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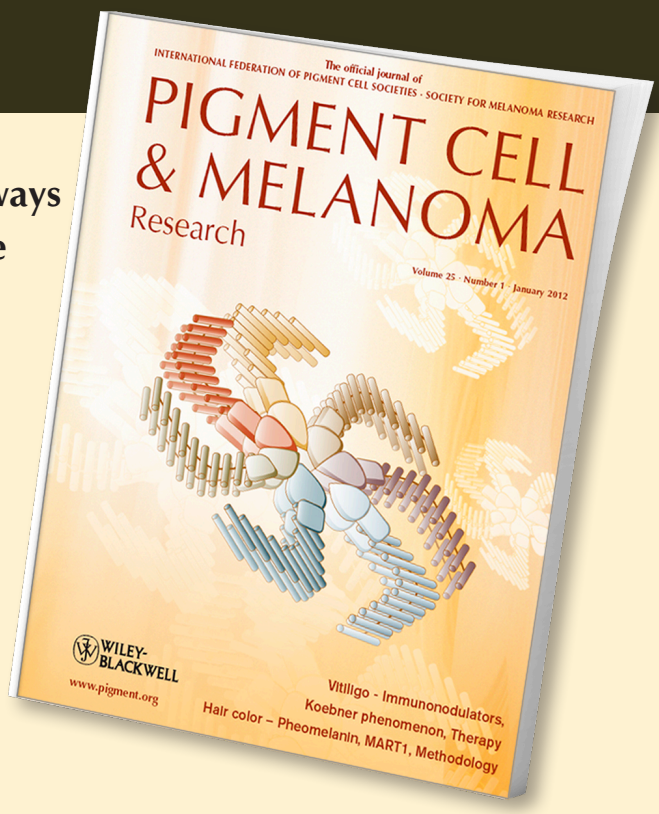

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MEK–ERK and heparin-susceptible signaling pathways are involved in cell-cycle entry of the wound edge retinal pigment epithelium cells in the adult newt

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Summary

The onset mechanism of proliferation in mitotically quiescent retinal pigment epithelium (RPE) cells is still obscure in humans and newts, although it can be a clinical target for manipulating both retinal diseases and regeneration. To address this issue, we investigated factors or signaling pathways involved in the first cell-cycle entry of RPE cells upon retinal injury using a newt retina-less eye-cup culture system in which the cells around the wound edge of the RPE exclusively enter the cell cycle. We found that MEK–ERK signaling is necessary for their cell-cycle entry, and signaling pathways whose activities can be modulated by heparin, such as Wnt-, Shh-, and thrombin-mediated pathways, are capable of regulating the cell-cycle entry. Furthermore, we found that the cells inside the RPE have low proliferation competence even in the presence of serum, suggesting inversely that a loss of cell-to-cell contact would allow the cells to enter the cell cycle.

Introduction

The retinal pigment epithelium (RPE), which is located between the neural retina and the choriocapillaris in the eye, is derived as a counterpart of the neural retina from the multipotent optic neuroepithelium. The mature RPE is a highly specialized monolayer with pigmented microvilli, supporting the neural retina and vision with various physiological roles (Marmor, 1998). Such RPE cells are as a rule mitotically inactive but, upon injury to the neural retina or the choroid, change their phenotypes and start to proliferate. In humans, RPE cell proliferation is a negative sign of the pathogenesis of retinal diseases such as proliferative vitreoretinopathy (PVR) or choroidal melanoma (Kim and Arroyo, 2002; Pastor, 1998).

In contrast to humans, newts can regenerate, even in adulthood, their entire retinas through proliferation and transdifferentiation of RPE cells (Chiba et al., 2006; Chiba and Mitashov, 2007; Haynes and Del Rio-Tsonis, 2004; Mitashov, 1996; Reh and Pittack, 1995; Tsonis, 2000; Tsonis and Del Rio-Tsonis, 2004). Therefore, the

Significance

In contrast to humans, newts can regenerate their retinas through proliferation and transdifferentiation of the RPE cells. Therefore, this system can be a good model to obtain insight into the treatment of retinal diseases such as proliferative vitreoretinopathy caused by proliferation and phenotype switching of RPE cells, and even for retinal regeneration in a patient's eyes. Three elements (the MEK–ERK pathway, heparin-susceptible pathways, and a pathway for liberation from contact inhibition), which we propose here to be involved in the first cell-cycle entry of RPE cells upon retinal injury, would undoubtedly be the next subjects to find the differences between retinal diseases and regeneration.

adult newt retinal regeneration system can be a good model to obtain insight into the treatment of retinal diseases caused by phenotype switching and proliferation of RPE cells, and even for retinal regeneration in patients' eyes.

The onset mechanism of proliferation in mitotically quiescent RPE cells is not fully understood in either humans or newts, although it can be a clinical target for manipulating both retinal diseases and regeneration. To address this issue, plenty studies have been conducted thus far using primary cultures of isolated RPE cells or cell lines established from those cells (Bryckaert et al., 2000; Hecquet et al., 2002a,b; Hollborn et al., 2006; Kaven et al., 2000; Lashkari et al., 1999; Pacheco-Domínguez et al., 2008; Palma-Nicolas et al., 2008; Parrales et al., 2010; Susaki and Chiba, 2007). However, these systems might not be suitable because isolated cells have already received some signals to become mitotically active during the enzymatic dissociation process. In fact, in adult newts, when RPE cells are isolated from the basement membrane (Bruch's membrane)-choroid tissue by an 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). So, to minimize this problem, in a previous study, we established an alternative system using the adult newt *Cynops pyrrhogaster*, which is a serum-free culture system of the posterior half of the eyeball from which the neural retina has been carefully removed, resulting in a tissue termed the 'retina-less eye-cup (RLEC)' (Susaki and Chiba, 2007). This tissue culture system would be more advantageous to explore factors or signals which initiate proliferation in RPE cells. Therefore, in the current study, we investigated, using this system, which factors or signals are involved in the first cell-cycle entry of RPE cells following removal of the neural retina.

Results

Wound edge RPE cells enter the S-phase of cell cycle under RLEC culture conditions

In adult newt eyes, proliferating cells have not been found in the intact RPE (Figure 1, and see Keefe, 1973). We first examined whether such RPE cells become mitotically active under RLEC culture conditions (Figure 2). RLECs were prepared in PBS as illustrated in Figure 2(A) (for a sample, see Figure 2B) and then cultured at 25°C in a newt MEM (80% L-15 medium containing 7.5 μg/ml heparin and 5 μg/ml BrdU). Under these culture conditions, the RPE appeared to maintain their original characteristics for 10 days; RPE cells retained melanin pigments, and their hexagonal morphology was almost unchanged (Figure 2C); at 10 days in culture, the number of RPE cells in the RLEC (1894 ± 114, n = 49) was not statistically different from that in the intact eye-cup (1804 ± 131, n = 10), and apoptotic cells were not observed in the TUNEL assay (n = 2). On the other hand, 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-neuron marker, acetylated tubulin (AT) (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 melanin pigments. Interestingly, most BrdU+ cells were located along the peripheral margin of the RPE sheet (Figure 2D, E). When the ratio of the BrdU+ cells was

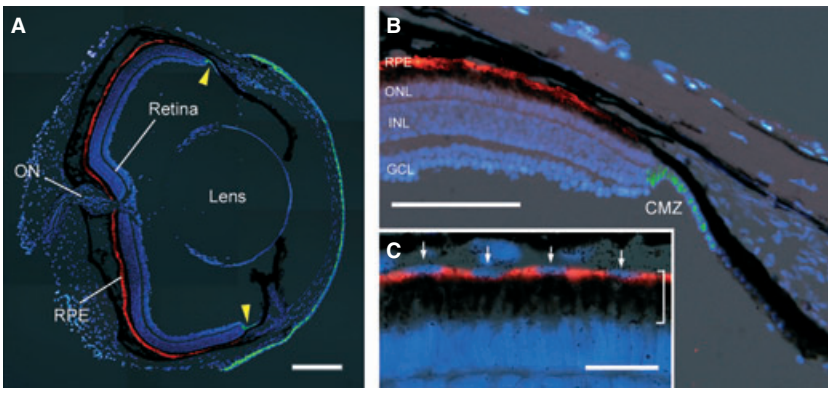

Figure 1. Mitotically quiescent retinal pigment epithelium (RPE) cells in the newt. (A) A section of the adult *Cynops pyrrhogaster* newt eye. Top: dorsal side. Red, RPE65 immunoreactivity; Green, proliferating cell nuclear antigen (PCNA) immunoreactivity; Blue, DAPI nuclear staining. Arrowheads: retinal stem/progenitor cells in the ciliary marginal zone (CMZ). ON, optic nerve. (B) A magnified view of the peripheral retina (dorsal side). ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. (C) A magnified view of a central part of the RPE. Arrows: 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. Scale bars: 400 μm (A); 200 μm (B); 40 μm (C). This figure was reproduced from original data in Chiba et al., 2006 with modifications.

