Studies on the Cell-Cycle Entry Mechanism of Retinal Pigment Epithelium Cells of the Adult Newt, *Cynops pyrrhogaster*

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1. Abbreviations

BrdU+: BrdU positive
CNS: central nervous system
DW: distilled water
DIV: days in vitro
ECM: extracellular matrix
EMT: epithelial-mesenchymal transition
FBS: fetal bovine serum
HSPG : heparan sulfate proteoglycan
MEM: minimum essential medium
NR: neural retina
PFA: paraformaldehyde
PCR: polymerase chain reaction
po: post operative
RLEC: retina-less eye-cup
RPE: retinal pigment epithelium
RT: room temperature

2. Abstract

The retinal pigment epithelium (RPE) is a highly specialized cell layer located between the neural retina and the choriocapillaris in the eye, providing essential support for the metabolism and physiology of the neural retina (NR). Mature RPE cells are mitotically inactive. However, they change their phenotype and proliferate when the retina is injured by ischemia or trauma. In humans, RPE cell proliferation typically results in retinal diseases and blindness. However, newts can regenerate their NR through proliferation and transdifferentiation of the RPE cells. Therefore, the adult newt retinal regeneration system may be a good model to obtain insight into the treatment of retinal diseases caused by proliferation of RPE cells, and may also help to establish regenerative therapies.

The mechanisms underlying proliferation in mitotically quiescent RPE cells are not fully understood, mainly because a suitable experimental system has not been established. Therefore, in the present study, I developed a new in vitro experimental system in the newt. Using this system, I investigated the mechanisms of the cell-cycle entry of RPE cells, following the removal of the NR.

I performed retinectomy, a surgical operation to remove the NR from the eye, in vitro and cultured the posterior half of the eyeball containing the RPE. Under this retina-less eye-cup (RLEC) culture condition, the RPE cells entered the S-phase of cell-cycle between 5 and 10 days in culture, but these cells were located around the wound edge of the RLEC. Cells inside the

tissue hardly entered the cell-cycle, which could not be explained by degeneration or cell death because a cell viability assay and intraocular transplantation of the RPE showed no significant degeneration or cell death of the RPE cells.

To determine the mechanism by which RPE cells around the wound edge enter the cell-cycle, I examined the effect of heparin because it is known to bind various soluble factors and support their action on receptors. As expected, heparin promoted the cell-cycle entry of RPE cells. Therefore, I next examined the heparin-binding factors FGF2, Wnt, Shh, and thrombin, which have been speculated to participate in body-parts regeneration in vertebrates. FGF2, Wnt, and Shh did not promote cell-cycle entry of RPE cells. In contrast, thrombin promoted cell-cycle entry of RPE cells only in the presence of serum, although thrombin itself showed an inhibitory effect.

Subsequently, I investigated factors that suppress the cell-cycle entry of RPE cells in the center of the RLEC. I found that when a small piece of RPE was removed from the center of the RLEC, the cells around the evulsed area entered the cell-cycle. I also found that EGTA treatment, which loosens cell-to-cell contact, but not basement membrane (Bruch's membrane) digestion, promotes the cell-cycle. These results suggest that the cell-cycle entry of the RPE cells is suppressed through cell-to-cell contact.

In the conclusion, in vitro retinectomy followed by RLEC culture is a good system to research the first cell-cycle entry of the RPE cells, following NR injury. Thrombin is a heparin-susceptible factor that stimulates the cell-cycle entry of RPE cells in combination with serum factors, and cell-to-cell contact

suppresses this process.

3. Introduction

The retinal pigment epithelium (RPE), which is located between the neural retina (NR) and the choriocapillaris in the eye (Figure 1), plays an essential role in vision. In the mature state, RPE cells are mitotically inactive. Once the retina is injured, the RPE cells start to proliferate. In humans, the proliferation of RPE cells is a sign of pathogenesis causing blindness.

In contrast to other vertebrates, newts can regenerate their entire retina through the proliferation and transdifferentiation of RPE cells, even in adulthood and even when the NR is completely removed by surgery (Chiba et al., 2006; Chiba and Mitashov, 2007; Haynes and Del Rio-Tsonis, 2004; Mitashov, 1996; Reh and Pittack, 1995; Reh and Pittack, 1997; Tsonis, 2000; Tsonis and Del Rio-Tsonis, 2004). Therefore, the adult newt retinal regeneration system may be a good model to obtain insights into the treatment of retinal diseases caused by phenotype switching and proliferation of RPE cells, and even retinal regeneration.

3.1. The RPE

The RPE, which is located between the NR and the choriocapillaris in the eye, is derived as a partner of the NR from the multipotent optic neuroepithelium. The mature RPE is a highly specialized monolayer with pigmented microvilli, and its apical membrane faces the photoreceptor outer

segments of the NR (Figure 2). Long apical microvilli surround the light-sensitive outer segments, establishing a complex with close structural interaction. With its basolateral membrane, the RPE faces the basement membrane (Bruch's membrane), which separates the RPE from the fenestrated endothelium of the choriocapillaris. The RPE provides an essential support for the NR and vision with various physiological roles (Marmor, 1998; Strauss, 2005). As a layer of pigmented cells, the RPE absorbs the light energy focused by the lens on the retina (Bok, 1993; Boulton and Dayhaw-Barker, 2001). The RPE transports ions, water, and metabolic end products from the subretinal space to the blood and delivers nutrients such as glucose, retinol, and fatty acids from the blood to the NR. In addition, the RPE plays an important role in the visual cycle of retina, in order to maintain photoreceptor activity. Furthermore, the RPE secretes a variety of growth factors or immunosuppressive factors to facilitate homeostasis of the NR and vision.

3.2. Phenotype switching and proliferation of RPE cells

The RPE cells are highly specialized and mitotically inactive. However, they change their phenotype and start to proliferate when the NR or the choroid is injured. In humans, RPE cell proliferation is a negative sign, indicating the pathogenesis of retinal diseases such as proliferative vitreoretinopathy (PVR) or choroidal melanoma (Kim and Arroyo, 2002; Pastor, 1998). In PVR, upon injury of the retina or the choroid, the RPE cells are exposed to the serum through damage to the blood–retinal barrier.

Subsequently, they are detached from the Bruch's membrane, lose their epithelial morphology, migrate into the vitreous cavity through a tear in the NR, and participate in the formation of epiretinal membranes on both the surfaces of the NR. The epiretinal membranes grow and contract, causing further retinal detachment and loss of vision.

In contrast to humans, newts can regenerate their entire retinas through the proliferation and transdifferentiation of the RPE cells (Reh and Pittack, 1995; Mitashov, 1996, 1997; Tsonis, 2000; Hynes and Del Rio-Tsonis, 2004; Tsonis and Del Rio-Tsonis, 2004; Chiba and Mitashov, 2007). In the adult newt, upon surgical removal of the NR, the RPE cells typically form layers and aggregate with a thickness of a few cells, possibly because of their migration and/or the shrinking of the vitreous chamber (Chiba et al., 2006). The cells become mitotically active during this process and then generate a bilayer of cells consisting of presumptive progenitor cells of the NR and the RPE (Chiba et al., 2006; Susaki and Chiba, 2007; Kaneko and Chiba, 2009). The cells of the inner cell layer facing the vitreous cavity continue to proliferate, producing various types of retinal neurons and glia to form visual circuitry, and finally regenerate a new functional NR (Negishi et al., 1992; Saito et al., 1994; Cheon et al., 1998; Chiba, 1998; Cheon and Saito, 1999; Chiba and Saito, 2000; Chiba et al., 1997, 2005, 2006; Sakakibara et al., 2002; Oi et al., 2003a, b; Nakamura and Chiba, 2007); however, the cells of the outer cell layer, along the Bruch's membrane, exit the cell-cycle earlier and differentiate to renew the RPE cell layer (Chiba et al., 2006).

3.3. Experimental systems to study RPE cell-cycle entry

The mechanism underlying the onset of proliferation in mitotically quiescent RPE cells following retinal injury is not fully understood in either humans or newts, although it may present a clinical target for analyzing retinal disease or regeneration. To address this issue, many studies have used primary cultures of isolated RPE cells or have performed retinectomy (Fredi-Reygrobellet et al., 1991; Kauffmann et al., 1994; Lashkari et al., 1999; Andrews et al., 1999; Bryckaert, et al., 2000; Kaven et al., 2000; Bian et al., 2001; Meitinger et al., 2001; Hecquet et al., 2002a,b; Nagineni et al., 2003; Hollborn et al., 2006; Susaki and Chiba, 2007; Pacheco-Domínguez et al., 2008; Palma-Nicolas et al., 2008). However, isolated cells or primary culture systems may not be suitable because isolated cells have already received signals to become mitotically active during the enzymatic dissociation process. In fact, in adult newts, when the RPE cells are isolated from the basement membrane (Bruch's membrane) of choroid tissue by enzyme treatment and then cultured in serum-free minimum essential medium (MEM), they enter the S-phase of the cell-cycle even in the absence of exogenous growth factors (Susaki and Chiba, 2007). However, retinectomy also presents problems with regard to experimentation. Wound opening, blood inflow, vascular expansion, and other disruptions resulting from the operation make the intraocular environment hard to control.

3.4. Objective of this study

The mechanism underlying RPE cell proliferation following retinal injury

is not fully understood in humans or newts, although it may provide new insights into clinical treatments for retinal diseases or retinal regeneration. One of the reasons why advances have been limited is the lack of a suitable experimental system. Therefore, in the present study, using adult newts of the species *Cynops pyrrhogaster*, I developed an in vitro retinectomy procedure followed by the RPE culture from the posterior half of the eye, termed the retina-less eye-cup (RLEC), to observe RPE cells with lower disruption than that observed in in vivo experiments. Using this system, I investigated the factors or signals involved in the first cell-cycle entry of RPE cells, following removal of the NR.

4. Materials and methods

4.1. Animals

Adult *Cynops pyrrhogaster* newts (total body-length: 8-10 cm) were purchased from local suppliers in Japan and housed in polyethylene containers with water at 18 °C under a natural light condition (Casco-Robles et al., 2010, 2011). The original research reported herein was performed under the guidelines established by the University of Tsukuba Animal Use and Care Committee.

4.2. RPE culture

4.2.1. In vitro retinectomy (RLEC culture)

Adult newts were anesthetized with 0.1 % FA-100 (4-allyl-2-methoexyphenol; Tanabe, Japan) in the dark for 2 h and then sacrificed. After rinsing the heads in 70 % ethanol, the eyeballs were placed, cornea side up, on a membrane filter (Millipore, Billerica, MA, USA) in a 35mm plastic dish (Becton Dickinson, Franklin Lakes, NJ, USA) at one eyeball per dish, and the eyeball was cut along the equator, its anterior half was carefully removed, the posterior half (i.e., the eye-cup) was soaked in PBS for ~1 h, and finally the NR was carefully removed to make a "retina-less eye-cup (RLEC)" (Figure 3).

4.2.2. RLEC culture

The RLEC on the filter was transferred into a chamber, a cap of the 1.5 ml sample-tube (Assist, Tokyo, Japan), containing 200 μl of a culture medium. After the chamber was closed with the sample-tube body, the tubes were was incubated at 25 °C. The culture was transferred into another chamber containing a fresh medium every 5 days. The newt MEM composed of 80 % L-15 (Invitrogen, Carisbad, CA, USA) diluted with distilled water (DW), 7.5 μg/ml heparin (heparin, sodium salt; 081-00136, Wako, Osaka, Japan) and 5 μg/ml BrdU (Sigma, St. Louis, MO, USA) was used as a standard culture medium.

In some experiments, the RLEC was cut into halves along a longitudinal axis through the optic disk with a razor blade immediately before culture (Figure 6A). In other experiments, a piece of the epithelium was removed from a central region of the RPE in the RLEC before culture as follows: the tip of a micropipette (0.2-10 μl, outer diameter: ~800 μm; BIO-BIK No. 1088, INA-OPTIKA, Osaka, Japan) was carefully put on the apical surface of the RPE under a stereomicroscope, applied with a small negative pressure, and then lifted up together with a piece of the epithelium attached (Figure 16A).

To investigate the participation of cell-to-ECM and cell to cell contact in the cell-cycle entry, RLECs were incubated in either 0.1% (w/v) elastase solution at 25 °C for 1 h or 10 mM EGTA solution at 25 °C for 1.5 h, rinsed twice (10 and 35 min) in 80% L-15, and then cultured in the basal medium. The elastase solution was prepared by diluting 1% stock [Elastase – high purity, porcine, EC134; Elastin Products Co., Owensville, MO, USA; stored

in 10 mM Tris-HCl (pH = 8.4) at 4 °C and used at room temperature (pH = 7.8)] in 80% L-15 and sterilized through a syringe filter (DISMIC-25cs, cellulose acetate, 0.2 μm pore size; Toyo Roshi Kaisha, Ltd., Tokyo, Japan) right before use. The EGTA solution contains 115 mM NaCl, 3.7 mM KCl, 10 mM EGTA, 18 mM D-glucose, 10 mM HEPES, and 0.001% phenol red (pH was adjusted to 7.5 with 0.3 N NaOH). For mock control of each treatment, 80% L-15 containing 1 mM Tris-HCl, or a newt saline [115 mM NaCl, 3.7 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 18 mM D-glucose, 10 mM HEPES, 0.001% phenol red; pH = 7.5] was used.

