Studies on the Expression of Pax6 during Reprogramming of Adult Newt Retinal Pigment Epithelium Cells

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Abbreviations

CL: cornea limbus

CMZ: ciliary marginal zone

DIV: days in vitro

EMT: epithelial-mesenchymal transition

HD: homeo-domain

HTH: helix-turn-helix

NR: neural retina

PD: paired-domain

PST: proline, serine and threonine

PVR: *proliferative vitreoretinopathy*

RLEC: retina-less eye-cup

RPE: retina pigment epithelium

RPESCs: RPE stem cells

Abstract

Adult newt retinal pigment epithelium (RPE) cells are reprogrammed into a unique state of multipotent cells at an early phase of retinal regeneration. Here, to understand the signal triggering of reprogramming, four classes (v1, v2, v3 and v4) of Pax6 variants in the eyes of adult newt were identified and their expression in RPE cells after retinectomy was investigated. The Pax6 *v1* and *v2* variants were newly expressed in RPE cells by 10 days after retinectomy, both *in vivo* and *in vitro*. *In vitro* examinations suggested that Pax6 expression is mediated through a pathway separate from the MEK-ERK pathway, which is required for cell cycle re-entry of RPE cells, but in a condition closer to the *in vivo* state, it was promoted by the activity of MEK. These findings predict the existence of a pathway that must be further pursued to understand the reprogramming of RPE cells during retinal regeneration.

1. Introduction

1.1. The RPE cells

Retina pigment epithelium (RPE) is a simple epithelium constituting the eyeballs of vertebrates. RPE surrounds the back of the neural retina (NR), which receives light. This tissue adheres to the basement membrane, which is located external to it, and the choroid membrane, whose blood vessels are distributed external to it. RPE tissue is comprised of one kind of single-layered epithelium cells named RPE cells. These cells, which accumulate melanin and have microvilli, play essential roles in early visual processing as a partner of the NR. Mature RPE cells do not divide and specialize morphologically and functionally (for reviews, see Fuhrmann et al., 2014; Strauss, 2005).

However, when the NR suffers from a traumatic injury, RPE cells lose their epithelial characteristics and undergo proliferation and transformation. As an example, there is *proliferative vitreoretinopathy* (PVR), which is a human disease. When RPE cells are exposed to serum, causing the detachment of retina and colloidal disorder, they can be isolated from the basement membrane and lose their epithelial structure. Moreover, these cells migrate into the vitreous through tears in the neural retina. Then, they form an epiretinal membrane on the surface of the NR, leading to the loss of vision. This process is a kind of epithelial-mesenchymal transition (EMT), a phenomenon in

which the epithelial cells detach from the underlying basement membrane and tranform into mesenchymal cells which involves enhanced migratory capacity and the production of extracellular matrix components (Chiba, 2014).

1.2. Newt RPE cells contribute to neural regeneration

On the other hand, in the newt, a group of the family Salamandridae in urodele amphibians, a similar change in RPE cells enables them to regenerate an entire retina, even as adults (Chiba et al., 2006a; Chiba and Mitashov, 2007; Chiba, 2014). In the adult newt, when the NR is completely removed from the eye by a surgical operation 'retinectomy' (Figure 1A,B), RPE cells are detached from each other as well as from the basement membrane, losing their epithelial characteristics to become cell aggregates, while reaching the S-phase of the cell-cycle (Figure 1C). Ten days after an operation, when these changes are observed, is defined as Stage 'E-1'. These RPE-derived cells are then sorted into two populations which form the prospective-NR and -RPE layers (pro-NR and pro-RPE layers, respectively) with correct polarity (Figure 1D). Cells in the pro-NR and pro-RPE layers start to proliferate and eventually regenerate new functional NR and RPE (Figure 1E). Therefore, newt retinal regeneration serves as a good model system to compare with RPE-mediated retinal disorders in humans, providing insight into medical treatments that would allow for *in vivo* retinal regeneration (Chiba, 2014).

1.3. The reprogramming of RPE cells

What changes occur to newt RPE cells that have lost their epithelium form? Recently, our laboratory has isolated RPE cells before retinectomy and at Stage E-1, and compared their gene expression profiles. This study demonstrated that RPE cells at Stage E-1 express three pluripotency factors, i.e., *c-Myc*, *Klf4* and *Sox2*, as well as factors involved in fate switching between the NR and RPE in embryonic/larval stages, namely *Pax6* and *Mitf* (Islam et al., 2014). Since these genes have not been confirmed as intact RPE cells, it is thought that they express newly in the process of regeneration. On the other hand, RPE cells at Stage E-1 preserve certain original characteristics such as the presence of microvilli and the expression of RPE65, which is an RPE cell-specific marker (Islam et al., 2014). Adult newt RPE cells are then reprogrammed into a unique multipotent state which has the potential to create neural retinas and is observed only in the regeneration process. The cells of this state are referred to as RPE stem cells (RPESCs). However, it remains uncertain what signals trigger the reprogramming of RPE cells as well as cell cycle re-entry.

1.4. Pax6 is expressed in RPESCs

In this study, this subject was attempted to approach by focusing on the expression of transcription factor Pax6. Pax6 is a well-known master control gene of eye morphogenesis and a highly conserved

transcription factor. It is a protein that is essential to the normal development of the retina from *Drosophila melanogaster* to humans (Baumer et al., 2002). In vertebrate development, Pax6 exists over a wide area, including the retina and RPE progenitor cells, at the early optic cup stage. During cell differentiation, Pax6 expression is limited and finally disappears in the mature RPE and neural retina except for amacrine, horizontal and ganglion cells (Davis-Silberman et al., 2005; Hsieh et al., 2009; Macdonald et al., 1997).

In the study of newt regeneration, it has been suggested that Pax6 is expressed in the eyeball of Stage E-1. Furthermore, the *Pax6* sequence was elucidated when genetic information about the newt was poor and Pax6 was the rare protein that an antibody equivalent was available. Expression of Pax6 in reprogramed cells was also confirmed in a recent study using isolated RPE cells. It is thought that *Pax6* mRNA expresses in all RPESCs of Stage E-1, and after two cell layers have arisen from RPESCs, pro-NR cells gradually change in Pax6-positive cells, as confirmed by immunohistochemistry (Islam et al., 2014). Therefore, Pax6 is a useful marker to trace the process of reprogramming in RPE cells.

1.5. Pax6 structure and variety

In studies involving Pax6, it is necessary to consider the function of multiple transcription products. Pax6 variants are derived from a difference in the transcription initiation site on the same locus, and

consist of many isoforms generated via post-transcriptional regulation such as alternative splicing. Several Pax6 variants with different structures have been reported (Bandah et al., 2007; Gorlov and Saunders, 2002; Kammandel et al., 1999; Kim and Lauderdale, 2006; Shaham et al., 2012; Short and Holland, 2008).

The first variant, named “canonical Pax6”, is the most universal and abundant of the Pax6 variants (Figure 2). This variant includes two DNA-binding paired-domain (PD) and homeo-domain (HD) in the N-terminal region. These two domains are separated by glycine-rich linker. The C-terminal region of Pax6 is enriched with proline, serine and threonine (PST) residues (Shaham et al., 2012). The PD consists of two subdomains named PAI and RED. PAI and the RED subdomain have an HTH motif each (Xu et al., 1995; Xu et al., 1999; Epstein et al., 1994a). The C-terminal region of Pax6 is enriched with PSTs and functions as a transcriptional transactivator (Czerny and Busslinger, 1995; Tang et al., 1998). These structures are widely conserved from flies to humans (Xu et al., 1995; Xu et al., 1999).

Secondly, in vertebrates, there is a Pax6(5a) variant that is made by post-transcriptional alternative splicing that inserts an additional exon 5a into canonical Pax6 (Figure 2; Walther and Gruss, 1991). Since the insertion site is the HTH motif in PAI, PAI loses its DNA binding capacity while that of RED is maintained (Epstein et al., 1994b). As a result, the DNA-recognition sequence of Pax6(5a) is different from canonical Pax6 (Epstein et al., 1994b). Different proportions of canonical Pax6 and

Pax6(5a) express in each tissue, and changes in their ratio cause pathological conditions and abrogated development (Epstein et al., 1994b; Pinson et al., 2005; Zhang et al., 2001).

The third Pax6 variant, which lacks the PD and is termed paired-less, is made by a promoter unlike canonical Pax6 and Pax6(5a) in vertebrates (Figure 2). The overexpression of this variant disturbs the development of the cornea and lens, and causes the microphthalmic phenotype (Kim and Lauderdale, 2006, 2008). However, its physiological activity in the eye is still unknown (Shaham et al., 2012).

In addition, many Pax6 variants have been reported. For example, in the PST region of amphibian and mouse Pax6, it has been reported that a part equivalent to exon 12 of human Pax6 is spliced out and subsequently shifts the sequence (Mizuno et al., 1997). A functional analysis of the majority of these variants has not been performed.