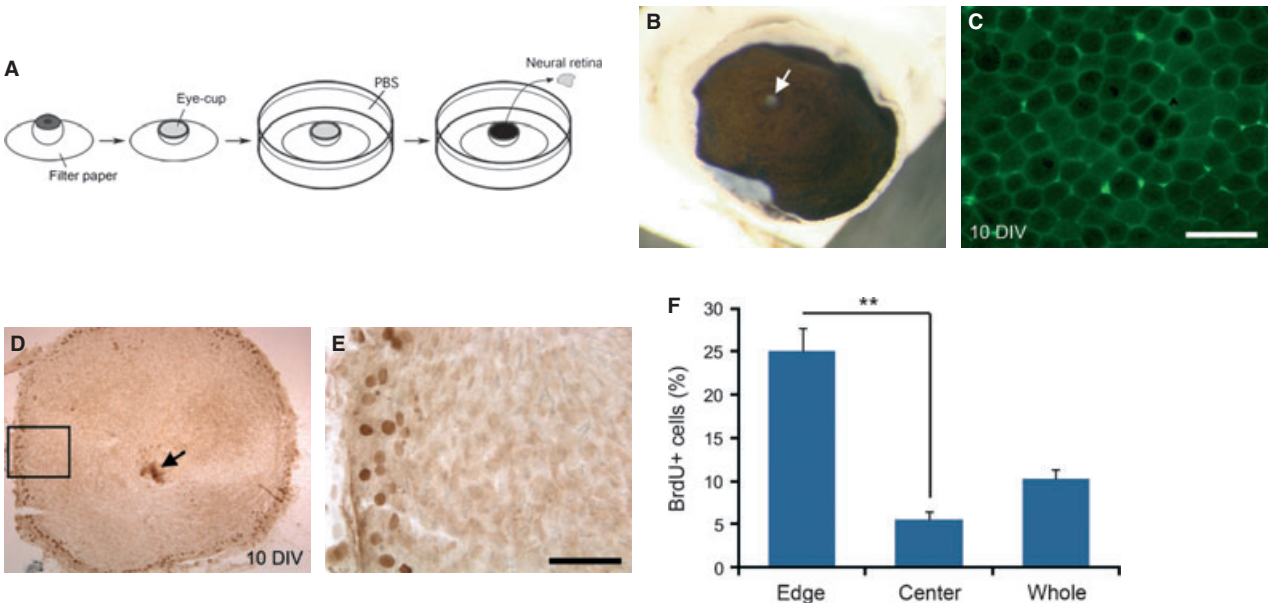

Figure 2. Cell-cycle entry of retinal pigment epithelium (RPE) cells in a retina-less eye-cup (RLEC) cultured in newt minimum essential medium. (A) Schematic showing a procedure to make a RLEC (see Methods). (B) A RLEC before culture. The RPE was well preserved in the cup. The arrow indicates a hole where the optic nerve exits. The amputated nerve remained. (C) Morphology of RPE cells after 10 days *in vitro* (DIV). The RPE sheet was separated together with the choroid tissue from the RLEC culture, placed and flattened on a glass slide, and then viewed under a fluorescence microscope (see Methods). Under these conditions, the number of RPE cells on the sheet was counted. (D) Distribution of BrdU+ RPE cells at 10 DIV. After the cell count was complete, BrdU+ nuclei on the sheet were visualized by bleaching melanin pigments. The BrdU+ nuclei were mostly located along the peripheral margin of the sheet. The arrow indicates non-specific staining of the remaining optic nerve. A region enclosed by the square is enlarged in panel E. Scale bars in C and E: 100 μm. (F) 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* (Sheffe's test following the Friedman test, **P < 0.01).

calculated in a 100-μm-wide region along the peripheral margin (defined as the 'Edge'), the value was significantly higher than that in the rest of the RPE sheet (defined as the 'Center') (Figure 2F). Mitotic figures were only rarely observed in either region: 2.4 ± 0.4% of BrdU+ cells in the Edge of 18 (36.7%) RPE sheets and 2.4 ± 0.5% of BrdU+ cells in the Center of 9 (18.4%) RPE sheets. It should be noted that no cells exhibiting mitotic figures were observed in RPE at 5 days. These results indicate that a population of RPE cells, mostly located on the Edge, enters the S-phase of the cell cycle but hardly proceeds into the M-phase within 10 days when cultured in the RLEC.

Such a distribution pattern of BrdU+ cells might reflect a difference in cell properties between the peripheral and central RPE or might indicate whether the cell-cycle entry is related to the wound edge of the RPE/RLEC. Therefore, we cut the RLEC longitudinally across the posterior pole into halves and cultured them separately for 10 days (Figure 3A). In every piece of the RPE sheet, after 10 days in culture, BrdU+ cells appeared along the newly formed longitudinal margins (Figure 3B, C). This result indicates that the cell-cycle entry is connected with the wound edge of the RPE/RLEC.

Heparin promotes cell-cycle entry of RPE cells

For the RLEC culture shown above, we used the newt MEM because this medium allowed us to carry out 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 glycosaminoglycans. In the previous study, in which we aimed to examine the effects of exogenously administered growth factors such as FGF2 on RPE cells *in vitro* (Susaki and Chiba, 2007), we added heparin to the basal medium (80% L-15 medium) as a supplement because heparin is known to bind various soluble factors including FGF2 and support their actions on receptors (DiGabriele et al., 1998; Kan et al., 1993; Yanon et al., 1991) as well as protect these factors against degradation (Gospodarowicz and Chen, 1986; Saksela et al., 1988). Therefore, we suspected that heparin assists some endogenous factors released from RLEC wounds.

To examine this possibility, we compared the results obtained in the 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 BrdU+ cells in the RPE increased significantly in the presence of 7.5 μg/ml heparin (i.e. in the standard

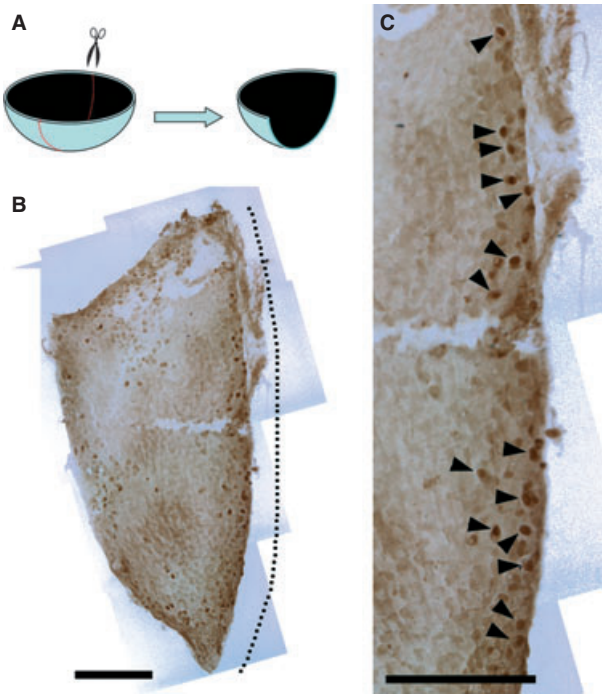

Figure 3. Wound edge–dependent cell-cycle entry of retinal pigment epithelium (RPE) cells. (A) Schematic showing a procedure to make a half retina-less eye-cup (1/2 RLEC) which has a wound along a longitudinal axis in addition to one along the equator (see Methods). (B) Distribution pattern of BrdU+ nuclei in the 1/2 RLEC cultured in the newt minimum essential medium for 10 days. The right-hand margin of the RPE, which is enlarged in panel C, corresponds to the wound made along the longitudinal axis (dotted line) of the RLEC. BrdU+ nuclei were located around the wound edge along the longitudinal axis (arrowheads in C) as well as around that along the equator. Scale bars: 200 μm (B); 130 μm (C).

newt MEM), although the distribution pattern did not change substantially (Figure 4A). However, it must be noted that the 10× higher concentration of heparin (75 μg/ml) did not elevate the ratio of BrdU+ cells further. Next we examined whether heparin affects the kinetics of the cell cycle (Figure 4B). In the presence of 7.5 μg/ml heparin, a small number of BrdU+ cells appeared in the RPE in 5-day culture (5–62 cells in the Whole), whereas in the basal medium (heparin-free), few positive cells were observed (<1 cell in the Whole), suggesting that this concentration of heparin may assist a process underlying the cell-cycle entry of RPE cells. MTT and TUNEL assays were carried out to evaluate cell viability, but the results were not different between the absence and presence of heparin (0.75–75 μg/ml; data not shown).

FGF2 is unlikely to be a trigger of cell-cycle entry of RPE cells

It is accepted that FGF2, whose actions *in vitro* can be supported by heparin, is a candidate trigger factor of transdifferentiation of the embryonic/larval RPE into the neural retina (Azuma et al., 2005; Galy et al., 2002; Nguyen and Arnheiter, 2000; Spence et al., 2007; Vergara and Del Rio-Tsonis, 2009). Also in the adult newt, this factor promotes neural transdifferentiation with AT expression in RPE cells *in vitro* (Susaki and Chiba, 2007). Therefore, we examined the effects of FGF2 on the cell-cycle entry of RPE cells; FGF2 was administered to RPE cells in the RLEC from the beginning of culture at 50 ng/ml; this concentration is enough to promote AT expression in RPE cells even in the Center of the RLEC (Susaki and Chiba, 2007). In the control culture with the standard newt MEM, RPE cells mostly started to enter the S-phase of the cell cycle later than 5 days ('heparin' in Figure 5A). FGF2 was unlikely to affect either the kinetics of the cell cycle or the distribution pattern and ratio of BrdU+ cells in the RPE ('heparin+ FGF2' in Figure 5A). To confirm this conclusion, we examined the effects of an FGF receptor–specific tyrosine kinase inhibitor, SU5402, on the cell-cycle entry of RPE cells; 25 μM SU5402 and its solvent 0.25% DMSO was administered to RPE cells in the RLEC from the beginning of culture. Expectedly, as compared to the solvent only (Mock), SU5402 was unlikely to affect the distribution pattern and ratio of BrdU+ cells (Figure 5B).