To investigate signaling pathways involved in the cell-cycle entry of RPE cells, the following reagents were tested: 50 ng/ml FGF2 (a synthetic *C. pyrrhogaster* FGF2; stock solution: 10 μg/ml in PBS, -20 °C; Susaki and Chiba, 2007), 5 μM U0126 [V1121, Promega, Madison, WI, USA; stock solution: 2 mM in DMSO (D2650, Sigma), -20 °C], 10 ng/ml Dkk-1 [1096-DK, R&D Systems, Minneapolis, MN, USA; stock solution: 10 μg/ml in PBS containing 0.1 % bovine serum albumin (BSA; A3294, Sigma), -20 °C], 500 ng/ml Shh [Human Sonic Hedgehog (C24II), amino terminal peptide, 1845-SH, R&D systems; stock solution: 20 μg/ml in PBS containing 0.1 % BSA, -20 °C], 40 μM KAAD (KAAD-Cyclopamine, K171000, Toronto Research Chemicals, North York, ON, Canada; stock solution: 10 mM in ethanol, -80 °C), 5 U/ml thrombin (a bovine thrombin, 605157, EMD Chemicals, Gibbstown, NJ, USA; stock solution: 500 U/ml in PBS, -20 °C), 20 μM PPACK (520222, Calbiochem, Darmstadt, Germany; stock solution: 9.54 mM in 5 % acetic acid solution, 4 °C; pH was adjusted to 7.4 with 2N NaOH

before administration) and 1 % fetal bovine serum (FBS; 26140-079, Gibco, Carisbad, CA, USA). These reagents were added to the culture medium from the beginning of culture except for U0126, KAAD and PPACK which were administered from the time at which the eye-cup is soaked in PBS. For mock control of each drug, only the solution to store the drug was administered at the corresponding concentration, pH and timing.

4.2.3. Intraocular RPE transplantation

RLECs were prepared as described above. For transplant, an RPE-choroid tissue was separated from the RLEC with a fine needle and kept in PBS. Adult newts were anesthetized with 0.1 % FA-100 (4-allyl-2-methoexyphenol; Tanabe, Japan) in the dark for 2 h and then placed under a binocular microscope. The dorsal half of the left eye was cut open along the corneal-scleral junction, and the NR together with the lens was carefully removed by a fine needle and forceps (retinectomy, Figure 3). During this operation, the vitreous chamber was gently rinsed with sterile newt saline solution (115 mM NaCl, 3.7 mM KCl, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 18 mM D-glucose, 5 mM HEPES, pH=7.5 adjusted with 0.3 N NaOH). Enucleated RPE-choroid tissue was put into the eye by spatula and fine needle.

After the operation, the eye flap, consisting of the iris and cornea, was carefully placed back in its original position. The operated animals were maintained in moist containers and allowed to recover at 22 °C (the day-night cycle was 12 h: 12 h). They were sacrificed on selected

post-operative (po) days under anesthesia.

Eyeballs of selected days po were fixed in 2 % paraformaldehyde (PFA)/ 0.2 % picric acid in phosphate-buffered saline (PBS, pH=7.5) for 6 h at 4 °C, washed thoroughly in PBS overnight at 4 °C, then cryosectioned transversely at 20 μm thickness. The samples were immersed into 1/50000 DAPI (Molecular prove,) in PBS for 3 h, then rinsed twice in PBS (5 min each).

4.3. Counting the cells entering S-phase of cell-cycle

RLEC cultures were fixed in 4 % PFA in PBS for 15 h at 4 °C, and washed thoroughly (PBS, 15 min → 0.5 % Triton X-100 in PBS, 15 min → PBS, 15 min). They were incubated in 0.3 % H_2O_2 diluted with PBS for 20 min, rinsed twice in PBS (5 min each), and then incubated in 2 N HCl for 2 h. After washed thoroughly, they were incubated in a blocking solution [3% goat normal serum (S-1000, Vector, Burlingame, CA, USA)/ 0.5% TritonX-100 in PBS] containing Avidin D (1:50; Avidin/Biotin blocking kit, SP-2001, Vector) for 2 h. After rinsed twice in PBS, they were incubated in a mouse anti-BrdU antibody (1:400; B2531-2ML, Sigma) diluted with the blocking solution containing Biotin (1:50; Avidin/Biotin blocking kit) for 15 h at 4 °C. After washed thoroughly, they were incubated in a biotinylated goat anti-mouse IgG antibody (1:400; BA-9200, Vector) diluted with the blocking solution for 4 h. After rinsed twice in PBS, they were incubated in a mixture of Avidin and Biotin Complex (Vectastain ABC Elite kit, PK-6100, Vector) for 2 h. After washed thoroughly, they were incubated in a DAB solution (DAB substrate kit, SK-4100, Vector) for 3 min. Finally the reaction was stopped by washing

them in DW for 15 min.

A total RPE-cell number in a RLEC culture was counted as follows: the culture was re-fixed in 4 % PFA in PBS for 20 min and rinsed with DW; the RPE-choroid tissue was separated from the sclera by fine forceps and pins under a stereomicroscope, and transferred onto a slide glass; the tissue was immersed into 90 % glycerol in PBS and mounted under a cover slip; the preparation was put on the stage of a fluorescence microscope (BX50; Olympus, Tokyo, Japan) and viewed through a filter set (excitation: 460-495 nm, emission: 510-550 nm; U-MWIBA/GFP; Olympus); RPE cells identified by their characteristic morphology appeared on a green autofluorescence of the choroid (see Figure 4B) were counted.

BrdU+ nuclei in the RPE were counted as follows: the cover slip mounted on the RPE-choroid tissue was removed, and the tissue was transferred into DW and rinsed well, incubated in 15 % H_2O_2/ 1.5 % sodium azide (197-11091, Wako) in PBS for overnight to bleach their melanin pigments, and rinsed twice in DW; the tissue was transferred into 90 % glycerol in PBS on a slide glass and mounted under a cover slip again; the preparation was put on the stage of the microscope and the number of brown nuclei (BrdU+) were counted under a transmitted light condition (Figure 4C, D).

4.4. Cell viability assay

For MTT assay, RLEC cultures were incubated in 80% L-15 medium containing 500 μg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT; Nacalai tesque, Tokyo, Japan) at 25 °C for 3 h, transferred into a

sample tube containing 200 μl DMSO, and sonicated for 3 min. The supernatant containing formazan was collected by centrifugation at 12000 rpm for 5 min, and examined for its absorbance at 570 nm (A570) by a spectrophotometer (D640; Beckman coulter, Brea, CA, USA). TUNEL assay to label apoptotic cells was carried out using Neuro TACS II kit (4823-30-K; Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions.

For TUNEL assay, RLEC cultures were fixed in 4 % PFA in PBS for 15 h at 4 °C, and washed thoroughly (PBS, 15 min → 0.5 % Triton X-100 in PBS, 15 min → PBS, 15 min). They were incubated in Neuropore solution (NeuroTACS II kit, 4823-30-K, TREVIGEN, Gaithersburg, MD, USA) for 30 min, rinsed twice in PBS (10 min each), and then incubated in 3 % H_2O_2 diluted with methanol for 5 min. After rinsing in PBS (5 min), they were incubated with TdT labeling buffer (NeuroTACS II kit) for 5 min, TdT reaction buffer (NeuroTACS II kit) for 1 h and TdT stop buffer (NeuroTACS II kit) for 5 min. After rinsing twice in PBS, the RLECs was incubated in 2 % Strep-HRP (NeuroTACS II kit) diluted with PBS for 10 min. After rinsing twice in PBS, they were incubated in 0.03 % H_2O_2, 0.5 % DAB (NeuroTACS II kit), 0.1 % DAB enhancer (NeuroTACS II kit) diluted with PBS for 7 min. Finally, the reaction was stopped by washing them in DW four times (10 min each).

The total cell number in a RLEC and apoptotic cell number in RPE were counted as same way as BrdU labeling (see Materials and methods 4.3.).

4.5. Polymerase chain reaction (PCR) analysis

Template cDNA pools were constructed from six RLECs collected on selected days po and six NR as described previously (Susaki and Chiba, 2007). One μl of template cDNA was used for 50 μl PCR solution. KOD Dash (LDP-101, Toyobo, Osaka, Japan) was used as the PCR enzyme. For *Wnt2b*, a primer set (sense, 5'- cacagccatacaagtgac-3'; antisense, 5'-cgactgtctcctcgcagtc-3') was designed to amplify a 314bp cDNA fragment. For Wnt5a, a primer set (sense, 5'-ccagaaagggtcctacgagagc-3'; antisense, 5'-cagcttcccccggctgttcagc-3') was designed to amplify a 235bp cDNA fragment. These primers were designed according to Hayashi et al., (2006).

4.6. Data analysis

Bright-light and fluorescence images of tissues were acquired using a personal computer and a color CCD camera (C4742-95 ORCA-ER system, Hamamatsu Photonics, Japan). Figures were prepared using Photoshop Extended CS5. Image, brightness, contrast, and sharpness were adjusted. Statistical data in the text were presented as the mean ± SEM (n: the number of RLECs examined) from more than two independent experiments (N). Non-parametric tests were carried out to evaluate the statistical significance of the data.

5. Results

5.1. Development of in vitro retinectomy and RLEC culture

5.1.1. Properties of RPE cells under RLEC culture conditions

In adult newt eyes, RPE cells entered the cell-cycle in 10 days when NR was surgically removed. I first examined whether the RPE cells become mitotically active under RLEC culture conditions (Figure 4). RLECs were prepared in PBS, as illustrated in Figure 4A, and then cultured in newt MEM (80% L-15 medium containing 7.5 μg/ml heparin and 5 μg/ml BrdU). Under this culture condition, the RPE appeared to maintain its original characteristics for 10 days; RPE cells retained their melanin pigment, and their hexagonal morphology was almost unchanged (Figure 4B). However, some indications of dedifferentiation appeared; microvilli on the apical surface of the RPE were not obvious, and most RPE cells apparently had an enlarged nucleus, and a small population of the RPE cells expressed a pan-retinal neuronal marker, acetylated tubulin (see Susaki and Chiba, 2007). During this 10-day culture, BrdU-positive (BrdU+) cells were observed in the RPE when BrdU-incorporated nuclei were immunostained and visualized by bleaching of the melanin pigments. Interestingly, most BrdU+ cells were located along the peripheral margin of the RPE sheet (Figure 4C, D). When the ratio of the BrdU+ cells was calculated in a 100-μm region along the peripheral margin (defined as 'Edge'), the value was significantly higher than that in the rest of the RPE sheet (defined as

'Center') (25.0 ± 2.8% in the edge, 5.6 ± 0.9% in the center, and 10.2 ± 1.2% in the whole area; Sheffe's test following the Friedman test, $p < 0.01$; n = 49, Figure 5). Mitosis was observed only rarely in each region: at the edge of 18 (36.7%) RPE sheets, 2.4 ± 0.4% BrdU+ cells and in the center of 9 (18.4%) RPE sheets, 2.4 ± 0.5 % BrdU+ cells. These results indicate that a population of RPE cells, mostly located in the edge, enters the S phase of the cell-cycle, but hardly proceeds into the M phase in 10 days, when cultured in the RLEC.

5.1.2. Wound-edge RPE cells enter the S phase of the cell-cycle under the RLEC culture condition

The distribution pattern of BrdU+ cells raises the question of whether there is a difference of cell properties between the peripheral and the central RPE or whether the cell-cycle entry is related to the wound edge of the RPE/RLEC. Therefore, I cut the RLEC longitudinally across the posterior pole into halves and cultured the halves separately for 10 days (Figure 6). In every piece of the RPE sheet after 10 days in culture, the BrdU+ cells appeared along the newly formed longitudinal margins (Figure 6B, C). These results indicate that cell-cycle entry is related to the wound-edge of the RPE/RLEC.

In contrast, the ratio of cells entering the cell-cycle inside the tissue was low, although no cell death was observed on performing the TUNEL assay (n = 2; data not shown). The number of RPE cells in the RLEC at 10 days in culture (1894 ± 114, n = 49) was not significantly different from that (1804 ± 131, n = 10) in the intact eye-cup. In addition, to examine whether the

cultured RPE cells maintained their regenerative ability, I transplanted an RPE-choroid tissue enucleated from a 10-day-cultured RLEC into the retinectomized eye of a living animal (see method) and found that an NR was regenerated from the transplanted tissue on the 40th day after the transplant (Figure 7). Thus, cell death or degeneration was unlikely to be the cause of the low ratio of cell-cycle entry inside the tissue.

These observations provided insight that a factor guiding RPE cells into the cell-cycle exists near the wound, and that the lack of this factor or the presence of a suppression mechanism is responsible for the low ratio of cell-cycle entry in RPE cells located at the center of the tissue. Therefore, in a subsequent study, I explored for these factors by using an in vitro retinectomy and a RLEC culture system.

5.2. Exploration of the factors that lead RPE cells into the cell-cycle

5.2.1. Heparin promotes the cell-cycle entry of RPE cells

For the RLEC culture shown above, newt MEM was used because this medium had been used for serum-free culture of newt isolated RPE cells in a previous study (Susaki and Chiba, 2007). However, this medium contained heparin, a member of the glycosaminoglycan family. In a previous study that aimed at examining the effects of exogenously administered growth factors such as FGF2 on RPE cells in vitro (Susaki and Chiba, 2007), heparin was added to the basal medium (80% L-15) as a supplement because it is known to bind various soluble factors, including FGF2 and support their effect on receptors (Cumberledge and Reichsman, 1997; Waksman and Herr, 1998; Tumova et al., 2000; Zhang et al., 2007) in addition to protecting the factors against degradation (Gospodarowicz and Chen, 1896; Saksela et al., 1988;

Tumova et al., 2000). Therefore, I hypothesized that heparin enhances the effect of endogenous factors released from an RLEC wound.