1.6. The newt Pax6

In the newt, two kinds of Pax6 splicing, namely the addition of a PD and the deletion of part of the PST, were discovered. Four kinds of variants (LL, LS, SL and SS) form from different combinations of this splicing. SL and LL are homologs of canonical Pax6 and Pax6(5a), respectively. SS and LS are variants in which the exon in the PST splices out from SL and LL (Mizuno et al., 1997). However, the paired-less variant has not been found and knowledge about Pax6 variants of the newt

is limited. In addition, there are few reports related to the expression and function of known Pax6 variants. Our previous study demonstrated that canonical Pax6 is newly expressed in RPESCs. However, it remains unknown which other variants are expressed in those cells.

Therefore, in the first part of this study, utilizing a transcriptome database IMORI (http://antler.is.utsunomiya-u.ac.jp/imori/), which our laboratory established to study newt retinal regeneration (Nakamura et al., 2014), Pax6 variants expressed in the adult newt eye were attempted to thoroughly identify. Then, in the second part of this study, on the basis of this information, the *Pax6* classes that are expressed in RPE cells upon retinectomy were explored. Finally, the pathways that triggered Pax6 expression were investigated.

1.7. The RLEC system

In newt retinal regeneration, it is challenging to study the molecular mechanism underlying the reprogramming of RPE cells through injection/implantation of chemical tools into the eye because i) the wound along the dorsal half of the eye, from which the NR is removed, has not closed until ~2 weeks in which reprogramming has been completed; ii) since RPE cells become solitary upon retinectomy and are then distributed in the vitreous cavity, the microenvironment (or niche) surrounding RPE cells is susceptible to physical perturbations, resulting in the abnormal regeneration of retinas, mostly giving rise to rosette-shaped tissues, even when using normal saline

only; iii) more essentially, the wound/injury itself, which is inevitably made for retinectomy or chemical treatments, may turn-on reprogramming signals. In this study, candidate pathways were attempted to identify by using the retina-less eye-cup (RLEC) system, an *in vitro* system which our laboratory has introduced to study the initial response of RPE cells in association with the wounding of eyes and retinectomy (Yohikawa et al., 2012). In this system, almost the same operation of retinectomy as that made *in vivo* was carried out *in vitro*. This ensured that the posterior half of the eyeball is missing the NR (i.e., the RLEC), which is then incubated in a minimal essential medium. In this condition, RPE cells do not appear to have died or have been seriously altered for as long as 10 days. In fact, when RPE-choroid tissues isolated on day-10 are implanted into the vitreous cavity of the eye of a living animal immediately after retinectomy, retinal tissue is regenerated from the implanted RPE (also see Chiba et al., 2006b). However, it is unknown whether the RLEC system can be used as a tool to check the signal(s) causing the reprogramming of RPE cells. After the style of Pax6 expression in the RLEC system and RPESCs were compared, and confirmed whether *in vitro* Pax6 expression reflects real regeneration, it was necessary to examine the factor(s) participating in the reprogramming of RPE cells.

In a previous paper, focusing on the wound edge, our laboratory reported that RPE cells in the RLEC enter the cell cycle with almost the same time course as that observed *in vivo* (Yoshikawa et al., 2012). Together with a subsequent paper (Mizuno et al., 2012), our laboratory discovered that

cell cycle re-entry of RPE cells in the RLEC requires a MEK1/2-ERK1/2-mediated intracellular signaling pathway, whose activity rises transiently within 30 min after retinectomy both *in vivo* and *in vitro*. Consequently, our laboratory proposed this as the putative pathway for the cell cycle re-entry of RPE cells. In this study, based on previous studies, the signaling pathways triggering Pax6 expression with a focus on the relationship with cell cycle re-entry were investigated.

2. Materials and Methods

2.1 Identification of the Pax6 variant expressed in the eyeballs of the adult newt

2.1.1 Animals

Japanese fire belly newts, *Cynops pyrrhogaster*, were purchased from local suppliers (Ouchi Kazuo Seibutsu Kyozai, Saitama, Japan/Aqua Grace, Yokohama, Japan). The newt for this experiment was originally captured from Chiba or Okayama prefecture, Japan. The animals had been reared in containers into which water had been poured to a depth of 5 cm, and placed at 18°C under natural light. They were fed daily with frozen mosquito larvae (Akamushi; Kyorin, Himegi, Japan), and the containers were kept clean at all times. To select those newts which had completely matured into adults, individuals of sufficient size (total body-length: 9–11 cm) were used. Before surgical operation or sacrifice, newts were anesthetized for 2 h with FA100 (1:1000; 4-allyl-2-methoexyphenol; DS Pharma Animal Health, Osaka, Japan) in the dark. All experiments were carried out in accordance with the guidelines approved by the University of Tsukuba Animal Use and Care Committee.

2.1.2. Transcriptome data

To search unknown *Pax6* isoforms expressed in the eyes of the adult newt, a *de novo* assembled transcriptome database (IMORI, http://antler.is.utsunomiya-u.ac.jp/imori/) from the eyes at 0-14 days after removal of both the neural retina and the lens was used. This database was created in our laboratory using a next-generation sequencer (Nakamura et al., 2014). At that time, three algorithms, Trinity, Trans-ABySS and Velvet-Oases, were applied during database assembly (Nakamura et al., 2014). Three assembled database was integrated into one as a sequence database, and blastn (version v2.2.26+, NCBI) was carried out using a known *Pax6* (Accession #: D88741) as the query sequence. The contigs which connected to unknown *Pax6* sequences were selected from the blastn results. An ensuing blastn, which used only unknown regions of *Pax6* as the query sequence, was performed. Until a newly unknown sequence was not provided, the search was repeated. Finally, some candidates of new *Pax6* classes were derived to connect them.

2.1.3. Design of primer sets

The candidates of new *Pax6* classes expected *in silico* had unique sequences upstream of the PD-coding region, including 5′ UTR. To confirm the existence of new *Pax6* isoforms in the eyes of adult newts and to decide their sequence information, forward primers located in the unique sequences of the 5′ UTR that could distinguish the different classes of *Pax6*, were designed. Furthermore, to design reverse primers located in known and common sequences of the 3′ UTR, the

primer sets were configured so as to amplify the ORF.

2.1.4. Sample preparation from parts of the normal eyeball

After the eyeball was excised, it was opened up along the equator in RNase-free PBS. The operation took place in this solution by manipulating fine forceps and scissors under a dissecting microscope. The ciliary marginal zone (CMZ), iris, lens, cornea limbus (CL) and cornea were isolated from the anterior half of the eye (Figure 3). NR and RPE cells, together with the choroid tissues (RPE-choroid) and sclera, were isolated from the posterior half of the eye (Figure 3). Using a thin needle that can peel each tissue, sclera and NR were removed to reveal the RPE-choroid. It is difficult to remove RPE cells from the basal membrane mechanically. The separation of RPE cells from RPE-choroid tissue was performed in a solution containing protease and chelate. After as many blood cells in the choroid were removed as possible by shaking the samples in the dish, samples were transferred into elastase (1 mg/ml; Porcine pancreas; Ref: 11027891001; Roche Diagnostics Japan, Tokyo, Japan) in EDTA solution (in mM: 115 NaCl, 3.7 KCl, 10 EGTA, 18 D-glucose, 10 HEPES, and 0.001% phenol red, pH 7.5 adjusted with 0.3N NaOH) and incubated for 90 min at 28°C. Subsequently the tissue samples were rinsed with RNase-free PBS several times and then RPE cells were separated by a current which was created by pipetting the tissues with a 3.5 ml transfer pipette (Sarsted, D-51588 Nümbrecht, Germany). The suspension was transferred to 1%

agarose-coated 35-mm plastic dishes (08-772A, Thermo Fisher Scientific, Waltham, MA, USA). After verifying that RPE cells were not contaminated by other tissues, they were used as the RPE sample. The residual tissue, which was confirmed to have completely separated RPE cells, was used as the colloid sample.

2.1.5. Molecular cloning

Total RNA was extracted from whole/tissues/cells collected from 3-5 eyeballs using a total RNA isolation kit (5185-6000, Agilent Technologies, Santa Clara, CA, USA), and template cDNAs were conventionally constructed using SuperScript II Reverse Transcriptase (18064-014, Thermo Fisher Scientific). Although the commercial kit was basically used according to the manufacturer's instructions, the reaction time of reverse transcriptase was extended to 90 min. PCR was performed using KOD FX (KFX-101, Toyobo, Tokyo, Japan). The confirmed PCR amplification product was cloned. To prevent PCR errors, the number of cycles was minimized to 25. The PCR products were electrophoresed by agarose gel (Solana Agarose STANDARD 01, Rikaken, Nagoya, Japan) electrophoresis and the bands of expected size were cut out.

After purifying DNA using a Geneclean Spin Kit (111101200, MP Biomedicals, Santa Ana, CA, USA) and having added adenine to the blunt 3′ end with advantage 2 (639206, Takara Bio, Otsu, Japan), TA was cloned with the TOPO TA Cloning Kit (K4560-01, Thermo Fisher Scientific). DNA

sequencing was carried out using ABI Prism 3130 (Thermo Fisher Scientific). For each class of *Pax6*, the nucleotide sequence of the longest variant was deposited in DDBJ/GenBank (Accession #: *Pax6 v2*, LC002642; *Pax6 v3*, 28 LC002643; *Pax6 v4*, LC002644).