In a previous study, we found that RPE cells in the RLEC can express AT during culture even in the absence of exogenous FGF2, and that the responsiveness of RPE cells to FGF2 with AT expression appears later than 5 days (Susaki and Chiba, 2007). Taken together, in the adult newt, FGF2 is unlikely to be a trigger of either cell-cycle entry or neural transdifferentiation of RPE cells. However, as demonstrated in isolated RPE cell culture, this factor might allow, after more than 10 days, the RPE cells to proceed to the M-phase and promote their subsequent proliferation (Susaki and Chiba, 2007).

MEK–ERK signaling is necessary for cell-cycle entry of RPE cells

The mitogen-activated protein kinase (MAPK) cascades are key intracellular signaling pathways involved in the regulation of normal cell proliferation, survival, and differentiation (Friday and Adjei, 2008; Raman et al., 2007; Zhang and Dong, 2007). ERK is a member of the MAPK family, and its activity is positively regulated by phosphorylation mediated by MEK. The MEK–ERK cascade is typically activated by growth factors that stimulate the receptor tyrosine kinases on the cell membrane. In the culture of RLEC with the newt MEM, it was suggested that MEK–ERK signaling in RPE cells is gradually increased, even in the Center of the RPE, from the beginning of culture (Susaki and Chiba, 2007). Therefore, we examined whether MEK–ERK signaling is involved in the cell-cycle entry of RPE cells (Figure 6); RLECs were prepared in PBS containing a MEK inhibitor, 5 μM U0126, and its solvent 0.25% DMSO within

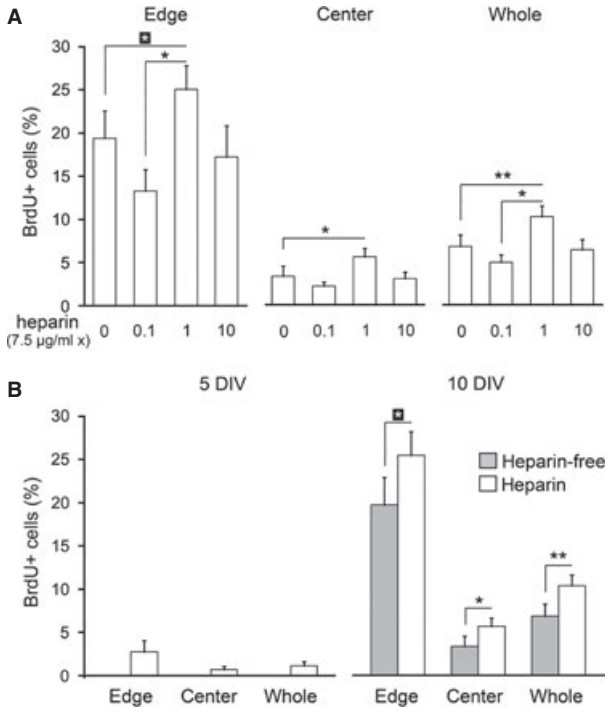

Figure 4. Effects of heparin on the cell-cycle entry of retinal pigment epithelium (RPE) cells. (A) Dose dependency. The ratio of BrdU+ cells in RPE was measured in retina-less eye-cup (RLEC) cultured for 10 days in 80% L-15 (+BrdU) medium containing different concentrations (0, 0.75, 7.5, and 75 μg/ml) of heparin. The value at 7.5 μg/ml heparin, i.e. in the standard newt minimum essential medium (MEM), (n = 49) was obviously higher than that at heparin-free, i.e. in the basal medium, (n = 39) or that at 0.75 μg/ml heparin (n = 11). Statistical differences are indicated by *P < 0.05 and **P < 0.01; for Mann–Whitney's *U*-test, black asterisks; for the Jonckheere–Terpstra test, white asterisks. Note that the value at 75 μg/ml heparin (n = 13) had no significant difference compared to that at 7.5 μg/ml heparin. (B) Changes in the ratio of BrdU+ cells in RPE during culture of the RLEC in either the basal medium (heparin-free) or the standard newt MEM (heparin). At five DIV, in heparin-free, only one BrdU+ cell was recognized only in the Edge in one of the total two RLECs examined (the mean value: 0.08% in the Edge; 0% in the Center; 0.02% in the Whole), whereas in *heparin*, a larger number (range: 5–62; n = 3) of cells became BrdU+ (the mean value: 2.8 ± 1.3% in the Edge; 0.7 ± 0.4% in the Center; 1.2 ± 0.5% in the Whole), implying a promotive effect of heparin on the cell-cycle entry of RPE cells. Consistently, by 10 DIV, the ratio of BrdU+ cells in the RPE under the *heparin* condition (n = 49) obviously increased to a significantly higher level than that in heparin-free (n = 39), regardless of their location. For definitions of *Edge*, *Center,* and *Whole*, see Figure 2.

1 h and cultured them in the newt MEM containing the same dosages for 10 days. Compared to the mock control (Mock) with solvent only, the ratio of BrdU+ cells at 10 days decreased significantly in every region of the RPE (Figure 6A). Cell viability assessed by MTT and TUNEL assays was not different between U0126 and Mock (data not shown). Unfortunately, we could not test higher concentrations of U0126 because higher concentrations of DMSO – required to dissolve U0126 – were highly toxic to RPE cells (see Susaki and Chiba, 2007). However, these results suggest that MEK–ERK signaling activity is necessary for cell-cycle entry of RPE cells.

Immunoblot analysis with antibodies against MEK, ERK, and their phosphorylated forms (p-MEK and p-ERK) was carried out using the protein samples prepared from RLECs immediately before culture (0-DIV) and those at 5 and 10 days in culture. The MEK and ERK antibodies can label their total proteins, including the phosphorylated proteins, while those against p-MEK and p-ERK are designed to be specific to them. In every sample, both MEK and p-MEK antibodies labeled two identical bands (~40 and ~38 kD) corresponding to MEK1 and MEK2, and both ERK and p-ERK antibodies also labeled two bands (~39 and ~37 kD) corresponding to ERK1 and ERK2, respectively. Figure 6(B) shows a particular data set. In this case, when the RLECs were cultured under control conditions (Mock), the phosphorylation levels of MEK2 and ERK1 seemed to increase in 5 days and then decreased. In the presence of 5 μM U0126, phosphorylation of ERK1 seemed to be partially suppressed before culture (0-DIV) as well as during 5-day culture. However, a semiquantitative analysis with three independent data sets (from 15 RLECs in total) suggested that during 10-day culture, the changes in the total amount of each kinase protein are not statistically significant (Figure 6C, D, E), whereas the ratio of the phosphorylated protein tends to decrease gradually (Figure 6F, G, H). In addition, it revealed that the inhibitory effect of 25 μM U0126 on the phosphorylation of either ERK1 or ERK2 was statistically significant before culture (0-DIV), suggesting that the activation of ERK1 and ERK2 mediated by MEK1/2 that is required for cell-cycle entry of RPE cells may occur during preparation of the RLEC that takes ~1 h.

We examined a possibility that heparin may influence MEK–ERK signaling activity during culture. As, for preparing RLECs, eye-cups were soaked in PBS (i.e. heparin-free solution), the activation of MEK–ERK signaling during preparation of the RLEC was unlikely to be associated with heparin. However, we wondered whether heparin might have helped with sustaining the MEK–ERK signaling activity during culture. Then, we cultured RLECs in the newt MEM (heparin+) or basal medium (heparin–) for 5 days and calculated the ratio of p-ERK to total ERK in each condition from immunoblot data. However, unexpectedly, in either ERK1 or ERK2, the values were not different between the presence and absence of heparin (Figure 6I). Consequently, heparin is unlikely to affect MEK–ERK signaling activity; in other words, the signaling through which heparin promotes the cell-cycle entry of RPE cells does not appear to be mediated by the MEK–ERK module.

