Studies on the Trigger Mechanism of Retinal Regeneration in the Adult Newt

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Abbreviations

α-SMA: α-smooth muscle actin

BrdU: 5-bromo-2'-deoxyuridine

DAB: 3,3'-Diaminobenzidine

DAPI: 4',6-diamidino-2-phenylindole

DMSO: Dimethyl sulfoxide

EGTA:

ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EMT: Epithelial to mesenchymal transition

ERK: Extracellular signal-regulated kinase

FGF: Fibroblast growth factor

GPCR: G protein-coupled receptor

IF: Immunofluorescence

IP: Immunoperoxidase

MAPK: Mitogen-activated protein kinase

MEK: Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase

MMP: Matrix metalloproteinase

NR: Neural retina

NSCM: Newt standard culture medium

PBS: Phosphate buffer solution

pERK: phosphorylated ERK

PVR: Proliferative vitreoretinopathy

RLEC: Retine-less eye-cup

RPE: Retinal pigment epithelium

RPESC: RPE-derived stem-like cell

TGF-β: Transforming growth factor-β

Abstract

Newts, urodele amphibians, have outstanding ability of regeneration and they can regenerate many body parts (i.e. limbs, tail, lens and retina in the eyeball) even in the adult stage. Although this remarkable ability has been investigated for long time, the detail molecular mechanisms, especially in the initial step of the regeneration, are still unclear.

Retinal regeneration is one of a suitable system to address this issue. Retina is composed by neural retina (NR), which senses light and transduces basic signals of visual system into brain, and retinal pigment epithelium (RPE), which supports such NR functions. Even if NR is removed, newts can regenerate complete retina from RPE cells. When NR is removed, RPE cells start losing their epithelial characteristics. Following it, they re-enter to DNA synthesis phase of cell cycle (cell cycle re-entry) and acquire multipotency. From the RPE-derived cells, new NR and RPE are generated. Since the resource of regeneration is restricted into single cell type, RPE cells, this system is advantageous to trace and identify the cells during the regeneration process. In addition, *in vitro* analysis methods are applicable. Therefore, it is possible to analyze signaling pathways/molecular mechanisms involved in the regeneration process.

Studies of the trigger mechanisms have been performed by using the cell cycle re-entry of RPE cells as an indicator. In previous studies, it was suggested that MEK [mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase] -ERK signaling was reinforced within 30 min after NR removal operation and participated into cell cycle re-entry of RPE cells. In addition, it was also suggested that attenuation of cell-cell contact was an important element for cell cycle re-entry of RPE cells. However, there still remained following three questions; (1) what is the causal stimuli reinforcing MEK-ERK signaling, (2) which is the signaling pathway(s) mediating promotion of cell cycle re-entry by attenuation of cell-cell contact, (3) how are these elements related each other. In this study, these questions were focused on and addressed using the *in vitro* system.

Firstly, in *in vitro* conditions, MEK-ERK signaling reinforcement was observed in whole area of the RPE sheet simultaneously and this reinforcement was not observed when NR was left on the RPE. Therefore, it was suggested that NR removal itself is a causal event for the MEK-ERK signaling reinforcement. Secondly, it was found that attenuation of cell-cell contact of RPE cells promoted β-catenin nuclear translocation and inhibition of β-catenin signaling significantly decreased cell cycle re-entry ratio of RPE cells. Also in *in vivo* retinal regeneration, β-catenin

nuclear translocation in RPE cells was observed at 3 days post operation when the cell-cell contact became loose. From these results, it was suggested that attenuation of the cell-cell contact promotes cell cycle re-entry of RPE cells via β-catenin signaling. Finally, it was found that inhibition of MEK-ERK signaling significantly decreased β-catenin nuclear translocation ratio. Therefore, it was suggested that MEK-ERK signaling is a prerequisite for nuclear translocation of β-catenin.

From the above, it was suggested that MEK-ERK signaling, which is stimulated by NR removal, and following β-catenin signaling, which is stimulated by attenuation of cell-cell contact, initiate cell cycle re-entry of RPE cells. And, together with previous studies, it became apparent that the early phase of newt retinal regeneration is regulated by multi-step triggers.

1. Introductions

1.1. Newt body parts regeneration

The newt is a urodele amphibian belonging to a group in salamandoridae. This animal has outstanding ability of body parts regeneration. The amazing regenerative ability of newts was firstly found by Lazzaro Spallanzani in 1768, and, for almost 250 years, countless investigation has been performed (Tsonis and Fox, 2009). As examples, the newt can regenerate limbs, tail (spinal code), jaws, lens and retina in the eyeball, brain, and heart (limb: Iten and Bryant, 1973; Tanaka et al., 2016; tail: Iten and Bryant, 1976; jaw: Goss and Stagg, 1958; Ferretti, 1996; lens: Tsonis et al., 2004; Eguchi et al., 2011; retina: Keefe, 1973a; Keefe, 1973b, Chiba and mitashov, 2007; brain: Minelli et al., 1987; Parish et al., 2007; heart: Oberpriller and Oberpriller, 1974; Singh et al., 2010). The most unique and remarkable characteristic of the regeneration is that they can regenerate even after metamorphosis, in other words, in the adult stage.

Also in other vertebrates, there exists the ability to regenerate multiplex body parts. However such regenerative ability depends on endogenous stem/progenitor cells and lost during individual maturation by decreasing the stem/progenitor cells except for physiological regeneration like maintenance of tissue function

(Seifert and Voss, 2013; Yun, 2015). Therefore, such regeneration is a temporal phenomenon which is observed only in the early phase of individual development.

In contrast, newts can regenerate multiplex body parts even after individual maturation. However, it does not mean that they have a lot of stem/progenitor cells in their body. The resource of adult newt regeneration is fully differentiated cell. When they injured, such fully differentiated cell are respond to it and change their characteristics into stem-like state (reprogramming). From these stem-like cells, lost body parts are regenerated through their proliferation, re-differentiation, and patterning. Among salamandridae, only newts have such adult-type regeneration [even axolotls do not have such ability (Sandoval-Guzmán et al., 2014)]. Therefore, this reprogramming-based body parts regeneration can be said as a newt specific ability acquired during evolution. By uncovering this mechanism, it becomes possible to compare with other vertebrates which cannot regenerate in the adult stage such as humans. It makes clear differences between these animals and how newts acquire such amazing ability. This information can contribute not only to biological fields but also to the medical treatment for traumatic injury. However, there still remain several mysterious points. Especially, detail molecular mechanisms of the early phase of the regeneration, in other words, how the regeneration is started is still unclear. To address this

issue, retinal regeneration system was focused on in this study.

1.2. Newt retinal regeneration

Retina is located inside back of eyeballs and functions in the early step of the visual system. Retina is composed by neural retina (NR) and retinal pigment epithelium (RPE) (Figure 1). NR makes multilayered neural circuit. NR senses light by photoreceptor cells and transduces basic signals of the visual system into brain. RPE is a highly pigmented epithelial tissue and supports such physiological functions of NR (Strauss, 2005; Fuhrmann et al., 2014). Even if the NR is removed, newts can regenerate complete functional retina from RPE cells. When NR is removed, RPE cells start losing their epithelial characteristics. Following it, RPE cells re-enter to the DNA synthesis phase (S-phase) of the cell cycle (cell cycle re-entry) and acquire multipotency between 5-10 days after NR removal. This RPE-derived cell is called as RPE stem-like cell (RPESC). The RPESCs are separated into two layers called pro-NR and pro-RPE, and new NR and RPE itself are generated from these layers respectively (Chiba et al., 2006; Chiba, 2014) (Figure 2). This regeneration system has two big advantages. First one is about the cell resource of the regeneration. In adult newt retinal regeneration, the resource of the regeneration is restricted into

single cell type, RPE cells. Therefore, this system is advantageous to trace and identify the cells during regeneration process. Second one is about the analysis method. In this system, *in vitro* analysis methods can be applicable for RPE cells (mentioned in detail below and in materials and methods). It allows us to investigate signaling pathways/molecular mechanisms involved in the regeneration process. From these reasons, retinal regeneration is a suitable system to analyze newt regeneration processes.

Interestingly, also in mammals, similar changes of RPE cells are observed after retinal injury. One of the examples is proliferative vitreoretinopathy (PVR). In PVR, after retinal injury, RPE cells start losing their epithelial characteristics and proceed into the epithelial-mesenchymal transition (EMT) process. During this process, RPE cells start proliferation, acquire multipotency, and transit to myo-fibroblastic state with expression of myo-fibroblastic markers like vimentin and α-smooth muscle actin (α-SMA). The RPE cell-derived myo-fibroblastic cells attach to remaining NR, generate fibrotic structure, and finally cause serious visual disorders and vision loss (Casaroli-Marano et al., 1999; Yang et al., 2015; Tamiya and Kaplan, 2016) (Figure 3). Like this, the behavior of RPE cells after retinal injury is quite similar between newts and mammals. However, in mammals, although RPE cells can acquire multipotency *in vitro* (Salero et al., 2012), they show metaplastic transformation and cannot

regenerate their retina *in vivo*. Unraveling the mechanisms underlying newt retinal regeneration also allows us to compare with mammalian traumatic retinal disorders. It can reveal differences between these two systems and how newts evolved such unique ability.

1.3. Retinectomy and *in vitro* retinectomy

There are two important methods to investigate newt retinal regeneration process, retinectomy and *in vitro* retinectomy (schematic diagram is in Figure 4 and 5).

Retinectomy is a surgical operation to remove NR from living newt eyeballs. In brief, firstly, dorsal half of the eyeball is cut along to the cornea-scrolal junction and the NR together with lens is carefully removed by gentle stream of normal newt saline. Then, the anterior half of the eyeball is carefully put back to its original position. Retinectomised animals are kept in moist containers at 22 ℃ until experiments are performed.

In *in vitro* retinectomy, firstly the eyeball is enucleated and, in buffer solution, cut open along equator to separate the anterior half from the posterior half. And then, from the posterior half of the eyeball, NR is carefully removed by a fine needle. This posterior half of the eyeball without NR is named retina-less eye-cup (RLEC). After preparation, RLECs are incubated in

culture medium at 25 ℃ until experiments are performed. In this condition, although the three-dimensional structure of retina still cannot be induced, RPE cells can re-enter the S-phase of the cell cycle and express some genes which expressed in *in vivo* retinal regeneration almost same time course as *in vivo* (Yoshikawa et al., 2012; Inami et al., 2016). Then, it is possible to analyze the early process of the regeneration. In addition to it, pharmacological experiments are applicable in this method. In *in vivo* conditions, it is difficult to apply such experiments because eye pressure pushes out drugs from the inside of eyeballs. In contrast, in this method, by adding reagents, such as inhibitors or activators, to the buffer solution and/or the culture medium, investigation of signaling pathways/molecular mechanisms involved in the regeneration process become possible. Therefore, this *in vitro* system is a suitable system to analyze responses of RPE cells after retinectomy, especially in the early phase.

1.4. Previous studies

Studies of mechanisms initiating the retinal regeneration have been advanced using cell cycle re-entry of RPE cells as an indicator. In previous studies, two important elements for cell cycle re-entry of RPE cells were found.

First one is MEK [mitogen-activated protein kinase

(MAPK)/extracellular signal-regulated kinase (ERK) kinase]-ERK signaling. MAPK signaling is a signaling pathway widely conserved from invertebrates to vertebrates and participates in a lot of biological phenomena, for example proliferation, differentiation and survival (Shaul and Seger, 2007). ERK belongs to MAPK family and is regulated by MEK, which belongs to MAPK kinase family. This MEK-ERK module is typically activated receptor tyrosine kinases or G protein-coupled receptors (GPCR) stimulated by several factors (REF). In previous studies, it was found that MEK-ERK signaling was temporally reinforced within 30 min after retinectomy. And, in the *in vitro* condition, MEK inhibitor U0126 treatment significantly decreased the proportion of cell cycle re-entry of RPE cells (Mizuno et al., 2012; Yoshikawa et al., 2012). Thus, it was suggested that this temporal reinforcement of MEK-ERK signaling is a requisite to cell cycle re-entry of RPE cells.

Second one is cell-cell contact of RPE cells. In the *in vitro* conditions, unlike *in vivo* retinal regeneration, cell cycle re-entry of RPE cells observed actively in the area near from the wound edge (the 'Edge' area, detail definition is in materials and methods). In contrast, in the area except for the Edge area (the 'Center' area, detail definition is in materials and methods), the cell cycle re-entry was hardly observed (Yoshikawa et al., 2012) (Figure 6). However, it was found that, when the cell-cell contact

was attenuated by calcium chelating or partial removal of the RPE tissue, cell cycle re-entry of RPE cells was promoted (Yoshikawa et al., 2012). Therefore, it was suggested that cell-cell contact of RPE cells has negative effects and, to enter cell cycle, relieve from such negative effects is required.

1.5. Aim of this study

Thus, the involvement of these elements was already suggested in previous studies. However, there still remained following three questions; (1) what is the causal stimuli reinforcing MEK-ERK signaling, (2) which is the signaling pathways mediating promotion of cell cycle re-entry by attenuation of cell-cell contact, (3) how are these elements related each other. In this study, these questions were addressed by using the *in vitro* system. And finally, together with previous studies, it was suggested that the early phase of retinal regeneration was regulated by multi-step trigger system.

2. Materials and methods

All methods were carried out in accordance with Regulations on the Handling of Animal Experiments in University of Tsukuba (RHAEUT). All experimental protocols were approved by the Committee for Animal Experiments in University of Tsukuba (CAEUT).

2.1. Animals

Adult *Cynopos pyrrhogaster* newts (total body length: 9-12 cm) were purchased from local suppliers in Japan (Aqua grace, Yokohama, Japan) and housed at 18 ℃ in containers under natural light conditions. Animals were used without distinction of sex. In all experiments, animals were anesthetized with 0.1% FA100 (4-allul-2-methoexyphenol; DS Pharma Animal Health, Osaka, Japan) before surgery or sacrifice.

2.2. Retinectomy and collection of eyeballs

Animals were anesthetized by 0.1% FA100 for 2 h in dark condition and carefully washed by tap water and then placed under stereo-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 gentle stream of sterilized newt saline solution [(mM) 115NaCl, 3.7 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 18 D-glucose, 5 HEPES, pH 7.5]. After operation, the eye flap consisting of the iris and cornea was carefully placed back in its original position. Operated animals were kept in moist containers at 22 ℃ (day/night cycle 12:12 h). They were sacrificed on appropriate days under anesthesia. After anesthetization, they were decapitated and, from the heads, normal or retinectomized eyeballs were collected.

2.3. Preparation and incubation of retina-less eye-cups (RLECs)

Animals were anesthetized by 0.1% FA100 for 2 h in dark condition and carefully washed by tap water. Anesthetized animals were decapitated and the heads were sterilized by 70% ethanol for around 60 sec. Then, under microscope, eyeballs were enucleated. The eyeballs were soaked in the order of 70% ethanol → phosphate buffer solution (PBS) → 70% ethanol → PBS for 20 sec each for sterilization and washing. Washed eyeball was placed cornea side up on a membrane filter (HAWP 013 00, Millipore, Billerica, MA, USA) and cut along the equator. And then, its anterior half was carefully removed. The posterior half (eyecup) was soaked in PBS for 10-20 min and the NR was carefully removed by using a fine needle to make retina-less eyecup (RLEC).