To examine this possibility, I compared the results obtained in newt MEM containing different concentrations of heparin (0.75, 7.5, and 75 μg/ml) with those in the basal medium (i.e., heparin-free). As expected, the ratio of the BrdU+ cells in the whole RLEC in the 7.5 μg/ml heparin condition (i.e., the standard newt MEM) was significantly higher than that in the basal medium (i.e., heparin-free) (10.2 ± 1.2 % in standard newt MEM, n = 49; 6.7 ± 1.4 % in the heparin-free medium, n = 13; Mann-Whitney U-test, $p < 0.01$, Figure 8) or that in the 0.75 mg/ml heparin condition (6.4 ± 1.2, n = 13; Mann-Whitney U-test, $p < 0.05$, Figure 8). However, it should be noted that a 10x higher concentration of heparin (75 μg/ml) did not further increase the ratio of BrdU+ cells (6.4 ± 1.2 %, n = 13, Figure 8). Next, I examined whether heparin influences the kinetics of the cell-cycle (Figure 9). At 5 DIV, in the heparin-free medium, only 1 BrdU+ cell was recognized in the edge of one of the total 2 RLECs examined (0.08% in the edge, 0% in the center, and 0.02% in the whole), whereas in the heparin-containing medium, a larger number (range: 5 – 62, n = 3) of cells became BrdU+ (2.8 ± 1.3% in the edge, 0.7 ± 0.4% in the center, and 1.2 ± 0.5% in the whole), suggesting that this concentration of heparin may facilitate the cell-cycle entry of the RPE cells. Consistently, by 10 DIV, the ratio of BrdU+ cells in the RPE under the heparin condition obviously increased to a significantly higher level than that in the heparin-free condition, regardless of the location (in the edge, 25.0 ± 2.8% in standard newt MEM, n = 49, 19.4 ± 3.2% in the heparin-free

medium, n = 39; Jonckheere-Terpstra test, $p < 0.05$; in the center, 5.6 ± 0.9% at the standard newt MEM, n = 49; 3.3 ± 1.2% at heparin-free, n = 39; Mann-Whitney U-test, $p < 0.05$, Figure 9). MTT and TUNEL assays were performed to evaluate cell viability, and the results were not different in the case of the absence and presence of heparin (data not shown).

5.2.2. The heparin-susceptible factors FGF2, Wnt, and Shh do not trigger cell-cycle entry of RPE cells

Subsequently, I examined signaling pathways whose activity can be modulated by heparin. Initially, I tested FGF2, whose actions in vitro are supported by heparin. FGF2 is a candidate trigger factor for the transdifferentiation of the embryonic/larval RPE into the NR (Nguyen and Arnheiter, 2000). In addition, in the adult newt, this factor promotes neural transdifferentiation with AT expression in the RPE cells in vitro (Susaki and Chiba, 2007).

I also analyzed Wnt and Shh because these factors are known to be heparin-binding proteins involved in the regeneration of various tissues (Cumberledge and Reichsman, 1997; Zhang et al., 2007).

5.2.2.1. FGF2

FGF2 was administered to RPE cells in the RLEC from the beginning of the culture at a concentration of 50 ng/ml. In the control culture with newt MEM ('Heparin' in Figure 10), the RPE cells mostly started entering the S phase of the cell-cycle after more than 5 days of beg. There was no significant difference between the ratio of BrdU+ cells in presence of FGF2 ('Heparin + FGF2' in Figure 10) and that of the control at both 5 DIV (in the edge, 0.1 ± 0.1% with heparin, n = 49, 0.2 ± 0.1% with heparin + FGF2, n = 42; in the

center, 0% with heparin, n = 49, 0% with heparin + FGF2, n = 42; in the whole, 0.02 ± 0.01 % with heparin, n = 49, 0.05 ± 0.3% with heparin + FGF2, n = 42, Figure 10) and 10 DIV (in the edge, 25.0 ± 2.8% with heparin, n = 49, 24.2 ± 3.2% with heparin + FGF2, n = 42; in the center, 5.6 ± 0.9% with heparin, n = 49, 6.0 ± 1.7% with heparin + FGF2, n = 42; in the whole, 0.02 ± 10.2 ± 1.2% with heparin, n = 49, 9.9 ± 1.8% with heparin + FGF2, n = 42, Figure 10). FGF2 did not affect either the kinetics of the cell-cycle or the distribution pattern and ratio of the BrdU+ cells in the RPE. To confirm this finding, I examined the effects of an FGF receptor–specific tyrosine kinase inhibitor, SU5402, on the cell-cycle entry of the RPE cells: 25 μM SU5402 dissolved in 0.25% DMSO was administered to RPE cells in the RLEC from the beginning of culture. As expected, compared with the solvent only (mock), SU5402 did not affect the distribution pattern and ratio of the BrdU+ cells (in the edge, 27.5 ± 6.8% in the mock condition, n = 21, 28.3 ± 4.5% with SU5402, n = 15; in the center, 5.0 ± 1.4% in the mock condition, n = 21, 7.4 ± 1.7% with SH5402, n = 15; in the whole, 11.1 ± 2.8% in the mock condition, n = 21, 11.7 ± 2.0% with SU5402, n = 15, Figure 11).

5.2.2.2 Wnt

I evaluated the secreted protein Dickkopf1 (Dkk-1), which can inhibit Wnt signaling by blocking the interactions of Wnt with the transmembrane co-receptors Frizzled and low-density lipoprotein receptor-related protein (Klaus and Birchmeier, 2008). When RLECs were cultured in newt MEM containing 10 ng/ml Dkk-1 for 10 days, the ratio of the BrdU+ cells increased only in the edge (in the edge, 22.2 ± 3.6% with heparin, n = 15, 33.6 ± 5.4% with heparin + Dkk-1, n = 15; Jonckheere-Terpstra test, $p < 0.05$; in the

center, 7.4 ± 3.9% with heparin, n = 15, 6.7 ± 2.5% with heparin + Dkk-1, n = 15; in the whole, 11.3 ± 3.0% with heparin, n = 15, 13.7 ± 2.6% with heparin + Dkk-1, n = 15, Figure 12A), assuming that endogenous Wnt may exert an inhibitory effect at the edge. I further examined the expression of Wnt ligands in the eye-cup tissue by performing RT-PCR. Here, I tested Wnt2b and Wnt5a and found that both of them are expressed in the NR, although no positive signal was detected in the RLECs (Figure 12B). Thus, Wnt signaling did not trigger or promote the cell-cycle entry of the RPE cells.

5.2.2.3. Shh

I tested KAAD, which blocks Shh signaling by inhibiting the G-protein-coupled receptor Smoothened (Smo), which sends a signal when Shh binds the receptor Patched (Ptch) and liberates Smo from repression by Ptch (Ng and Curran, 2011). RLECs were cultured in newt MEM containing 40 μM KAAD dissolved in 0.4% EtOH for 10 days. Unexpectedly, the ratio of the BrdU+ cells did not change in any region of the RPE (in the edge, 24.3 ± 10.2% with heparin, n = 9, 14.3 ± 2.6% with heparin + KAAD, n = 11; in the center, 3.0 ± 0.7% with heparin, n = 9, 4.9 ± 1.6% in KAAD, n = 11; in the whole, 6.6 ± 1.1 with heparin, n = 9, 7.9 ± 1.5% with heparin + KAAD, n = 11, Figure 13A). Thus, Shh signaling was also not involved in the heparin-promoted cell-cycle entry of the RPE cells.

Next I examined the effect of Shh. RLECs were cultured in newt MEM (with heparin) containing 0.5 μg/ml Shh for 10 days. In this condition, Shh did not affect the cell-cycle entry of the RPE cells (in the edge, 25.0 ± 2.8% with heparin, n = 49, 31.7 ± 4.4% with Shh, n = 23; in the center, 5.6 ± 0.9% with heparin, n = 49, 3.7 ± 1.4% with Shh, n = 23; in the whole, 10.2 ± 1.2% with heparin, n = 49, 8.5 ± 1.6% with Shh, n = 23, Figure 13B). However, because heparin is also known to perturb the interaction of Shh with Ptch by binding to Shh (Carrasco et al., 2005), I further examined this possibility. RLECs were cultured in the basal medium containing the same

concentration of Shh for 10 days. The ratio of BrdU+ cells only increased in the edge (in the edge, 19.4 ± 3.2% without Shh, n = 39, 35.8 ± 8.3% with Shh, n = 6; Jonckheere-Terpstra test, $p < 0.05$; in the center, 3.3 ± 1.2% with no Shh, n = 39, 3.8 ± 1.4% with Shh, n = 6; in the whole, 6.7 ± 1.4% with Shh, n = 39, 11.5 ± 2.9% at Shh, n = 6, Figure 13C), suggesting that Shh may promote cell-cycle entry at the edge, but this effect is suppressed by heparin. However, because KAAD did affect the ratio of the BrdU+ cells even in the absence of heparin (in the edge, 4.9 ± 2.4% without KAAD, n = 6, 5.6 ± 1.7% at KAAD, n = 6; in the center, 2.2 ± 1.2% without KAAD, n = 6, 1.4 ± 0.6% with KAAD, n = 6; in the whole, 2.7 ± 1.0% without KAAD, n = 6, 2.1 ± 0.7% at KAAD, n = 6, Figure 13D), Shh may not be a component of the endogenously activated signaling that allows RPE cells in the RLEC to enter the cell-cycle in the absence of heparin.

5.2.3. The heparin-susceptible factor thrombin promotes cell-cycle entry of RPE cells depending on the presence of serum

< An omission >

5.3. Exploration of factors that suppress cell-cycle entry of RPE cells

5.3.1. Removal of a small piece of the RPE induces cell-cycle entry into surrounding cells

As shown above, the ratios of BrdU+ cells were very low in the center of the RLEC, even in the presence of both thrombin and FBS (Figure 15). This observation led to the next hypothesis that the RPE cells in the center of the RLEC may have limited susceptibility to mitotic factors.

I suspected that cell-to-cell contact may be responsible for the inhibition of cell-cycle entry in the center because many studies have suggested that

contact inhibition suppresses the proliferation of cells (Matsumoto et al., 1990; Tezel and Del Priore, 1996; Liu et al., 2010). Therefore, I removed a small piece of the epithelium from the center of the RPE in the RLEC by using a micropipette tip under a stereomicroscope (Figure 16A; see Materials and Methods) and cultured the RLEC. After 10 days in culture, BrdU+ nuclei were observed on Bruch's membrane inside the area without epithelium and also around its circumference (Figure 16B-E).

5.3.2. Digestion of Bruch's membrane does not promote the cell-cycle entry of the RPE cells

I examined the participation of cell-to-extracellular matrix (ECM) contact in the inhibition of the cell-cycle entry of the RPE cells; RLECs were incubated in a basal medium containing 0.1% elastase, a protease that efficiently digests Bruch's membrane beneath the RPE (Susaki and Chiba, 2007), for 1 h prior to 10 days of culture in the basal medium. This elastase treatment significantly reduced the ratio of BrdU+ cells in the edge (in the edge, 26.2 ± 4.8% in the mock condition, n = 13, 11.4 ± 3.0% with elastase, n = 13; Jonckheere-Terpstra test, $p < 0.05$; in the center, 2.9 ± 0.7% in the mock condition, n = 14, 1.3 ± 0.4% with elastase, n = 14; in the whole, 8.1 ± 1.2% in the mock condition, n = 13, 3.8 ± 0.9% with elastase, Figure 17A), although the total cell number was not affected in any region of the RLEC (in the edge, 247 ± 24 cells in the mock condition, n = 13, 251 ± 16 cells with elastase, n = 13; in the center, 897 ± 91 cells in the mock condition, n = 14, 754 ± 72 cells with elastase, n = 13; in the whole, 1144 ± 109 cells in the mock condition, n =

13, 1006 ± 85 cells with elastase, Figure 17B).

5.3.3. The calcium chelator EGTA promotes the cell-cycle entry of the RPE cells

Next, I evaluated the effect of EGTA which weaken cell-to-cell contact. RLECs were incubated in 10 mM EGTA (see Materials and Methods) for 1.5 h before the 10-day culturing in the basal medium. As expected, EGTA treatment increased the ratio of the BrdU+ cells in the whole and in the edge (in the edge, 32.5 ± 4.5% in the control, n = 9, 47.9 ± 6.1% with EGTA, n = 9; Sheffe's test following the Friedman test, $p < 0.01$; in the center, 9.1 ± 3.1% in the control, n = 9, 14.1 ± 3.2% with EGTA, n = 9; in the whole, 13.5 ± 3.2% in the control, n = 9, 21.4 ± 3.5% with EGTA, n = 9; Jonckheere-Terpstra test, $p < 0.05$, Figure 18A). In the center, however, the increase was not statistically significant, possibly because the cells in the center were more susceptible to this treatment and many of them left the RLEC (in the edge, 312 ± 31 cells in the control, n = 9, 257 ± 30 cells with EGTA, n = 9; Jonckheere-Terpstra test, $p < 0.05$; in the center, 1355 ± 168 cells in the control, n = 9, 969 ± 119 cells with EGTA, n = 9; Sheffe's test following the Friedman test, $p < 0.01$; in the whole, 1667 ± 193 cells in the control, n = 9, 1226 ± 148 cells with EGTA, n = 9; Sheffe's test following the Friedman test, $p < 0.01$, Figure 18B).

These results support the hypothesis that cell-to-cell contact, rather than cell-to-ECM contact, is involved in the inhibition of the cell-cycle entry of RPE cells.

6. Discussion

Because of the similarity of the behavior of the RPE cells in the initial process of human PVR and that of newt retinal regeneration, identification of the mechanisms underlying these processes is important for both the development of retinal disease treatment and establishment of regenerative therapy. RPE cells detach from each other, migrate, and start to proliferate after retinal injury in both humans and newts (Kim and Arroyo, 2002; Pastor, 1998; Chiba and Mitashov, 2007). To date, however, how the mature mitotically inactive RPE cells enter the cell-cycle has not really been investigated, mainly because of the lack of a suitable experimental system.

Therefore, in the current study, I developed an in vitro retinectomy and RLEC culture system by using adult *Cynops pyrrhogaster* newts and investigated the factors and signals involved in the proliferation of mitotically quiescent RPE cells.

6.1. Factors that lead RPE cells into the cell-cycle

Under the RLEC culture condition, the RPE cells entered the cell-cycle between 5 and 10 days in culture, similar to the in vivo situation, and these cells are localized around the wound edge of the epithelium. These results provided insight into the existence of factors that lead RPE cells from around the wound into the cell-cycle.

In the current study, I found that heparin promotes the cell-cycle entry of the RPE cells in the RLEC. As heparin is known to interact with various

molecules (Bradley and Brown, 1990; DiGabriele et al., 1998; Kan et al., 1993; Li et al., 1974; Tumova et al., 2000; Yanon et al., 1991; Zhang et al., 2007), I suspected that it may indirectly affect cell-cycle entry through modifications of diffusible factors. Here, I examined FGF2, Wnt, Shh, and thrombin as candidate factors and provided evidence suggesting that endogenous thrombin in RLEC culture may participate in the heparin-promoted cell-cycle entry of the RPE cells.