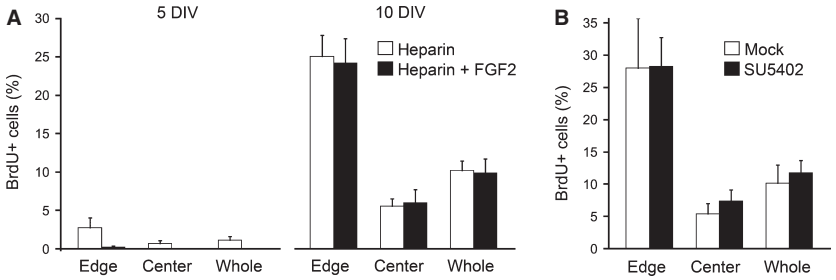

Figure 5. Participation of FGF2 in the cell-cycle entry of retinal pigment epithelium (RPE) cells. (A) Effects of FGF2. Changes in the ratio of BrdU+ cells in RPE during culture of the retina-less eye-cups (RLECs) were examined in either newt minimum essential medium (MEM) (heparin) or newt MEM to which 50 ng/ml FGF2 was added (heparin+ FGF2). In either five DIV (heparin, n = 3; heparin+ FGF2, n = 5) or 10 DIV (heparin, n = 49; heparin+ FGF2, n = 42), the values were unlikely to be different between the absence and presence of FGF2. (B) Effects of an FGF receptor–specific tyrosine kinase inhibitor SU5402. 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), or newt MEM containing the solvent only (Mock). Cell-cycle entry of RPE cells was unlikely to be affected by SU5402 regardless of their location. For definitions of *Edge*, *Center,* and *Whole*, see Figure 2.

Heparin-susceptible signaling pathways such as Wnt-, Shh-, and thrombin-mediated pathways might be involved in the cell-cycle entry of RPE cells

Subsequently, we examined signaling pathways whose activity can be modulated by heparin but which do not contain a path through the MEK–ERK module. Here we selected Wnt and Shh because these factors are known to be heparin-binding proteins involved in the regeneration of retinas (Bradley and Brown, 1990; Osakada et al., 2007; Spence et al., 2004; Zhang et al., 2007). In addition, we examined a serine protease thrombin which is also known to bind to heparin (Li et al., 1974). In the newt, thrombin activation is crucial for cell-cycle entry of iris pigment epithelium cells and regeneration of the lens (Imokawa and Brockes, 2003), and a thrombin-activated factor present in the serum is known to promote cell-cycle reentry of myotubes derived from a newt limb regeneration blastema cell line (Tanaka et al., 1999). On the other hand, thrombin activity is inhibited by heparin; heparin activates a protein anti-thrombin which inactivates several enzymes – including thrombin – in the coagulation system (Li et al., 2004). In humans, heparin is generally used during surgical operations as an anticoagulant. Therefore, it is interesting to examine whether thrombin is involved in the heparin-promoted cell-cycle entry of RPE cells.

Wnt signaling

We tested the secreted protein Dickkopf1 (Dkk1) that can inhibit Wnt signaling by blocking the interactions of Wnt with transmembrane co-receptors Frizzled and a low-density lipoprotein receptor–related protein (Klaus and Birchmeier, 2008). When RLECs were cultured in newt MEM containing 10 ng/ml Dkk1 for 10 days, the ratio of BrdU+ cells tended to increase only in the Edge (Figure 7A), creating the perception that Wnt might exert an inhibitory effect in the Edge. We have recently observed that inhibition of the Wnt canonical pathway during embryonic chick retina regeneration is sufficient to induce RPE transdifferentiation, supporting our current results using newt RLEC cultures (K. Del Rio-Tsonis, unpublished data). It has been shown that in different cell contexts, heparin can be either a positive or a negative regulator of the canonical Wnt signaling pathway (Ai et al., 2003; Colombres et al., 2008; Ling et al., 2010). However, as higher concentrations of heparin were unlikely to promote the cell-cycle entry of RPE cells further (Figure 4A) as Dkk-1 did here, we may exclude at least a possibility that heparin binding to Wnt ligands may make, as a negative regulator, the Wnts unavailable to the RPE cells, allowing them to enter the cell cycle. We further examined the expression of Wnt ligands in the eye-cup tissues by RT-PCR. Here we tested Wnt2b, Wnt3a, and Wnt5a and found that both Wnt2b and Wnt5a are expressed in the neural retina, although no positive signal was detected in the RLECs (Figure 7B).

Shh signaling

We tested 3-Keto-N-(aminoethyl-aminocaproyl-dihydrocinnamoyl)-Cyclopamine (KAAD), which can block Shh signaling by inhibiting a G-protein-coupled receptor Smoothened (Smo), which sends a signal when Shh binds to 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 and its solvent (0.4% EtOH) for 10 days. Unexpectedly, the ratio of BrdU+ cells did not change in any region of the RPE (Figure 8A). Thus, Shh signaling was also unlikely to be involved in the heparin-promoted cell-cycle entry of RPE cells.

Next we examined the effect of Shh; RLECs were cultured in newt MEM (with heparin) containing 0.5 μg/ml Shh for 10 days. Under this condition, Shh did not affect the cell-cycle entry of RPE cells (Figure 8B). However, as heparin is also known to reduce Shh activity through binding to Shh (Carrasco et al.,