2.1.6. Antibodies

Chicken polyclonal anti-Pax6 antibody PA1-801 (1:1,000; Thermo Fisher Scientific) and rabbit polyclonal anti-Pax6 antibody PRB-278P (1:1,000; Covance, Princeton, NJ, USA) were used as the primary antibodies. PA1-801, which can recognize a sequence (REEKLRNQRRQASNTPSHI) neighboring the 3′ end of HD in the newt, was applied to immunoblotting. This epitope is present in all classes of Pax6 variants but this antibody was inadequate for immunohistochemistry. Therefore, PRB-278P was applied to immunohistochemistry. This antibody has high reactivity and recognizes the C-terminal (QVPGSEPDLSQYWPRIQ) of LL and SL forms of Pax6 and the –L form of paired-less Pax6. Biotinylated goat anti-chicken IgG antibody (1:500; BA-9010, Vector Laboratories, Burlingame, CA, USA) and anti-rabbit IgG antibody (1:500; BA-1000, Vector Laboratories) were used as the secondary antibodies. The negative controls were obtained by omitting the primary antibodies.

2.1.7. Immunohistochemistry

Normal eyeballs were fixed in 2% paraformaldehyde / 0.2% picric acid in PBS (pH 7.5) for 5-6 h at 4°C and cryosectioned at a thickness of ~20 μm after washing away the fixative solution. Tissue sections were rinsed thoroughly (PBS, 1% TritonX-100 in PBS, PBS, 15 min each rinse), treated with 3.3% H_2O_2 in methanol for 20 min, washed thoroughly, incubated in a blocking solution [3% normal goat serum (S-1000, Vector Laboratories) / 1% TritonX-100 in PBS] containing Avidin D (1:50; Avidin/Biotin Blocking kit, SP-2001, Vector Laboratories) for 2 h, washed in PBS twice, and then incubated in the primary antibody diluted with the blocking solution containing Biotin (1:50; Avidin/Biotin Blocking kit) for 72 h at 4°C. After washing thoroughly, the samples were incubated in the biotinylated secondary antibody diluted with the blocking solution for 4 h at room temperature, washed thoroughly, incubated in a mixture of Avidin and Biotin Complex (Vectastain ABC Elite kit, PK-6100, Vector Laboratories) for 2 h, washed thoroughly, and then incubated in a DAB solution (DAB substrate kit, SK-4100; Vector Laboratories) for 6 min. After the samples were washed, melanin pigments in the tissues were bleached, and the nuclei of RPE cells were stained with DAPI (1:50,000; D1306, Thermo Fisher Scientific).

Images of tissues were acquired using a CCD camera system [C4742-95 ORCA-ER system (Hamamatsu Photonics, Hamamatsu, Japan) or a DP73 system (Olympus, Tokyo, Japan)]. Figures were prepared using Photoshop Extended CS5 (Adobe, San Jose, CA, USA).

2.1.8. Discrimination of the classes of Pax6 variants in normal eyes

PCR was performed using KOD FX and the primer sets distinguished the new and known classes of *Pax6* against several cDNAs from each ocular part of the eye. The number of cycles was increased compare with the cloning step (PCR conditions, see Table 2). To evaluate the integrity of cDNAs, the expression of EF1-α (Accession #: AB005588, sense, 5′-gacctttgcccccagtaacgtaaccac-3′; antisense, 5′-actgggtgttgctggcgctacttcttg-3′; 573-bp) was confirmed. An MJ Mini Gradient Thermal Cycler (PTC-1148, Bio-Rad, Hercules, CA, USA) was routinely used. Exceptionally, to examine *Pax6 v2* transcripts, a Takara PCR Thermal Cycler 1 MP (TP3000, Takara, Otsu, Japan) was also used.

2.1.9. Immunoblotting

Sample tissues were prepared from five anterior halves of eyeballs after removing the lens and adding 75 µl of lysis buffer [25 mM Tris-HCl (pH = 7.5), 150 mM NaCl, 1 mM EDTA-2Na, 1% Igepal CA-630 (56741, Sigma-Aldrich, St. Louis, MO, USA), 1% protease inhibitor cocktail (P8340, Sigma-Aldrich), 1% sodium deoxycholate (190-08313, Wako Pure Chemicals Industries, Ltd., Osaka, Japan), and 0.1% SDS (191-07145, Wako)] chilled on ice. Samples in the sample tube were frozen in liquid nitrogen and sonicated in chilled water for 5 min. The frozen sample was gradually dissolved. After this step was repeated once more, the suspension was centrifuged at 7000 g for 10 min by a

cooled centrifuge at 4°C. 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)] and immediately heated for 5 min in a water bath at 98°C.

Protein samples (μl/lane) were separated on a 10% Mini-PROTEAN TGX Precast Gel (456-1033, Bio-Rad, Hercules, CA, USA) by SDS-PAGE and electrophoretically transferred onto an Immun-Blot PVDF membrane (1620174, Bio-Rad) using a Mini transblot cell (1703930JA, Bio-Rad).

The blots were incubated in blocking solution [5% non-fat skimmed milk (198-10605, Wako) in TBST [100 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween20]] containing Avidin D (1:50; Avidin/Biotin Blocking kit) for 1 h, rinsed with TBST twice, and then incubated overnight at 4°C in the primary antibody diluted with the staining system [Can Get Signal (NKB-101T, Toyobo)] containing Biotin (1:50; Avidin/Biotin Blocking kit). After washing three times in TBST for 15 min each wash, the blots were incubated in biotinylated secondary antibody diluted with the staining system [Can Get Signal] for 90 min at room temperature, washed three times in TBST, incubated in a mixture of Avidin and Biotin Complex (Vectastain ABC Elite kit) for 90 min, washed six times in TBST, and then incubated in a DAB solution (DAB substrate kit) until the bands were visualized. The negative control was obtained by omitting the primary antibody.

2.2. RPE cells newly express *Pax6 v1* and *v2* variants upon retinectomy

2.2.1 Retinectomy

To induce retinal regeneration, living newts were held in an operating chamber. The dorsal half of the left eye was cut open along the position slightly below the boundary between the cornea and sclera using a blade and fine scissors. While sterile saline solution (in mM: NaCl, 115; KCl, 3.7; $CaCl_2$, 3; $MgCl_2$, 1; D-glucose, 18; HEPES, 5; pH 7.5 adjusted with 0.3N NaOH) was infused into the vitreous chamber through the slit by an injection needle (27Gx3/40, NN-2719S, Terumo, Tokyo, Japan) which was connected to a syringe (1 ml, SS-01T, Terumo) via a filter cassette (0.20 mm pore size, Cellulose acetate, DISMIC-25CS, ADVANTEC, Japan), both the neural retina and lens were carefully removed by same needle and forceps. After operation, the slit was carefully placed back to its original position. The retinectomized animals were maintained in a moist container at 22°C (the day-night cycle was 12 h:12 h). The container was kept clean and the animals were not fed to control the speed of regeneration.

2.2.2. Discrimination of the classes of *Pax6* variants in RPESCs

When almost all RPE cells reached Stage E-1 at 10 days after the operation (Chiba 2006; Islam et al., 2014), the eyeball was extracted. In the same timing as normal eyeballs, five tissues were

collected. These were treated more carefully because the connection between the regenerating cells and the basal membrane was weak. The eyeball was opened up along the equator, and the posterior side of the wound created during retinectomy, as well as the part which not affected by it, were collected. The existence of RPESCs was confirmed inside each hemisphere and separated, together with the colloid, from the sclera. Using the RPESCs with a colloid (RPESCs-colloid), template cDNAs were constructed using the same procedure described in 2.1.5.

Exceptionally, template cDNAs were prepared from isolated RPESCs as follows.

Cells on the basal membrane were isolated by elastase in EDTA solution as described in section 2.1.4. The isolated cell suspension was transferred to 1% agarose-coated 35-mm plastic dishes placed on ice.

To avoid contamination of the basement membrane and colloid tissue that became fragile during regeneration, solitary RPE cells or RPE-derived cells were identified by their morphological characteristics (Figure 13). Then 100 cells were selected and placed into a PCR tube (No T-02F, Ina Optica, Osaka, Japan) using a microtip (703Y, Ina Optika) attached to a micropipette (set at 0.5 ml; Pipetman P20; Gilson, WI 53562-0027, USA) under a dissecting microscope. The cells were accumulated at the bottom of the tube after centrifugation for 1 min at 3,000 g. Because the amount of mRNA in a single cell is small, two-step pre-amplification of the cDNA was carried out. Total RNA was obtained and cDNA was constructed using TaKaRa CellAmp Whole Transcriptome

Amplification Kits (Real Time) Ver.2 (3730; Takara). In this step, the resulting cDNAs were pre-amplified non-selectively by PCR (21 cycles). Furthermore, cDNAs were pre-amplified by PCR (65 cycles) using KOD FX with the class-specific primer sets. PCR products were separated by agarose gel electrophoresis, and those corresponding to the size of *Pax6* variants were excised and purified using a Geneclean Spin Kit. Pre-amplified cDNAs were prepared for all classes of *Pax6*, and used as the templates.