The RLECs were incubated in newt standard culture medium (NSCM) [80% L-15 medium (41300-039, Thermo Fisher Scientific, Waltham MA, USA; pH 7.5) containing 1% penicillin-streptomycin (15140-122, Thermo Fisher Scientific) and 1% fetal bovine serum (26140079, Lot 1024914, Thermo Fisher Scientific)]. The medium was refreshed on day-5 of incubation. To observe the cell cycle re-entry of the RPE cells, 5 μg/ml BrdU (B5002, Sigma-Aldrich, St Louis, MO, USA) was added to the NSCM. In experiments to observe MEK-ERK signaling activity, RLECs or eyecups were incubated 80% L-15 medium containing 1% penicillin-streptomycin (pH 7.5). In some experiments, 10mM EGTA solution [(in mM) 115 NaCl, 3.7 KCl, 10 EGTA, 18 D-glucose, 10 HEPES, and 0.001% phenol red; pH 7.5] was treated to RLECs for 60 min immediately after removal of NR to attenuate cell-cell contact. In this case, as a control, modified newt saline [(in mM) 115 NaCl, 3.7 KCl, 3 $CaCl_2$, 1 MgC_2, 18 D-glucose, 10 HEPES, and 0.001% phenol red; pH 7.5] was used. After treatment, RLECs were washed by 80% L-15 medium for 15 min twice, transferred into culture medium, and incubated. In signal inhibitor experiments, ß-catenin signal inhibitor XAV939 which was dissolved in DMSO (D2650, Sigma-Aldrich) at 10 mM or MEK 1/2-specific inhibitor U0126 (V1121, Promega, Fitchburg, WI, USA) which dissolved in DMSO at 2 mM immediately before use was administrated at a final concentration 10 μM and 5 μM

respectively from the time point at which eye-cup was soaked in PBS. In XAV939 treatment, 0.1% DMSO was used as a control. In U0126 treatment, U0124 (an inactive analog of U0126; 662006, Millipore) was used at same concentration to U0126 as a negative control. To examine the effect of the fluid in vitreous cavity of intact/regenerating eyeballs on cell cycle re-entry, eyeballs were enucleated from intact animals or animals at 2 days after retinectomy. The intact/regenerating eyeballs were made a cut (almost half of circumference) carefully not to lose the fluid. Then, they were transferred into 80% L-15 medium containing 1% penicillin-streptomycin (1 eyeball/200 μml) and incubated for 1 h. After incubation, the eyeballs were removed and the conditioned medium was used to incubate the RLECs. As a control, the RLECs were incubated in 80% L-15 medium containing 1% penicillin-streptomycin. To examine the effect of newt serum on RLECs, newt blood was collected from the surgical site of decapitated animals. Collected blood was leaved until serum was separated from other components by blood coagulation. About ~20 μl serum could be collected from one animal. The serum was administrated to 80% L-15 medium containing 1% penicillin-streptomycin at 10% concentration and, using this culture medium, RLECs were incubated. As a control, RLECs were incubated in 80% L-15 medium containing 1% penicillin-streptomycin.

2.5. Immunohistochemistry

2.5.1. Antibodies

Rabbit polyclonal anti-phospho-ERK1/2 antibody (1:150; Phospho-p44/p42 MAP Kinase antibody, 9101S, Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-N-cadherin antibody (1:200; ab12221, Abcam, Cambridge, UK), rabbit polyclonal anti-α smooth muscle actin (α-SMA) antibody (1:200; ab 137734, Abcam), mouse monoclonal anti-β-catenin antibody (1:1000; C7207, Sigma-Aldrich), mouse monoclonal anti-vimentin antibody (1:200; XL-VIM-14.13, PROGEN Biotechnik GmbH, Heidelberg, Germany), and mouse monoclonal anti-RPE65 antibody (1:1000; MAB5428, Millipore, MA, USA) were used as the primary antibodies. Biotinylated goat anti-rabbit IgG antibody (1:400; BA-1000, Vector laboratories, Burlingame, CA, USA), biotinylated goat anti-mouse IgG antibody (1:400; Vector laboratories), Alexa-488-conjugated goat anti-rabbit IgG antibody (1:500; A-11008, Thermo Fisher Scientific), tetramethylrhodamine-conjugated goat anti-mouse IgG antibody (1:200; T2762, Life Technolocies, MD 20850, USA) were used as secondary antibodies.

2.5.2. Preparation of samples

For immunohistochemistry, the normal and retinectomized eyeballs were fixed in 2% paraformaldehyde/0.2% picric acid in PBS (pH 7.5) for 5-6 h at 4 ℃, washed thoroughly in PBS overnight 4 ℃, then cryosectioned transversely at ~20 μm thickness. RLEC preparations were fixed in 4% paraformaldehyde in PBS (pH 7.5) overnight for BrdU immunostaining or were fixed in 2% paraformaldehyde/0.2% picric acid in PBS (pH 7.5) for 3-4 h at 4 ℃ for other experiments. After fixation, RLECs were washed thoroughly in PBS. Then, they were cryosectioned transversely ~20 μm thickness or moved into whole-mount immunostaining process.

2.5.3. Immunofluorescence (IF)

Immunofluorescence (IF) labeling was performed as follows. Samples were washed in PBS and incubated in the blocking solution [5% bovine serum albumin (A3294, Sigma-Aldrich), 0.2% TritonX-100 diluted in PBS] containing 2% normal goat serum (S-1000, Vector laboratories, Burlingame, CA, USA) for 2 h. After rinsing in PBS, they were incubated in a primary antibody diluted with the blocking solution for overnight at 4 ℃. After

washing thoroughly in PBS, they were incubated in secondary antibodies diluted with the blocking solution for 4 h and then washed in PBS. In double labeling with primary antibodies derived from different hosts (mouse and rabbit), they were applied together and labeled appropriate secondary antibodies. In double labeling with primary antibodies both derived from mouse, additional blocking process was added. After labeling of first target, samples were incubated in a normal mouse IgG (1:200; 15381, Sigma-Aldrich) for 3h. After washing thoroughly in PBS, they were incubated in goat anti-mouse IgG Fab fragment (1:100; 115-007-003, Jackson Immuno Reserch, West Grove, USA) for overnight at 4 ℃. After washing thoroughly in PBS, another primary antibody and secondary antibody were applied as described above.

2.5.4. Immunoperoxidase (IP)

Immunoperoxidase (IP) labeling was performed as follows. The samples were washed in PBS, incubated in 0.3% H_2O_2 in PBS for 30 min, rinsed twice in PBS and then incubated in the blocking solution containing 2% normal goat serum and 2% AvidinD (Avidin / Biotin Blocking kit; SP-2001, Vector laboratories) for 2 h. After rinsing twice in PBS, they were incubated in a primary

antibody diluted with the blocking solution containing 2% Biotin (Avidin / Biotin Blocking kit; Vector laboratories) overnight at 4 ℃. After washing thoroughly, they were incubated in a biotinylated secondary antibody diluted with the blocking solution for 4 h. After rinsing twice in PBS, they were incubated in a mixture Avidin and Biotin Complex (Vectastain ABC Elite kit; PK-6100, Vector; prepared 30 min before use) for 2 h. After washing thoroughly, they were incubated in DAB solution (DAB substrate kit; SK-4100, Vector) up to 3 min. Finally, the reaction was stopped by washing them in PBS thoroughly. In double labeling with primary antibodies derived from different hosts (mouse and rabbit), they were applied together and labeled appropriate secondary antibodies. In double labeling with primary antibodies both derived from mouse, additional blocking process was added after biotinylated secondary antibody reaction as described above. After the reaction of another primary and secondary antibody, Avidin-Biotin complex solution and DAB solution were applied as described above. Finally, the samples were washed in PBS thoroughly. Only in BrdU immunostaining, RLECs were incubated in 2 N HCl for 2 h at room temperature after H_2O_2 treatment to denature DNA and increase antibody affinity to BrdU. In IP of phosphorylated ERK (pERK) and β-catenin in tissue section, antigen retrieval step was added before immunolabeling. In the eyeball or RLEC sections, slits

were made along the inside margin of the cornea and sclera to separate the iris and retinal tissues from those connective tissues (by manipulating a blade under a stereo-microscope). They were rinsed in PBS for 15 min, incubated in a sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) at 90 ℃ for 10 min and then rinsed twice in PBS. During the incubation, the corneal and scleral tissues became shrunk and detached into the buffer solution. This treatment clearly decreased the background staining of the tissues while sustaining immunoreactivity, which increased the signal to noise ratio.

After IF or IP labeling, the samples were fixed again by 4% paraformaldehyde in PBS for 5 min, washed in PBS and then incubated in 15% H_2O_2/1.5% sodium azide in PBS [reaction time; tissue section: up to 2 h, RLEC (IF): up to 8 h, RLEC (IP): over-night] to bleach their melanin pigments. After rinsing twice in PBS, the nuclei were visualized by DAPI (1:50000; D1306, Thermo Fisher Scientific) or TO-PRO®-3 (1:1000; T3605, Thermo Fisher Scientific) dissolved in PBS for 1 h. After rinsing in distilled water, each sample was mounted in 90% glycerol under a cover slip.

2.6. Calculation of pERK+ RPE cell nuclear ratio, BrdU+ RPE cell ratio, and ß-catenin+ RPE cell nuclear ratio

pERK+ RPE cell nuclear ratio was calculated as follows. After immunostaining, the number of total RPE cells was counted by DAPI staining in RPE65+ cells. And then, the number of pERK+ cell nucleus was counted by pERK immunoreactivity co-localized with DAPI staining in the RPE65+ cells. From these values, pERK+ RPE cell nuclear ratio in a tissue section was calculated. And, average of three sections (from same RLEC or eyecup, distanced five sections each) was regarded as pERK+ RPE cell nuclear ratio in a sample.

BrdU+ RPE cell ratio and ß-catenin+ RPE cell nuclear ratio was calculated as follows. Before bleaching of melanin pigment, the PRE-choroid tissue was carefully separated from the sclera by using a fine needle and transferred into 90% glycerol on a grass slide, and mounted under a coverslip. RPE cells can be identified by their characteristic morphology observed over green autofluorescence of the choroid and the total RPE cell number was counted. After counting, the cover slip mounted on the RPE-choroid tissue was carefully removed and the tissues were transferred into PBS to rinse. And then, melanin pigment was bleached by 15% H_2O_2/1.5% sodium azide in PBS. After bleaching, the tissues were rinsed by PBS twice, transferred into 90%

glycerol on a grass slides, and mounted under a cover slip. And then, BrdU+ nuclear or ß-catenin+ nuclear was counted under a microscope through transmission light. According these values, BrdU+ RPE cell ratio and ß-catenin+ RPE cell nuclear ratio were calculated. In cell and nuclear counting, firstly the cell number in a whole RPE sheet was counted. Then the cell number within 100 μm from wound edge of RPE sheet was counted (defined as 'Edge'). The cell and nuclear number in area except the Edge area was calculated by subtraction the Edge area from the whole area (defined as 'Center').

2.7. Digital images

Bright light and fluorescence images of tissues were acquired using a CCD camera system [a DP73 system (Olympus, Tokyo, Japan)] attached onto a fluorescence microscope (BX50, Olympus). Confocal microscopic images were acquired using a confocal microscope system (LSM510, Carlzeiss, Oberkochen, Germany). Only in Figure 10, the pictures were taken using digital camera (C-5060, Olympus) attached onto a dissecting microscope (M165 FC, Leica Microsystem, Wetzlar, Germany) to take living RPE cells.

2.8. Statistical analysis

All statistical analysis were performed pairwise manner (one eyeball was used as test sample and another eyeball was used as control in one animal) to decrease effect of individual difference. Cell cycle re-entry of RPE cells was occurred almost same frequency in RLECs from same animals (Figure 7, Spearman's rank correlation coefficient, $P = 0.0009$, $R^2 = 0.802$). Statistical data were presented as a line graph connecting pairwise values. Non-parametric tests were carried out to evaluate the statistical significance of the data using Sheffe's test following Freidman's test.

3. Results

3.1. NR removal and MEK-ERK signaling

As mentioned above, although it was suggested that MEK-ERK signaling was required for cell cycle re-entry of RPE cells, it was still uncertain if removal of NR was a causal event or not. There are several manipulations prior to NR removal (incision into sclera/choroid or removal of the anterior half of the eyeballs include lens). Thus far, it was difficult to exclude the possibility that such manipulations respond to MEK-ERK signaling reinforcement. Therefore, firstly, I confirmed this using *in vitro* system.

If surgical incision into the sclera/choroid itself and/or the factors come from the incision site reinforces MEK-ERK signaling, the time course and distribution pattern of the reinforcement would show difference between the incision margin and the central region of RPE sheet. To examine this possibility, I visualized pERK on whole mount preparation of the RPE sheet by immunostaining. In *in vitro* condition, when the eyeball was incised to make the posterior eyecup and the NR was removed, MEK-ERK signaling activity in RPE cells was increased within 30-60 min as indicated by nuclear translocation of pERK (Figure 8). This was consistent with our previous observation *in vivo*

(Mizuno et al., 2012), although progress was slightly slow in the current condition. Importantly, the change of MEK-ERK signaling activity took place simultaneously and uniformly throughout the RPE sheet. Therefore, the possibility that the surgical incision into the sclera/choroid might be a causal event for MEK-ERK signaling reinforcement in RPE cells could be excluded.

Next possibility was effect of the anterior eyecup including the lens (Figure 9). If such anterior eyecup had inhibitory effects to MEK-ERK signaling and removal of it is responsible, MEK-ERK signaling would be reinforced regardless the presence or absence of the NR. When the NR was removed *in vitro* (retinectomy+), pERK nuclear translocation was obviously occurred in 72-83% (80.3 ± 2.2%, n = 5) of RPE cells. In contrast, when the eye-cup which the NR had been left as intact was incubated in the same condition (retinectomy-), pERK+ RPE cell nuclear was decreased to 19-38% (27.9 ± 3.3%, n = 5). This result indicated that NR removal is essential event for induction of MEK-ERK signaling reinforcement in RPE cells.

These two results, together with our previous findings, suggested that the removal of NR, but not other surgical operation processes, can be a trigger for reinforcement of MEK-ERK signaling and consequently for cell cycle re-entry of RPE cells and retinal regeneration.

3.2. Attenuation of the cell-cell contact and the downstream signaling pathway

Importantly, in *in vitro* conditions, RPE cells in the Center hardly re-enter to cell cycle even though retinectomy is carried out (Yoshikawa et al., 2012) (Figure 6). However, in a previous study, it was found that cell cycle re-entry of RPE cells was promoted along incised margin of the RPE sheet and stimulated either by removal of a piece of the RPE tissue from the Center or by treatment of EGTA solution, which attenuates cell-cell contact mediated by cadherin, a calcium-dependent cell adhesion molecule (Tamiya et al., 2010; Yoshikawa et al., 2012). Therefore, it had been suggested that cell-cell contact of RPE cells is responsible to this inhibition. Actually, in amniotes, including humans, liberation from contact inhibition is an essential step for mature RPE cells to re-enter the cell cycle (Tamiya et al., 2010; Chen et al., 2012). Probably, in the RLEC *in vitro* conditions, factors which reduce cell-cell contact in the RPE are lacking unlike *in vivo* conditions.

In the present study, I examined signaling pathways that are activated by attenuation of cell-cell contact. For this, I firstly determined a condition of EGTA treatment. In control condition treated modified normal newt saline solution, RPE cells were attached each other and kept its epithelial morphology (Figure

10A). However, when treated EGTA solution for 60 min, each RPE cells could become recognized and cell-cell contact seemed to be loose (left-hand image in Figure 10B). In 120 min treatment, cell-cell contact becomes loose more and some RPE cells started to detach from Bruch's membrane (right-hand image in Figure 10B). In a previous study, RPE cells were detached from Bruch's membrane and decreased the number during the incubation time by 90 min treatment of EGTA solution. Therefore, in the present study, I set 60 min for treatment time. Next, I confirmed the effect of this condition on cell cycle re-entry of RPE cells (Figure 11). In this condition, the proportion of cells which had re-entered the S-phase of the cell cycle in the Center within 10 days was on average about three times higher (range:2.2-36.3%; 20.5 ± 5.2%, n = 6) than the control. However, the effect of EGTA treatment varied between individuals [RPE in 3 (50%) of 6 animals obviously responded]. This can be due to the threshold. The EGTA treatment condition that adopted in this study was mild than the previous one. Therefore, it was thought that the present condition provided a stimulus slightly above the threshold for cell cycle re-entry of RPE cells. However, since cell cycle re-entry ratio was promoted in total, I used this set of condition in following studies.