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My current results imply that Wnt and Shh may also participate in the cell-cycle entry of the RPE cells with inhibitory and excitatory effects, respectively. The Wnt function was not affected by heparin, but the effect of Shh was disturbed by heparin. In the current study, I was unable to fully investigate the participation of endogenous Wnts in the RLEC culture because of limited genetic information, although expression of Wnt2b and Wnt5a were observed in the NR. My current data with KAAD revealed that Shh is not an endogenous factor in RLEC culture. In fact, in the newt eye, Shh is present in the NR, whereas its receptors, Ptch1 and Ptch2, are found in the RPE (Takabatake et al., 1997).

Heparin is closely related in structure to heparan sulfate, which is present as a proteoglycan [i.e., heparan sulfate proteoglycan (HSPG)] on the cell surface and in the ECM; therefore, heparin-binding factors can also bind to HSPG (Tumova et al., 2000). It has been suggested that HSPG in the ECM

accumulates factors released by surrounding or remote cells in addition to cells in the blood and regulates their functions (Bernfield et al., 1999; Tumova et al., 2000; Zhang et al., 2007). In the current study, digestion of Bruch's membrane by elastase treatment resulted in a significant decrease in the ratio of BrdU+ cells, suggesting the importance of the ECM for the cell-cycle entry of the RPE cells. To determine the roles of heparin-binding factors, including Wnt, Shh, and thrombin, in the first cell-cycle entry of the RPE cells, the dynamics of HSPG and the location of these molecules in the RLEC (containing Bruch's membrane) should also be addressed.

6.2. Factors that suppress the cell-cycle entry of the RPE cells

The RPE cells inside the epithelium (i.e., cells surrounded by neighboring cells) hardly enter the cell-cycle even in the presence of a mixture of thrombin and serum. In contrast, in isolated cell culture, the cell-cycle entry of RPE cells is promoted by the presence of FBS (Susaki and Chiba, 2007). Contact inhibition may explain this difference in the competency of the RPE cells.

Contact inhibition underlies wound healing and tissue repair; when a portion of the tissue is missing after a traumatic injury, the cells surrounding the wound start to proliferate, and as they occupy the wound, cell growth is terminated by inhibitory signals through cell-to-cell contact (Jacinto et al., 2001; Lanosa and Colombo, 2008). Similar phenomena are observed in culture. For example, in chick embryos, when isolated RPE cells are cultured in a dish, they attach onto the bottom of the dish and start to

proliferate, and as the cells become confluent, they leave the cell-cycle and take on epithelial morphology while growing pigmented microvilli (Cahn and Cahn, 1966). The cell-to-cell contact in the RPE may suppress the competence of the cells to respond to mitotic factors by cell-cycle entry, allowing them to maintain a differentiated state. Thus, loss of cell-to-cell contact may be a requisite for cell-cycle entry of the RPE cells. My cell-removal experiments support this hypothesis.

Under the current culture conditions, the RPE kept its epithelial morphology for 10 days. In contrast, in the eye of a living animal, within ~2 h after removal of the NR, the RPE cells started to detach from neighboring cells and changed their morphology into a spherical shape, leaving Bruch's membrane as single cells. A similar process, known as the epithelial-mesenchymal transition (EMT), is also an important step in PVR (Grisanti and Guidry, 1995; Lee et al., 2001; Liu et al., 2010; Pratt et al., 2008; Tamiya et al., 2010). Therefore, the loss of epithelial characteristics may be a key event that liberates RPE cells from contact inhibition, allowing them to enter the cell-cycle in both retinal regeneration and PVR. Here, I demonstrated that EGTA treatment, which is known to disrupt cadherin ligation by chelating calcium (Tamiya et al., 2010), obviously increased the cell-cycle entry of the RPE cells. In a future study, the mechanisms of contact inhibition and EMT in newt RPE cells in comparison with those in mammals need to be addressed.

6.3. Conclusions

In the current study, I developed an in vitro retinectomy and RLEC culture system in newts. Using this system, I demonstrated that thrombin and other heparin-susceptible factors may be involved in the cell-cycle entry of the RPE cells. Furthermore, I demonstrated that cell-to-cell contact could suppress the cell-cycle entry of the RPE cells. Because there has been no suitable experimental system to observe the initial process of phenotype change in RPE cells following retinal injury, limited information is available regarding the underlying mechanisms. In vitro retinectomy and RLEC culture may be an effective option to investigate these mechanisms. Furthermore, the identification of factors that participate in the cell-cycle entry of the RPE cells is a new finding in the fields of retinal regeneration and traumatic retinal diseases. Because these factors may participate in the induction of retinal regeneration in newts, they have to be analyzed in greater depth. In humans, no comparative information is available; therefore, this mechanism should be investigated by constructing a parallel experimental system with newts. It is interesting to determine whether the mechanism of cell-cycle entry of the RPE cells is common between newts and humans.

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8. References

Bernfield, M., Gotte, M., Park, P.W., Reizes, O., Fitzgerald, M.L., Lincecum, J., and Zako, M. (1999). Functions of cell surface heparin sulfate proteoglycans. Annu. Rev. Biochem. 68, 729–777.

Bok D. (1993).The retinal pigment epithelium: a versatile partner in vision. J. Cell. Sci. Suppl. 17: 189–195

BoultonMand Dayhaw-Barker P. (2001). The role of the retinal pigment epithelium: topographical variation and ageing changes. Eye 15: 384–389

Bradley, R.S., and Brown, A.M.C. (1990). The proto-oncogene int-1 encodes a secreted protein associated with the extracellular matrix. EMBO J. 9, 1569–1575.

Bryckaert, M., Guillonneau, X., Hecquet, C., Perani, P., Courtois, Y., and Mascarelli, M. (2000). Regulation of proliferation-survival decisions is controlled by FGF1 secretion in retinal pigmented epithelial cells. Oncogene 19, 4917–4929.

Cahn, R.D., and Cahn, M.D. (1966). Heritability of cellular differentiation: clonal growth and expression of differentiation in retinal pigment cells in vitro. Proc. Natl Acad. Sci. U.S.A. 55, 106–114.

Carrasco, H., Olivares, G.H., Faunes, F., Oliva, C., and Larraı´n, J. (2005). Heparan sulfate proteoglycans exert positive and negative effects in Shh activity. J. Cell. Biochem. 96, 831–838.

Casco-Robles, M.M., Yamada, S., Miura, T., and Chiba, C. (2010). Simple and efficient transgenesis with I-SceI meganuclease in the newt, Cynops pyrrhogaster. Dev. Dyn. 239, 3275–3284.

Casco-Robles, M.M., Yamada, S., Miura, T., Nakamura, K., Haynes, T., Maki, N., Del Rio-Tsonis, K., Tsonis, P.A., and Chiba, C. (2011). Expressing exogenous genes in newts by transgenesis. Nat. Protoc. 6, 600–608.

Cheon, E.W., and Saito, T. (1999). Choline acetyltransferase and acetylcholinesterase in the normal, developing and regenerating newt retinas. Dev. Brain Res. 116, 97–109.

Cheon, E.W., Kaneko, Y., and Saito, T. (1998). Regeneration of the newt retina: order of appearance of photoreceptors and ganglion cells. J. Comp. Neurol. 396, 267–274.

Chiba, C. (1998). Appearance of glutamate-like immunoreactivity during retinal regeneration in the adult newt. Brain Res. 785, 171–177.

Chiba, C., and Mitashov, V.I. (2007). Cellular and molecular events in the adult newt retinal regeneration. In The Strategies for Retinal Tissue Repair and Regeneration in Vertebrates: From Fish to Human 2007, C., Chiba, ed. (Kerala, India: Research Signpost), pp. 15–33.

Chiba, C., and Saito, T. (2000). Gap junctional coupling between progenitor cells of regenerating retina in the adult newt. J. Neurobiol. 42, 258–269.

Chiba, C., Matsushima, O., Muneoka, Y., and Saito, T. (1997). Time course of appearance of GABA and GABA receptors during retinal regeneration in the adult newt. Dev. Brain Res. 98, 204–210.

Chiba, C., Oi, H., and Saito, T. (2005). Changes in somatic sodium currents of ganglion cells during retinal regeneration in the adult newt. Dev. Brain Res. 154, 25–34.

Chiba, C., Hoshino, A., Nakamura, K., Susaki, K., Yamano, Y., Kaneko, Y., Kuwata, O., Maruo, F., and Saito, T. (2006). Visual cycle protein RPE65 persists in new retinal cells during retinal regeneration of adult newt. J. Comp. Neurol. 495, 391–407.

DiGabriele, A.D., Lax, I., Chen, D.I., Svahn, C.M., Jaye, M., Schlessinger, J., and Hendrickson, W.A. (1998). Structure of a heparinlinked biologically active dimer of fibroblast growth factor. Nature 393, 812–817.

Gospodarowicz, D., and Chen, J. (1986). Heparin protects acidic and basic FGF from inactivation. J. Cell. Physiol. 128, 475–484.

Grisanti, S., and Guidry, C. (1995). Transdifferentiation of retinal pigment epithelial cells from epithelial to mesenchymal phenotype. Invest. Ophthalmol. Vis. Sci. 36, 391–405.

Hayashi, T., Mizuno, N., Takada, R., Takada, S., and Hisato, K. (2006). Determinative role of Wnt signals in dorsal iris-derived lens regeneration in newt eye. Mech. Dev. 123, 793–800.

Haynes, T., and Del Rio-Tsonis, K. (2004). Retina repair, stem cells and beyond. Curr. Neurovasc. Res. 1, 231–239.

Hecquet, C., Lefevre, G., Valtink, M., Engelmann, K., and Mascarelli, F. (2002a). Activation and role of MAP kinase-dependent pathways in retinal pigment epithelial cells: ERK and RPE cell proliferation. Invest. Ophthalmol. Vis. Sci. 43, 3091–3098.

Hecquet, C., Lefevre, G., Valtink, M., Engelmann, K., and Mascarelli, F. (2002b). cAMP inhibits the proliferation of retinal pigmented epithelial cells through the inhibition of ERK1／2 in a PKA-independent manner. Oncogene 21, 6101–6112.

Hollborn, M., Bringmann, A., Faude, F., Wiedemann, P., and Kohen, L. (2006). Signaling pathways involved in PDGF-evoked cellular responses in human RPE cells. Biochem. Biophys. Res. Comm. 344, 912–919.

Imokawa, Y., and Brockes, J.P. (2003). Selective activation of thrombin is a critical determinant for vertebrate lens regeneration. Curr. Biol. 13, 877–881.

Jacinto, A., Martinez-Arias, A., and Martin, P. (2001). Mechanisms of epithelial fusion and repair. Nat. Cell Biol. 3, E117–E123.

Kan, M., Wang, F., Xu, J., Crabb, J.W., Hou, J., and McKeehan, W.L. (1993). An essential heparin-binding domain in the fibroblast growth factor receptor kinase. Science 259, 1918–1921.

Kaneko, J., and Chiba, C. (2009). Immunohistochemical analysis of Musashi-1 expression during retinal regeneration of adult newt. Neurosci. Lett. 450, 252–257.

Kaven, C., Spraul, C.W., Zavazava, N., Lang, G.K., and Lang, G.E. (2000). Growth factor combinations modulate human retinal pigment epithelial cell proliferation. Curr. Eye Res. 20, 480–487.

Kim, L.K., and Arroyo, J.G. (2002). Mechanisms in proliferative vitreoretinopathy. Ophthalmol. Clin. North Am. 15, 81–86.

Klaus, A., and Birchmeier, W. (2008). Wnt signalling and its impacton development and cancer. Nat. Rev. Cancer 8, 387–398.

Lanosa, X.A., and Colombo, J.A. (2008). Cell contact-inhibition signaling as part of wound-healing processes in brain. Neuron Glia Biol. 4, 27–34.

Lashkari, K., Rahimi, N., and Kazlauskas, A. (1999). Hepatocyte growth factor receptor in human RPE cells: implications in proliferativevitreoretinopathy. Invest. Ophthalmol. Vis. Sci. 40, 149–156.

Lee, S.C., Kim, S.H., Koh, H.J., and Kwon, W. (2001). TGF-bs synthesized by RPE cell have autocrine activity on mesenchymal transformation and cell proliferation. Yonsei Med. J. 42, 271–277.

Li, E.H.H., Orton, C., and Feinman, R.D. (1974). The interaction of thrombin and heparin. Proflavine dye binding studies?. Biochemistry 13, 5012–5017.

Liu, Y., Xin, Y., Ye, F., Wang, W., Lu, Q., Kaplan, H.J., and Dean, D.C. (2010). Taz-Tead1 links cell-cell contact to Zeb1 expression, proliferation, and dedifferentiation in retinal pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. 51, 3372–3378.

Marmor, M.F. (1998). Structure, function and disease of the retinal pigment epithelium. In The Retinal Pigment Epithelium. Function and Disease, M.F., Marmor, and T.J., Wolfensberger, ed. (New York: Oxford University), pp. 3–9.

Matsumoto, B., Guerin, C.J., and Anderson, D.H. (1990). Association for research in vision and ophthalmology cytoskeletal redifferentiation of feline, monkey, and human RPE cells in culture. Invest. Ophthalmol. Vis. Sci. 31, 879–889.

Mitashov, V.I. (1996). Mechanisms of retina regeneration in urodeles. Int. J. Dev. Biol. 40, 833–844.

Mitashov, V.I. (1997). Retinal regeneration in amphibians. Int. J. Dev. Biol. 43, 893–905.

Nakamura, K., and Chiba, C. (2007). Evidence for Notch signaling involvement in retinal regeneration of adult newt. Brain Res. 1136, 28–42.

Ng, J.M.Y., and Curran, T. (2011). The hedgehog's tale: developing strategies for targeting cancer. Nat. Rev. Cancer 11, 493– 501.

Nguyen, M.-T.T., and Arnheiter, H. (2000). Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. Development 127, 3581–3591.

