are no longer able to heal the wound normally. Either no closure is observed, presumably because of the lack of proliferation or migration ability, or a multilayered RPE can be seen, presumably because of the lack of contact inhibition. In the latter case, as shown in figure 8h and 8i, the defect is covered by the fibroblastic tissue, and this seems to be mainly consisting of the elongated spreading cells. Since SRT laser does not destroy Bruch's membrane, these covering cells are most likely spreading RPE cells. With this experiment, we could demonstrate an obvious

functional difference between younger and older cultures. Six-day-old culture is no longer able to close the wound appropriately.

Additionally, we investigated VEGF secretion as a functional aspect of RPE cells in the perfusion culture. In the living eye, VEGF is produced mainly by RPE cells, additionally to ganglion cells and Müller cells of the retina, which express low levels of VEGF [30, 31]. Western blot analysis confirms the expression of VEGF proteins in RPE-choroid cells and the retina (data not shown). In cell culture, RPE cells have been reported to produce considerable quantities of VEGF [32], which is secreted into the medium, and hence is easily accessible for quantification. In cell cultures, regulative factors from the surrounding tissue are missing. The neighboring choroidal tissues could influence VEGF expression in tissue culture, and therefore the quantities or the changes of VEGF level that are detected in our tissue culture experiments might be closer to the in vivo situation, compared to RPE cell culture experiments. Blaauwgeers et al. [33] suggest that a high degree of differentiation of RPE cells in vitro is reflected by a high production of VEGF. After a latency period of 24 h, approximately 620 pg/ml VEGF is produced from 6 tissue cultures (6 rings). The next day, the secretion decreases to the level of 437.9 pg/ml and the level was stable between 400 and 500 pg/ml during the next 4 days. The high level of VEGF secretion on day 1 might be the reflection of the cellular damage at the edge lesion. The explants are fixed by two rings from both sides, therefore edge damage is inevitable. The stable secretion from day 2 to day 5 suggests that the RPE is viable and functionally stable over this period of time. However, starting on day 6, VEGF secretion increases significantly. Previous reports proved that hypoxia or oxidative stress induce VEGF secretion [34, 35]. Therefore, the cellular stress in the RPE-choroid culture could be the major stimulator of VEGF secretion. The stable amount of VEGF secretion until day 5 is an indicator of tissue stability, and the increase after day 6 is thought to correlate with the cellular degenerative changes, in which cells are exposed to a variety of stress. Since the morphological degenerative changes develop in parallel with the increase of VEGF secretion, we consider that significant degeneration starts around day 7. Independent of the main purpose of this study, this time point might be also an interesting time point of investigation for degenerative chorioretinal disorders.

In summary, the analysis of morphological and functional changes demonstrated that the RPE-choroid tissue in perfusion culture is stable until day 5 of cultivation and that the best time period to conduct experiments seems to be between day 2 and day 5 of cultivation. The prominent degenerative change is thought to start around day 7. In case of longer cultivation, degenerative changes have to be taken into consideration.

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