Under this set of conditions, I examined the effect of attenuation of cell-cell contact on the activation of ß-catenin signaling. It is known that ß-catenin, which associate with the intracellular

domain of cadherin on the cell membrane, is released from cadherin and sends a signal to the nucleus when cadherin-mediated cell-cell contact is disrupted by calcium depletion (Peluso et al., 2000). In the newt, RPE cells express N-cadherin (Nakamura et al., 2014; see below). When the RLEC was treated with EGTA solution for 60min and then incubated in NSCM for 5 days, a proportion of ß-catenin+ nuclei in the Center (range: 3.1-22.3%; 10.9 ± 3.2%, n = 5) increased significantly compared to the control without EGTA treatment (range: 1.9-9.3%; 5.5% ± 1.5%, n = 5) (Figure 12 and Figure 13). In this study, I chose this time point (day-5) because a majority of the RPE cells have not re-entered the cell cycle *in vivo* (Chiba, 2014; Islam et al., 2014) and in the Edge of the control (Yoshikawa et al., 2012). In the control condition, ß-catenin immunoreactivity was mostly localized on the cell membrane along the cell-cell contact region (Center in Figure 12A), as observed in either the intact RPE or the RPE immediately after retinectomy (see below). On the other hand, the EGTA treatment condition, nuclear translocation of ß-catenin was frequently observed in the area where RPE cell changed their hexagonal shape to rhombus or fusiform shape (Center in Figure 12B). Probably the decrease of cell-cell adhesion allowed the cells to change their structure. The distribution pattern of such areas in the Center was different among RLECs. Note that those areas rarely appeared in the

control condition.

To confirm whether β-catenin signaling was involved in cell cycle re-entry of RPE cells, I examined the effect of an inhibitor of β-catenin signaling, XAV939, on cell cycle re-entry of RPE cells in the EGTA treatment condition (Figure 14). When the EGTA-treated RLEC was incubated in the presence of XAV939 for 10 days, the proportion of BrdU+ cells in the Center (range: 0-19.8%; 4.5 ± 2.1%, n = 9) decreased significantly compared to the mock control, which only contained solvent (range: 0.4-34.6%; 9.7 ± 3.5%, n = 9). Taken together, attenuation of cell-cell contact is likely to activate β-catenin signaling, which is involved in cell cycle re-entry of RPE cells.

Followed by *in vitro* studies, to confirm if β-catenin signaling in RPE cells was activated in *in vivo*, I examined changes in the subcellular localization of β-catenin at the early phase of the retinal regeneration by immunohistochemistry. Figure 15 illustrates a summary of events that take place during retinal regeneration (Mizuno et al., 2012; Islam et al., 2014). In this process, I chose the period when RPE cells lose their epithelial characteristics. Firstly, I investigated the cell-cell contact condition in the early phase using N-cadherin as an indicator. In both intact RPE cells and RPE cells immediately after retinectomy (DAY 0), intense immunoreactivity of N-cadherin was detected at the site of the cell-cell contact (Figure 16A and B). Also

in Day 1, RPE cells still keep their epithelial morphology and N-cadherin immunoreactivity (Figure 16C). However, in Day 3, RPE cells started to change their shape and, in some population, the decrease of N-cadherin immunoreactivity was observed (Figure 16D). In addition, it was observed that some RPE cells was detached from each other and floated into the vitreous cavity. Thus, in this time point, cell-cell and cell-Bruch's membrane attachment of the RPE cells seemed to be loose although most of the cells still stayed on Bruch's membrane.

During this process, I examined the subcellular localization of β-catenin. In both intact RPE cells and Day 0 RPE cells, β-catenin was mostly located on the cell membrane along the region of cell-cell contact where N-cadherin was co-localized (compare Figure 17B with Figure 16B). Also in Day 1, β-catenin immunoreactivity was observed only at the cell-cell contact region. After retinectomy, nuclear translocation of β-catenin was first recognized in RPE cells (71.5 ± 2.3%, n = 6) on the Day 3 (Figure 17D), corresponding to the decrease of N-cadherin immunoreactivity.

These results suggested that β-catenin signaling in RPE cells is also activated in association with a decrease of their cell-cell contact *in vivo*, consistent with *in vitro* observation. As mentioned above, reinforcement of MEK-ERK signaling takes place within 30-60 min after retinectomy (Figure 8). Hence, activation of

β-catenin signaling seemed to take place later than reinforcement of MEK-ERK signaling.

3.3. Relationships between two elements

After retinectomy, it was suggested that MEK-ERK signaling reinforcement took place within 30-60 min and β-catenin signaling activation took place at Day 3. Therefore, I hypothesized that MEK-ERK signaling was involved in the activation of β-catenin signaling. To confirm this, I examined the relationships between these signaling pathways using the *in vitro* system. For this, I administrated a MEK inhibitor, U0126, from the time point when the eyeball was incised into the eyecup. In the presence of U0126, I carried out retinectomy, treated the resulting RLECs with EGTA solution for 60 min, and incubated them in NSCM (Figure 18). The concentration of U0126 was 5 μM which can inhibit the initial activation of ERK1/2 mediated by MEK1/2 up to ~50% (Yoshikawa et al., 2012). In this set of conditions, the proportion of BrdU+ cells in the Center at 10 days (range: 0-3.9%; 1.1 ± 0.4%, n = 11) was significantly lower than the mock control with U0124, which inactive analogue of U0126 (range: 1.1-15.2%; 5.8 ± 1.3%) (Figure 18B). This observation was consistent with previous results (Yoshikawa et al., 2012). In the same set of conditions, I examined β-catenin signaling after

incubation for 5 days, and found that nuclear translocation of β-catenin was significantly decreased (range: 0.7-2.7%; 1.2 ± 0.5%, n = 4) compared to the mock control condition (range: 5.1-20.9%; 10.9% ± 3.4%, n = 4) (Figure 18C). These results indicated that MEK-ERK signaling strengthened by NR removal is a prerequisite for nuclear translocation of β-catenin or β-catenin signaling, which stimulated by the attenuation of cell-cell contact.

3.4. Results obtained from the Edge area

So far, I focused on the Center area, where cell cycle re-entry is hardly occurred. I also investigated the effects of the above conditions in the Edge area. In this area, as explained in introductory part, the RPE cells spontaneously re-enter the S-phase of the cell cycle unlike the Center area (Yoshikawa et al., 2012). It is thought that, in this area, cell-cell contact is impaired by physical stimuli of the incision to the RPE sheet. As shown in Figure 19, I obtained almost same results in the Edge. Inhibition of MEK-ERK signaling was significantly decreased cell cycle re-entry of RPE cells and nuclear translocation of β-catenin and inhibition of β-catenin signaling was also decreased the cell cycle re-entry ratio (Figure 19B, C, and D). Only about nuclear translocation of β-catenin under EGTA treatment conditions, the significant change was not observed unlike the Center area

(Figure 19A). It is conceivable that, as I expected, since cell-cell contact of RPE cells was already impaired by the incision, EGTA treatment could not affect to nuclear translocation of β-catenin. In fact, the ratio of nuclear translocation of β-catenin in the Edge (range: 41.9-81.4%; 55.9 ± 6.8%, n = 5; Figure 19A) was higher than in the Center (range: 1.9-9.3%; 5.5 ± 1.5%; n = 5; Figure 13). According to these results, it was suggested that, also in the Edge area, RPE cells re-enter the cell cycle as same manner to the Center area. This information supports the observation in the Center area.

4. Discussion

The newt is one of the greatest models of *in vivo* regeneration and they can regenerate multiplex body parts even after individual maturation through reprogramming of fully differentiated cells. Although this newt-specific ability has been investigated for long time, the detail molecular mechanisms, especially the initial step of regeneration process, is still uncertain. In this study, focusing on cell cycle re-entry of RPE cells in the retinal regeneration, the trigger mechanism was investigated.

To address this issue, in this study, the *in vitro* retinecotomy method was applied. Under this *in vitro* condition, although three-dimensional structure of retina still cannot be induced, RPE cells re-enter to S-phase of the cell cycle in same time course as *in vivo*. In addition, unlike *in vivo* conditions, pharmacological experiments can be applied. Thus, the present *in vitro* system is one of suitable the models to investigate the molecular mechanisms of the initial phase of retinal regeneration.

In present study, it was suggested that a combination of NR removal, which stimulates MEK-ERK signaling, and attenuation of cell-cell contact, which stimulates nuclear translocation of β-catenin (i.e. β-catenin signaling), is necessary for cell cycle re-entry of RPE cells. And, together with previous studies,

multi-step regulation underlying the early phase of the retinal regeneration was implied.

4.1. The first step trigger

In the present *in vitro* condition, reinforcement of MEK-ERK signaling took place within 30-60 min after retinectomy, although it was slightly slow compared to previous *in vivo* condition (Figure 8) (Mizuno et al., 2012). The important point is that this reinforcement was observed simultaneously and uniformly in whole area of the RPE sheet. This means that incision to the RPE sheet and/or the factors which come from the incision site are not causal events to the signaling reinforcement. In addition, this reinforcement was not observed if NR was left on the RPE tissue (retinectomy- condition) during incubation (Figure 9). These results suggest that removal of NR, but not other surgical manipulations, is a causal event to trigger cell cycle re-entry of RPE cells and consequent retinal regeneration. However, it is still unsolved how MEK-ERK signaling is stimulated after NR removal. One conceivable possibility is that RPE cells are relieved from inhibitory effects mediated either by direct contact of RPE cells with the NR via photoreceptor outer segment or by factors released from the NR in physiological conditions (Grigoryan., 2012; Pastor et al., 2016). Another possibility is excitatory factors

which are released from the NR and/or the RPE itself, when the NR is separated from the RPE cells.

MEK-ERK signaling is also interested in mammalian RPE cell proliferation and EMT. Intriguingly, similar to newt RPE, it was reported that MEK-ERK signaling is activated within 15min after retinal detachment even in mammalian RPE cells (Geller et al., 2001), although, same as this study, the mechanisms are still uncertain. In *in vitro* PVR model, many factors which stimulate RPE cell proliferation were reported including cytokines (PDGF, FGF, EGF, IGF, VEGF, and HGF) and thrombin, which is a blood coagulation factor (Chiba, 2014; Kuznetsova et al., 2014; Chen et al., 2015; Yang et al., 2015; Pator et al., 2016). Interestingly, these factors can activate the pathways converge into MEK-ERK module. In addition, retinal detachment can produce reactive oxygen species (ROS). Since MAPK signaling is also involved in oxidative stress-induced response in RPE cells, it is one important possibility (Garg and Chang, 2003; Kyosseva, 2016).

In the future studies, considering the time course of MEK-ERK signaling augmentation, it is necessary to determine the mechanisms/factors of the first-step trigger for the RPE cell proliferation process in the adult newt.

4.2. The second step trigger

In present study, EGTA treatment for 60 min promoted the cell cycle re-entry of the RPE cells and nuclear translocation of β-catenin (Figure 11, 12, and 13). And inhibition of β-catenin signaling was significantly decreased the cell cycle re-entry ratio of RPE cells (Figure 14). These results suggest that attenuation of cell-cell contact promotes cell cycle re-entry of RPE cells through β-catenin signaling activation.

Also in mammalian RPE cells, cell-cell contact is one of an important factor for cell proliferation. It is well known that cell density in the cell culture and cell-cell contact conditions affect to the cell proliferation (Kamei et al., 1996; Tamiya et al., 2010; Stern and Temple, 2015). And, in the RPE sheet culture, similar to the present *in vitro* conditions, cell proliferation is observed in the margin of the sheet and hardly occurred in the central area (Tamiya et al., 2010). In such conditions, respond to loss or attenuation of cell-cell contact by scratch of the sheet or EGTA treatment, RPE cells enter the cell cycle and start proliferation and EMT (Kamei et al., 1996; Kaida et al., 2000; Tamiya et al., 2010; Chen et al., 2012).

β-catenin is well known factor which have an important role for cell adhesion. It binds to cadherin cytoplasmic domain and composes cell adhesion complex (Ozawa et al., 1989; Niessen and

Gottardi, 2008; Valenta et al., 2012). In addition to it, β-catenin has another role as a transcription coactivator in cell nuclei. According to disruption of cadherin mediated cell-cell contact, β-catenin is released into cytoplasm. And such 'free' β-catenin is translocated into cell nuclei respond to several stimuli (MacDonald et al., 2009; Valenta et al., 2012). In RPE cell nuclei, β-catenin interacts with T-cell specific transcription factor (TCF) and promotes the transcription of genes include Cyclin D1 and c-Myc, leading activation of cyclin-dependent kinases responsible for the cell cycle progression through the G1-phase to the S-phase (Valenta et al., 2012; Kuznetsova et al., 2014). In human PVR model established in culture, β-catenin is translocated into nuclei during EMT, which is the process inducing loss of epithelial characteristics, and promotes cell proliferation and expression of mesenchymal markers such as vimentin and α-SMA (Chen et al., 2012; Umazume et al., 2014).

Thus, it is suggested that newts and mammals have similar players in cell proliferation of RPE cells. However, in mammals, RPE cells start expression of myo-fibroblastic markers like vimentin and α-SMA during EMT process. In contrast, in the newt, such marker expression was not observed in this study (Figure 20). Interestingly, when Pax6, which expressed in RPESCs, is knockdown during normal reprogramming process in retinal regeneration, RPE-derived cells finally differentiated into

myo-fibroblast-like cells, which express α-SMA and vimentin, as well as N-cadherin (Casco-robles et al., 2016). This suggests that the reprogramming ability of newt RPE cells is not acquired independently but acquired by modification of our injury response system. Therefore, it is conceivable that the factors involved in EMT process of mammalian RPE cells participate to the newt retinal regeneration process. In fact, similar morphological change was observed even in this study. In the early phase of the retinal regeneration, there was a stage when N-cadherin immunoreactivity was decreased in the RPE cells and cell-cell or cell-Bruch's attachment seemed to be loose coincident with ß-catenin nuclear translocation (Figure 16 and 17).

Currently, in mammalian RPE cells, TGF-ß, especially TGF-ß2 is regarded as the most important player in EMT of RPE cells, as well as their proliferation (Kuznetsova et al., 2014; Chen et al., 2015; Yang et al., 2015; Pator et al., 2016). TGF-ß2 induces the loss of epithelial markers such as E/P-cadherin, zonula occludens-1 (ZO-1), both of which is involved in cell-cell contact, and stimulates an increase of mesenchymal (myo-fibroblastic) markers such as vimentin, α-SMA, fibronectin, and collagen type Ⅳ. The decrease of E/P-cadherin leads an increase of the amount of 'free' ß-catenin in the cytoplasm and allows ß-catenin to translocate into nucleus (Kuznetsova et al., 2014).

In current situation, in the adult newt, it is difficult to address if

TGF-β participates to disruption of cell-cell contact inhibition and/or loss of epithelial characteristics, or if it is a factor for the second-step trigger of the RPE cell proliferation because analytical tools are limited in this animal. However, during present study, I found that the fluid in the vitreous cavity of regenerating newt eyeballs effectively increased cell cycle re-entry of RPE cells in the Center area *in vitro* (Figure 21). And, I also found that administration of newt serum in culture medium induced morphological change of the RPE cells during culture period (Figure 22). Although it is still unknown if they really participate to the disruption of the cell-cell contact inhibition, such factors produced after injury can be one of important candidates.

4.3. Multi-step trigger model in the newt retinal regeneration

In the early phase of the retinal regeneration *in vivo*, the reinforcement of MEK-ERK signaling activity took place within 30 min and nuclear translocation of β-catenin took place at day 3 after retinectomy. Additionally, in the *in vitro* condition, inhibition of MEK-ERK signaling significantly decreased β-catenin nuclear translocation (Figure 18). These results suggested that MEK-ERK signaling, which is stimulated by NR removal, and β-catenin signaling, which is stimulated by

attenuation of cell-cell contact, are not independent each other but lined serially and MEK-ERK signaling is a prerequisite for β-catenin signaling activation (Figure 23).

This mechanism explains well the phenomenon that, if isolated from eye-cup, RPE cells start to re-enter the S-phase of the cell cycle without any exogenous factors (Susaki and Chiba, 2007). And, so far, the reason why cell cycle re-entry of newt RPE cells require such long periods (5-10 days) after reinforcement of MEK-ERK signaling activity has not been known. This also may be explained by the necessity of the second-step trigger that connects these events. However, it is still unknown how MEK-ERK signaling participates to β-catenin signaling activation. In future studies, it is necessary to examine pre- and post-signaling reinforcement conditions in RPE cells by omics analysis and investigate the connection between MEK-ERK signaling and β-catenin signaling.

As mentioned above, it is known that isolated newt RPE cells can re-enter the S-phase of the cell cycle spontaneously. However, to proceed the mitotic phase and following proliferation stage, they require exogenous factors (Susaki and Chiba, 2007). Candidates for those factors are FGF2 which activates MEK-ERK signaling, and other serum-containing factors that synergistically promote the effect of FGF2 (Susaki and Chiba, 2007). On the other hand, for acquisition of multipotency, another independent

pathway has been expected. In *in vitro*, Pax6 is expressed spontaneously and uniformly in the whole area of RPE sheet. In addition, MEK inhibitor, U0126, was not affected to the expression of Pax6 (Inami et al., 2016). This might mean that, for the expression of Pax6 and probably for acquisition of multipotency, RPE cells require different pathways stimulated by retinectomy. Taken together, it is implied that the early phase of newt retinal regeneration regulated by a multi-step trigger system (Figure 24).