Oi, H., Chiba, C., and Saito, T. (2003a). The appearance and maturation of excitatory and inhibitory neurotransmitter sensitivity during retinal regeneration of the adult newt. Neurosci. Res. 47, 117–129.

Oi, H., Chiba, C., and Saito, T. (2003b). A decay of gap junctions associated with ganglion cell differentiation during retinal regeneration of the adult newt. Vis. Res. 43, 2847–2859.

Pacheco-Domı´nguez, R.L., Palma-Nicolas, J.P., Lo´ pez, E., and Lo´ pez-Colome´ , A.M. (2008). The activation of MEK-ERK1 /2 by glutamate receptor-stimulation is involved in the regulation of RPE proliferation and morphologic transformation. Exp. Eye Res. 86, 207–219.

Palma-Nicolas, J.P., Lo´ pez, E., and Lo´ pez- Colome´ , A.M. (2008). PKC isoenzymes differentially modulate the effect of thrombin on MAPK-dependent RPE proliferation. Biosci. Rep. 28, 307–317.

Parrales, A., Palma-Nicola´ s, J.P., Lo´ pez, E., and Lo´ pez-Colome´ , A.M. (2010). Thrombin stimulates RPE cell proliferation by promoting c-Fos-mediated Cyclin D1 expression. J. Cell. Physiol. 222, 302–312.

Pastor, J.C. (1998). Proliferative vitreoretinopathy: an overview. Surv. Ophthalmol. 43, 3–18.

Pratt, C.H., Vadigepalli, R., Chakravarthula, P., Gonye, G.E., Philp, N.J., and Grunwald, G.B. (2008). Transcriptional regulatory network analysis during epithelial-mesenchymal transformation of retinal pigment epithelium. Mol. Vis. 14, 1414–1428.

Reh, T.A., and Pittack, C. (1995). Transdifferentiation and retinal regeneration. Seminars Cell Biol. 6, 137–142.

Saito, T., Kaneko, Y., Maruo, F., Niino, M., and Sakaki, Y. (1994). Study of the regenerating newt retina by electrophysiology and immunohistochemistry (bipolar- and cone-specific antigen localization). J. Exp. Zool. 270, 491–500.

Sakakibara, S., Hiramatsu, H., Takahashi, Y., Hisatomi, O., Kobayashi, Y., Sakami, S., Saito, T., and Tokunaga, F. (2002). Opsin expression in adult, developing, and regenerating newt retinas. Mol. Brain Res. 103, 28–35.

Saksela, O., Moscatelli, D., Sommer, A., and Rifkin, D.B. (1988). Endothelial cell-derived heparin sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. J. Cell Biol. 107, 743–751.

Susaki, K., and Chiba, C. (2007). MEK mediates in vitro neural transdifferentiation of the adult newt retinal pigment epithelium cells: is FGF2 an induction factor? Pigment Cell Res. 20, 364–379.

Tamiya, S., Liu, L., and Kaplan, H.J. (2010). Epithelial-mesenchymal transition and proliferation of retinal pigment epithelial cells initiated upon loss of cell-cell contact. Invest. Ophthalmol. Vis. Sci. 51, 2755–2763.

Tanaka, E.M., Drechsel, D.N., and Brockes, J.P. (1999). Thrombin regulates S-phase re-entry by cultured newt myotubes. Curr. Biol. 9, 792–799.

Tezel, T.H., and Del Priore, L.V. (1996). Density-dependent growth regulation of pig retinal pigment epithelial cells in vitro. Graefe's Arch. Clin. Exp. Ophthalmol. 234, S89–S95.

Tsonis, P.A. (2000). Regeneration in vertebrates. Dev. Biol. 221, 273–284.

Tsonis, P.A., and Del Rio-Tsonis, K. (2004). Lens and retina regeneration: transdifferentiation, stem cells and clinical applications. Exp. Eye Res. 78, 161–172.

Tumova, S., Woods, A., and Couchman, J.R. (2000). Heparan sulfate proteoglycans on the cell surface: versatile coordinators of cellular functions. Int. J. Biochem. Cell Biol. 32, 269–288.

Yanon, A., Klagsbrun, M., Esko, J.D., Leder, P., and Ornitz, D.M. (1991). Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64, 841–848.

Zhang, F., McLellan, J.S., Ayala, A.M., Leahy, D.J., and Linhardt, R.J. (2007). Kinetic and structural studies on interactions between heparin or heparan sulfate and proteins of the hedgehog signaling pathway. Biochemistry 46, 3933–3941.

9. Figures and Legends

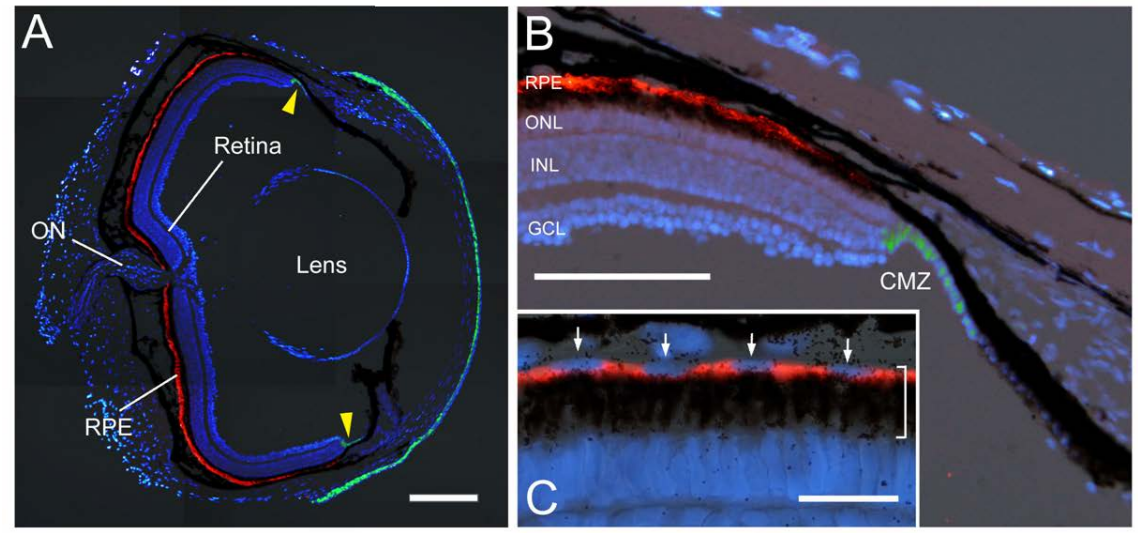

Figure 1. Structure of newt eyeball.

(A) A section of adult *Cynops phrrhogaster* newt eye. Top: dorsal side. Red, RPE65 immunoreactivity; Green, proliferating cell nuclear antigen (PCNA) immunoreactivity; Blue, DAPI nuclear staining. The arrowheads indicate retinal stem/progenitor cells in the ciliary marginal zone (CMZ). (B) A magnified view of the peripheral retina (dorsal side). (C) A magnified view of a central part of the RPE. The arrows indicate nuclei of the RPE cells. Parenthesis indicates the thickness of the RPE including the microvilli. Note that PCNA signal is never observed in the RPE in either the central or peripheral retina, but in the CMZ. This figure was reproduced from original data in Chiba et al., 2006 with modifications.

ON: optic nerve, ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer.

Scale bars in (A); 400 μm (B); 200 μm (C); 40 μm.

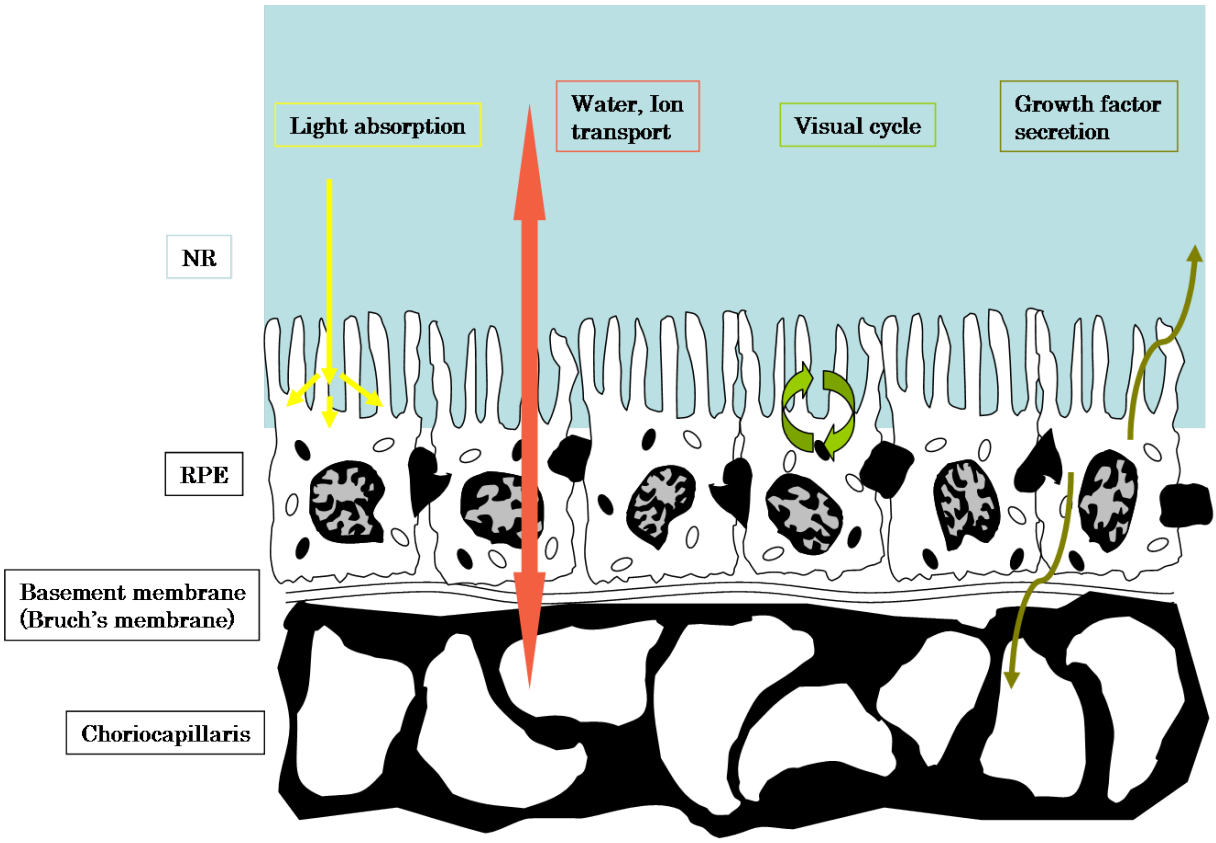

Figure 2. The Retinal Pigment Epithelium (RPE).

Schematic diagram showing the RPE. Modified from strauss 2005.

NR: neural retina.

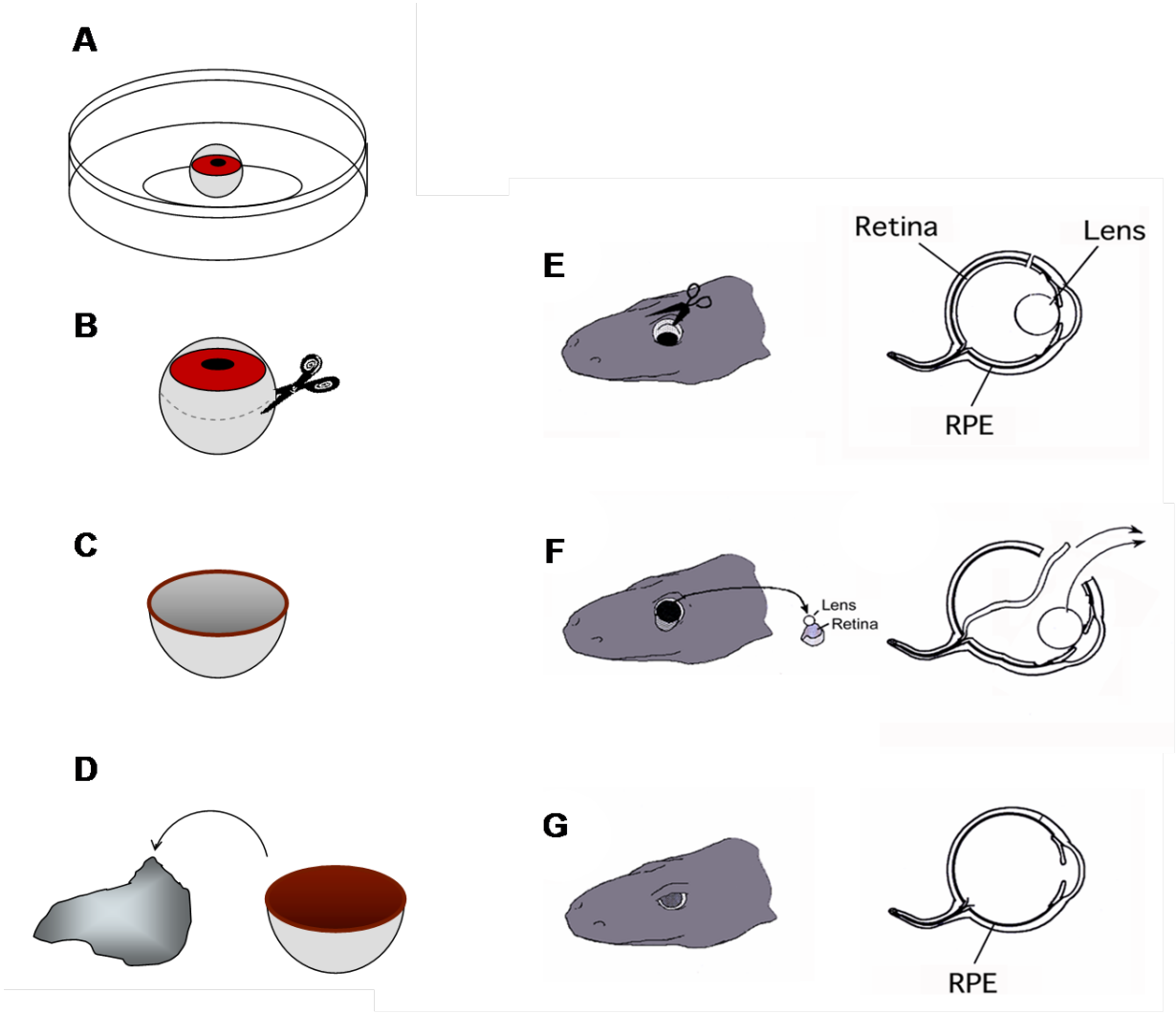

Figure 3. Outline of in vitro retinectomy and retinectomy.