Discrimination of the classes of *Pax6* variant was performed against cDNA from RPESCs-colloid and isolated RPESCs in the same way as indicated in section 2.1.8.

2.3. Estimation of the signal pathway causing the reprogramming of RPE cells

2.3.1. Preparation and incubation of RLECs

After the eyeball was excised, the muscle which was attached to the sclera, was carefully removed. It was sterilized twice for 20 sec by 70% ethanol, placed cornea side up on a membrane filter (HAWP 013 00, Millipore, Billerica, MA, USA), and cut along the equator. The anterior half was carefully removed and the posterior half (eye-cup) was soaked in PBS for 20-30 min to create a loose connection between NR and RPE. Then NR was peeled off by a needle and forceps to create a RLEC, and the optic nerve was cut out. An RLEC of 0 DIV was used for analyses. The other RLEC was incubated in 80% L-15 medium (41300-039, Thermo Fisher Scientific) containing 2%

penicillin-streptomycin liquid (15140-122, Thermo Fisher Scientific) at 25°C for up to 10 days. The medium was refreshed on day-5 of incubation (Figure 18).

The following factors were added to culture medium. MEK1/2-specific inhibitor U0126 (V1121; Promega, Fitchburg, WI, USA), which was dissolved in DMSO (D2650; Sigma-Aldrich) at 2 mM immediately before use, was administered at a final concentration of 5 μM from the time point at which the eye-cup was soaked in PBS and the culture medium. Heparin, sodium salt (081-00136, Wako) was added to the medium at a concentration of 7.5 μg/ml from the beginning of incubation. The newt FGF2 (see Susaki and Chiba, 2007) was tested by adding it to the heparin-containing medium at a concentration of 50 ng/ml.

When the medium was replaced, fresh factors were added.

2.3.2. PCR analysis

RLECs, which were incubated for 10 days or simply removed from normal eyeballs, were transferred to RNase-free PBS. RPE cells with colloid tissue were peeled off from the remainder of RLECs. In the same manner as described in section 2.2.2., sample collection, cDNA synthesis and discrimination of the classes of *Pax6* variant were carried out.

2.3.3. Antibodies

Rabbit polyclonal anti-Pax6 antibody PRB-278P (1:500; Covance) and mouse monoclonal anti-RPE65 antibody (1:500; MAB5428, Millipore) were used as the primary antibodies. Biotinylated goat anti-rabbit IgG antibody (1:500) and Alexa-488-comjugated goat anti-mouse IgG antibody (1:500; A-11008, Thermo Fisher Scientific) were used as the secondary antibodies. To confirm that there was no nonspecific adsorption of the primary antibody, the anti-Pax6 antibody in which epitope was blocked in the target amino acid sequence itself was used as the negative control. PRB-278P was pre-adsorbed with a synthetic peptide (QVPGSEPDLSQYWPRIQ) corresponding to the newt Pax6 to make the negative control. The antibody to which the peptide (50 μg/ml) was added was placed overnight at 4°C before use. When the negative control was used, the test antibody was also dispensed to maintain the same conditions.

2.3.4. Immunohistochemistry

RLECs attached to the sclera, as well as the membrane used in culture, were fixed in 4% paraformaldehyde in PBS for 2.5 h at 4°C. The fixative solution was washed off. The tissue sections were sliced to a thickness of ~20 μm and stained as in section 2.1.7. When double staining was performed, two antibodies were simultaneously added to the solution.

For whole-mount staining, RLEC preparations were rinsed thoroughly (PBS, 1% TritonX-100 in PBS, PBS, 15 min each), treated with 3.3% H_2O_2 in methanol for 20 min on ice, washed thoroughly,

incubated in a blocking solution [3% normal goat serum (S-1000, Vector Laboratories) / 1 % TritonX-100 in PBS] containing Avidin D (1:50; Avidin/Biotin Blocking kit) for 2 h, washed in PBS twice, and then incubated in the primary antibody diluted with blocking solution containing Biotin (1:50; Avidin/Biotin Blocking kit) for 72 h at 4°C. After washing thoroughly, the samples were incubated in the biotinylated secondary antibody diluted with the blocking solution for 4 h, washed thoroughly, incubated in a mixture of Avidin and Biotin Complex (Vectastain ABC Elite kit) for 2 h, washed thoroughly, and then incubated in a DAB solution (DAB substrate kit) for 6 min. The reaction was stopped by washing samples in distilled water. After the DAB solution was washed away, melanin pigments in the tissues were bleached by incubating in 15% H_2O_2 / 1.5% sodium azide (197-11091, Wako) in PBS overnight. After the bleaching solution was washed away, the nuclei of RPE cells were stained with DAPI (1:50,000; D1306, Thermo Fisher Scientific) for 6 h. The RPE-choroid tissue was separated from the sclera, placed RPE-side up on a glass slide, immersed into 90% glycerol in PBS and finally mounted under a cover slip. Bright-light and fluorescence images of tissues were acquired using the same CCD camera systems used in section 2.1.7.

2.3.5. Cell counting

Total RPE cells in whole-mount preparations were identified and counted by observing their

characteristically flat and oval nucleus on the surface of the basement membrane in fluorescence field. Moreover, the amount of immunostaining that overlapped with nuclear staining was assessed in bright field as positive cells. In this study, total and Pax6-immunoreactive nuclei of RPE cells were counted in an area further than 50 μm from the peripheral margin of the RPE sheet, because non-specific staining along the margin, which was due to the primary antibody (rabbit IgG), was serious under the current conditions.

2.3.6. Data analysis

Data were presented as the mean ± SEM (n: the number of RLECs) from more than three independent rounds of experiments. Normality and non-parametric tests (Mann-Whitney's U-test and Jonckheere-Terpstra test) were carried out to evaluate the statistical significance of the data, using Ekuseru-Toukei 2008 software (Social Survey Research Information, Tokyo, Japan).

3. Results

3.1 Identification of Pax6 variants expressing in the eyeballs of adult newts

3.1.1. Search for unknown *Pax6*

To search for classes of unknown *Pax6* expressed in the normal eye and regenerated retina of the adult newt, transcriptome data which our laboratory made in the past was used. This data was made using the total RNA obtained from the posterior half of the normal eye from which the retina and regenerating eyeball were removed at 0-14 days after retinectomy (Nakamura et al., 2014). After paired end sequencing was carried out by a next generation sequencer, the fragmentary sequence information was connected by assembly software. In this process, three algorithms were applied and three databases were produced because there is no consensus about the best *de novo* assembly software. In this study, three *de novo* assembled databases were integrated and used to search widely for unknown *Pax6*variants.

By repeating the local blast analysis that used known *Pax6* as the query sequence, three new classes of *Pax6* transcripts were identified. In this study, these new *Pax6* were named *Pax6* variant2~4 (*v2~4*) while the known *Pax6* was named variant1 (*v1*). The characteristic common to the structure of all the new *Pax6*'s searched *in silico* was a unique sequence upstream of the PD-coding

region. Their unique 5′ regions are shown in Figure 4 and the full sequences are displayed in Figures 5, 6 and 7. *Pax6 v3* has a long unique sequence with partially homogeneity to the *Pax6* regulatory region of other animals whereas the unique sequence of *Pax6 v2* is short. These different unique sequences continue to share the same point where is just before the PD-coding region. On the other hand, in *v4*, the sequence extended to inside of the PD-coding region. This resulted in the lack of a 5′ part (127 bp) of the PD-coding region (i.e., a neighbouring sequence of the 5′ end of exon 5a) and the addition of a 92-bp extra sequence after the 3′ end of exon 5a. This class has an ATG start codon after the PD-coding region. Therefore, it is expected to encode paired-less forms of *Pax6*. Unlike these varieties of unique sequences, the sequence downstream of the PD-coding region has high homogeneity with known *Pax6*.

Based on the above data, whether these *Pax6* were expressed in the eyeballs of adult newts was checked. Primers were designed using sequences that are specific for each class of *Pax6* and PCR was performed using a cDNA template which was reverse transcribed from mRNA extracted from whole adult newt eyeballs. Four kinds of *Pax6* expected *in silico*, including three new classes, were detected.

Splice variants in each class were also confirmed. Using RT-PCR, four variants of *Pax6 v1* and *v2* (LL, SL, LS and SS) were confirmed. In contrast, only two variants (SL and SS) lacking 5a were identified in *v3*. In class *v4*, two variants probably encoding two paired-less forms (-L and -S) were

confirmed. Figure 8 shows the structure of each class, including splice variants. Thus, 12 variants of known or new Pax6 expressed in normal newt eyeballs with unique 5′ sequences were detected.

3.1.2. Distribution of Pax6 variants in the adult newt eye

Next, immunohistochemistry using antibody PRB-278P, which recognizes all classes of Pax6, was performed to confirm which parts of the normal eyeball express Pax6. The results of immunohistochemistry, which are shown in Figures 9 and 10, suggest that Pax6 is expressed in epithelial cells of the cornea, CL, lens and iris as well as in cells of the CMZ and in amacrine and ganglion cells in the NR, but not in the RPE, choroid or sclera.