4.4. Conclusion

In this study, I found that a combination of MEK-ERK signaling, which is stimulated by NR removal, and subsequent β-catenin signaling, which is stimulated by attenuation of the cell-cell contact, is involved in cell cycle re-entry of RPE cells during newt retinal regeneration. Moreover, together with previous studies, I proposed a multi-step trigger model in the initial step of the retinal regeneration. Also in other body parts regeneration in the adult newts, injury response of the cells, histolysis (cell-cell detachment and disruption of tissue structure), and transition to mesenchymal state are reported as important events (Hay and Fischman, 1961; Vinarsky et al., 2005; Yun et al., 2014; Wang et al., 2015). But, in these cases, studies by high temporal resolution,

like this study, are not performed because of several technical barriers. From present findings, it is thought that such multi-step regulation also participate to other body parts regeneration. Indeed, also in limb regeneration of the newt, it was implied that proceeding to M-phase requires independent factors to S-phase entry (Mescher and Tassava, 1978). However, even in the retinal regeneration, there still remain several questions as mentioned above. To understand the newt regeneration mechanisms, further studies are needed focusing such questions in the future.

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Figures and legends

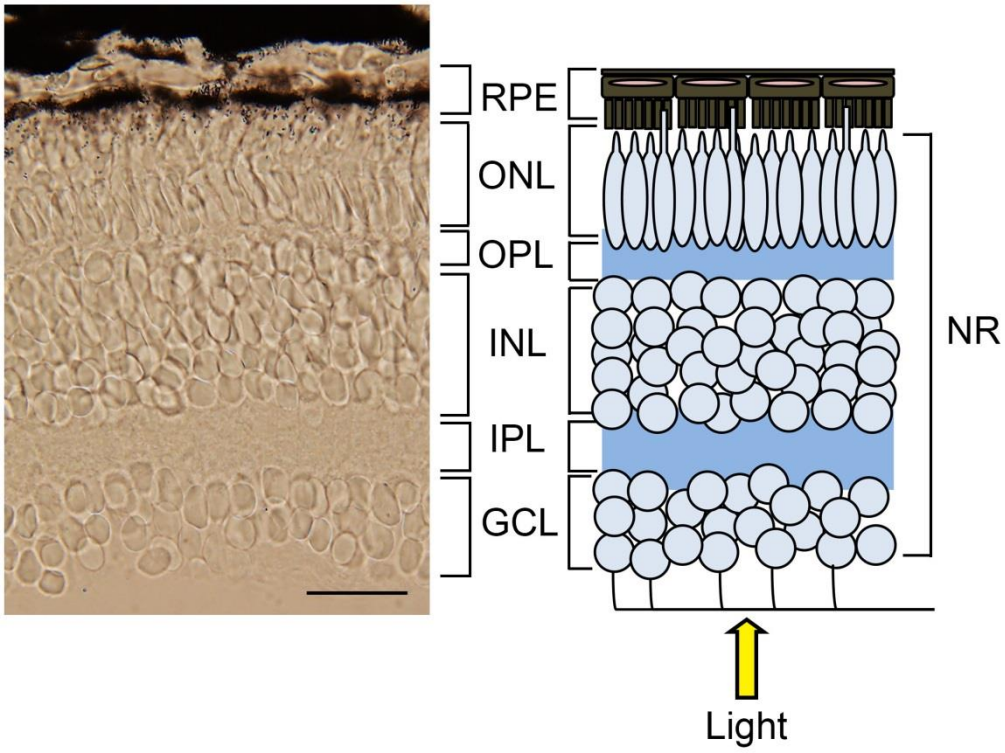


Figure 1. Structures of newt retina

A micrograph of newt retina (left-hand image) and schematic diagram of newt retinal structure (right-hand image). The upper and lower side of images are consistent to posterior (Bruch's membrane) and anterior (cornea and lens) side respectively. Retina is composed by NR and RPE. ONL: outer nuclear layer. OPL: outer plexiform layer. INL: inner nuclear layer. IPL: inner plexiform layer. GCL: ganglion cell layer.

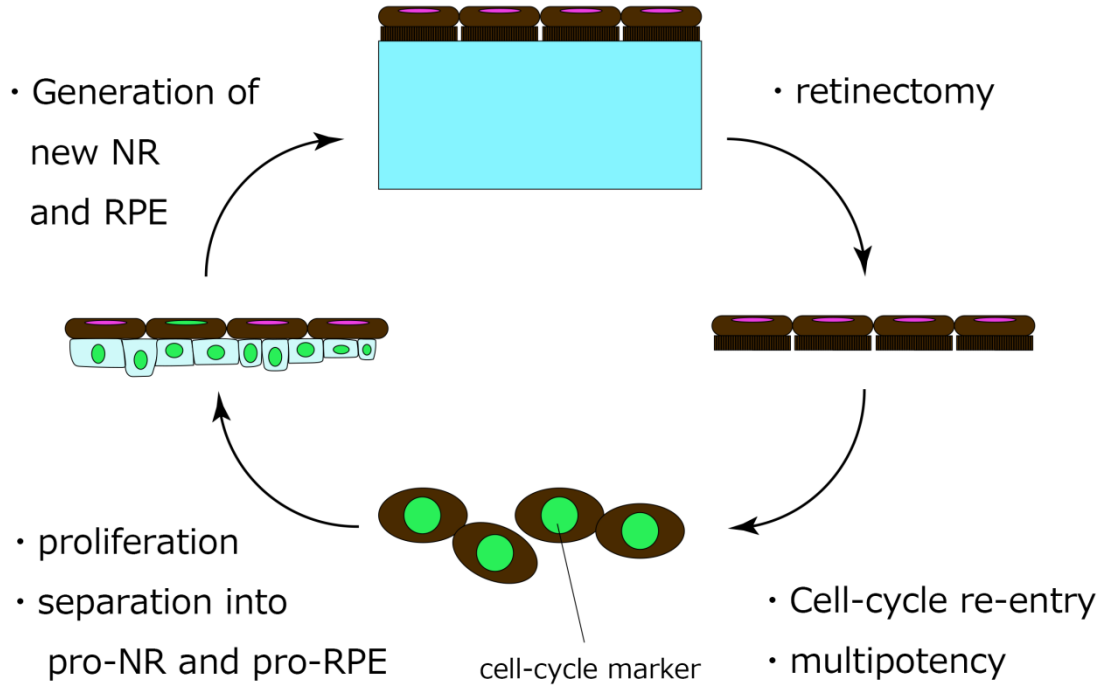


Figure 2. Process of the newt retinal regeneration

Schematic diagram of the newt retinal regeneration process. After retinectomy, RPE cells lose their epithelial characteristics. Then, they re-enter the S-phase of the cell cycle and acquire multipotency between 5-10 days after retinectomy (RPESC). The RPESCs are separated into two layers, pro-NR and pro-RPE, and proliferate to generate new RPE and NR layers.

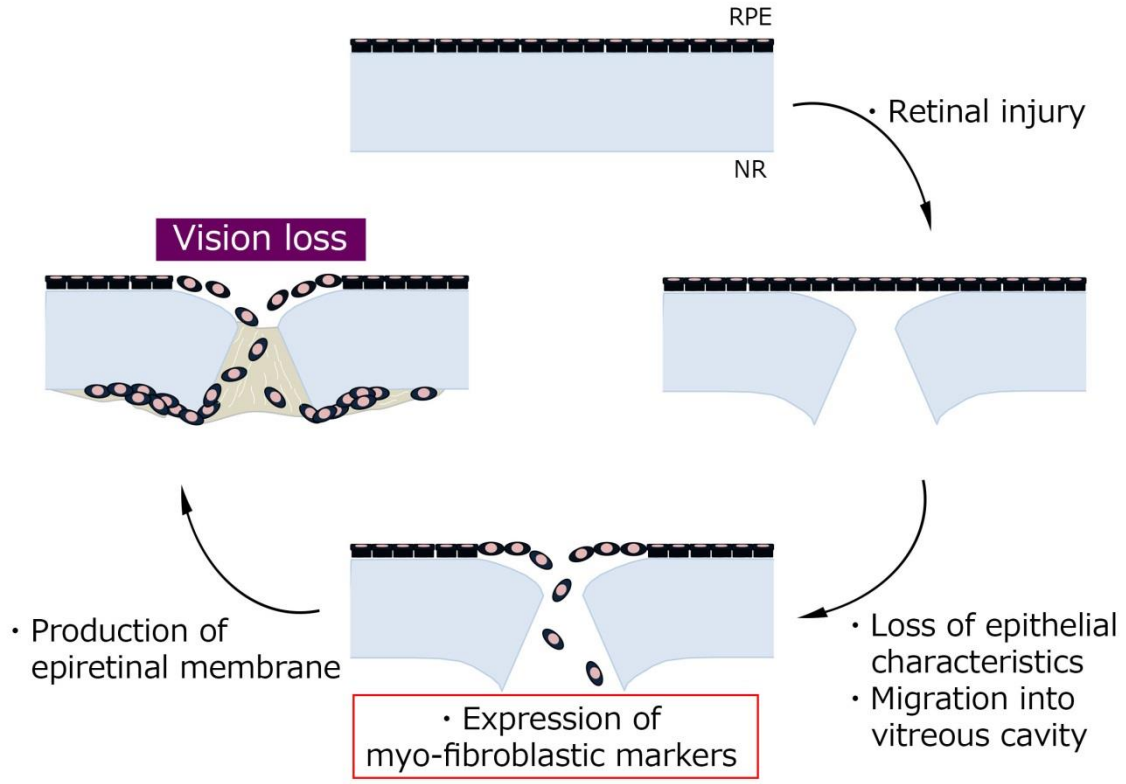


Figure 3. Process of proliferative vitreoretinopathy (PVR)

Schematic diagram of PVR process. After injury to retina, RPE cells lose their epithelial characteristics, start proliferation, and migrate into vitreous cavity. These RPE-derived cells start expression of myo-fibroblastic markers and transit into myo-fibroblastic state. Finally, the RPE-derived myo-fibroblastic cells attach to the NR and cause serious visual disorders and vision loss.

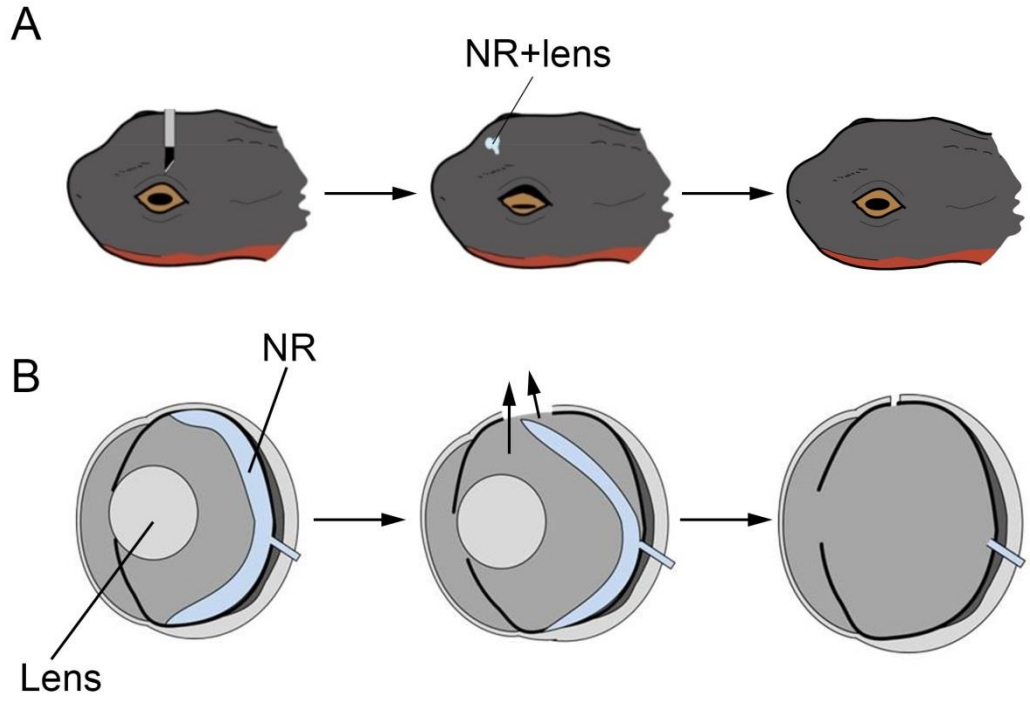


Figure 4. *In vivo* retinectomy

Schematic diagram of a newt head (A) and an eyeball (B). Firstly, small slit is made at the dorsal sclera of the eyeball by a fine blade. Then, the slit is extended by micro scissors. From the space, the NR together with the lens is removed carefully by gentle stream of normal newt saline. Finally, the eyeball is placed back its original position. The operated animals are kept in moist containers until experiments are performed.

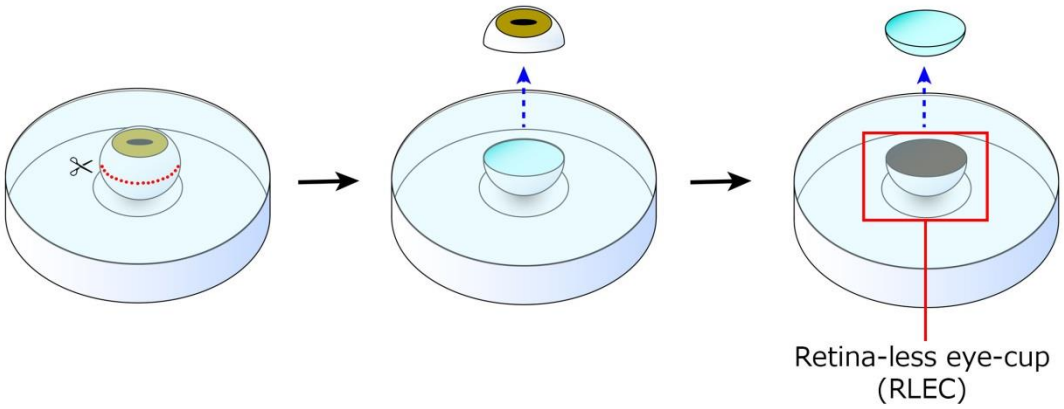


Figure 5. *In vitro* retinectomy

Schematic diagram of *in vitro* retinectomy. Firstly, the eyeballs are enucleated from sacrificed animals. The eyeball is cut open along equator in buffer solution and the anterior half is removed. From the posterior half, the NR is carefully removed to make retina-less eye-cup (RLEC). The RLECs are incubated culture medium until experiments are performed.

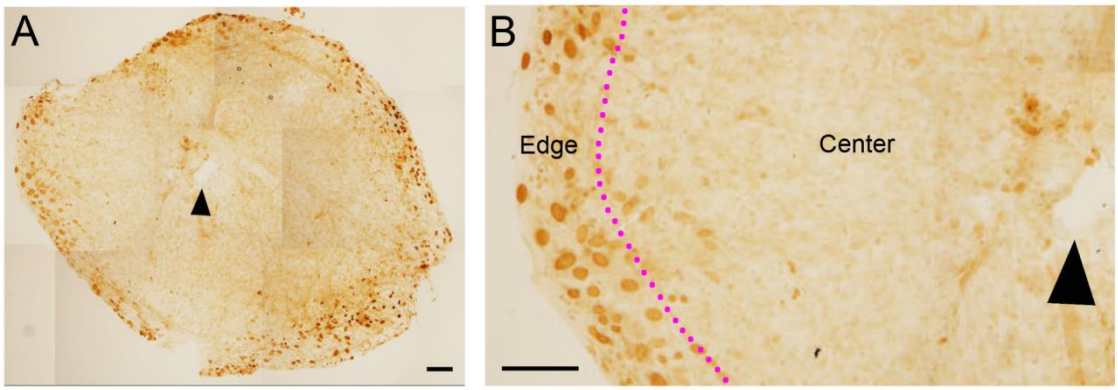


Figure 6. The distribution pattern of cell cycle re-entry of RPE cells after 10 days incubation *in vitro*

(A) Representative picture of the RPE sheet after 10 days incubation *in vitro*. Brown staining indicates BrdU+ RPE cell nuclear. BrdU+ RPE cell nuclei are mainly distributed at the incised margin of the RPE sheet. (B) Magnification image of (A). The area within 100 μm from the margin of the sheet is defined as 'Edge' and other area as 'Center'. Scale = 100 μm. Arrowhead: The hole where the optic nerve existed. Adopted from Yasumuro et al., Biomedicines. 2017 May 20;5(2). pii: E25.