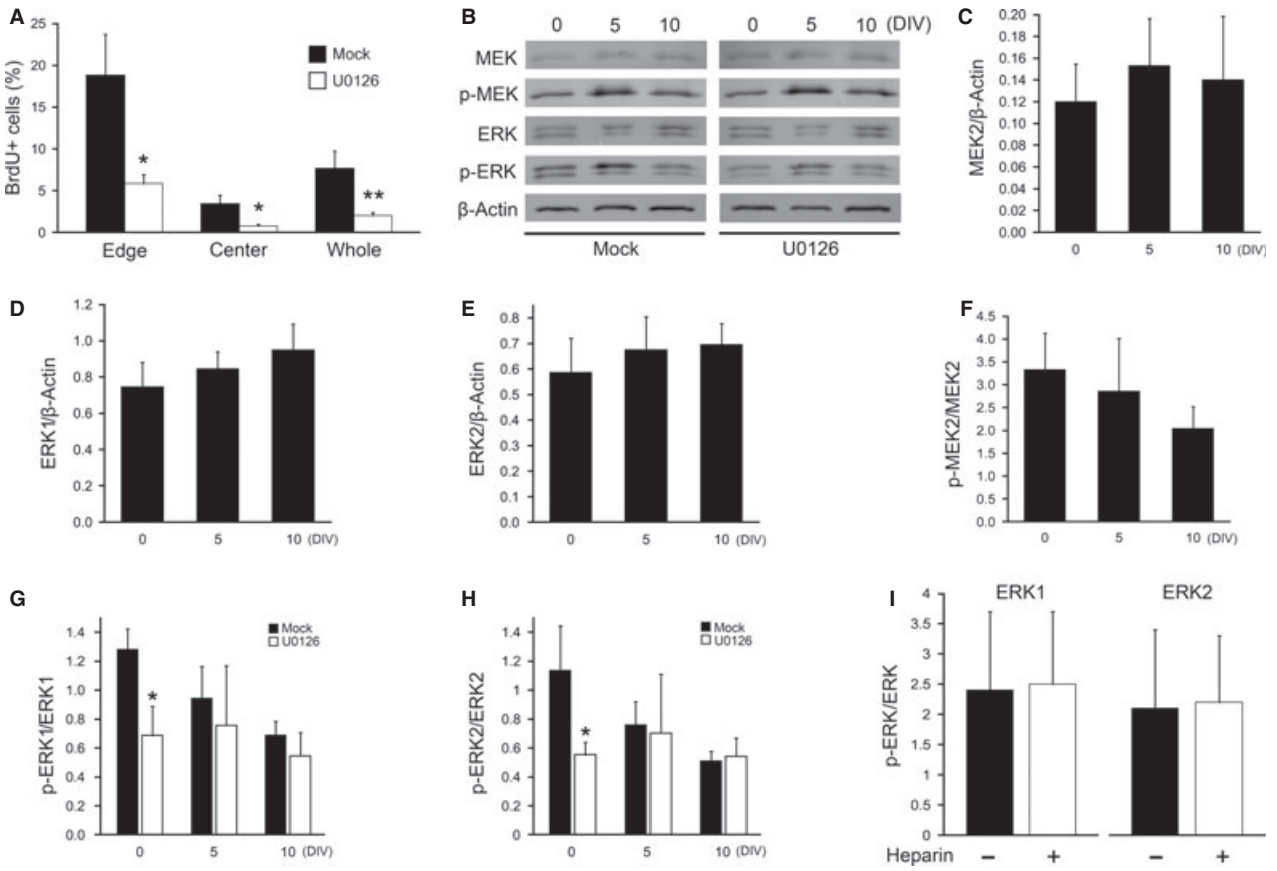

Figure 6. Involvement of MEK–ERK signaling in the cell-cycle entry of retinal pigment epithelium (RPE) cells. (A) Effects of a MEK inhibitor U0126 on the cell-cycle entry of RPE cells. Retina-less eye-cups (RLECs) were cultured in newt minimum essential medium (MEM) to which either 5 μM U0126 + 0.25% DMSO (U0126, n = 25) or 0.25% DMSO only (Mock, n = 31) was added. The ratio of BrdU+ cells in every region at 10 days in culture decreased significantly in the presence of U0126. For definitions of *Edge*, *Center,* and *Whole*, see Figure 2. (B) An example immunoblot data set showing expression and phosphorylation of MEK and ERK proteins and the effects of U0126 on MEK–ERK activity during RLEC culture performed as in A. Protein samples were extracted from 5 RLECs at 0 (immediately before culture), 5, and 10 DIV. Both MEK and p-MEK antibodies labeled two bands (~40 and ~38 kD) corresponding to MEK1 and MEK2, respectively, although the MEK1 bands were very faint. Both ERK and p-ERK antibodies also labeled two bands (~39 and ~37 kD) corresponding to ERK1 and ERK2, respectively. β-Actin: internal control. (C-E) Changes in the expression of MEK2, ERK1, and ERK2 proteins (normalized by β-actin) during RLEC culture performed as *Mock* in A. No significant change was detected in each protein. (F–H) Changes in the phosphorylation level of MEK2, ERK1, and ERK2 proteins during RLEC culture performed as *Mock* in A. The phosphorylation level (the ratio of a phosphorylated protein to the total protein containing the phosphorylated one) of each protein tended to decline throughout culture (Jonckheere–Terpstra test: for MEK2 in F, P = 0.0743; for ERK1 in G, P = 0.0295; for ERK2 in H, P = 0.0295). (G, H) Effects of U0126 on the phosphorylation of ERK1 and ERK2 during culture. In both proteins, the phosphorylation level was significantly decreased in 0 DIV. In C–H, the values at each culture day were deduced from three immunoblot data sets, as shown in B, obtained from three independent rounds of RLEC culture (five RLECs each). (I) Effects of heparin on the phosphorylation of ERK during RLEC culture. RLECs were cultured in either basal medium (heparin–) or standard newt MEM (heparin+). Protein samples were extracted from 3 RLECs at 5 days in culture and analyzed. The phosphorylation levels of ERK1 and ERK2 were estimated from three independent trials as carried out in G and H. In both ERK1 and ERK2, there were no significant differences between the values in heparin– and heparin+. Statistical differences according to the Mann–Whitney's *U*-test are indicated by *P < 0.05 and **P < 0.01.

2005), we further examined this possibility; RLECs were cultured in basal medium (no heparin) containing the same concentration of Shh for 10 days. Interestingly, the ratio of BrdU+ cells tended to increase only in the Edge (Figure 8C), suggesting that Shh promotes cell-cycle entry in the Edge, although this effect is suppressed by heparin. However, as even in the absence of heparin, KAAD was unlikely to affect the ratio of BrdU+ cells (data not shown), Shh might not be a component of the endogenously activated signaling which allows the RPE cells in the RLEC to enter the cell cycle in the absence of heparin.

Thrombin signaling

We tested a thrombin-specific inhibitor PPACK; RLECs were cultured in newt MEM containing 20 μM PPACK and 1.74 mM sodium acetate (see Methods) for 10 days. Intriguingly, the ratio of BrdU+ cells tended to

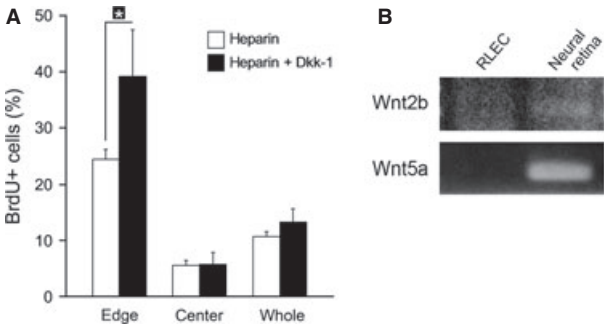

Figure 7. Participation of Wnt-mediated signaling pathways in the heparin-promoted cell-cycle entry of retinal pigment epithelium (RPE) cells. (A) Effects of a Wnt signaling blocker Dkk-1. Retina-less eye-cups (RLECs) were cultured in either newt minimum essential medium (MEM) (*heparin*, n = 88) or newt MEM to which 10 ng/ml Dkk-1 was added (heparin+ Dkk-1, n = 15). The ratio of BrdU+ cells in each condition was measured at 10 days in culture. Statistical differences according to the Jonckheere–Terpstra test are indicated by *P < 0.05. For definitions of *Edge*, *Center,* and *Whole*, see Figure 2. (B) Expression of *Wnts* in the eye-cup tissues. PCR products corresponding to Wnt2b (314 bp) and Wnt5a (235 bp) were detected in the neural retina, but not in the RLEC.

decrease only in the Edge (Figure 9A), suggesting that endogenous thrombin in the RLEC may be involved in the heparin-promoted cell-cycle entry of RPE cells in the Edge.

To confirm this conclusion, we examined the effect of thrombin; RLECs were cultured in newt MEM containing 5 U/ml thrombin for 10 days. However, unexpectedly, the ratio of BrdU+ cells in the RPE decreased significantly except in the Edge (Figure 9B). To evaluate the participation of heparin in this thrombin-mediated inhibitory effect, we cultured RLECs in basal medium containing the same concentration of thrombin for 10 days. Under this condition, thrombin did not affect the cell-cycle entry of RPE cells in every region (Figure 9C). Taken together, these results suggest that thrombin is capable of exerting both promoting and inhibitory effects on the cell-cycle entry of RPE cells, depending on the presence of heparin.

Cells inside the epithelial sheet have less competence to respond to serum-containing factors for the cell-cycle entry

As shown above, cells in the Center of the RPE did not seem to respond to Shh even in the absence of heparin (Figure 8C). This observation questions whether RPE cells in the Center have limited competence to respond to mitotic factors. To address this question, we examined the effects of fetal bovine serum (FBS) on RPE cells in the RLEC, because FBS is known to promote proliferation of isolated RPE cells; in the presence of 1% FBS, ~80% of isolated RPE cells start to proliferate in 10 days (Susaki and Chiba, 2007). We cultured RLECs in basal medium containing 1% FBS for 10 days. As expected, the distribution pattern of BrdU+ cells in the RPE was substantially unchanged (Figure 10A), suggesting that cell-cycle entry in the Center is suppressed.

We suspected that cell-to-cell contact may be responsible for the inhibition of cell-cycle entry in the Center, because many *in vitro* studies have suggested that contact inhibition suppresses cell-cycle entry of RPE cells and contributes to keeping their state of differentiation (Cahn and Cahn, 1966; Crawford, 1980; Liu et al., 2010; Matsumoto et al., 1990; Tezel and Del Priore, 1996). Therefore, we removed a small piece of epithelium from the Center of the RPE in the RLEC using a micropipette tip under a stereomicroscope (Figure 10B; see Methods) and cultured the RLEC in basal medium containing 1% FBS. At 10 days in culture, BrdU+ nuclei appeared to be dispersed in the region from which the epithelium had been removed and around its circumference (Figure 10C, D). Their cytoplasm was labeled with an RPE marker, RPE65 antibody (data not shown). These results indicate that the cells inside the epithelial sheet, maintaining cell-to-cell contact with each other, have less competence to respond to mitotic factors with the cell-cycle entry, suggesting inversely that a loss of the cell-to-cell contact, which occurs around the wound edge, might allow the cells to enter the cell cycle.

To confirm this hypothesis, we evaluated participation of the cell-to-extracellular matrix (ECM) contact in the

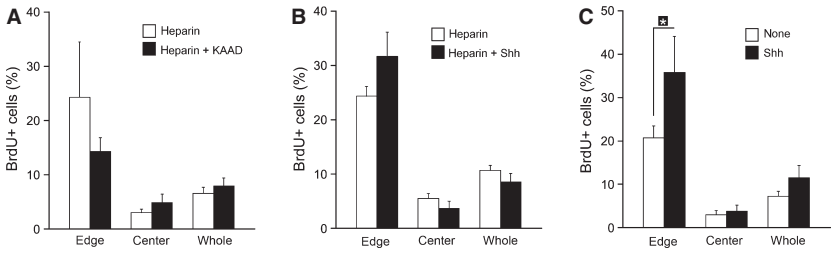

Figure 8. Participation of Shh-mediated signaling pathways in the heparin-promoted cell-cycle entry of retinal pigment epithelium (RPE) cells. (A) Effects of a Shh signaling blocker KAAD. Retina-less eye-cups (RLECs) were cultured in newt minimum essential medium (MEM) to which either 0.4% EtOH (heparin, 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 = 88) 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 = 46) or 0.5 μg/ml Shh was added (Shh, n = 6). The ratio of BrdU+ cells in each condition was measured at 10 days in culture. Statistical differences according to the Jonckheere–Terpstra test are indicated by *P < 0.05. For definitions of *Edge*, *Center,* and *Whole*, see Figure 2.