On the basis of this observation, tissue samples were isolated from each part of the eyeballs and the expression of *Pax6* in them was examined by PCR with class-specific primer sets. Furthermore, splice variants were distinguished based on band size. PCR products were detected in most eye tissues except for the RPE, choroid and sclera (Figure 11). This is consistent with the immunohistochemical evidence. Previous studies reported that there is no *Pax6 v1* in RPE cells (Chiba et al., 2006a; Islam et al., 2014; Nakamura et al., 2014). This experiment supports this claim and reveals that other classes of *Pax6* are also not expressed in RPE cells. Table 1 summarizes the *Pax6* variants that were identified in Figure 11. The NR had only four variants of *Pax6 v1*. In contrast, additional classes of *Pax6* were confirmed in other parts. The CMZ and iris expressed both

Pax6 v1 and *v2* but their expression patterns were different. In the CMZ, there were four variants for each class, but in the iris, only LS and SS were detected. The lens, CL and cornea expressed SL and SS of *Pax6 v3* and -L and -S of *Pax6 v4* as well as four variants of *Pax6 v1* and *v2*, although PCR signals for *Pax6 v3* in the lens and cornea were relatively weak (Figure11).

To confirm the presence of Pax6 proteins, western blotting was carried out using anterior eye tissues where it is expected that all four classes of Pax6 exist. As indicated in Figure 12, two protein bands at around 50 kD, as well as bands at ~31.6 kD and ~26.4 kD, were recognized. The arrow in Figure 12 shows bands which were specifically labelled by Pax6 antibody while the arrowhead shows a non-specific band which appeared even when the primary antibody was omitted, i.e., in the control. The upper two bands are likely the merges of longer forms (LL) of Pax6 v1, v2 and v3. The bands were no longer able to separate because their structures and molecular weights were similar to each other. The lower bands corresponded to the -L and -S forms of Pax6 v4 because this is paired-less Pax6 with a small molecular weight.

Taken together, these results suggest that Pax6 variants are expressed in many kinds of tissues in the eye in different combinations but not in intact RPE cells.

3.2. RPE cells newly express *Pax6 v1* and *v2* variants upon retinectomy

No class of *Pax6* was expressed in intact RPE cells. However, past studies revealed that RPE cells

are reprogrammed into a unique multipotent state and newly express *Pax6* in neural retina regeneration. Therefore, which *Pax6* variants were expressed in RPESCs was analysed.

At first, PCR was carried out with class-specific primer sets and RPESCs-choroid tissues collected from posterior halves of the eyeballs at 10 days after retinectomy. Figure 13 is an image of a collected sample that contains aggregates of RPESCs, which are RPE-derived cells which just entered Stage E-1. In this experiment, PCR conditions were changed and implemented for all subsequent analyses. Figure 14 shows two typical PCR results. On the left, product bands corresponding to LL, SL, LS and SS of *Pax6 v1*, and to LL of *Pax6 v2*, were recognized. On the right, three other variants of *Pax6 v2* were also detected in the same template when the annealing temperature of PCR was changed. All four variants of *Pax6 v1* and *v2* were identified, but no variants for *Pax6 v3* or *v4* were detected (Figure 15). On the other hand, no product bands were detected in the choroid tissues after RPE-derived cells had been removed (Figure 16). The same result was obtained in three independent rounds of experiments.

For further confirmation, RPESCs were isolated from the choroid by protease and a chelator. While confirming cell shape under a microscope, RPESCs were selected from pieces of other pigmented organized structures (Figure 13). Single-cell PCR with 100 isolated RPESCs was then attempted, and expression of the same classes of *Pax6* was confirmed and compared with RPESCs-choroid tissue (Figure 17). However, under this condition, variants of detectable transcripts were limited to

SS for *Pax6 v1* and SL for *Pax6 v2*. When PCR conditions were changed, additional variants were not detected. This result may reflect a difference in the stage of regeneration between both samples. A recent study indicated that it is possible for individual RPE cells to be reprogramed at a slightly different timing in the E-1 stage (Islam et al., 2014). In isolated RPESCs, uniform cells that had just begun reprogramming, were selected. Of note, the RPESCs-choroid tissue contained cells with an advanced stage of regeneration.

Taken together, these results suggest that transcript variants, possibly LL, LS, SL and SS, of two classes of *Pax6* (i.e., *v1* and *v2*) are expressed in RPE-derived cells in Stage E-1.

3.3. Estimate of the signal pathway which causes the reprogramming of RPE cells

3.3.1. RPE cells also express *Pax6 v1* and *v2* variants in RLECs

The signals that may have caused the reprogramming of RPE cells were estimated by using Pax6 expression as the marker of this phenomenon. Therefore, in this study, the RLEC system that was established to check the response of the RPE cells in our laboratory was used. Figure 18 shows the procedure of how to make the RLEC system. The newt eyeball was extracted and sterilized. The anterior hemisphere (the lens side) was cut off. The exposed NR was removed from the remaining posterior half of the eyeball in PBS and the RLEC was incubated in 80% L-15 medium at 25°C. L-15 medium is minimal essential medium consisting of amino acids, vitamins, inorganic salts,

galactose and sodium pyruvate. Since L-15 medium does not include any growth factors, it was suitable for this experiment to apply any factor.

At first, it was confirmed whether this system could be adapted to the analysis of Pax6 expression. The existence of the target protein was confirmed by using anti-Pax6 antibody, which recognized all variants of Pax6. The results of whole-mount staining are shown in Figure 19. Images of the RPE cell layer represent a top view. New Pax6 antibody-positive cells were observed in the RLEC system after incubation for 10 days. The right-hand panel represents the negative control on day-10. The antibody preadsorbed with the peptide which was specific for the epitope was used as the negative control to completely remove non-specific staining of the primary antibody as well as the secondary antibody. Pax6-immunoreactivity in RPE cells was confirmed by double staining of a section of a day-10 RLEC with RPE65 antibody (Figure 20). Images of the RPE cell layer were viewed from the side. RPE cells were identified by RPE65-immunofluorescence (Chiba et al., 2006a). The nuclei of RPE cells were labelled with Pax6 antibody (arrows). Horizontal bars indicate the thickness of the RPE layer, and asterisks indicate small nuclei in the choroid, which displayed nonspecific staining as well as autofluorescence under these conditions. These two immunohistochemical results of RLECs revealed that Pax6 protein exists in several nuclei of RPE cells on day-10.

PCR analyses were then carried out with RPE-choroid tissues collected from RLECs immediately after the NR was removed (day-0; d0) and with RLECs after 10 days of incubation (day-10; d10) *in*

vitro (Figure 21). No *Pax6* variant was detected on day-0 and all four variants of *Pax6 v1* and *v2* were newly expressed by day-10 while *Pax6 v3* and *v4* variants were not. These results were consistent with the *in vivo* results.

These results indicate that RPE cells incubated in the RLEC system express *Pax6* as the *in vivo* state. In particular, because variants expressed in RLEC and *in vivo* are common, regulation is suggest to be equal, which expands the applicability of the RLEC system. Thus, this system was subsequently used for the analysis of Pax6 expression signals.

3.3.2. RPE cells expressing Pax6 versus RPE cells entering cell-cycle

Interestingly, even though no external factors were added to cultures in the period of incubation, Pax6 was expressed in RLEC RPE cells. RLEC was incubated in minimal medium (Figure 19). Furthermore, the conditions of RPE cells in which Pax6 was expressed were different from those of RPE cells entering the cell cycle (Yoshikawa et al., 2012). Figure 22 indicates the ratio of Pax6-imunoreactive (Pax6+) nuclei in the RPE sheet derived from RLEC which was incubated without any factors. Pax6+ nuclei appeared within 5 days and increased significantly between 5 and 10 days. The Pax6+ nuclei were distributed uniformly (Figure 19). The number of nuclei was counted in the area farther than 50 μm from the peripheral margin of the RPE to avoid non-specific staining. The timing of Pax6 expression differed from the cell-cycle re-entry observed from day-5 to

10 (Yoshikawa et al., 2012). In addition, Pax6-positive cells and cells entering the cell cycle also showed a variable distribution pattern (Figure 23). Pax6 was expressed uniformly in RLEC, but after entering the cell cycle, expression concentrated in the area near the Edge of RLEC (Yoshikawa et al., 2012). It is possible that Pax6 expression and cell cycle re-entry are independently controlled in the RLEC system.

3.3.3. Pax6 expression in RPE cells is not affected by a MEK1/2 inhibitor U0126

As described in the introduction, our laboratory discovered that the MEK1/2-ERK1/2 intracellular signaling cascade, which was activated within 30 min after retinectomy, is necessary for the first cell cycle entry of RPE cells in the RLEC system (Mizuno et al., 2012). Therefore, the relationship of the MEK1/2-ERK1/2 signaling cascade and Pax6 expression in RPE cells was examined.