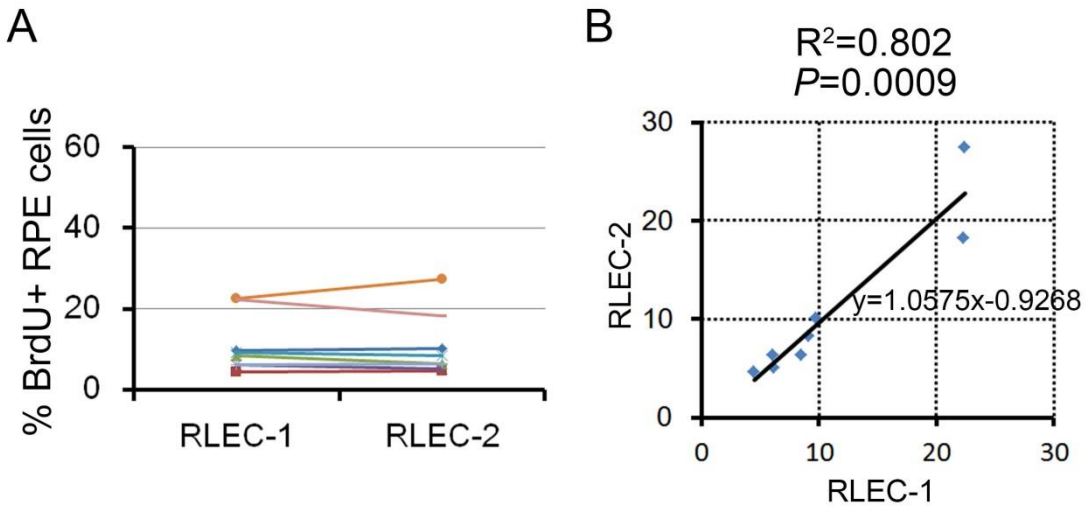
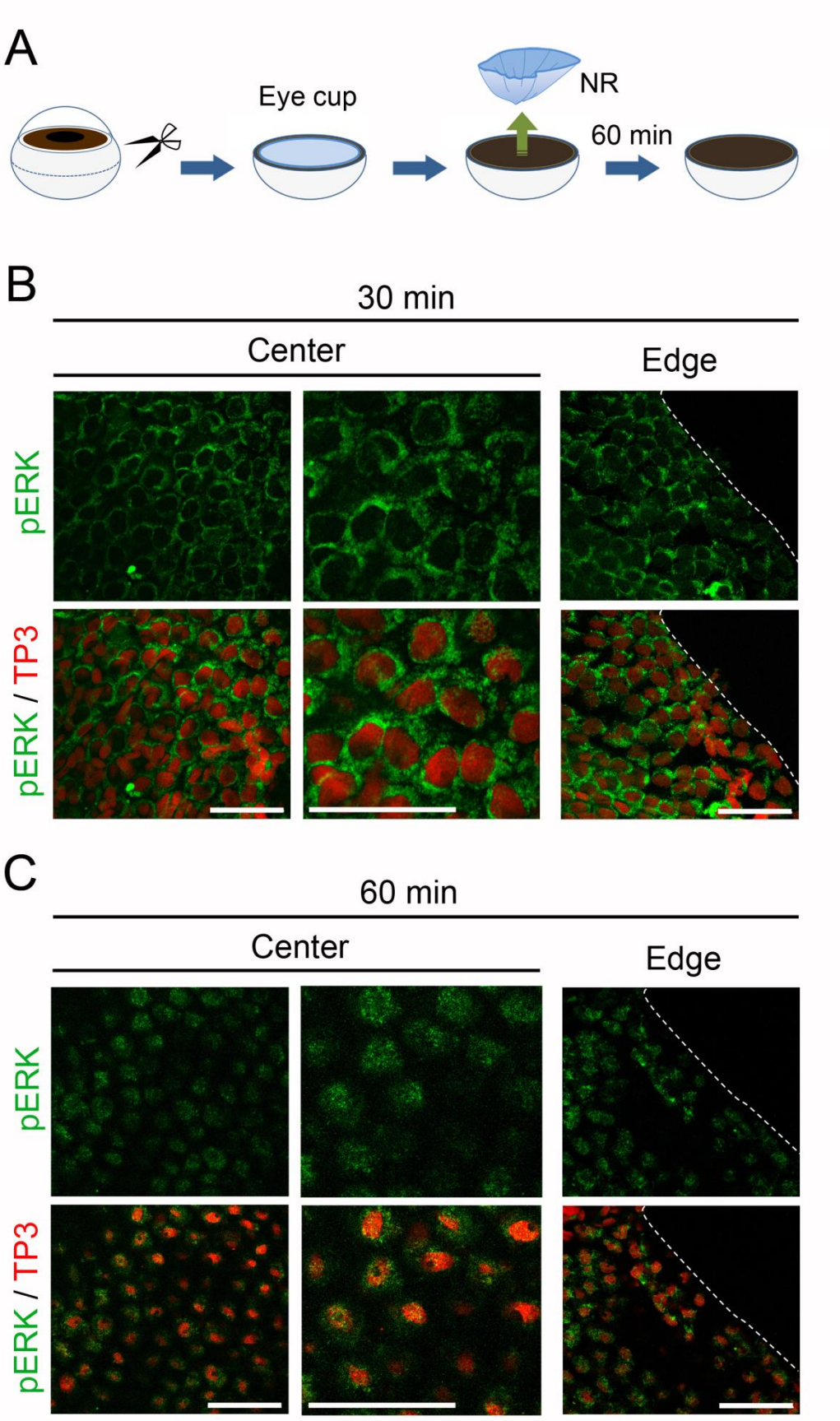


Figure 7. Correlation of the cell cycle re-entry ratio between the eyeballs from same animals

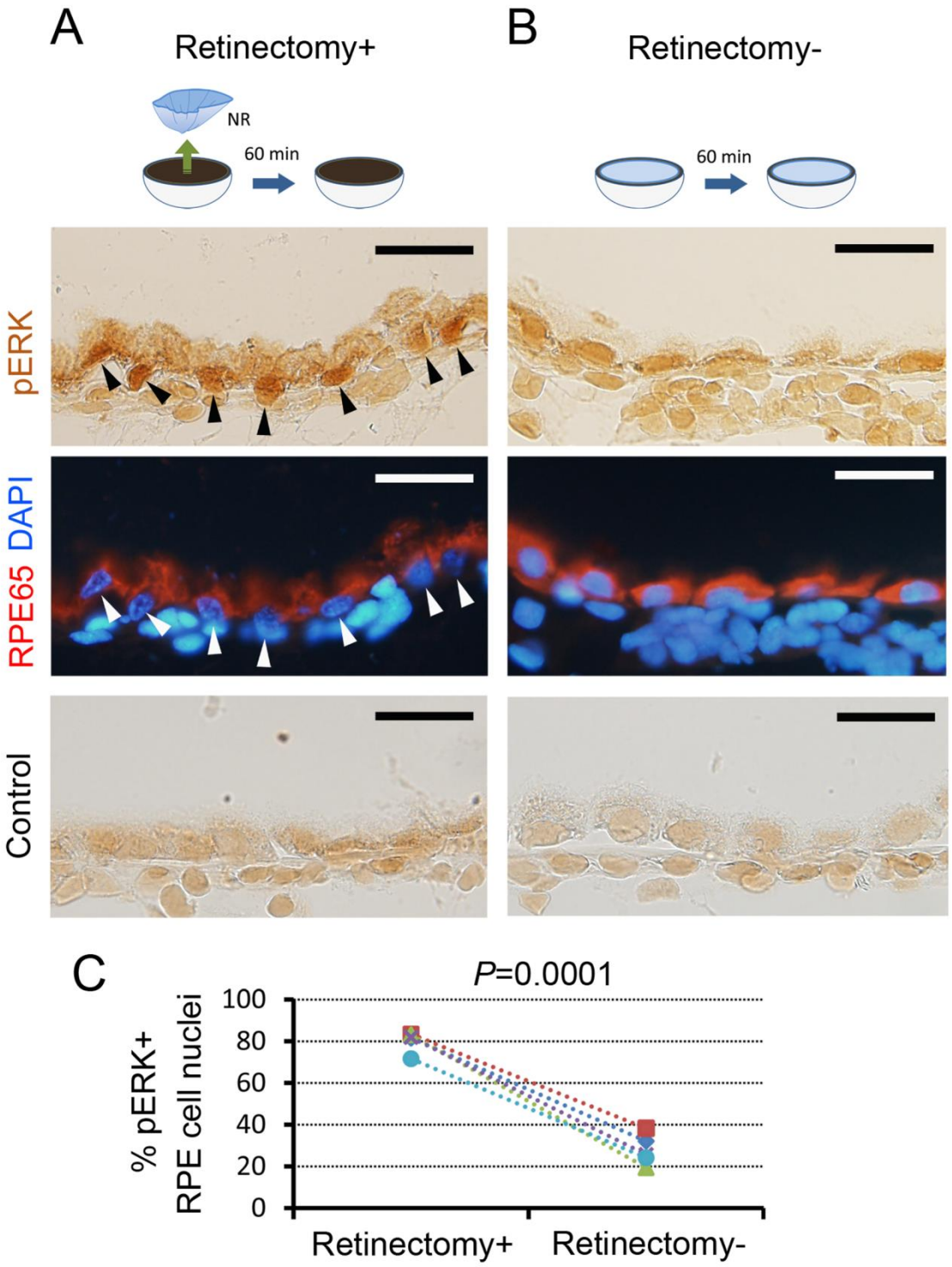
(A) Comparison of the cell cycle re-entry ratio between the RLECs obtained from same animal (n = 8). The samples obtained from same animal are connected by lines. The connected samples show almost same values. (B) Plot of the cell cycle re-entry ratio. Between two RLECs from same animal, significant correlation was present (Spearman's rank correlation coefficient, $P = 0.0009$, $R^2 = 0.802$).


A
Eye cup
NR
60 min
B
30 min
Center
Edge
pERK
pERK / TP3
C
60 min
Center
Edge
pERK
pERK / TP3

(Continued)

Figure 8. Whole mount staining of pERK in RELCs

(A) Schematic showing experimental paradigm. RLECs were incubated in culture medium for 30 or 60 min. (B,C) Nuclear translocation of pERK in RPE cells after retinectomy *in vitro*. The Center and the Edge in the RPE sheet are shown. These are representative images (n = 3 each). Right-hand panels in the Center show magnified images of corresponding left-hand panels. TP3: nuclear stain by TO-PRO®-3 iodide. pERK immunoreactivity (green), which was observed in the cytoplasm of most RPE cells at 30 min after retinectomy (B), became distributed to the nucleus (red) in the following 30 min (C). Note that in these confocal microscopic images (optical slices), RPE cell nuclei at 60 min after retinectomy seemed to be smaller than those at 30 min, because the shape of the RPE cell nuclei, which was as flat as in intact cells at 30 min after retinectomy, changed into a spheroid within 60 min. Such change of pERK immunoreactivity was observed simultaneously and uniformly throughout the RPE sheet. Scale = 100 μm. Adopted from Yasumuro et al., 2017.


A
Retinectomy+
NR
60 min
B
Retinectomy-
60 min
pERK
RPE65 DAPI
Control
C
P=0.0001
% pERK+ RPE cell nuclei
100
80
60
40
20
0
Retinectomy+
Retinectomy-

(Continued)

Figure 9. Immunohistochemistry of pERK with/without removal of NR

(A,B) Representative showing the effect of NR removal on ERK activity in RPE cells (5 newts). Arrowheads indicate pERK+ nuclei. Note that, for immunohistochemistry, the eye-cups after 60 min incubation (retinectomy-) were fixed after removal of the NR. RPE cells were identified by RPE65 immunoreactivity (red). DAPI (blue): nuclei. To control immunoreactivity (lowest panels), pERK antibody was replaced with control IgG. Scale = 50 μm. (C) Proportion of pERK+ RPE cell nuclei in the Center. In retinectomy+, ~80% of RPE cell nuclei showed pERK+, whereas, in retinectomy-, the number of pERK+ RPE cell nuclei was decreased significantly to less than 40% (Student's *t*-test, P = 0.0001, 5 newts). Adopted from Yasumuro et al., 2017.

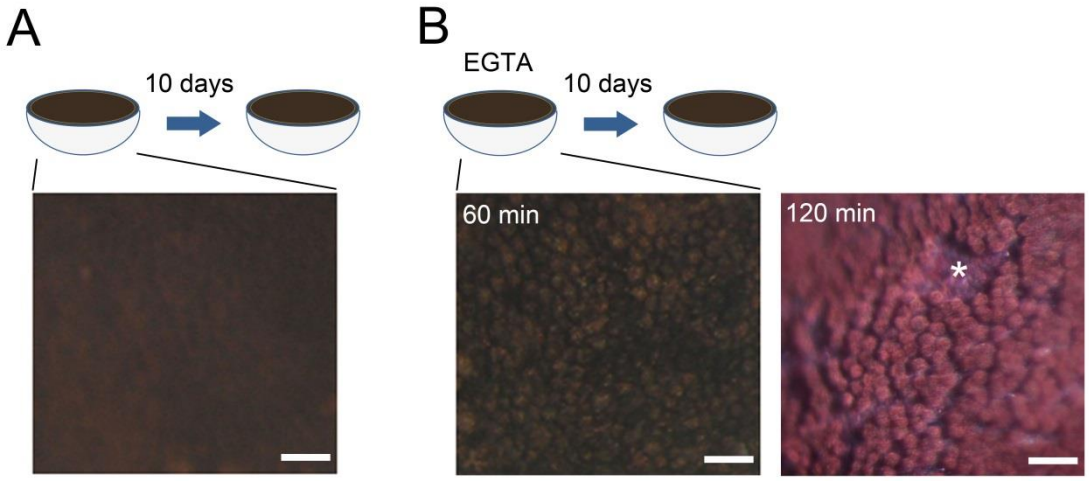
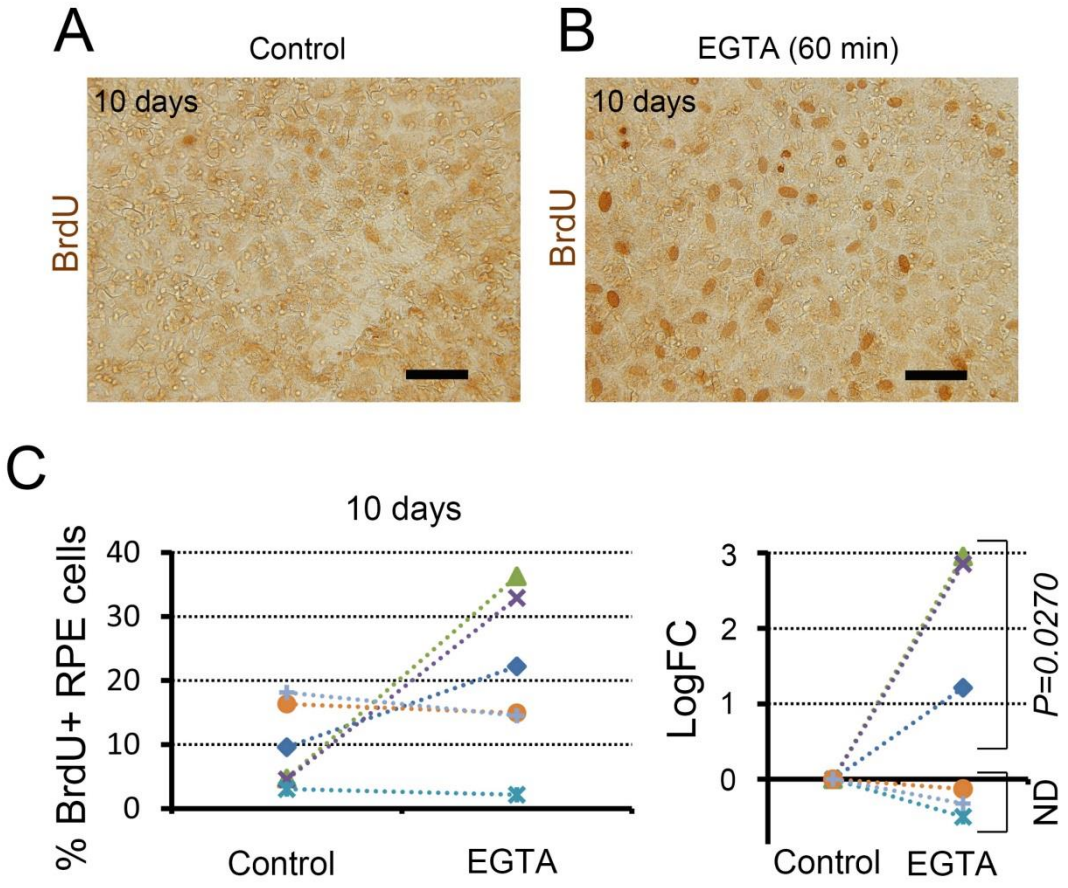


Figure 10. Condition setting for EGTA treatment

After removal of the NR from eye-cups, resulting RLECs were incubated in either normal newt saline or 10 mM EGTA solution. (A,B) Representative images of the RPE after incubation in normal saline and EGTA solution, respectively (6 newts). (B) In EGTA treatment for 60 min (left image), cell-cell attachment in the RPE decreased, allowing us to view the shape of the cells under a dissecting microscope. The right-hand image shows an example of the RPE after longer incubation in EGTA solution. In this condition (120 min), the space between neighboring cells became more obvious but the cells were sometimes dissociated from Bruch's membrane (asterisk). Scale = 100 μm. Modified from Yasumuro et al., 2017.