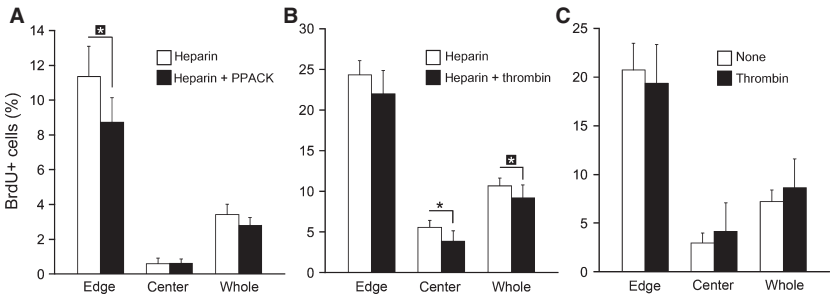

Figure 9. Participation of thrombin in the heparin-promoted cell-cycle entry of retinal pigment epithelium (RPE) cells. (A) Effects of a thrombin inhibitor PPACK. Retina-less eye-cups (RLECs) were cultured in newt minimum essential medium (MEM) to which either 1.74 mM sodium acetate (heparin, n = 24) or 20 μM PPACK + 1.74 mM sodium acetate (heparin+ PPACK, n = 29) were added. (B) Effects of thrombin. RLECs were cultured in either newt MEM (heparin, n = 88) or newt MEM to which 5 U/ml thrombin was added (heparin+ thrombin, n = 39). (C) Effects of thrombin in the absence of heparin. RLECs were cultured in BrdU containing 80% L-15 medium to which nothing (none, n = 46) or 5 U/ml thrombin was added (thrombin, n = 15). The ratio of BrdU+ cells in each condition was measured at 10 days in culture. Statistical differences are indicated by *P < 0.05; for the Mann–Whitney's *U*-test, black asterisks; for the Jonckheere–Terpstra test, white asterisks. For definitions of *Edge*, *Center,* and *Whole*, see Figure 2.

inhibition on the cell-cycle entry of RPE cells; RLECs were incubated in the basal medium containing 0.1% elastase, a protease that can efficiently digest Bruch's membrane beneath the RPE (Susaki and Chiba, 2007), for 1 h before the following 10-day culture in the basal medium. This elastase treatment significantly reduced the ratio of BrdU+ cells in the Edge (Figure 11A), although the total cell number was unlikely to be affected in any region of the RLEC (Figure 11B). Next we tested an EGTA solution to make cell-to-cell contact looser; RLECs were incubated in 10 mM EGTA solution (see Methods) for 1.5 h before the following 10-day culture in basal medium. Expectedly, EGTA treatment obviously elevated the ratio of BrdU+ cells in the Whole as well as in the Edge (Figure 11C). 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 (Figure 11D). Taken together, supporting our hypothesis, these results suggest that cell-to-cell contact, rather than cell-to-ECM contact, participates in the inhibition of cell-cycle entry of RPE cells.

Discussion

Retinal pigment epithelium cells in adult vertebrates are in general mitotically inactive under physiological conditions. In humans, proliferation of RPE cells is an indication of the pathogenesis of retinal diseases such as PVR or choroidal melanoma (Kim and Arroyo, 2002; Pastor, 1998). PVR is a major cause of failure in retinal surgery to treat retinal detachment or severe ocular trauma and has been clinically considered as a defective scarring process. In PVR, upon injury of the retina/choroid, RPE cells are exposed to the serum which comes through an altered blood–retina barrier. Subsequently, they detach from Bruch's membrane, lose their epithelial morphology, migrate into the vitreous through a tear in the neural retina, and participate in the formation of epiretinal membranes on both surfaces of the neural retina. The epiretinal membranes grow and contract, in a wound healing-like response, causing further retinal detachment and loss of vision. This process involves an epithelial-mesenchymal transition (EMT) through which RPE cells become fibroblast-like cells, proliferate, and acquire the ability to migrate (Grisanti and Guidry, 1995; Lee et al., 2001). The EMT has been considered as a variant of transdifferentiation (Iwano et al., 2002).

In contrast to humans, newts can regenerate their entire retinas through proliferation and transdifferentiation of RPE cells (Chiba and Mitashov, 2007; Haynes and Del Rio-Tsonis, 2004; Mitashov, 1996, 1997; Reh and Pittack, 1995; Tsonis, 2000; Tsonis and Del Rio-Tsonis, 2004). In the adult *C. pyrrhogaster* newt, upon surgical removal of the neural retina, RPE cells undergo a remodeling process during the early stages of transdifferentiation that is similar to the one taking place in EMT observed in PVR, where cells lie on each other's surface to form a layer/aggregate a few cells thick possibly owing to their migration and/or shrinkage of the vitreous chamber (Chiba et al., 2006). In the newt, however, the cells become mitotically active during this process and then generate a bilayer of cells consisting of presumptive progenitor cells of the neural retina and the RPE (Chiba et al., 2006; Kaneko and Chiba, 2009; Susaki and Chiba, 2007). The cells of the inner cell layer facing the vitreous cavity continue to proliferate, produce various types of retinal neurons and glia to form a visual circuitry, and finally regenerate a new functional neural retina (Cheon and Saito, 1999; Cheon et al., 1998; Chiba, 1998; Chiba and Saito, 2000; Chiba et al., 1997, 2005, 2006; Nakamura and Chiba, 2007; Negishi et al., 1992; Oi et al., 2003a,b; Saito et al., 1994; Sakakibara et al., 2002), while those of the outer cell layer along Bruch's membrane exit the cell cycle earlier and differentiate to renew the RPE cell layer (Chiba et al., 2006).

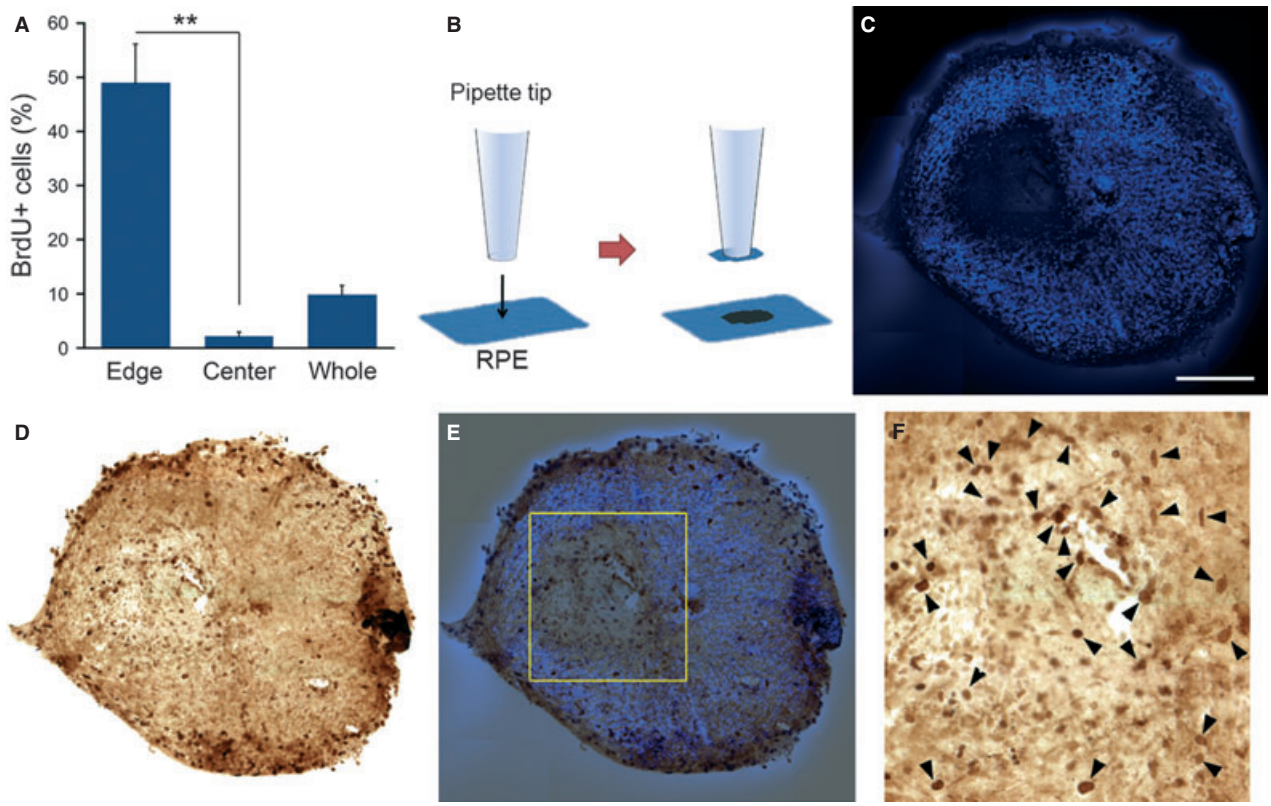

Figure 10. Low competence of cells located inside the epithelium to respond to serum-containing factors with cell-cycle entry. (A) Effects of a fetal bovine serum (FBS) on the cell-cycle entry of retinal pigment epithelium (RPE) cells. Retina-less eye-cups (RLECs) were cultured in BrdU containing 80% L-15 medium to which 1% FBS was added. The ratio of BrdU+ cells was measured at 10 days in culture. For definitions of *Edge*, *Center,* and *Whole*, see Figure 2. Statistical differences in Mann–Whitney's *U*-test are indicated by **$P < 0.01$. (B–F) Effects of the removal of a small piece of the epithelium from the *Center* of the RPE. A small piece of the epithelium was removed from the *Center* of the RPE in the RLEC by a micropipette tip (B; see Methods), and the operated RLEC was cultured as in A. BrdU+ nuclei in the RPE were visualized at 10 days in culture. (C) 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. (D) BrdU+ nuclei. (E) Merged image of C and D. The area enclosed by a square is enlarged in panel F. Arrowheads in F indicate sample BrdU+ nuclei. Scale bars: 400 μm (C); 170 μm (F).