RLECs were incubated in the presence of an MEK1/2 inhibitor U0126 at a concentration of 5 μM, which can inhibit up to ~50% of the initial activation of ERK1/2 and decrease the number of BrdU+ cells by 10 days by as much as ~25% (Yoshikawa et al., 2012). However, this treatment did not affect the number of Pax6+ RPE cells at 5 days compared to the solvent (0.25% DMSO) only (Figure 24). This result suggests that Pax6 can be expressed without the participation of the MEK1/2-ERK1/2 signaling cascade and that Pax6 expression in RPE cells may be independent of their cell cycle re-entry.

3.3.4 Heparin promotes the expression of Pax6 with the help of the MEK1/2-mediated pathway

Pax6 expression and cell cycle re-entry of RPE cells take place in the same period after retinectomy *in vivo* (Islam et al., 2014). An endogenous factor mediating these independent phenomena exists *in vivo*, but is exhausted *in vitro* was hypothesized.

Heparin, a glycosaminoglycan that is ubiquitously distributed in the body, and is known to bind various soluble factors and support their actions on receptors as well as protect these factors against degradation, served as a supplement. In fact, in the newt, heparin promotes the cell cycle re-entry of RPE cells in the RLEC system through a pathway that influences the area downstream of the MEK1/2-ERK1/2 signaling module (Yoshikawa et al., 2012). Therefore, heparin was added as an endogenous factor to activate RLEC.

Interestingly, when the RLEC was incubated for 5 days in the presence of heparin (7.5 μg/ml), the number of Pax6+ RPE cells tended to increase (Figure 25). Next the effects of U0126 (5 μM) on the promotion of Pax6 expression by heparin were examined (Figure 25). In this condition U0126 was tested. The number of Pax6+ RPE cells at 5 days after incubation had decreased significantly. These results suggest that heparin promotes Pax6 expression with the help of a MEK1/2-mediated pathway.

Finally, FGF2 was tested. FGF2 is a heparin-associated growth factor that is reportedly related to

regeneration. FGF2 is known to up-regulate Pax6 expression through the MEK-ERK pathway in chick embryonic RPE cells (Luz-Madrigal et al., 2014; Spence et al., 2007). However, administration of 50 ng/ml FGF2, a concentration used in previous experiments using RLEC (Susaki and Chiba, 2007), did not influence the effect of heparin on Pax6 expression in RPE cells after 10 days of incubation (Figure 26).

4. Discussion

4.1. Several kinds of Pax6 variants

To date, a wide variety of *Pax6* transcripts has been detected in many vertebrates, from fish to mammals. In addition, it is known that transcriptional initiation sites on the locus and post-transcriptional and post-translational modifications that derive various isoforms are spatiotemporally regulated in normal development and adult neurogenesis (Bandah et al., 2007; Gorlov and Saunders, 2002; Kammandel et al., 1999; Kim et al., 2008; Lakowski et al., 2007; Mizuno et al., 1997; Shaham et al., 2012; Walther and Gruss, 1991; Zhang et al., 2010). In the newt *C. pyrrhogaster*, a *Pax6* gene corresponding to Small eye (Sey) *Pax6* has been reported (Islam et al., 2014; Mizuno et al., 1997). In this study, new three classes of *Pax6* were discovered. The sequence of the conserved region of these *Pax6* is extremely similar. These sequence similarities suggest that these different classes of transcripts originate from the same locus. On the other hand, each class of *Pax6* has a unique sequence upstream of the PD-coding region, including the 5′ UTR (Figure 4), suggesting differences in transcriptional regulation among these classes.

These classes of *Pax6* are expressed in the intact eye tissues of the adult newt in different combinations, except in the RPE, choroid and sclera, as shown in this thesis. These findings may

provide suitable ground work for a study on the regulation and roles of the different classes of Pax6 variants in the newt. In this study, sequence information of the Pax6 variants was indispensable for determining the class of *Pax6* expressed in RPE cells after retinectomy.

4.2. RPESCs and CMZ express common *Pax6* variants

As described in the introduction, after retinectomy, adult newt RPE cells undergo a loss of their membrane attachment, forming cell aggregates in 10 days (Stage E-1 [4]). During this process, RPE cells are reprogrammed into a unique multipotent state with newly expressed *c-Myc, Klf4, Sox2, Mitf* and *Pax6*, while preserving their original characteristics (Islam et al., 2014). In this study, the class of *Pax6* expressed in such RPE-derived cells in Stage E-1 was determined, namely *Pax6 v1* and *v2*, both of which give rise to four splice variants LL, SL, LS and SS. It is still uncertain whether Pax6 works as a reprogramming factor in this system. However, it is possible that the expression of Pax6 in RPE-derived cells represents the reprogramming of RPE cells.

These should have the potency to form either the pro-NR or pro-RPE layer, as in cells in the early optic vesicle which also express c-Myc, Klf4, Sox2, Mitf and Pax6 (Islam et al., 2014).

In early eye development, Pax6 and Mitf are known to be involved in fate decision of cells in the optic vesicle to form either pro-NR or pro-RPE cells as the optic vesicle invaginates to form the optic cup (Fuhrmann et al., 2014; Martínez-Morales et al., 2004). Up-regulation of Pax6 or

down-regulation of Mitf biases the fate of cells toward the retinal stem/progenitor cells (Azuma et al., 2005; Fuhrmann et al., 2014; Nguyen and Arnheiter, 2000).

In the regeneration of the adult newt retina, up-regulation of Pax6 was observed in a population of RPE-derived cells that formed the pro-NR layer in the next stage (Islam et al., 2014). Intriguingly, in this study, the same set of *Pax6* variants as those in RPE-derived cells are expressed in the CMZ where retinal stem/progenitor cells have been harbored since the embryonic stage was found. Therefore, as an analogy of early eye development, one hypothesis may be that these classes of Pax6 variants express in the cells that have potency, producing neural retina. However, it is still uncertain whether Pax6 works as a reprogramming factor in this system.

4.3. The signal which produces RPESCs

In this study, Pax6 was examined as a marker of the reprogramed signal of RPE cells using the RLEC system. Interestingly, Pax6 expression did not require the administration of exogenous factors. Immunohistochemical analysis revealed that, in the absence of exogenously administered factors, Pax6+ cells in the RPE sheet appeared within 5 days after retinectomy and showed a gradual increase in number up to ~27 % (on average) of all RPE cells by 10 days, and that the Pax6+ cells on day-10 are distributed almost uniformly in the sheet. In addition, the appearance and increment of Pax6+ cells in the RPE sheet was not affected by U0126, a specific blocker against

MEK1/2-mediated signaling. In a previous study (Yoshikawa et al., 2012), it was shown that RPE cells enter the cell cycle even if factors were not provided in the RLEC system. Cell cycle re-entry and Pax6 expression were observed at the same time during Stage E1 *in vivo* regeneration. A previous study (Yoshikawa et al., 2012) indicated that the cell cycle re-entry of RPE cells in RLEC conditions is highly restricted to a zone of ~100 μm width along the peripheral margin of the RPE sheet, i.e., along the wound edge of the RLEC, an area that was defined as the 'Edge' . This event is obviously regulated by a MEK1/2-ERK1/2 module whose activity is transiently strengthened within 30 min after retinectomy (Mizuno et al., 2012). Furthermore, the cells which reside in the central area of the RPE sheet, defined as the 'Center' in Yoshikawa et al. (2012), hardly enter the cell cycle, possibly through a mechanism equivalent to contact inhibition (Yoshikawa et al., 2012), although the activity of the MEK1/2-ERK1/2 module in the 'Center' is as elevated as in the 'Edge' (unpublished data provided by H. Yasumuro).

Taken together, Pax6+ cells and cells which re-enter the cell cycle on the same RPE sheet appear to show a different spatial distribution pattern and requirement of the activity of MEK1/2, suggesting that the expression of Pax6 in RPE cells after retinectomy can be triggered independently of their cell cycle re-entry.

In the eye, Pax6 expression and cell cycle re-entry of RPE cells take place simultaneously (Islam et al., 2012). Other factors may dry up in the RLEC system but that mediate between these two

phenomena may be independently controlled *in vivo*. To reinforce this possibility, attention was paid to heparin, which activates the function of various growth factors. Heparin is the glycoprotein which is strongly negatively charged and combines with many growth factors such as FGFs, TGF-β and BMPs, to activate the function of these factors. Furthermore, evidence of the influence of the dosage of heparin in RPE cells entering the cell cycle was provided in past studies (Yoshikawa et al., 2012). Therefore, in this experiment, heparin was added as an endogenous factor in RLEC activation. Treatment of RLECs with heparin promoted Pax6 expression in RPE cells but this effect was inhibited by U0126.

This result indicates that Pax6 expression promoted by heparin administration, but depends upon the activity of MEK1/2. In other words, heparin administration seems to coordinate the pathway for Pax6 expression with a pathway mediated by MEK1/2 which is involved in the cell cycle re-entry. This pathway is driven by heparin or by heparin-associated factors which might be released from tissues in the RLEC.

Figure 27 indicates hypothetical pathways of molecular networks involved in the initial processes of retinal regeneration, including findings of cell cycle re-entry which were observed in parallel (red and green lines are aspects that became clear in this study). At least three intracellular signalling pathways in RPE cells after retinectomy were predicted:

1) A pathway for Pax6 expression. This is the signal system which is MEK1/2-ERK1/2- and heparin-

independent.