A
Control
10 days
BrdU
B
EGTA (60 min)
10 days
BrdU
C
10 days
% BrdU+ RPE cells
40
30
20
10
0
Control
EGTA
LogFC
3
2
1
0
P=0.0270
ND
Control
EGTA

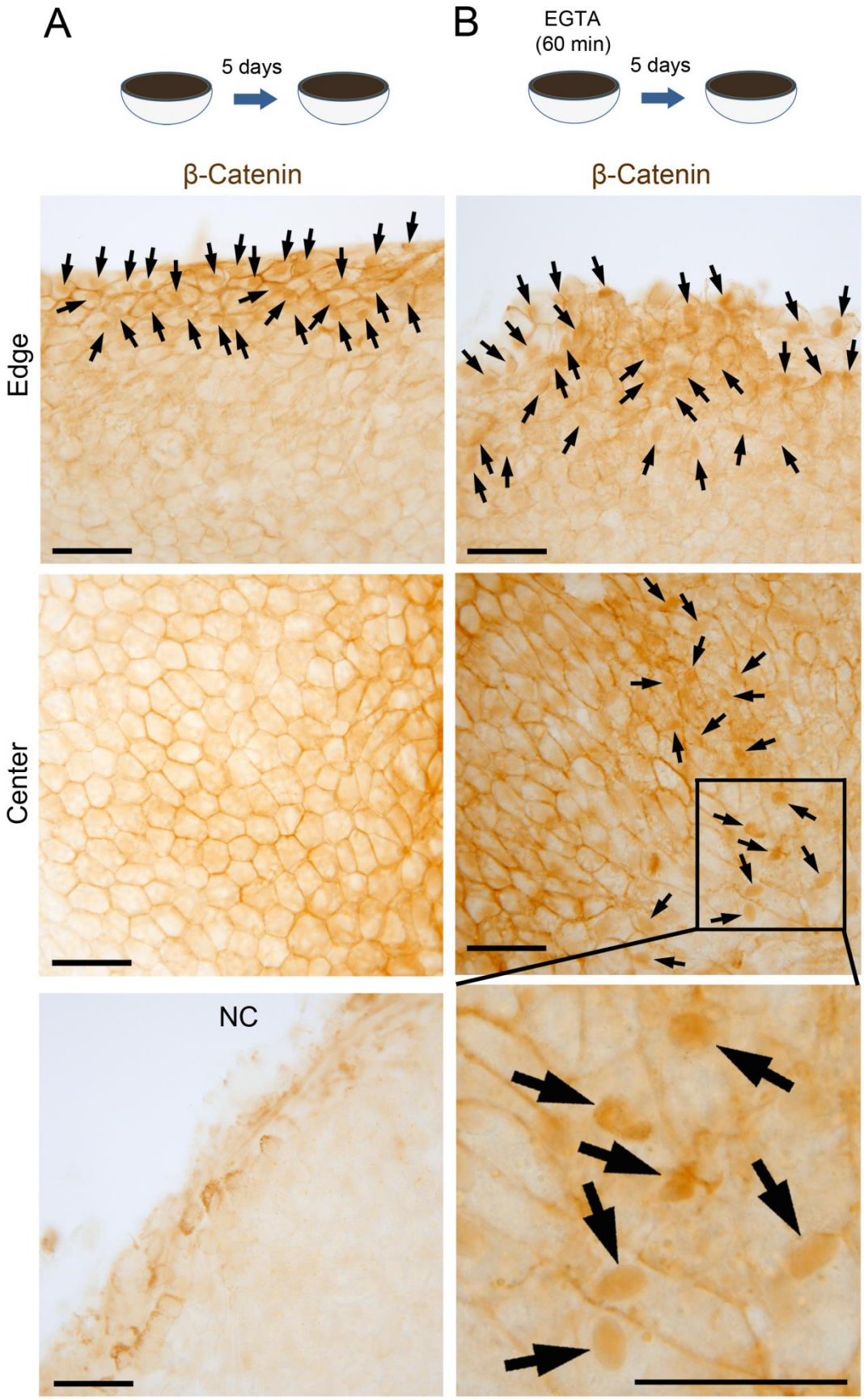
(Continued)

Figure 11. The effect of cell-cell contact attenuation on cell cycle re-entry of RPE cells

After EGTA treatment, RLECs were incubated in BrdU-containing NSCM for 10 days. (A,B) Sample images showing BrdU immunoreactivity in the RPE at 10 days after 60 min incubation in normal saline (control) and EGTA solution respectively. In this case, as indicated by BrdU-labeled nuclei (brown), a large number of RPE cells re-entered the S-phase of the cell cycle in EGTA treatment (B). The proportion of BrdU+ cells is shown in (C) as a purple X symbol. Scale = 100 μm. (C) Differences in the ratio of RPE cells which had re-entered the cell cycle in 10 days between normal saline (control) and EGTA treatment for 60 min. I counted nuclei labeled with BrdU in the RPE on day 10 and calculated the proportion of BrdU+ cells in the Center (left-hand gragh). The right-hand graph shows the relative change after EGTA treatment, and was plotted as $\log_2$ (fold change). Symbols linked by a dotted line show the data from the eyes of the same animal. I examined a total of 6 newts. In three of them, the values increased significantly after EGTA treatment (Student's *t*-test, $P = 0.0270$), although the other three did not show significant changes (ND). On average, the value was about three times (3.3 ± 1.4 times, n = 6) higher in EGTA treatment.

(Continued)

Note that RPE cells did not exhibit mitotic figures in 10 days as previously reported either *in vitro* (Yoshikawa et al., 2012) or *in vivo* (Islam et al., 2014). Modified from Yasumuro et al., 2017.


A
5 days
B
EGTA
(60 min)
5 days
β-Catenin
β-Catenin
Edge
Center
NC

(Continued)

Figure 12. The effect of cell-cell contact attenuation on nuclear translocation of β-catenin

(A,B) Representative images showing β-catenin immunoreactivity in the RPE (bleached) on day 5 (5 newts). In the Edge, many nuclei showed intense immunoreactivity (arrows) in both conditions. In the Center, in the control condition; (A) intense immunoreactivity was localized along the cell membrane which was in contact with neighboring cells, while in the EGTA treatment; (B) it was observed in many nuclei (arrows) as well as along the cell membrane in the area where RPE cells changed their hexagonal shape to a rhombus or fusiform shape. NC: representative staining with control IgG as the primary antibody (n = 3). Note that weak nonspecific staining was observed in intercellular substances along the incised margin. Scale = 100 μm. Adopted from Yasumuro et al., 2017.

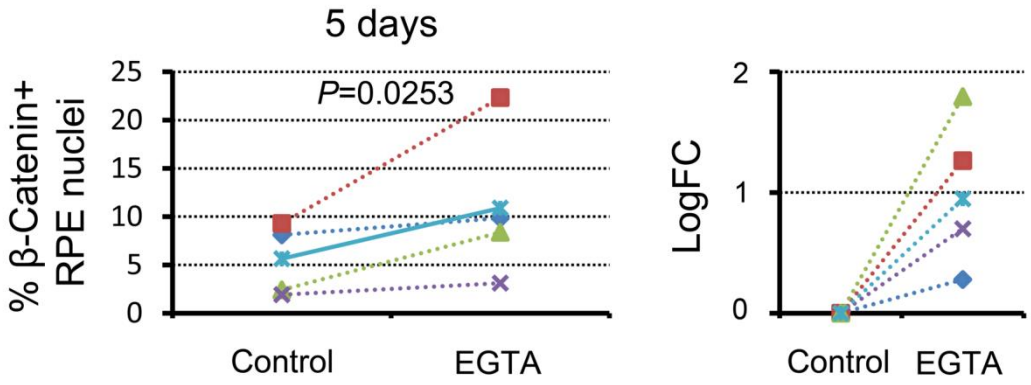


Figure 13. Proportion of β-catenin nuclear translocation under the cell-cell contact attenuation condition

Differences in the ratio of β-catenin+ nuclei in the Center of the RPE on day 5 between normal saline (control) and EGTA treatment. The right-hand graph shows the relative changes after EGTA treatment, and was plotted in $\log_2$ (fold change). The value increased significantly (Sheffe's pairwise comparison test following the Friedman test, $P = 0.0253$) after EGTA treatment. Modified from Yasumuro et al., 2017.

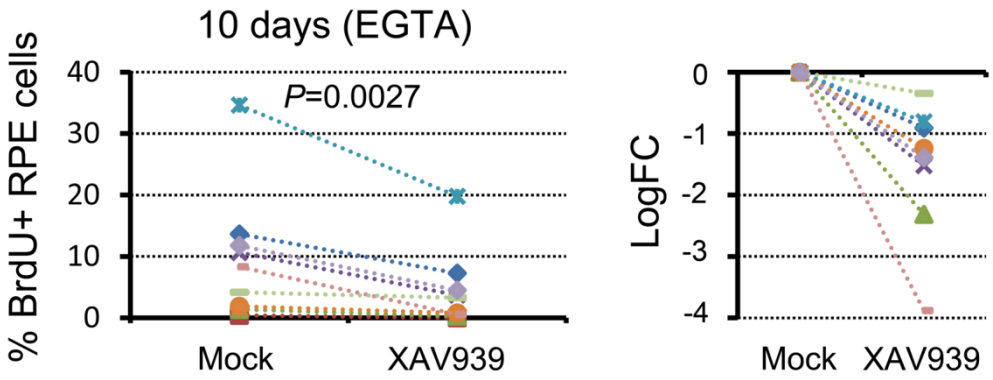


Figure 14. Effect of ß-catenin signal inhibitor, XAV939, on cell cycle re-entry of RPE cells

Effect of a ß-catenin signaling inhibitor, XAV939, on cell cycle re-entry of RPE cells in the condition of EGTA treatment (9 newts). The left-hand graph shows the proportion of BrdU+ cells in the Center of the RPE on day 10, and right-hand graph shows the relative changes in the presence of XAV939. The value decreased significantly (Sheffe's pairwise comparison test following the Friedman test, P = 0.0027) in the presence of XAV939. Modified from Yasumuro et al., 2017.

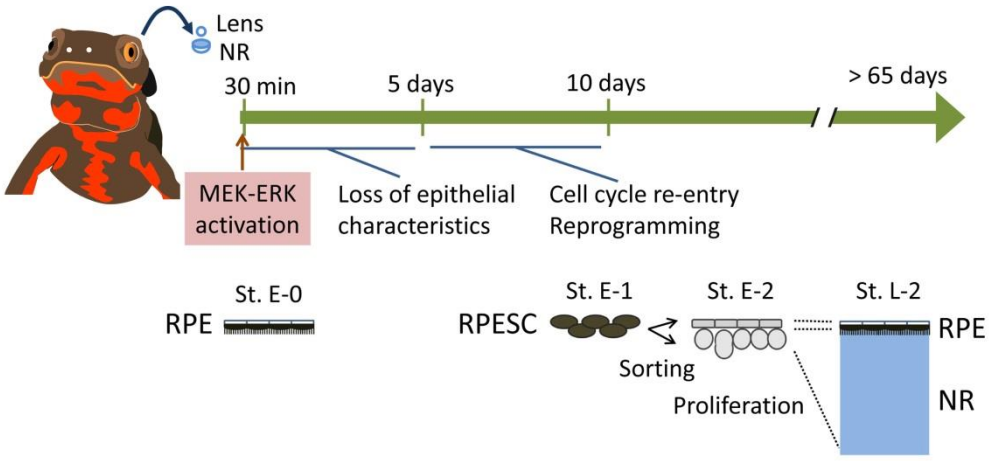
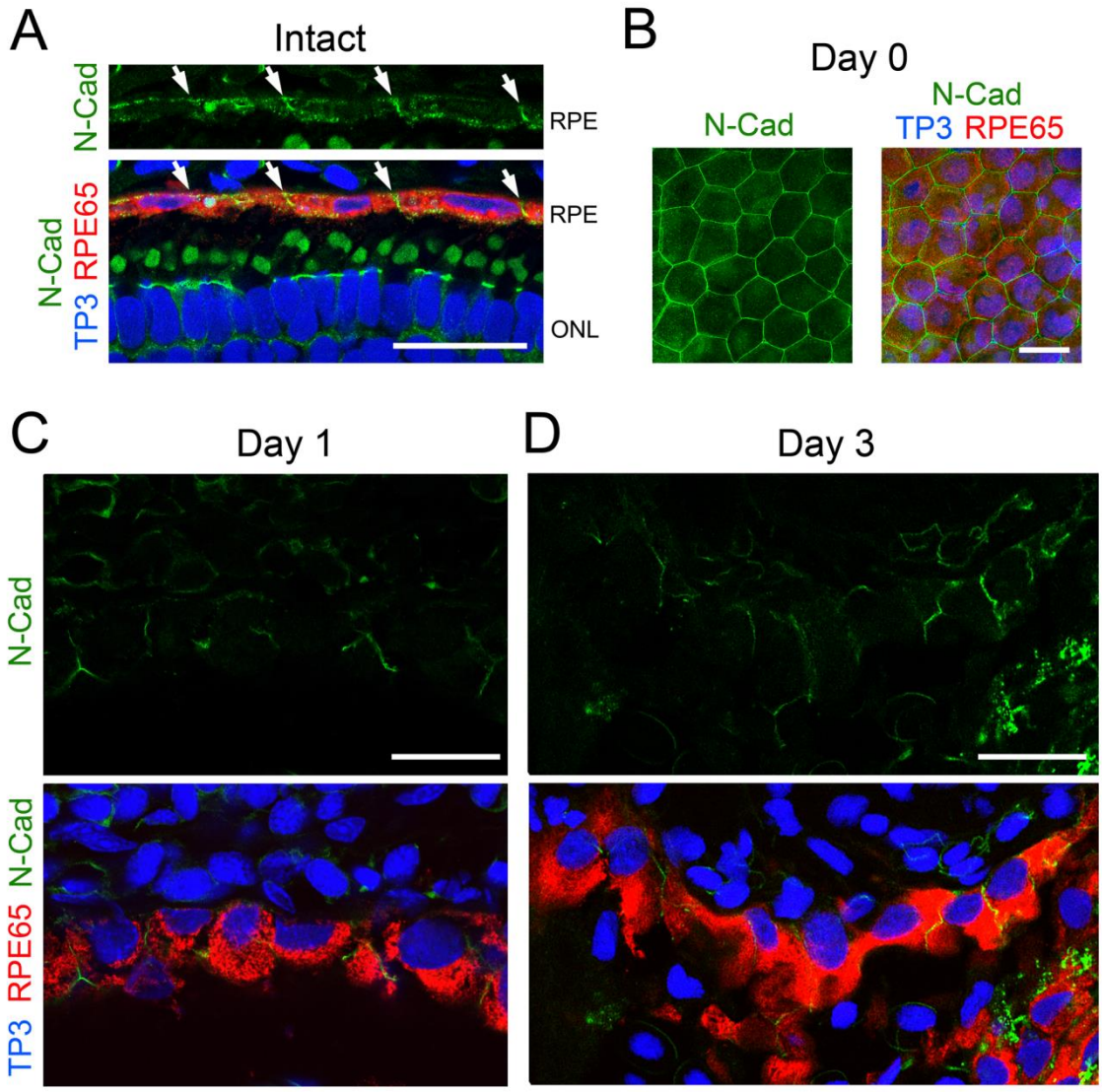