Thus, the behavior of RPE cells in an early process of the human PVR appears to be similar to that of newt retinal regeneration, although the consequence is quite different. Therefore, comparisons between these systems would allow us to obtain insight to treat retinal diseases and regenerate a functional retina. In the current study, using the RLEC culture system of the adult *C. pyrrhogaster* newt, we investigated the onset mechanism of proliferation in mitotically quiescent RPE cells and found three important elements to be involved in the mechanism: (i) the MEK–ERK pathway, (ii) heparin-susceptible pathways, and (iii) a pathway for liberation from contact inhibition.

MEK–ERK pathway

We found that immediate early activation of MEK–ERK signaling, which occurs during preparation of the RLEC (within 1 h), is essential for the first cell-cycle entry of RPE cells in both the Edge and Center. In general, the MEK–ERK cascade is activated by growth factors such as FGFs which stimulate the receptor tyrosine kinases on the cell membrane (Friday and Adjei, 2008; Raman et al., 2007; Zhang and Dong, 2007). In the current study, we examined FGF2 and an FGF receptor–specific tyrosine kinase inhibitor, but either had no effect on the cell-cycle entry of RPE cells under the RLEC culture conditions.

In the adult newt, when RPE cells are isolated from Bruch's membrane-choroid tissue by an enzyme treatment and then cultured in MEM alone, they enter the S-phase of the cell cycle but do not reach the M-phase (Susaki and Chiba, 2007). This suggests a possibility that the factors that regulate the first entry of RPE cells to the S-phase may be different from those for the progression to the M-phase and subsequent proliferation. In fact, FGF2 allows the S-phase RPE cells to proceed to the M-phase and promotes proliferation through activation of the MEK–ERK cascade *in vitro* (Susaki and Chiba, 2007).

In adult mammals, many *in vitro* studies with isolated RPE cells or cell lines established from those cells

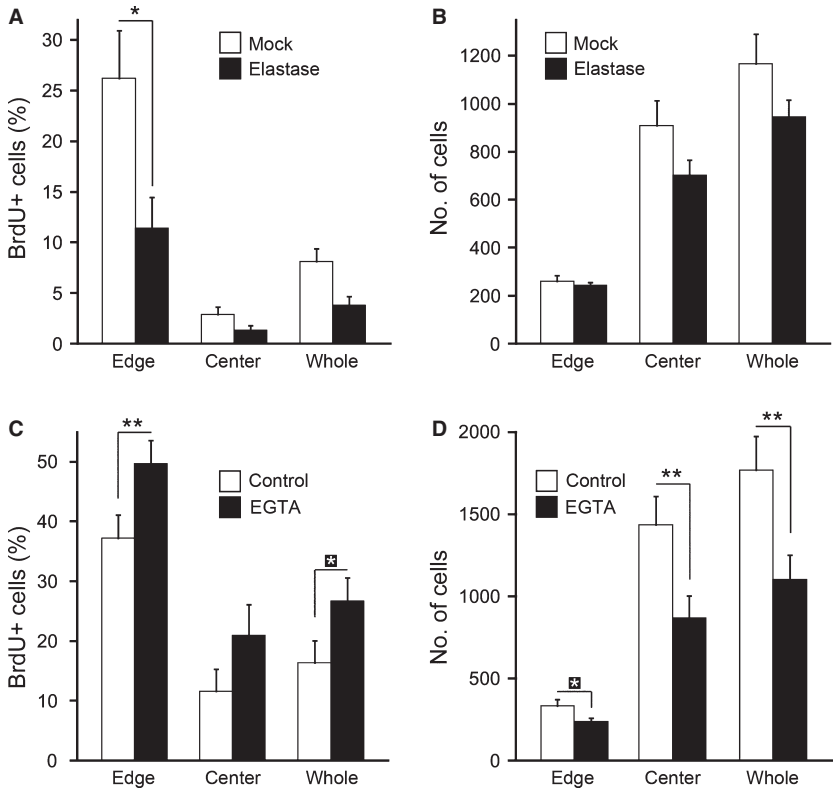

Figure 11. Participation of cell-to-ECM contact and cell-to-cell contact in the cell-cycle entry of retinal pigment epithelium (RPE) cells. (A, B) Effects of elastase, a protease that digests Bruch's membrane efficiently, on the ratio of BrdU+ cells (A) and total cell number in the RPE (B). Retina-less 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). (C, D) Effects of EGTA, a calcium chelator that makes cell-to-cell contact looser, on the ratio of BrdU+ cells (C) and total cell number in the RPE (D). 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). The ratio of BrdU+ cells in each condition was measured at 10 days in culture. In these experiments, two eyeballs of the same animal were used for the test and control experiments. Statistical differences are indicated by *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. For definitions of *Edge*, *Center* and *Whole*, see Figure 2.

suggest that a broad spectrum of serum-containing factors are capable of promoting proliferation of RPE cells, for example, PDGF, FGF, EGF, IGF, VEGF, HGF and TGFβ (Bryckaert et al., 2000; Hecquet et al., 2002a,b; Hollborn et al., 2006; Kaven et al., 2000; Lashkari et al., 1999), glutamate (Pacheco-Domínguez et al., 2008), and thrombin (Palma-Nicolas et al., 2008; Parrales et al., 2010). Intriguingly, signaling pathways activated by these factors seem to converge to the MEK–ERK module. Probably, also in the adult newt, various kinds of factors, including FGF2, may be involved in RPE cell proliferation. So far, IGF1 has also been reported to have a proliferative effect on adult newt RPE cells (Susaki and Chiba, 2007).

Taken together, activation of the MEK–ERK module is crucial for both the first cell-cycle entry and subsequent proliferation of RPE cells. In both newts and humans, we need to identify the molecular pathways that activate the MEK–ERK module in each process.

Heparin-susceptible pathways

We found that heparin promotes cell-cycle entry of RPE cells in the RLEC. This finding was surprising because heparin is widely used for cell culture and in surgical operations as an anticoagulant. As the effect of heparin was unlikely to be associated with the activation of the MEK–ERK module, heparin may regulate cell-cycle entry in parallel with the MEK–ERK–mediated pathway. As heparin is known to interact with various kinds of 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), we suspected that it may indirectly affect the cell-cycle entry through modifications of some diffusible factors. In the current study, we examined Wnt, Shh, and thrombin as the candidate factors and provided evidence suggesting that endogenous thrombin in RLEC culture may participate in the heparin-promoted cell-cycle entry of RPE cells.

We found that thrombin is capable of exerting both promoting and inhibitory effects on the cell-cycle entry

of RPE cells, depending on the presence of heparin. It is known that thrombin can directly or indirectly regulate cell-cycle entry by activating the protease-activated receptors (PARs) on the cell membrane (Palma-Nicolas et al., 2008; Parrales et al., 2010) or serum-containing factors (Tanaka et al., 1999) by cleaving them as a serine protease. We speculate that the endogenous thrombin in the RLEC of newts might activate some serum-containing factors in the choroid, and those factors might diffuse from the wound of the RLEC with the help of heparin, promoting the cell-cycle entry of RPE cells in the Edge, whereas exogenously administered thrombin might directly inhibit the cell-cycle entry of RPE cells in every region, neutralizing the promoting effect of activated serum-containing factors in the Edge. In any case, further analysis of the mechanism underlying the heparin-dependent effects of thrombin is required.

Our current results imply that Wnt and Shh might also participate in the cell-cycle entry of RPE cells with inhibitory and excitatory effects, respectively. The Wnt function was unlikely to be affected by heparin, but in the case of Shh, the action was perturbed by heparin. In the current study, we were unable to fully investigate the participation of endogenous Wnts in the RLEC culture because of limited gene information, although we found that Wnt2b and Wnt5a are expressed in the neural retina. On the other hand, our current data with KAAD revealed that Shh is unlikely to be an endogenous factor in RLEC culture. In fact, in the newt eye, Shh is present in the neural retina, while its receptors, Ptch1 and Ptch2, are in the RPE (Takabatake et al., 1997).

Heparin is a closely related molecule in structure to heparan sulfate which is present as a form of proteoglycan [i.e. heparan sulfate proteoglycan (HSPG)] on the cell surface and in the ECM, and therefore heparin-binding factors can also bind HSPG (Tumova et al., 2000). It has been suggested that HSPG in the ECM accumulates the factors released by cells around or even far from there as well as those in 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 (Figure 11A), suggesting the importance of the ECM on the cell-cycle entry of RPE cells. To understand the roles of heparin-binding factors, including Wnt, Shh, and thrombin, in the first cell-cycle entry of RPE cells, the dynamics of HSPG as well as the locations of these molecules in the RLEC (containing Bruch's membrane) should also be addressed.

Liberation from contact inhibition

We found that the RPE cells entering the cell cycle are mostly located around the wound edge of the epithelium; in other words, cells inside the epithelium (i.e. cells surrounded by neighboring cells) hardly enter the cell cycle. This phenomenon cannot be explained solely by endogenous factors diffused from the wound of the RLEC, because cells inside the epithelium have low competence of proliferation even in the presence of FBS, which can robustly promote proliferation in most isolated RPE cells (Susaki and Chiba, 2007). A plausible mechanism may be contact inhibition.