Schematic showing the procedures of in vitro retinectomy (A) – (D) and in vivo retinectomy (E) – (G). (A) Enucleated eyeball is put on the filter membrane conea side up and soaked in PBS. (B) Eyeball is cut along the equator (eye-cup). (C) After 10 min incubation in PBS, (D) neural retina is removed. This tissue, posterior half of the eyeball, was termed retina-less eye-cup (RLEC). (E) Dorsal half of the eyeball was cut open along the corneal-scleral junction. (F) The neural retina and the lens were carefully removed under infusion with saline solution. (G) The eye flap consisting of the iris and the cornea was placed back onto its original position.

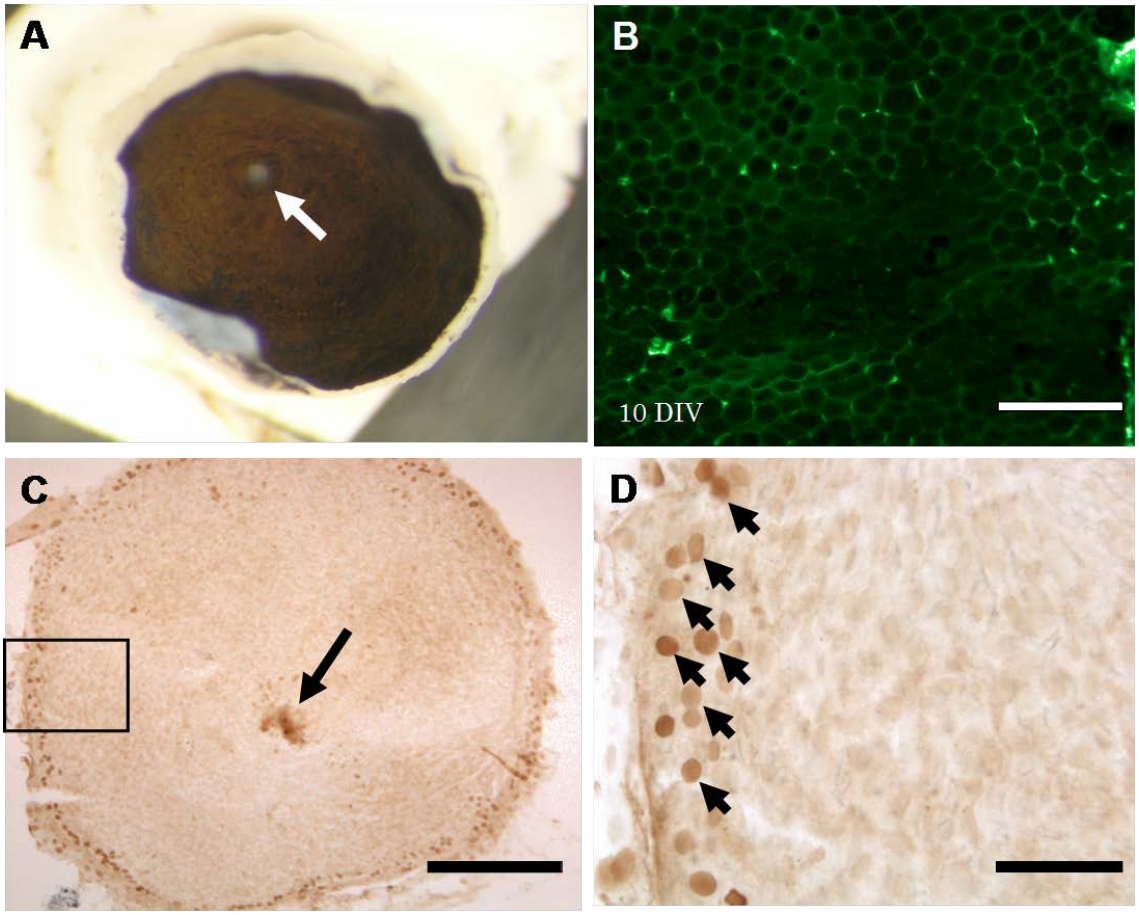

Figure 4. Morphology of RPE cells in a RLEC cultured in newt MEM.

(A) A RLEC before culture. The arrow indicates a hole where the optic nerve exits. The amputated nerve remained. (B) Morphology of RPE cells after 10 days in vitro (DIV). Green, autofluorescence of the choroid. (C) (D) Distribution of BrdU+ RPE cells at 10 DIV. The arrows indicate non-specific staining of the remaining optic nerve (C) and BrdU+ cells (D). A region enclosed by the square is enlarged in panel (D).

Scale bars in (B): 100 μm, (C): 500 μm, (D): 100 μm.

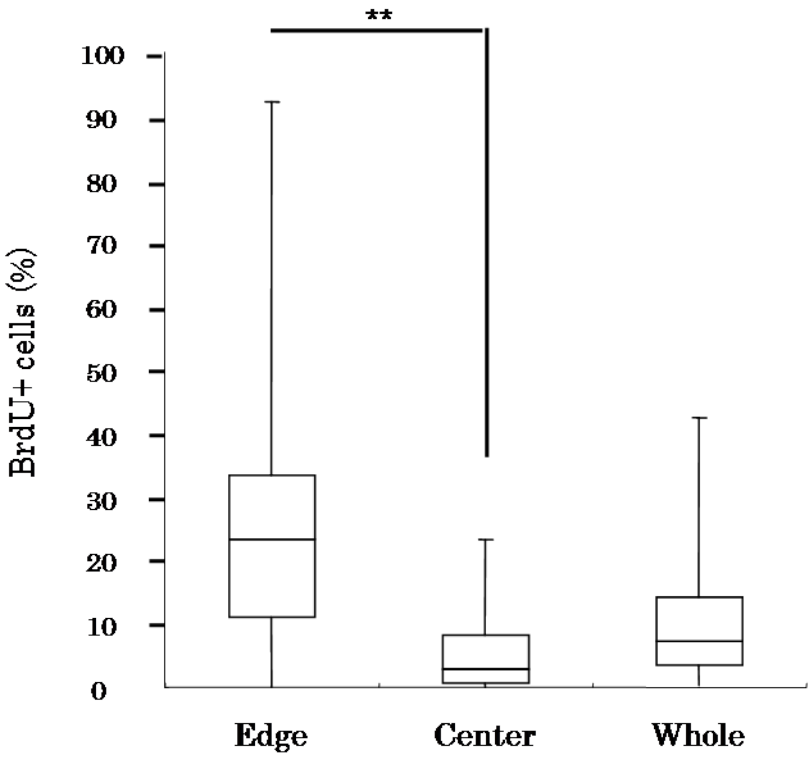

Figure 5. Wound-edge RPE cells enter the cell-cycle under RLEC condition.

The ratio of BrdU+ RPE cells at 10 DIV. In a whole RPE sheet, 10.2 ± 1.2% (n = 49) of cells were BrdU+ (Whole). When the sheet was partitioned into a region 100 μm wide along the peripheral margin (Edge) and the rest (Center), the value of the Edge (25.0 ± 2.8%) was significantly higher than that (5.6 ± 0.9%) of the Center.

$^{**}p < 0.01$, Sheffe's test following the Friedman test

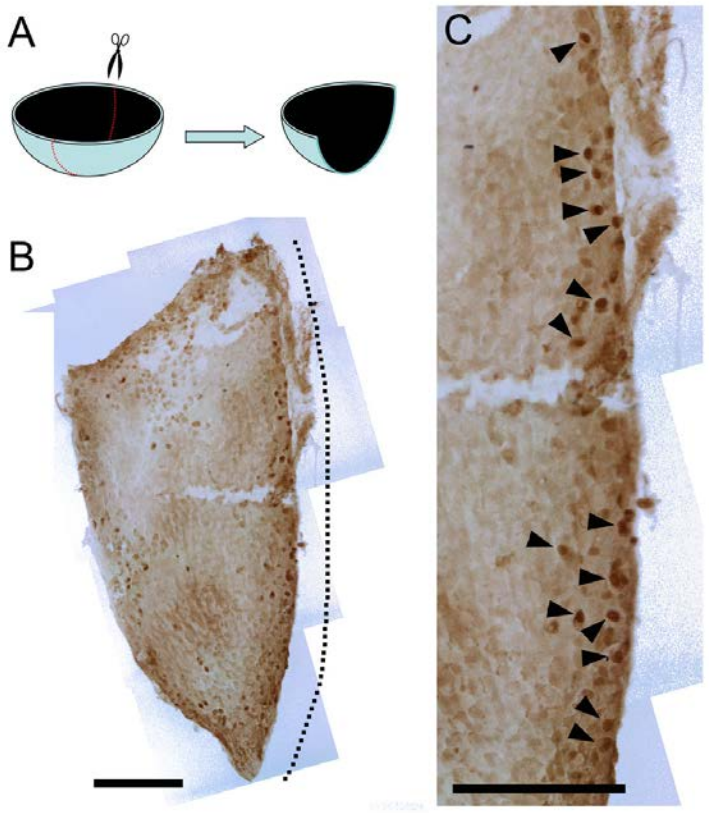

Figure 6. Wound edge–dependent cell-cycle entry of retinal pigment epithelium (RPE) cells.

(A) Schematic showing a procedure to make a half retina-less eye-cup (1/2 RLEC) which has a wound along a longitudinal axis in addition to one along the equator (see Methods). (B) Distribution pattern of BrdU+ nuclei in the 1/2 RLEC cultured in the newt MEM for 10 days. Dotted line shows the wound made along the longitudinal axis of the RLEC. The right-hand margin of the RPE is enlarged in panel (C). The arrowheads indicate BrdU+ nuclei were located around the wound edge along the longitudinal axis.

Scale bars in (B): 200 μm, (C): 130 μm.

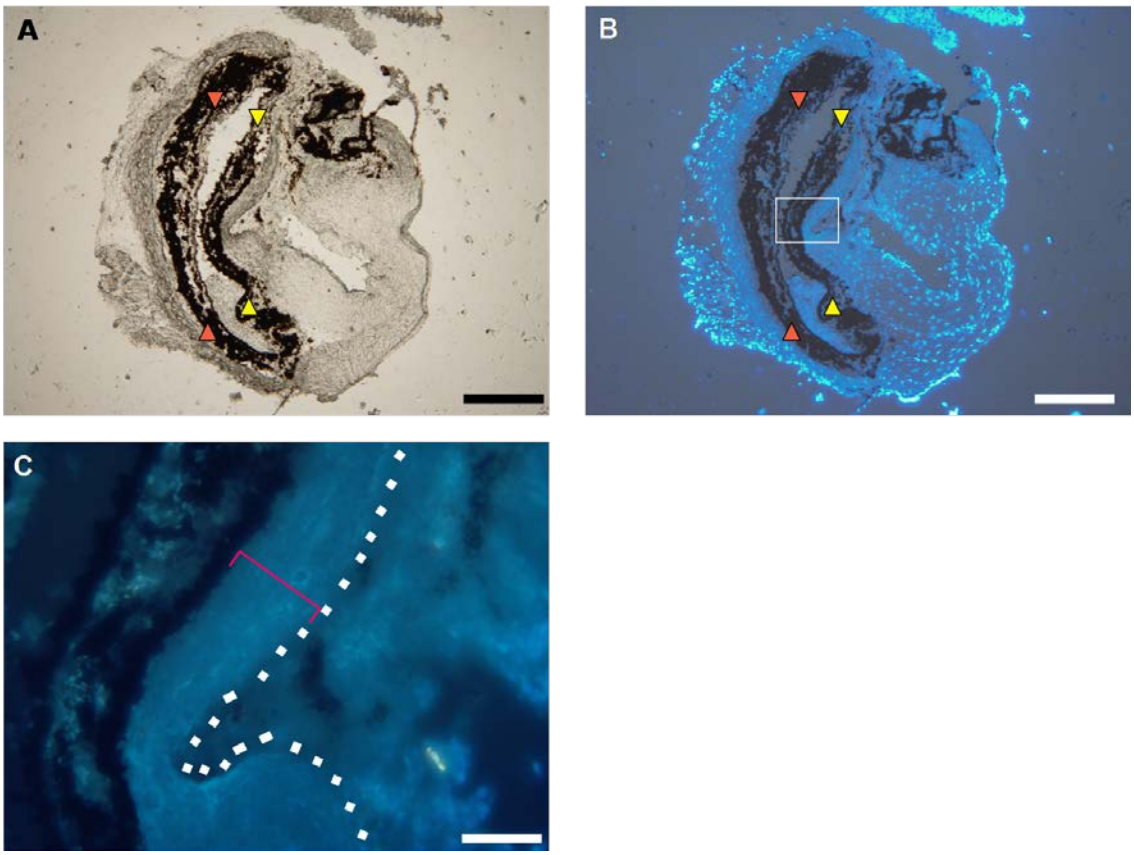

Figure 7. Regenerate neural retina from transplanted RPE-choroid tissue.

A section of newt eyeball within transplanted RPE-choroid (40 days po) under bright-light (A) and fluorescence (B), (C). RPE-choroid tissue was enucleated from RLEC which had been cultured for 10 days and transplanted into retinectomized eyeball. Blue, DAPI nuclear staining. The arrowheads indicate the original RPE (red) and transplanted RPE (yellow). A region enclosed by the square is enlarged in panel (C). The dotted line is along with regenerate NR from transplanted RPE. The hooked red bar indicates thickness of regenerate NR.