Figure 15. Events during the newt retinal regeneration

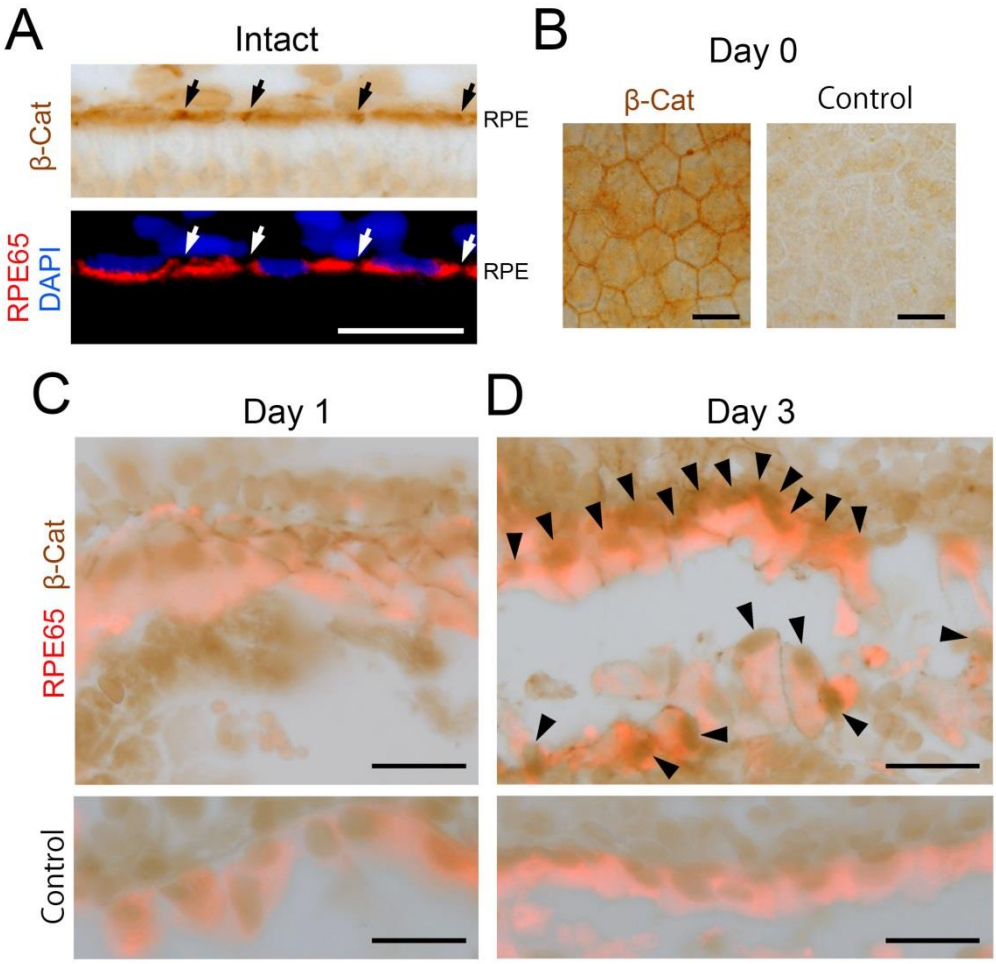
After retinectomy, within 30 min, MEK-ERK signaling reinforcement takes place. Then, until around 5 days, RPE cells lose their epithelial morphology. Between 5 to 10 days after retinectomy, RPE cells re-enter the S-phase of the cell cycle and acquire multipotency (RPESC). The RPECs are separated into two layers. From these layers, new NR and RPE itself are generated. Modified from Yasumuro et al., 2017.


A
Intact
N-Cad
RPE
N-Cad
TP3 RPE65
RPE
ONL
B
Day 0
N-Cad
N-Cad
TP3 RPE65
C
Day 1
D
Day 3
N-Cad
TP3 RPE65 N-Cad

(Continued)

Figure 16. Immunohistochemistry of N-cadherin in the early phase of retinal regeneration

(A) Representative N-cadherin immunoreactivity along the RPE layer in the intact eye (n = 3). Intense immunoreactivity was observed in the region of cell-cell contact in the RPE (arrows). Lower panel: merge of triple stain. RPE65 (red): RPE cells. TO-PRO®-3 (TP3; blue): nuclei. ONL: outer nuclear layer; (B) Representative N-cadherin immunoreactivity in the RPE sheet of the eye immediately after retinectomy (Day 0) (n = 3). Intense immunoreactivity was observed along the cell membrane which was in contact with neighboring cells. Right-hand panel: merge of the triple stain; (C) Representative N-cadherin immunoreactivity in RPE cells at 1 day after retinectomy (n = 5). Lower panel: merge of the triple stain. At this stage, RPE cells still lined along Bruch's membrane. N-cadherin immunoreactivity was recognized in the region of cell-cell contact; (D) Representative N-cadherin immunoreactivity in RPE cells/RPE-derived mesenchymal-like cells at 3 days after retinectomy (n = 5). Lower panel: merge of the triple stain. At this stage, cell-cell attachment in the RPE became loose but most of the cells still lay on Bruch's membrane. In those cells, N-cadherin immunoreactivity was recognized along the cell membrane but in most cells the signal was low. Scale = 50 μm. Adopted from Yasumuro et al., 2017.


A
Intact
β-Cat
RPE
RPE65
DAPI
RPE
B
Day 0
β-Cat
Control
C
Day 1
D
Day 3
RPE65 β-Cat
Control

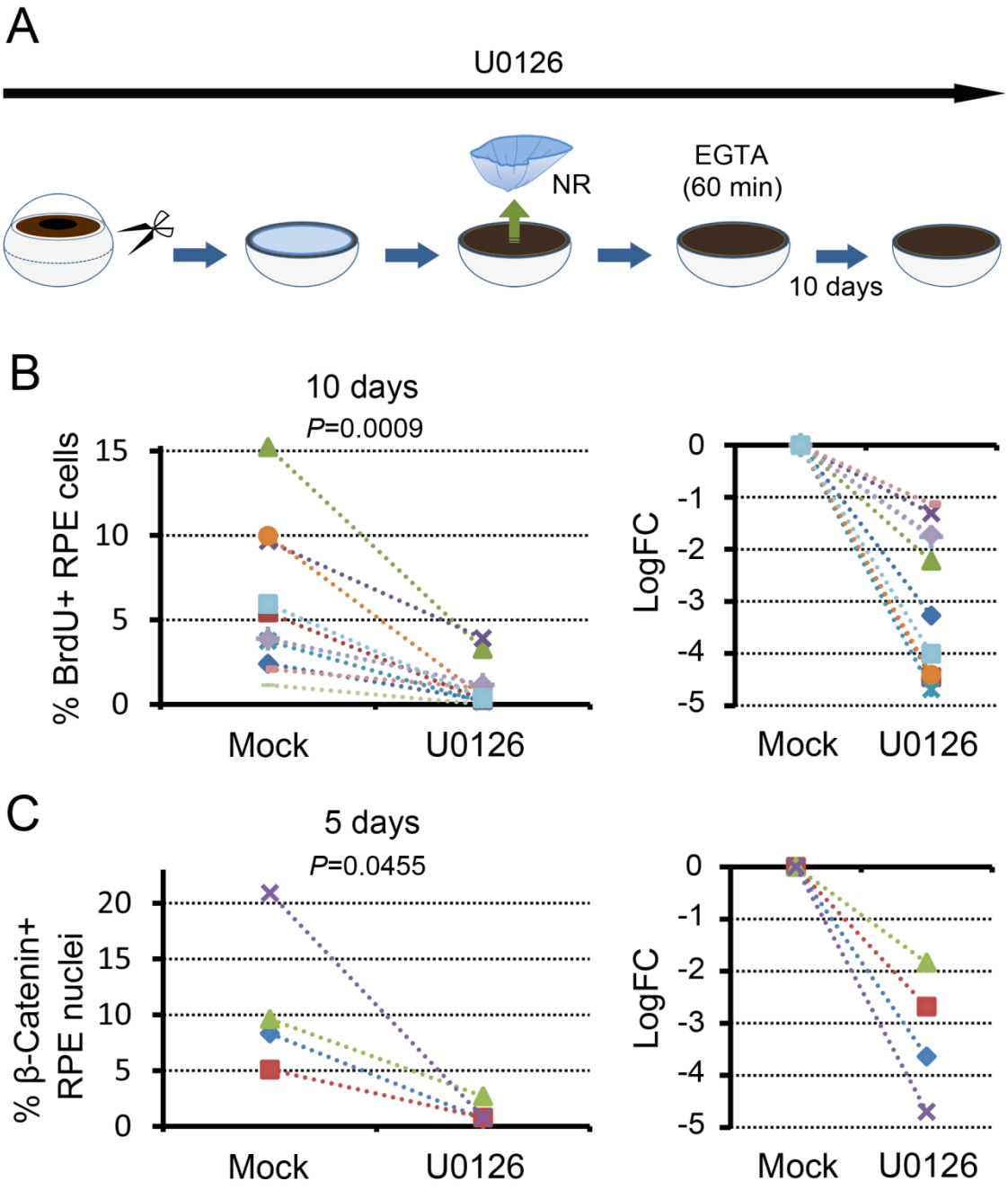
(Continued)

Figure 17. Immunohistochemistry of β-catenin in the early phase of retinal regeneration

(A) Representative β-catenin immunoreactivity along the RPE layer in the intact eye (n = 3). The tissue was bleached. Intense immunoreactivity was observed in the region of cell-cell contact in the RPE (black and white arrows). Lower panel: RPE65 immunoreactivity merged with DAPI nuclear stain in the same region; (B) Representative β-catenin immunoreactivity in the RPE sheet of the eye immediately after retinectomy (Day 0) (n = 3). Intense immunoreactivity was observed along the cell membrane which was in contact with neighboring cells. Right-hand panel: representative staining with control IgG as the primary antibody (n = 3); (C) Representative β-catenin immunoreactivity in the RPE cells at 1 day after retinectomy (n = 3). The tissue was double stained with RPE65 antibody and bleached. At this stage, immunoreactivity was not detected in the nuclei of RPE cells; (D) Representative β-catenin immunoreactivity in RPE cells/RPE-derived mesenchymal-like cells at 3 days after retinectomy (n = 3). Intense immunoreactivity was observed in the nuclei of RPE cells/RPE-derived mesenchymal-like cells (arrowheads), suggesting the activation of β-catenin signaling in this stage. Lower panels in (C) and (D):

(Continued)

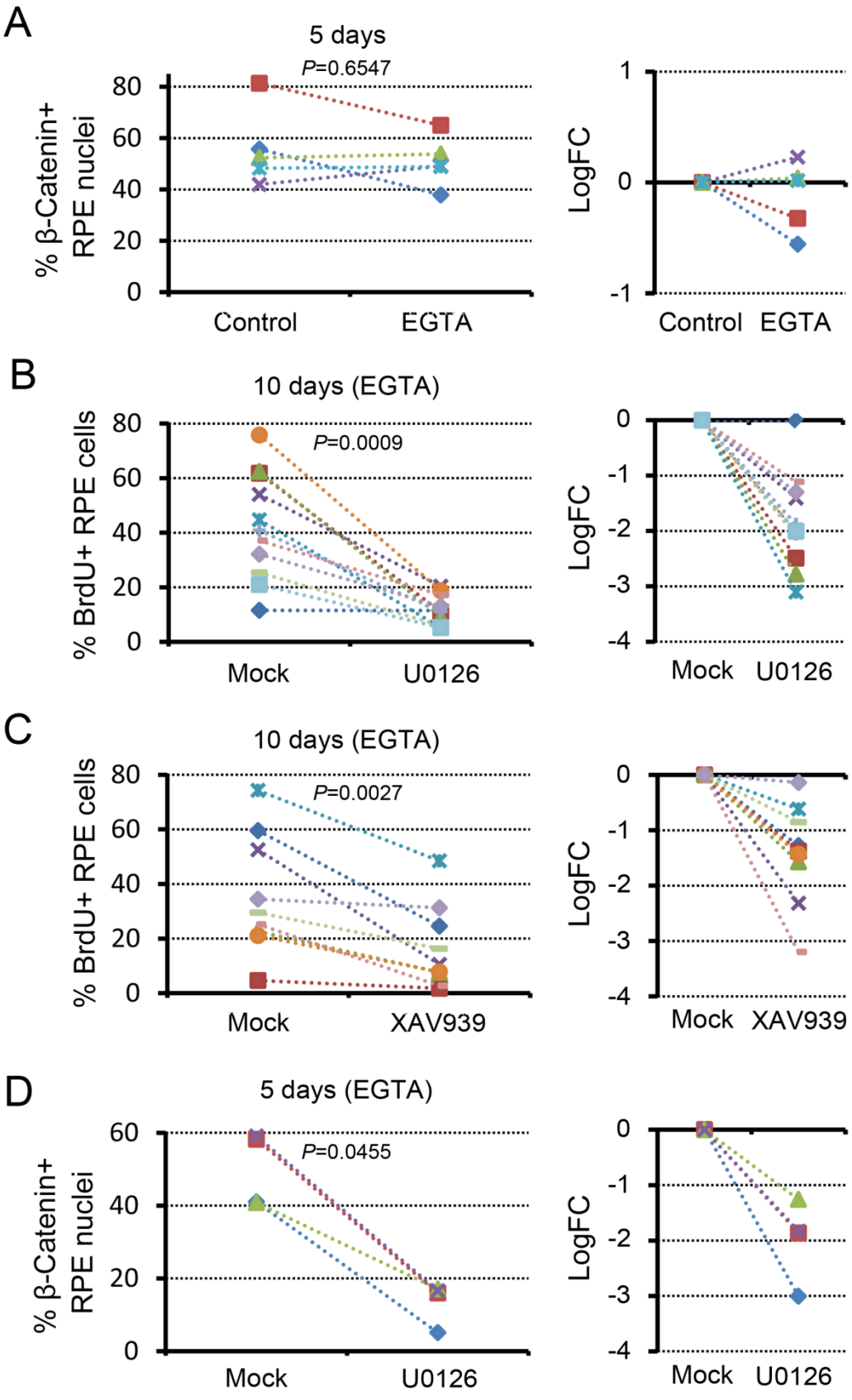
representative staining of the same stage of tissue using control IgG instead of ß-catenin antibody (n = 3 each). Scale = 50 μm. Modified from Yasumuro et al., 2017.


A
U0126
NR
EGTA
(60 min)
10 days
B
10 days
P=0.0009
% BrdU+ RPE cells
15
10
5
0
Mock
U0126
LogFC
0
-1
-2
-3
-4
-5
Mock
U0126
C
5 days
P=0.0455
% β-Catenin+
RPE nuclei
20
15
10
5
0
Mock
U0126
LogFC
0
-1
-2
-3
-4
-5
Mock
U0126

(Continued)

Figure 18. The effects of MEK inhibitor, U0126, on cell cycle re-entry of RPE cells and nuclear translocation of β-catenin

(A) Schematic showing an experimental paradigm. U0126 was administrated to medium from the time point when the eyeballs were cut open to make eye-cup. Under presence of U0126, RLECs were treated by EGTA solution and incubated; (B) Effect of U0126 on cell cycle re-entry of RPE cells that was promoted by EGTA treatment (11 newts). The left-hand graph shows the proportion of BrdU+ cells in the Center of the RPE on day 10, and the right-hand graph shows the relative changes in the presence of U0126. The value was significantly lower (Sheffer's pairwise comparison test following the Friedman test, $P = 0.0009$) in the presence of U0126; (C) Effect of U0126 on nuclear translocation of β-catenin in RPE cells that was promoted by EGTA treatment (4 newts). The left-hand graph shows the proportion of β-catenin+ nuclei in the Center of the RPE on day 5, and the right-hand graph shows the relative changes in the presence of U0126. The value decreased significantly (Sheffer's pairwise comparison test following the Friedman test, $P = 0.0455$) in the presence of U0126. Adopted from Yasumuro et al., 2017.