Contact inhibition underlies wound healing and the repair of tissues; when a part of the tissue is missing after a traumatic injury, cells surrounding the wound start to proliferate, and as the wound is occupied by them, 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 form the epithelial morphology while growing pigmented microvilli (Cahn and Cahn, 1966). Interestingly, there are many reports suggesting that the MEK–ERK module and heparin may be involved in contact inhibition pathways (Block et al., 2010; Iwamoto and Mekada, 2000; Iwamoto et al., 1999; Nanba and Higashiyama, 2004). The cell-to-cell contact in the RPE might suppress the competence of cells to respond to mitotic factors with the cell-cycle entry, allowing them to maintain a differentiated state. In other words, a loss of cell-to-cell contact might be a requisite for cell-cycle entry of RPE cells. Our cell-removal experiments (Figure 10) support this hypothesis.

Under the current culture conditions, the RPE kept its epithelial morphology for 10 days (Figure 2C). On the other hand, in the eye of a living animal, in ~2 h after removal of the neural retina, RPE cells started to detach from neighboring cells and changed their morphology into a spherical shape, leaving Bruch's membrane as single cells (i.e. EMT of RPE cells starts). This suggests the presence of a mechanism promoting EMT *in vivo*. A similar phenomenon to EMT, termed histolysis, has been reported in amphibian limb regeneration (Stocum and Cameron, 2011). In this case, the matrix metalloproteinase (MMP) family proteins have been suggested to be involved. Intriguingly, 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, EMT may be a key event that liberates the RPE cells from contact inhibition, allowing them to enter the cell cycle in both retinal regeneration and PVR. In mammals, important findings on the molecules involved in the regulation of the epithelial characteristics and EMT in RPE cells are increasing: for example, a cell adhesion molecule, cadherin, whose disruption results in the loss of cell-to-cell contact leading to EMT and proliferation (Tamiya et al., 2010), microRNAs miR-204/211 whose reduction triggers dedifferentiation or disruption of physiological functions (e.g. epithelial barrier function

via tight junction) as well as proliferation via upregulation of growth factor/cytokine-mediated signaling such as that mediated by TGF-βR2 (Wang et al., 2010), and a transcriptional repressor Zeb1, whose expression is reduced by cell-to-cell contact but in association with the EMT undergoes upregulation which is required for dedifferentiation and proliferation (Liu et al., 2009). Consistently, also in the newt, EGTA treatment, known to disrupt cadherin ligation by chelating calcium (Tamiya et al., 2010), obviously increased cell-cycle entry of RPE cells (Figure 11B). In a future study, we need to address the mechanisms of contact inhibition and EMT in the newt RPE cells in comparison with those in mammals. Such a comparative study would undoubtedly be informative and might shed light on our ability to harness the regenerative capacity of the mammalian RPE.

Methods

Adult *C. pyrrhogaster* newts (total body length: 9–12 cm) were purchased from local suppliers in Japan and housed in polyethylene containers with water at 18°C under natural light (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.

RLEC culture

Retina-less eye-cups were prepared as described previously (Susaki and Chiba, 2007; also see Figure 2A). Briefly, eyeballs were placed, cornea side up, on a membrane filter (Millipore, Billerica, MA, USA) in a 35-mm plastic dish (Becton Dickinson, Franklin Lakes, NJ, USA) at one eyeball per dish. 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 neural retina was carefully removed to make a RLEC. The RLEC on the filter was transferred into a chamber – a cap of a 1.5-ml sample tube (Assist, Tokyo, Japan) – containing 200 μl of culture medium. After the chamber was closed with the sample-tube body, it was incubated at 25°C. The culture was transferred into another chamber to which fresh medium was added every 5 days. The newt MEM was composed of 80% L-15 (Invitrogen, Carlsbad, CA, USA), 7.5 μg/ml heparin (heparin, sodium salt; 081-00136, Wako, Osaka, Japan), and 5 μg/ml BrdU (Sigma-Aldrich, St. Louis, MO, USA) and was used as the standard culture medium.

In some experiments, the RLEC was cut in half along a longitudinal axis through the optic disk with a razor blade immediately before culture (Figure 3A). In other experiments, a piece of the epithelium was removed from the 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 placed on the apical surface of the RPE under a stereomicroscope, a small negative pressure was applied, and then lifted up together with a piece of the epithelium attached (Figure 10B).

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 (in mM) 115 NaCl, 3.7 KCl, 10 EGTA, 18 D-glucose, 10 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 [(in mM) 115 NaCl, 3.7 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 18 D-glucose, 10 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-Aldrich), −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-Aldrich), −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), and 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 2 N NaOH before administration). These reagents were added to the culture medium from the beginning of culture except for U0126 which was administered from the time at which the eye-cup was 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.

Counting the BrdU+ cells

Retina-less eye-cup cultures were fixed in 4% paraformaldehyde (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 washing 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 rinsing twice in PBS, they were incubated in a mouse anti-BrdU antibody (1:400; B2531-2ML, Sigma-Aldrich) diluted with the blocking solution containing Biotin (1:50; Avidin/Biotin blocking kit; Vector) for 15 h at 4°C. After washing 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 rinsing 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 washing 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 distilled water (DW) for 15 min.

The total RPE cell number in a RLEC culture was counted as follows: the culture was refixed in 4% PFA in PBS for 20 min and rinsed in DW; the RPE-choroid tissue was separated from the sclera by fine forceps and pins under a stereomicroscope and transferred onto a glass slide; the tissue was immersed into 90% glycerol in PBS and mounted under a cover slip; the preparation was placed 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); and RPE cells, which could be identified by their characteristic morphology observed over green autofluorescence of the choroid (see Figure 2C), 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 overnight to bleach their melanin pigments, and rinsed twice in DW; they were transferred into 90% glycerol in PBS on a glass slide and mounted under a cover slip; and the preparation was placed on a microscope stage and the number of brown nuclei (BrdU+) was counted under transmitted light (Figure 2D, E).

Immunoblotting

Retina-less eye-cup cultures were collected in a tube containing PBS on ice. After the solution in the tube was removed carefully, a lysis buffer [25 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 1 mM EDTA-2Na, 1% Igepal CA-630 (56741, Sigma-Aldrich), 1% protease inhibitor cocktail (P8340, Sigma-Aldrich), 1% sodium deoxycholate (190-08313, Wako), 0.1% SDS (191-07145, Wako)] chilled on ice was poured into the tube at 7 μl/RLEC. The sample was frozen in liquid nitrogen and sonicated at 46 kHz, while thawed, in chilled water for 5 min. After the freeze/thaw/sonication cycle was repeated again, the suspension was centrifuged at 7000 *g* (4°C) for 10 min. The supernatant was mixed with the same amount of SDS sample buffer [0.5 M Tris-HCl (pH = 6.8), 10% (w/v) SDS, 10% 2-mercaptoethanol (M3148, Sigma-Aldrich), 20% glycerol, 0.5% (w/v) bromophenol blue (021-02911, Wako)], heat-denatured for 5 min, and stored at −20°C until use.

SDS-PAGE and immunoblotting were carried out as described previously (Susaki and Chiba, 2007). The primary antibodies were rabbit anti-MEK1/2 polyclonal antibody (1:400; 9122, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-MEK1/2 monoclonal antibody (1:500; 9154S, Cell Signaling Technology), rabbit anti-ERK1/2 polyclonal antibody (1:300; p44/42 MAP Kinase antibody, 9102, Cell Signaling Technology), rabbit anti-phospho-ERK1/2 polyclonal antibody (1:500; Phospho-p44/42 MAP Kinase antibody, 9101S; Cell Signaling Technology), and mouse anti-β-actin monoclonal antibody (1:3000; ab6276, Abcam, Cambridge, UK). The secondary antibodies were biotinylated goat anti-rabbit IgG antibody (1:300; BA-1000, Vector) and biotinylated goat anti-mouse IgG antibody (1:300; BA-9200, Vector).

For semiquantitative analysis of protein expression and phosphorylation/activation, the values were measured using a function of Photoshop Extended CS5 (Adobe, San Jose, CA, USA): The mean signal intensity of the protein band against the background was measured in a gray scale image of the immunoblotted membrane with inverted gradation.

PCR analysis

Polymerase chain reaction was carried out with cDNA pools constructed from RLECs and the neural retinas as described previously (Susaki and Chiba, 2007). As gene information of Wnts is very limited in the newts, only three Wnts (Wnt2b, Wnt3a, and Wnt5a) were examined here. Primers were designed according to Hayashi et al. (2006).

Cell viability assays

For the 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 12 000 rpm for 5 min and examined for its absorbance at 570 nm by a spectrophotometer (D640; Beckman Coulter, Brea, CA, USA). The TUNEL assay, used to label apoptotic cells, was carried out using the Neuro TACS II kit (4823-30-K, Trevigen, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Data analysis

Bright-light and fluorescence images of tissues were acquired using a color CCD camera (C4742-95 ORCA-ER

system; Hamamatsu Photonics, Hamamatsu, Japan) connected to a personal computer. 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 rounds. Non-parametric tests (Sheffe's test following the Friedman test; Mann–Whitney's *U*-test; Jonckheere–Terpstra test) were carried out to evaluate the statistical significance of the data, using software Ekuseru-Toukei 2008 (Social Survey Research Information, Tokyo, Japan).

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