2) A MEK1/2-ERK1/2 pathway which is primarily involved in cell cycle re-entry. This is more likely to receive contact inhibition.

3) A pathway driven by heparin or heparin-associated factors. This is located downstream of the MEK1/2-ERK1/2 pathway and mediates between it and Pax6 expression.

4.4. Differences in RPE cells between humans and newts

In humans, when the NR suffers a traumatic injury, RPE cells – as in the newt – start to lose their epithelial morphology while acquiring the ability to migrate and proliferate. However, unlike the newt, these finally withdraw the NR by contraction, leading to a loss of vision (Chiba, 2014). In this process of transformation, it has been suggested that RPE cells pass through a multipotent state and such multipotent RPE cells were named as human RPESCs. Human RPESCs are known to express *Pax6* as well as *c-Myc*, *Klf4*, *Sox2* and *Mitf* (Salero et al., 2012) as in newt RPESCs (Islam et al., 2014) although the expression levels of *Pax6* seem to vary depending on the RPE cell line from which the RPESCs are derived, or on the culture conditions (Salero et al., 2012). In the adult newt, Pax6 presumably plays important roles in retinal regeneration because its transcription is elaborately and dynamically regulated during retinal regeneration, as demonstrated in a previous study (Islam et al., 2014) and in the present findings. Therefore, knowledge of Pax6 regulation for retinal

regeneration of the adult newt, which was obtained in this study, would be a base to generate a neural retina *in vitro* or to regenerate an entire retina *in vivo* from RPE cells or RPESCs in humans.

4.5. Conclusions and future directions

In this thesis, the signaling pathways involved in the expression of Pax6 in adult newt RPE cells in a bid to identify the signal that is employed to trigger RPE cell reprogramming for retinal regeneration were investigated. As a result, the following aspects became clear:

1) *Pax6 v1* and *v2* express in RPESCs;

2) Pax6 expression is independent of entering the cell cycle;

3) Pax6 expression and cell cycle entry are conjugated by heparin or heparin-associated factors.

Future studies should perform proteomic analysis and identify factors that influence Pax6 expression. In these experiments, Pax6 expression was independent of MEK1/2-ERK1/2, an aspect that requires further detailed verification. In addition, our laboratory developed a newt transgenic technique with greater efficiency (Casco-Robles et al., 2011; 2014).

The functional analysis of Pax6 based on knowledge of its variants and the signal system that was employed in this study will deepen our understanding of newt retina regeneration.

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6. Figures and Legends

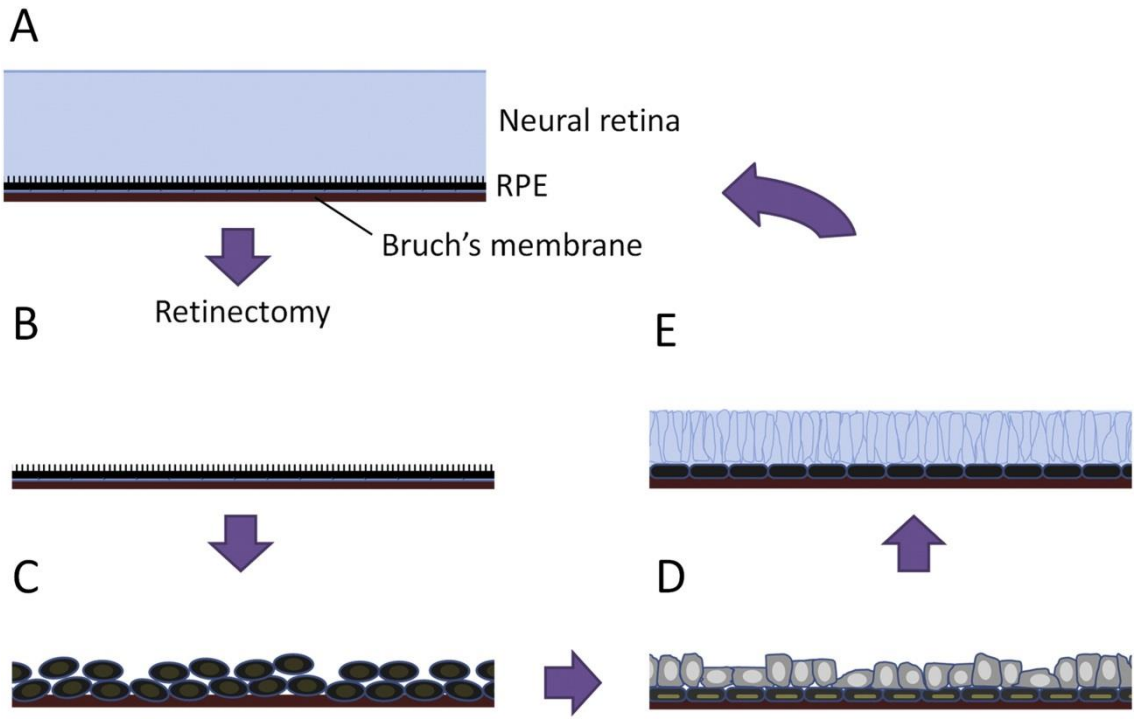

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Figure 1. Retinal regeneration in the adult newt *Cynops pyrrhogaster* by Chiba, 2014.
(A-B) The NR is completely removed from the eye by a surgical operation 'retinectomy'. (C) RPE cells are detached from each other as well as from the basement membrane, losing their epithelial characteristics (Stage 'E-1'). (D) RPE-derived cells are then sorted into two populations which form the prospective-NR and -RPE layers. (E) These Cells start to proliferate and eventually regenerate new functional NR and RPE.

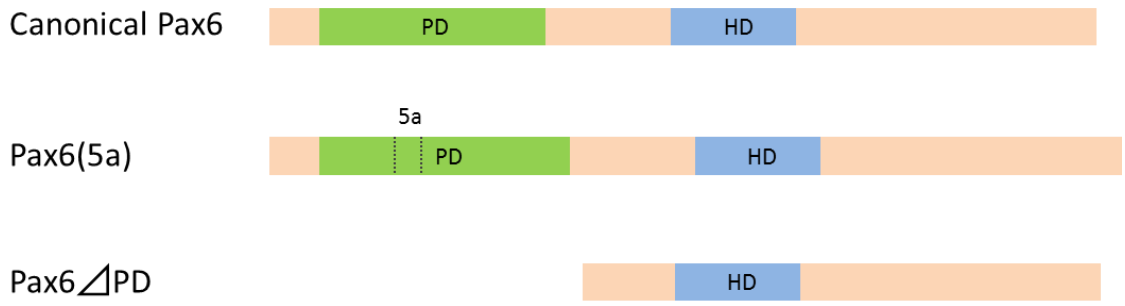


Figure 2. The structure of three typical Pax6 variants in vertebrates.

PD:paired-domein. HD: homeo-domain. Canonical Pax6 is the most universal Pax6 variants. Pax6(5a) is made by inserts an additional exon 5a into canonical Pax6. Pax6⊿PD lacks the PD and is termed paired-less.