A
5 days
P=0.6547
% β-Catenin+ RPE nuclei
80
60
40
20
0
Control
EGTA
LogFC
1
0
-1
Control
EGTA
B
10 days (EGTA)
P=0.0009
% BrdU+ RPE cells
80
60
40
20
0
Mock
U0126
LogFC
0
-1
-2
-3
-4
Mock
U0126
C
10 days (EGTA)
P=0.0027
% BrdU+ RPE cells
80
60
40
20
0
Mock
XAV939
LogFC
0
-1
-2
-3
-4
Mock
XAV939
D
5 days (EGTA)
P=0.0455
% β-Catenin+ RPE nuclei
60
40
20
0
Mock
U0126
LogFC
0
-1
-2
-3
-4
Mock
U0126

(Continued)

Figure 19. Results obtained from the Edge area of the RPE in RLECs

(A) Effect of EGTA treatment on nuclear translocation of ß-catenin in the RPE cells. The data were obtained from same samples (5 newts) used in Figure 4A; (B) Effect of a MEK inhibitor U0126 on cell cycle re-entry of RPE cells that was promoted by EGTA treatment. The data were obtained from the same samples (11 newts) used in Figure 7B; (C) Effect of a ß-catenin signaling inhibitor XAV939 on cell cycle re-entry of RPE cells that was promoted by EGTA treatment. The data obtained from the same samples (9 newts) used in Figure 4B; (D) Effect of U0126 on nuclear translocation of ß-catenin in RPE cells that was promoted by EGTA treatment. The data were obtained from the same samples (4 newts) used in Figure 7C. Data are presented in the same manner as in corresponding data obtained from the Center. Statistical analysis was performed by Sheffe's pairwise comparison test following the Friedman test. Adopted from Yasumuro et al., 2017.

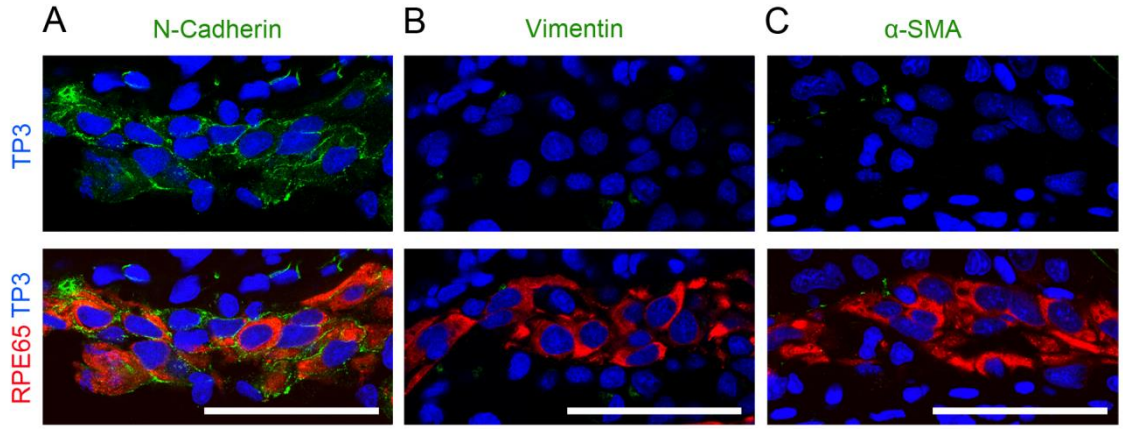
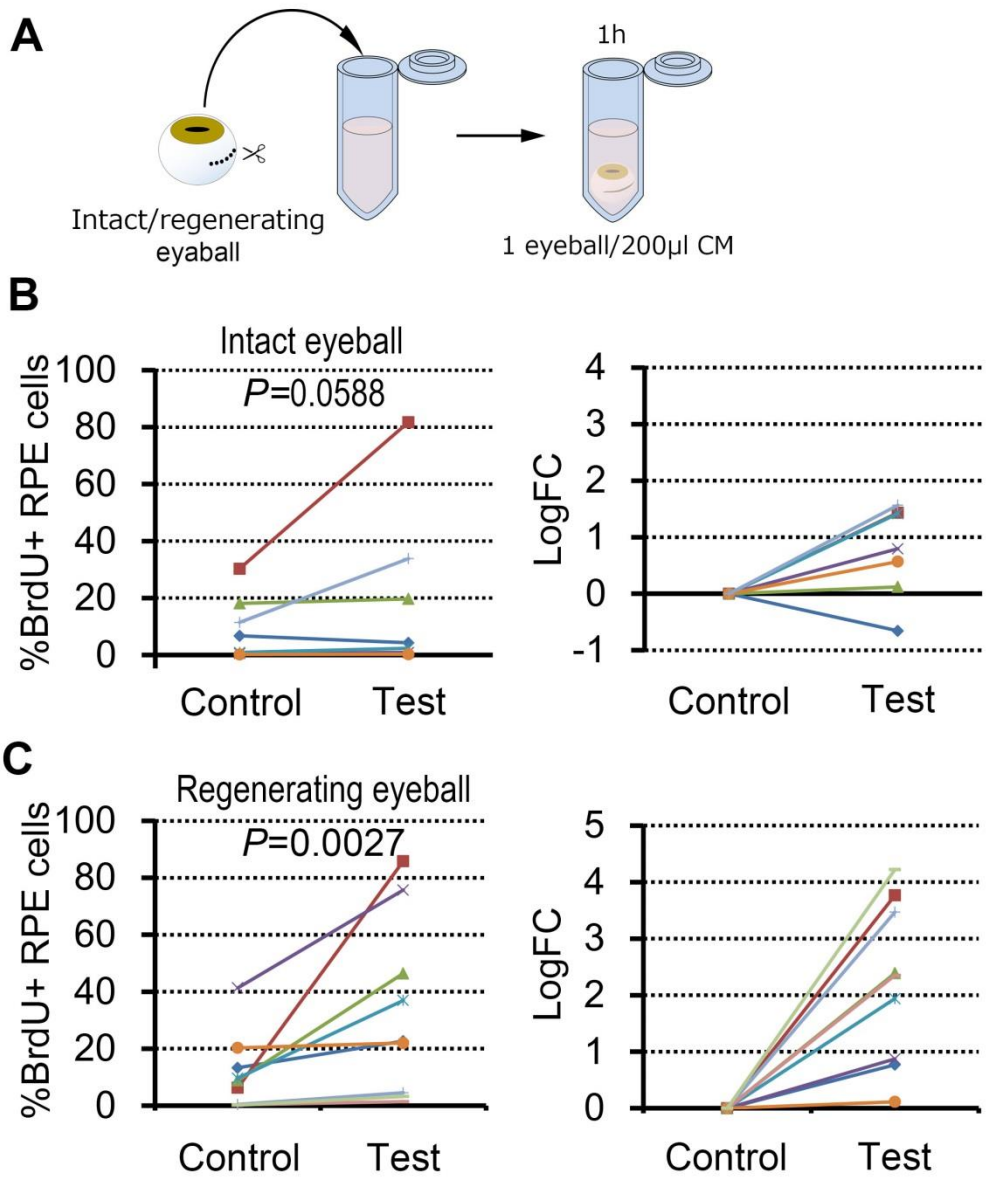


Figure 20. Immunolabeling of mesenchymal markers in RPE-derived mesenchymal-like cells at 10 days after retinectomy in the adult newt.

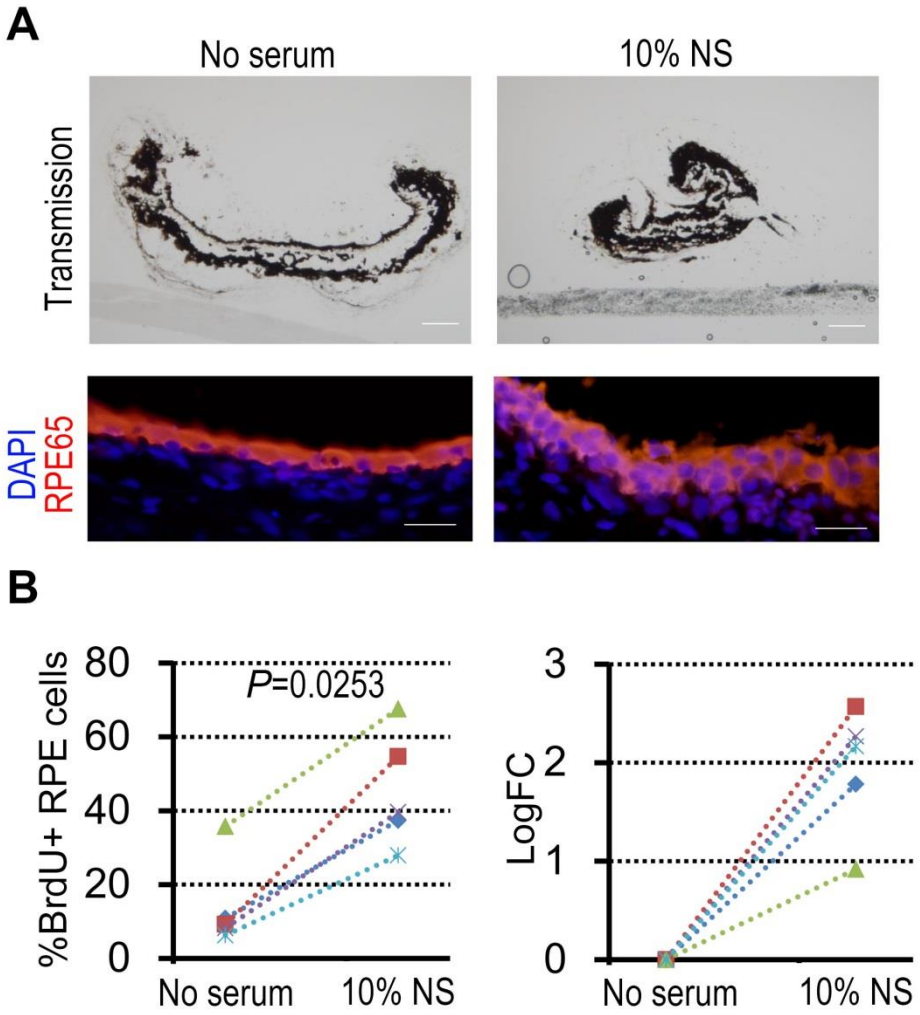
(A) Representative N-cadherin immunoreactivity (n = 3); (B) Representative vimentin immunoreactivity (n = 3); (C) Representative α-SMA immunoreactivity (n = 3). N-cadherin immunoreactivity was observed along the cell membrane of the RPE-derived cells which had formed aggregates in the vitreous cavity, whereas immunoreactivity to other markers were not detected in the RPE-derived cells. TP3 (blue): nuclei. RPE65 (red): RPE-derived cells. Scale = 100 μm. Adopted from Yasumuro et al., 2017.


A
1h
Intact/regenerating
eyaball
1 eyeball/200µl CM
B
Intact eyeball
P=0.0588
%BrdU+ RPE cells
100
80
60
40
20
0
Control
Test
LogFC
4
3
2
1
0
-1
Control
Test
C
Regenerating eyeball
P=0.0027
%BrdU+ RPE cells
100
80
60
40
20
0
Control
Test
LogFC
5
4
3
2
1
0
Control
Test

(Continued)

Figure 21. Effects of Fluid in the Vitreous cavity from intact/regenerating eyeballs on the cell cycle re-entry

(A) Schematic showing an experimental paradigm. Intact or regenerating eyeballs are made a cut (almost half of circumference) along equator and incubated in culture medium (CM) for 1 h. Then, the eyeballs were removed and the conditioned medium was used to incubate RLECs. (B,C) Proportion of cell cycle re-entry of RPE cells after 10 days incubation. (B) Conditioned medium with intact eyeballs. (C) Conditioned medium with regenerating eyeballs. In the former case, although the cell cycle re-entry ratio was increased in total (1.9 times higher than control on average), there was no significant difference (P= 0.0588, n = 7). In contrast, in the latter case, the cell cycle re-entry ratio significantly and effectively increased (5.5 times higher than control on average, P= 0.0027, n = 9).


A
No serum
10% NS
Transmission
DAPI
RPE65
B
%BrdU+ RPE cells
80
60
40
20
0
P=0.0253
No serum
10% NS
LogFC
3
2
1
0
No serum
10% NS

(Continued)

Figure 22. Effect of newt serum on the RPE cell morphology and cell cycle re-entry of RPE cells

(A) Representative sections of RLECs after 10 days incubation with/without newt serum (NS). In lower panels, the RPE cells and the nuclei are visualized by red (RPE65) and blue (DAPI) florescence respectively. Without serum, RPE cells kept their morphology during incubation periods. In contrast, with 10% NS, RLECs were shrunk might because RPE cells were tend to make aggregate-like structure. Additionally, RPE cells were enlarged and, in some population, multilayer-like structure seemed to be formed. Scale = 100 μm (upper panels) and 50 μm (lower panels). (B) Graphs of the cell cycle re-entry ratio (5 newts). By administration of 10% NS, cell cycle re-entry of RPE cells was significantly increased (range: 27.9-67.6%; 45.4 ± 7.0%, P = 0.0253) than without serum condition (range: 6.2-35.8%; 14.1 ± 5.5%).

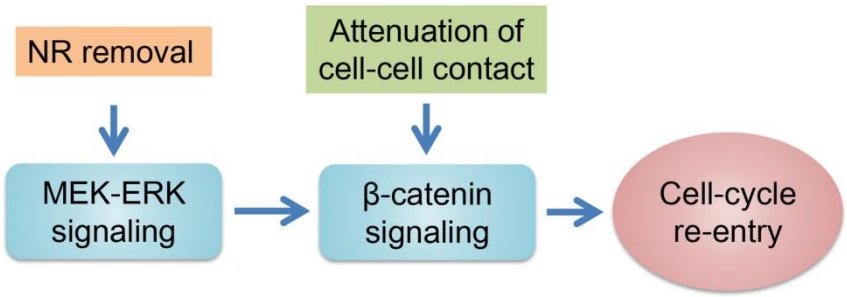


Figure 23. The model of cell cycle re-entry of RPE cells

After NR removal, MEK-ERK signaling is reinforced within 30-60 min. Under this condition, by attenuation of cell-cell contact of RPE cells, β-catenin signaling is activated. And then, β-catenin signaling initiates cell cycle re-entry of RPE cells.

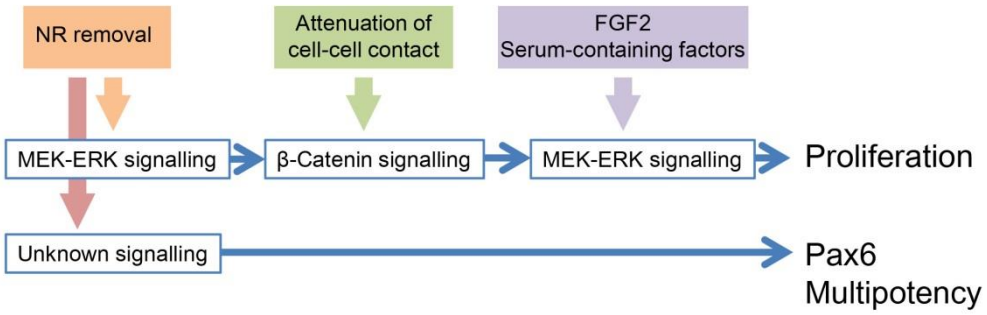


Figure 24. Multi-step trigger model of newt retinal regeneration

A combination of MEK-ERK signaling, which is stimulated by NR removal, and ß-catenin signaling, which is stimulated by attenuation of cell-cell contact, allows RPE cells to re-enter the S-phase of the cell cycle. And then, by FGF2 and serum containing factors, RPE cells proceeded into the M-phase of the cell cycle. In contrast, for expression of Pax6 and acquisition of multipotency, independent pathways are expected. Modified from Yasumuro et al., 2017.