Scale bars in (A) and (B): 500 μm, (C): 100μm

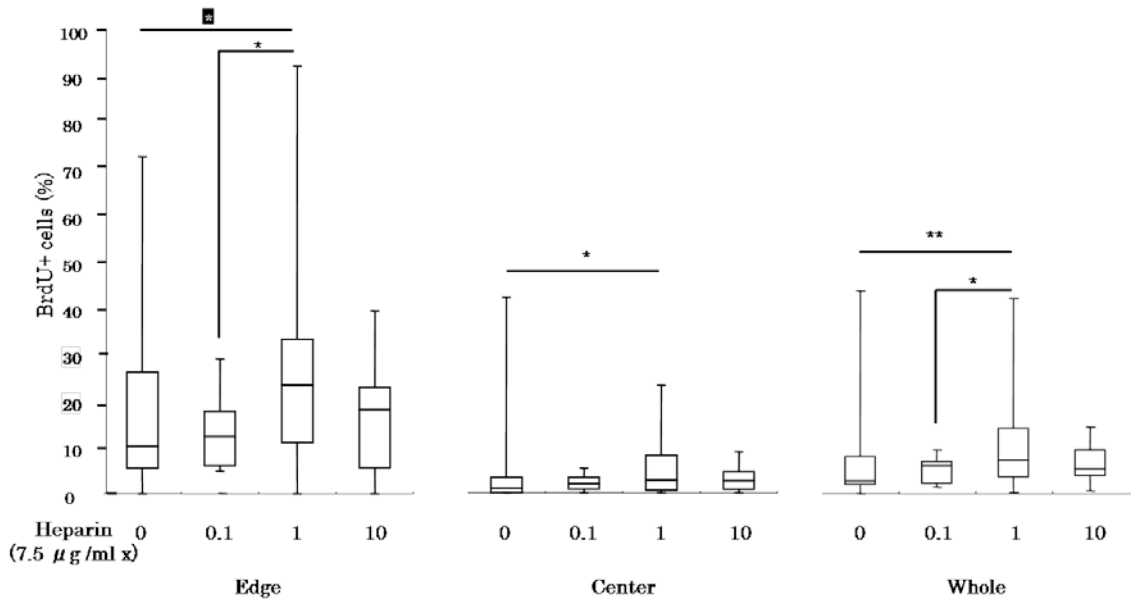

Figure 8. Dose dependence effects of heparin on the cell-cycle entry of RPE cells.

The ratio of BrdU+ cells in RPE was measured in RLEC cultured for 10 days in 80% L-15 (+BrdU) medium containing different concentrations (0, 0.75, 7.5 and 75 μg/ml) of heparin (n = 39 at 0 μg/ml, n = 11 at 0.75 μg/ml, n = 49 at 7.5 μg/ml, n = 13 at 75 μg/ml). For definitions of Edge, Center, and Whole, see Figure 7.

*p < 0.05 and **p < 0.01; for Mann–Whitney's U-test, black asterisks; for the Jonckheere–Terpstra test, white asterisks.

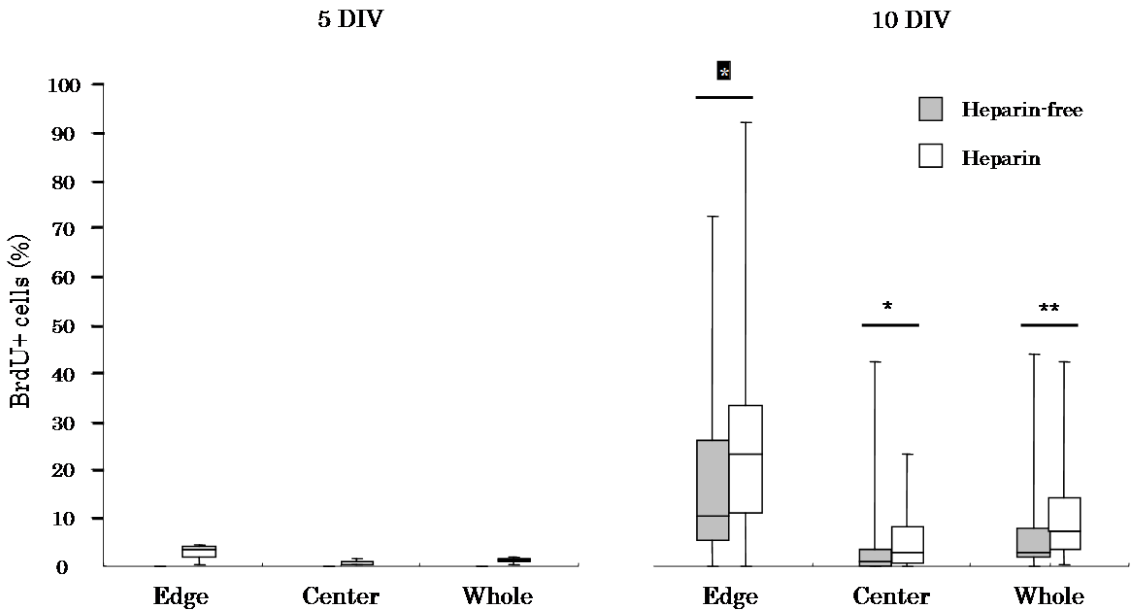

Figure 9. Effects of heparin on kinetic changes of the cell-cycle entry of RPE cells.
Changes in the ratio of BrdU+ cells in RPE during culture of the RLEC in either the basal medium (heparin-free, n = 2 at 5 DIV, n = 39 at 10 DIV) or the standard newt MEM (7.5 μg/ml heparin, n = 3 at 5 DIV, n = 49 at 10 DIV). For definitions of Edge, Center, and Whole, see Figure 7.
*p < 0.05 and **p < 0.01; for Mann–Whitney's U-test, black asterisks; for the Jonckheere–Terpstra test, white asterisks.

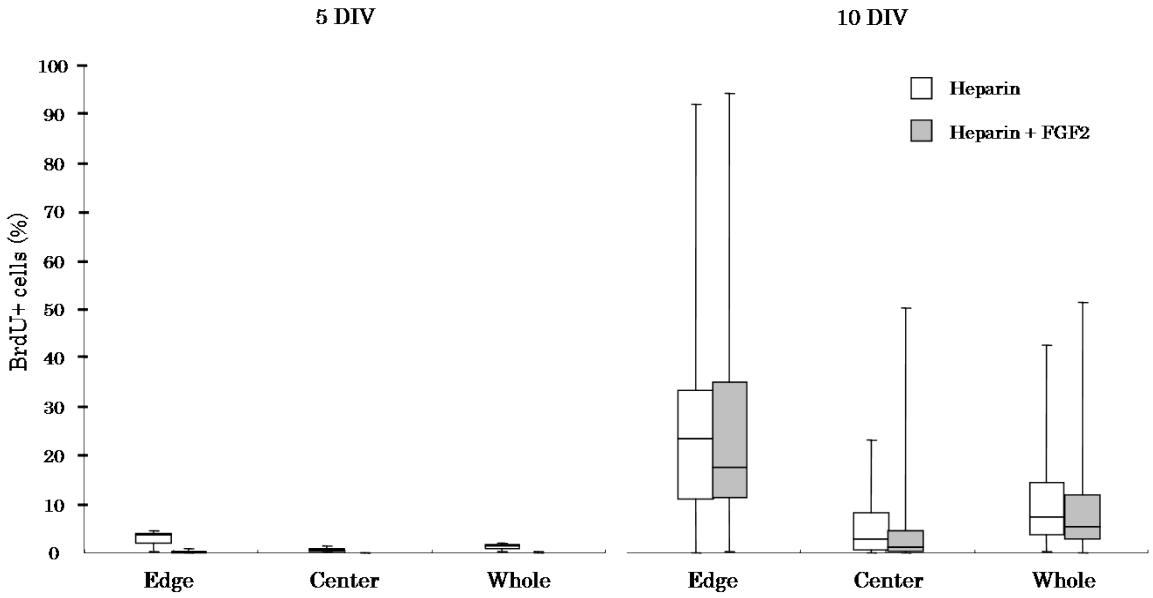

Figure 10. Participation of FGF2 in the cell-cycle entry of RPE cells.

Changes in the ratio of BrdU+ cells in RPE during culture of the RLECs were examined in either newt MEM (heparin, n = 3 at 5 DIV, n = 49 at 10 DIV) or newt MEM to which 50 ng/ml FGF2 was added (heparin+ FGF2, n = 5 at 5 DIV, n = 42 at 10 DIV). For definitions of Edge, Center, and Whole, see Figure 7.

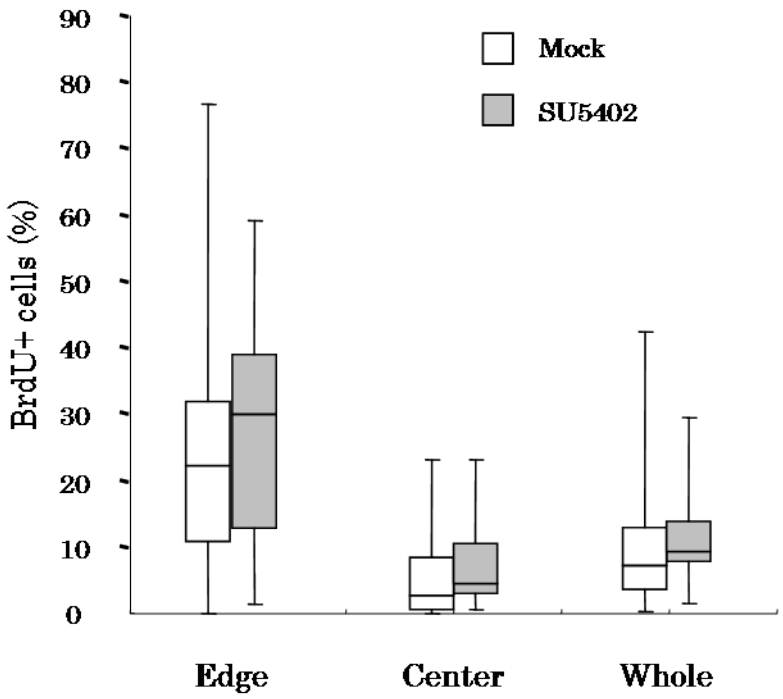

Figure 11. Effects of an FGF receptor–specific tyrosine kinase inhibitor SU5402 on the cell-cycle entry of RPE cells.

The ratio of BrdU+ cells in RPE was measured in the RLEC cultured for 10 days in either newt MEM containing 25 μM SU5402 and its solvent 0.25% DMSO (SU5402, n = 15), or newt MEM containing the solvent only (Mock, n = 21). For definitions of Edge, Center, and Whole, see Figure 7.

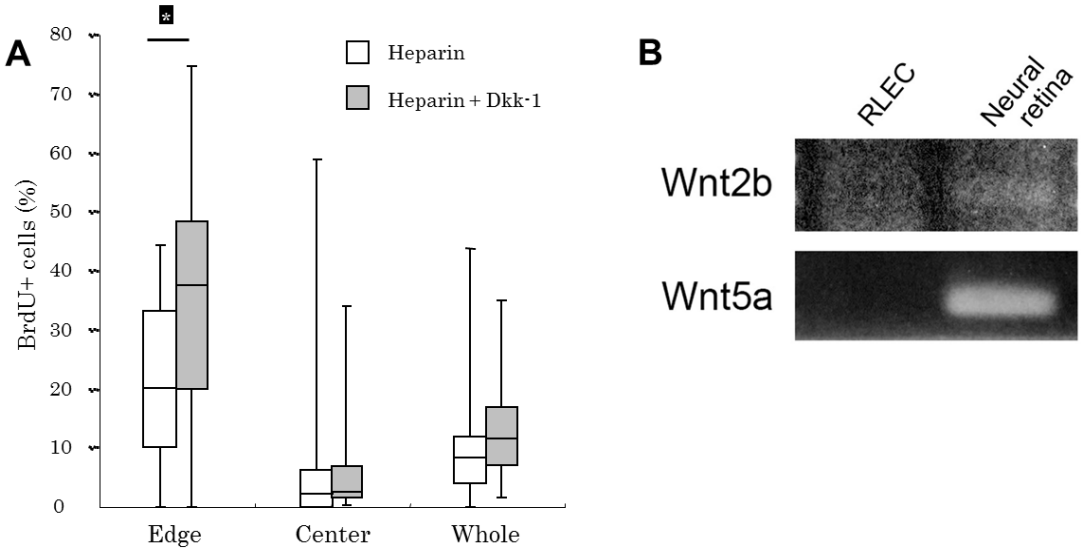

Figure 12. Participation of Wnt-mediated signaling pathways in the heparin-promoted cell-cycle entry of RPE cells.

(A) Effect of Wnt signaling blocker Dkk-1. RLECs were cultured in either newt MEM (heparin, n = 15) or newt MEM to which 10 ng/ml Dkk-1 was added (heparin+ Dkk-1, n = 15). For definitions of Edge, Center, and Whole, see Figure 7. (B) Expression of Wnts in the eye-cup tissue.

*$p < 0.05$, Jonckheere–Terpstra test.

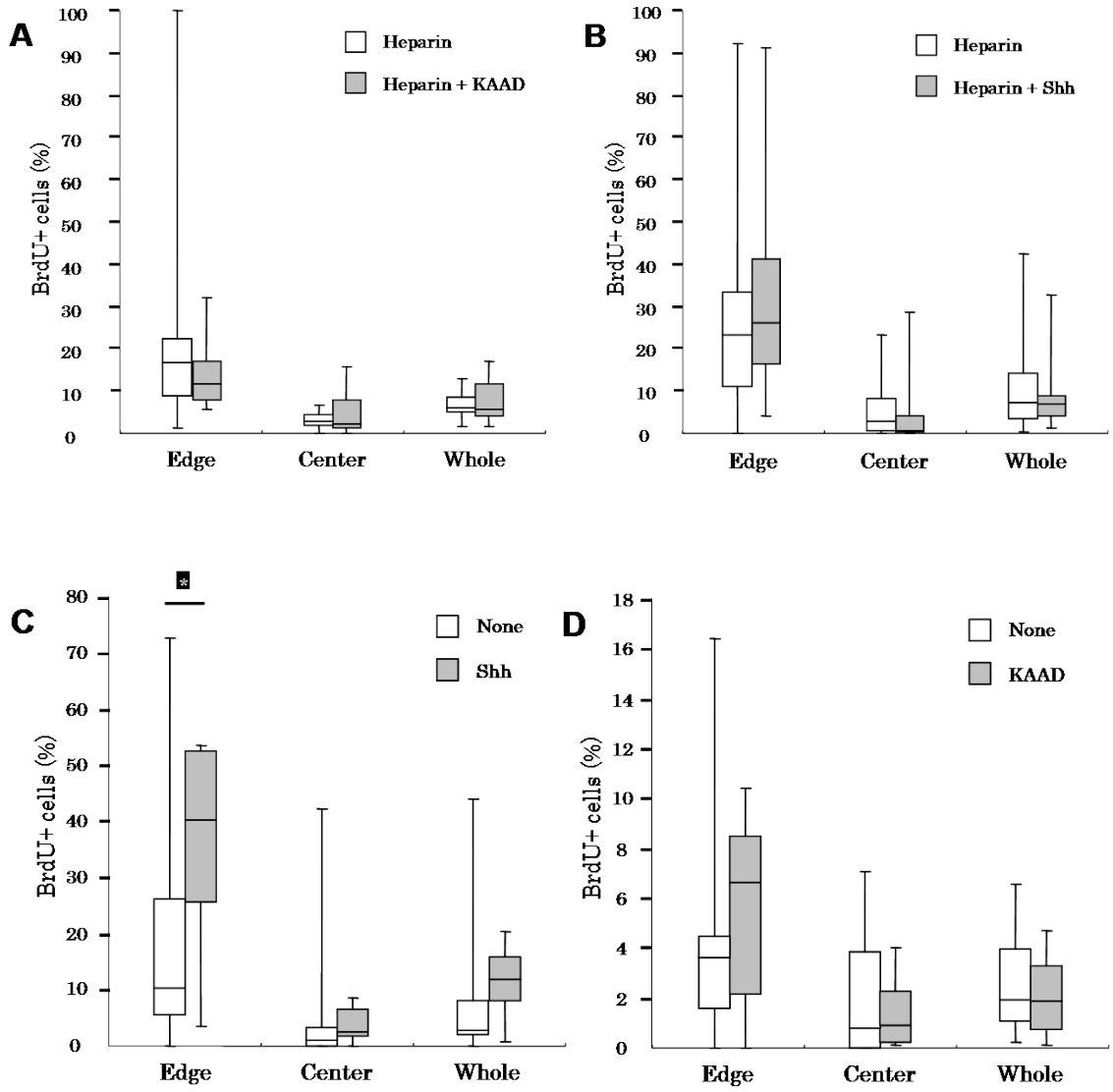

Figure 13. Participation of Shh-mediated signaling pathways in the heparin-promoted cell-cycle entry of RPE cells.

(A) Effects of a Shh signaling blocker KAAD. RLECs were cultured in newt MEM to which either 0.4% EtOH (heparin, n = 9) or 40 μM KAAD + 0.4% EtOH (heparin+ KAAD, n = 11) were added. (B) Effects of Shh. RLECs were cultured in either newt MEM (heparin, n = 49) or newt MEM to which 0.5 μg/ml Shh was added (heparin+ Shh, n = 23). (C) Effects of Shh in the absence of heparin. RLECs were cultured in basal medium to which either nothing (none, n = 39) or 0.5 μg/ml Shh was added (Shh, n = 6).

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(D) Effects of KAAD in the absence of heparin. RLECs were cultured in basal medium to which 0.4 % EtOH (None, n = 6) or 40 μM KAAD + 0.4% EtOH (KAAD, n = 6) were added. For definitions of Edge, Center, and Whole, see Figure 7.
*$p < 0.05$, Jonckheere–Terpstra test

Figure 14. Participation of thrombin in the heparin-promoted cell-cycle entry of RPE cells.

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Figure 15. Participation of fetal bovine serum (FBS) with thrombin on cell-cycle entry of RPE cells.

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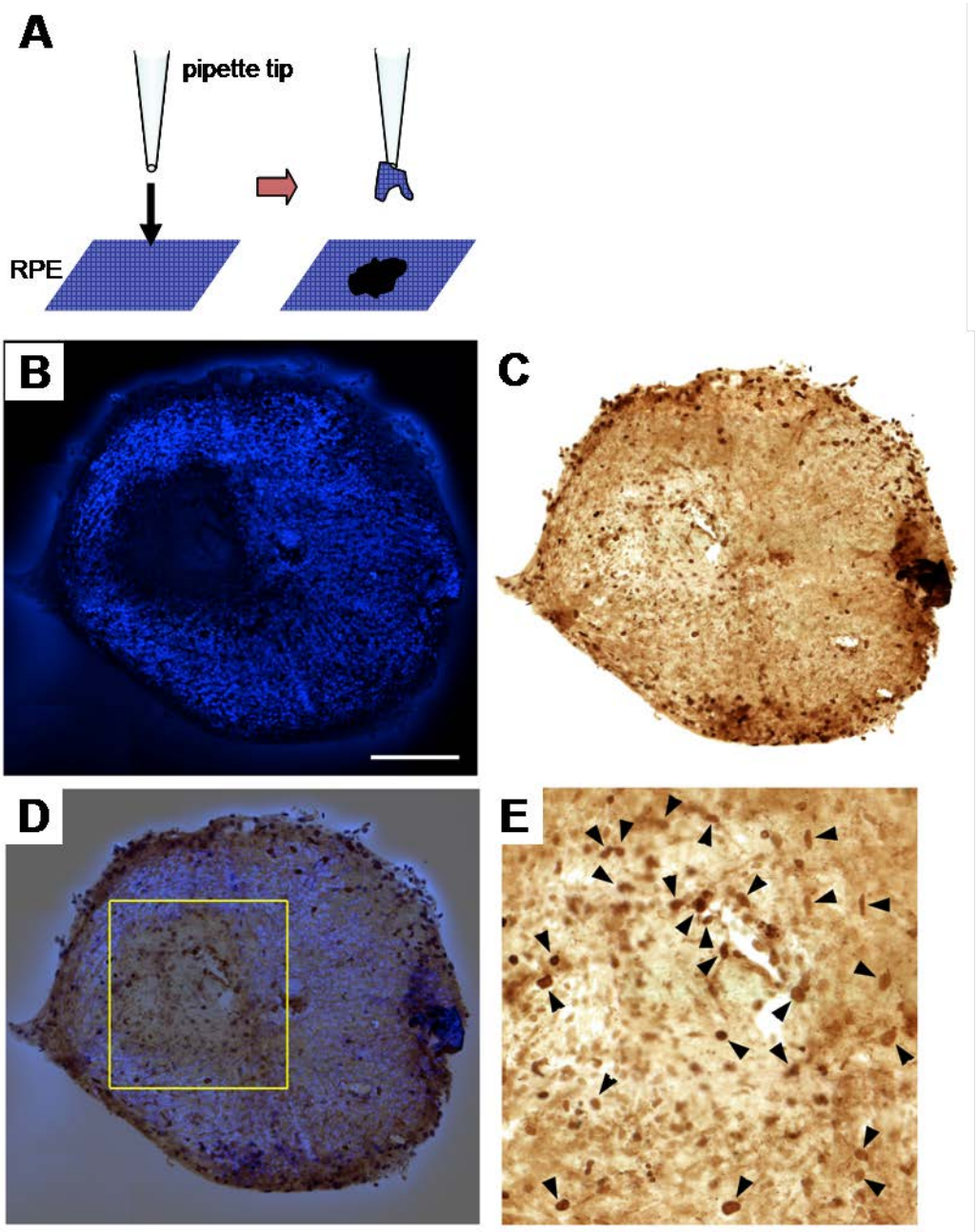

Figure 16. Effects of the removal of a small piece of the epithelium from the Center of the RPE.

(A) Schematic showing the procedures of removal of a small piece of the epithelium from the Center of the RPE in the RLEC by a micropipette tip. Then the operated RLEC was cultured and BrdU+ nuclei in the RPE were visualized at 10 days in culture.

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(continued)

(B) DAPI staining of nuclei. The area from which a piece of the epithelium had been removed was dark with a low density of nuclei when observed under a fluorescence microscope. (C) BrdU+ nuclei. (D) Merged image of (B) and (C). The area enclosed by a square is enlarged in panel (E). The arrowheads indicate sample BrdU+ nuclei. Scale bar in (A): 400 μm.

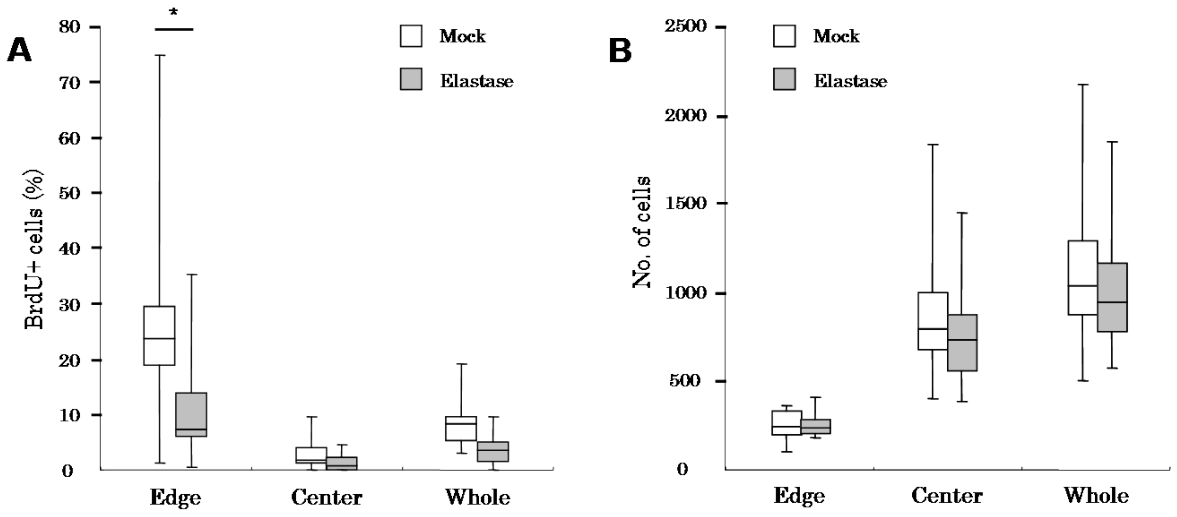

Figure 17. Participation of cell-to-extracellular-matrix (ECM) contact and in the cell-cycle entry of RPE cells.

Effects of elastase, a protease that digests Bruch's membrane efficiently, on the ratio of BrdU+ cells (A) and total cell number in the RPE (B). Retina-less eye-cups (RLECs) were incubated in either 80 % L-15 medium containing 0.1 % elastase/1 mM Tris-HCl (Elastase, n = 13), or that containing 1 mM Tris-HCl only (Mock, n = 13) for 1 h prior to the culture in the basal medium (see Methods). In these experiments, two eyeballs of the same animal were used for the test and control experiments. For definitions of Edge, Center and Whole, see Figure 7.

*$p < 0.05$; Sheffe's test following the Friedman test

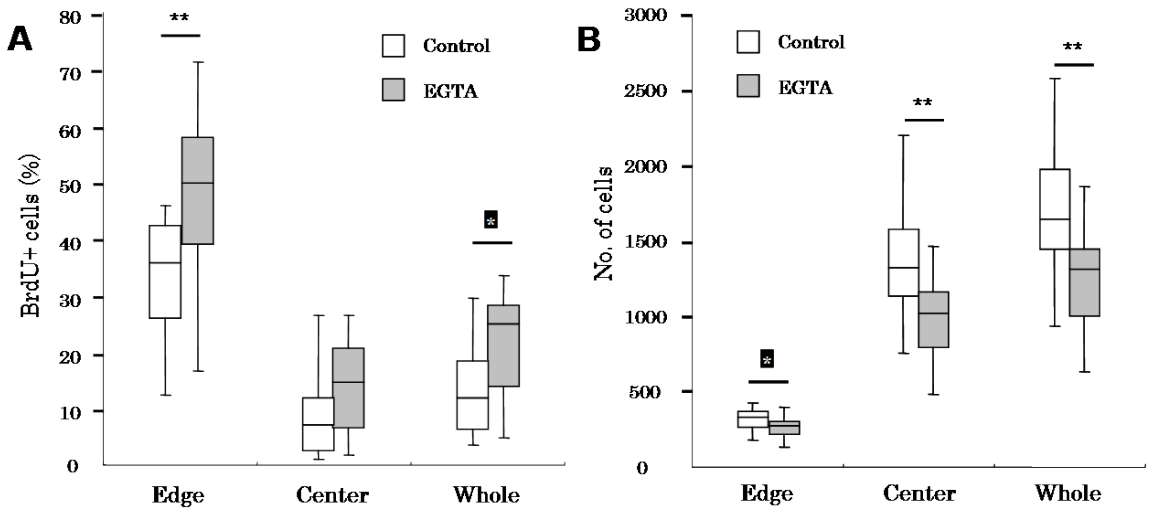

Figure 18. Participation of cell-to-cell contact in the cell-cycle entry of RPE cells.
Effects of EGTA, a calcium chelator that makes cell-to-cell contact looser, on the ratio of BrdU+ cells (A) and total cell number in the RPE (B). RLECs were incubated in either 10 mM EGTA solution (EGTA, n = 8) or a newt saline solution (Control, n = 8) for 1.5 h prior to the culture in the basal medium (see Methods). In these experiments, two eyeballs of the same animal were used for the test and control experiments. For definitions of Edge, Center and Whole, see Figure 7.
*$p < 0.05$ and **$p < 0.01$; for the Sheffe's test following the Friedman test, black asterisks; for the Jonckheere–Terpstra test, white asterisks.

Figure 19. Hypothesis of effect of thrombin.

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