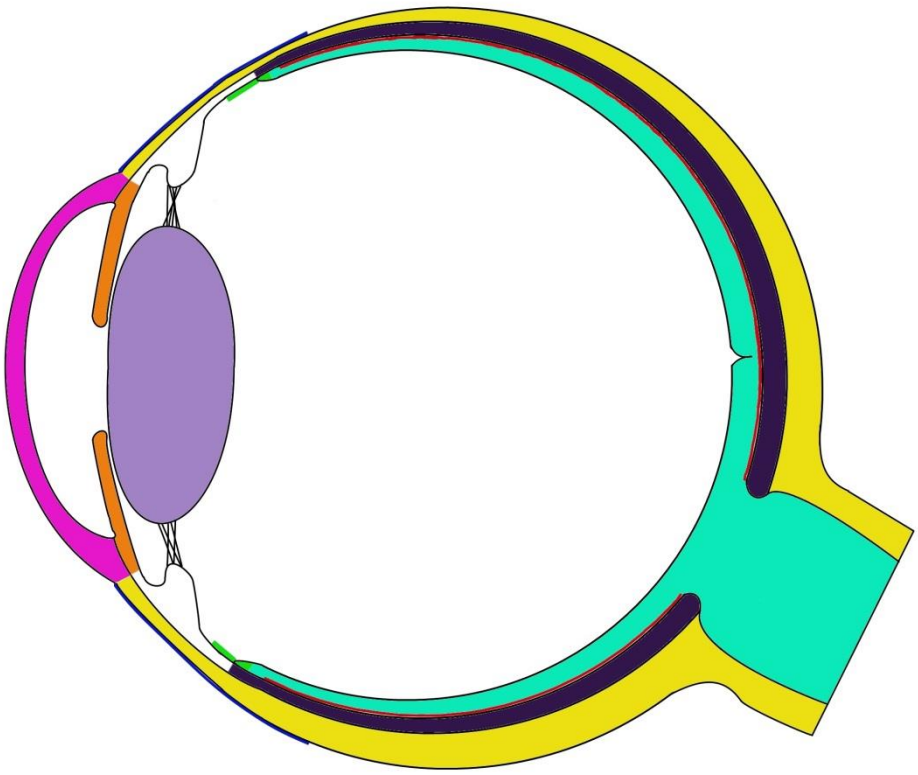
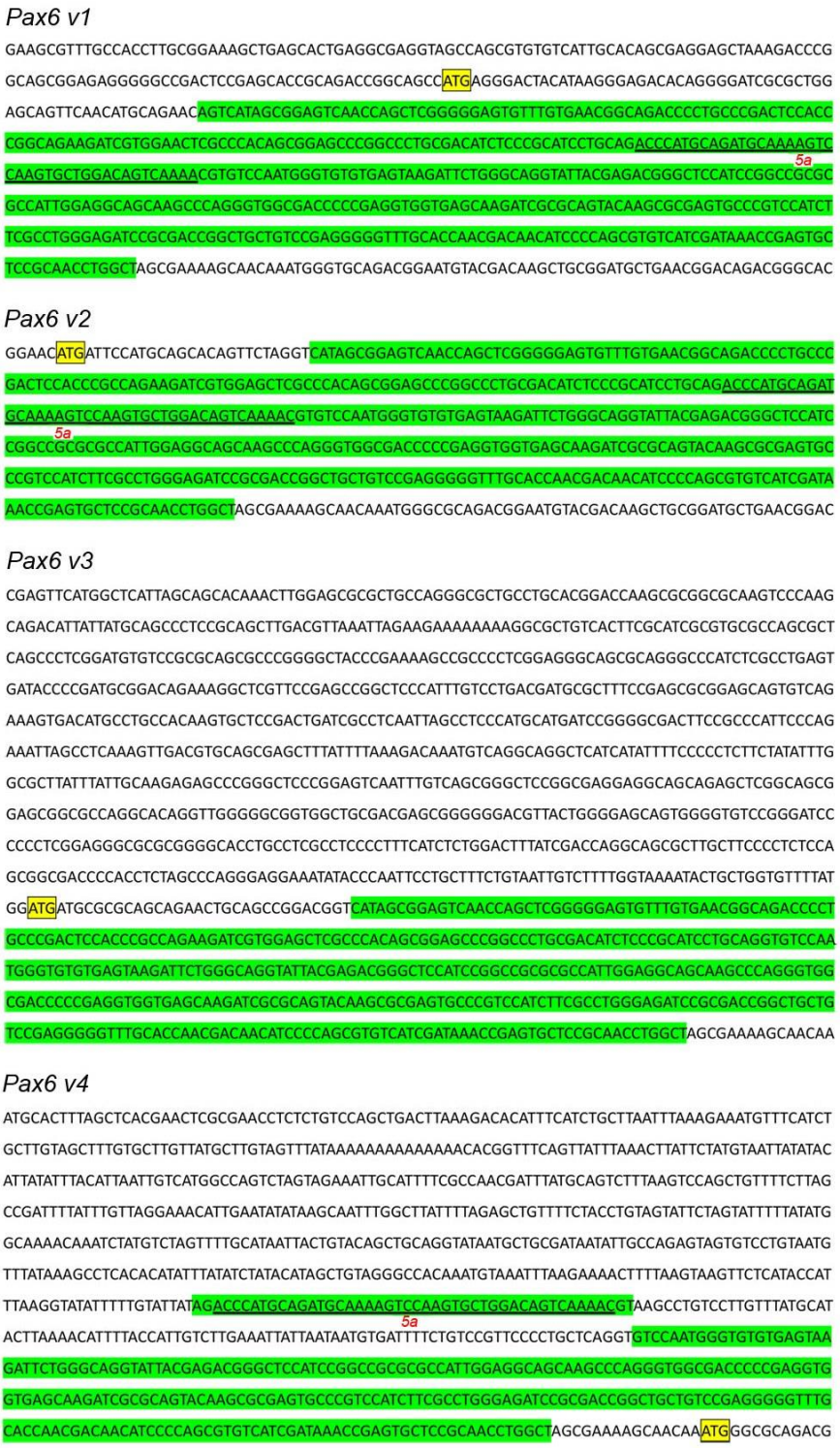


Figure 3. Position of the tissues used for sample preparation in the normal eyeball.

Green: ciliary marginal zone (CMZ), Orange: iris, Purple: lens, Blue: cornea limbus (CL), Pink: cornea, Light blue: NR, Red: RPE cells, Deep blue: choroid.



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(continued to next page)

Figure 4. *Pax6* variants in the newt, *C. pyrrhogaster*.

Their 5′ regions are shown here; yellow: start codon; green: sequence encoding the paired-domain (PD); Black underline: splicing region. 5′ untranslated region (5′UTR) was unique for each class of *Pax6*. The PD-coding sequence, except for that of *Pax6 v3*, had a splice region corresponding to '5a' of the vertebrate *Pax6*. In *Pax6 v4*, however, the PD was not translated because the start codon appears after the PD-coding sequence. *Pax6 v1* and *v2* had four variants [*Pax6 v1*: LL (1,492 bp), SL (1,450 bp), LS (1,341 bp), SS (1,299 bp); *Pax6 v2*: LL (1,496 bp), SL (1,454 bp), LS (1,345 bp), SS (1,303 bp)], and *Pax6 v3* and *v4* had two variants [*Pax6 v3*: SL (2,328 bp), SS (2,177 bp); *Pax6 v4*: -L (2,001 bp), -S (1,850 bp)].

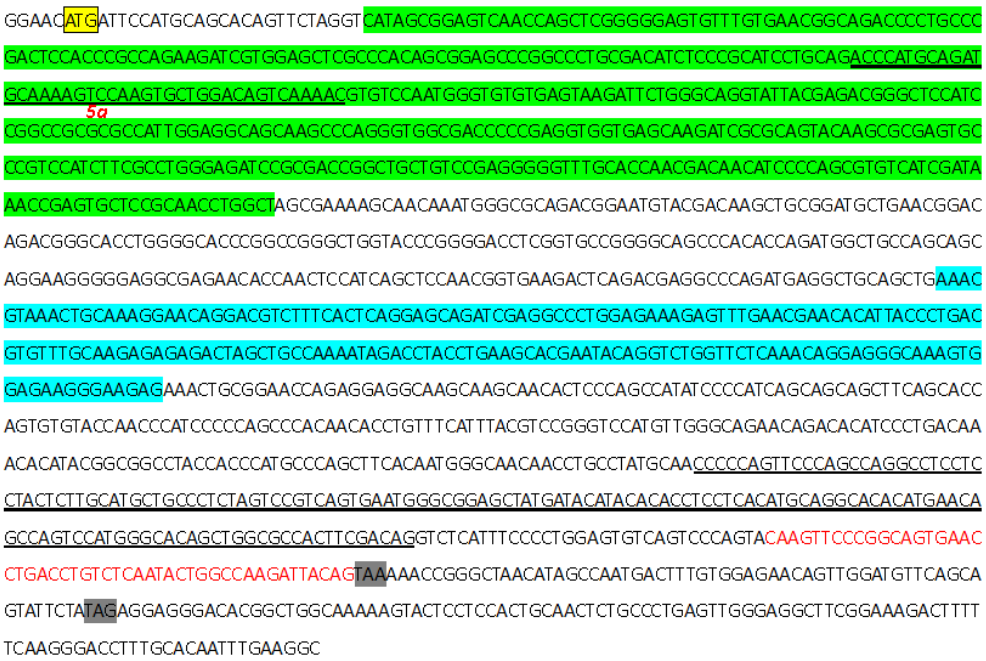

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Figure 5. *Pax6 v2* transcripts in the newt *C. pyrrhogaster*.

Yellow: start codon; grey: stop codon; green: sequence encoding the paired-domain (PD); blue: sequence encoding the homeo-domain (HD); Black underline: splicing region.

Inami W., Islam M.R., Nakamura K., Yoshikawa T., Yasumuro H., Casco-Robles M.M., Toyama F., Maruo F., and Chiba C. (2016) Expression of two classes of *Pax6* transcripts in reprogramming retinal pigment epithelium cells of the adult newt. Zool. Sci. 33:21-30

Figure 6. *Pax6 v3* transcripts in the newt *C. pyrrhogaster*.

Yellow: start codon; grey: stop codon; green: sequence encoding the paired-domain (PD); blue: sequence encoding the homeo-domain (HD); Black underline: splicing region.

Inami W., Islam M.R., Nakamura K., Yoshikawa T., Yasumuro H., Casco-Robles M.M., Toyama F., Maruo F., and Chiba C. (2016) Expression of two classes of *Pax6* transcripts in reprogramming retinal pigment epithelium cells of the adult newt. Zool. Sci. 33:21-30

Figure 7. *Pax6 v4* transcripts in the newt *C. pyrrhogaster*.

Yellow: start codon; grey: stop codon; green: sequence encoding the paired-domain (PD); blue: sequence encoding the homeo-domain (HD); Black underline: splicing region.

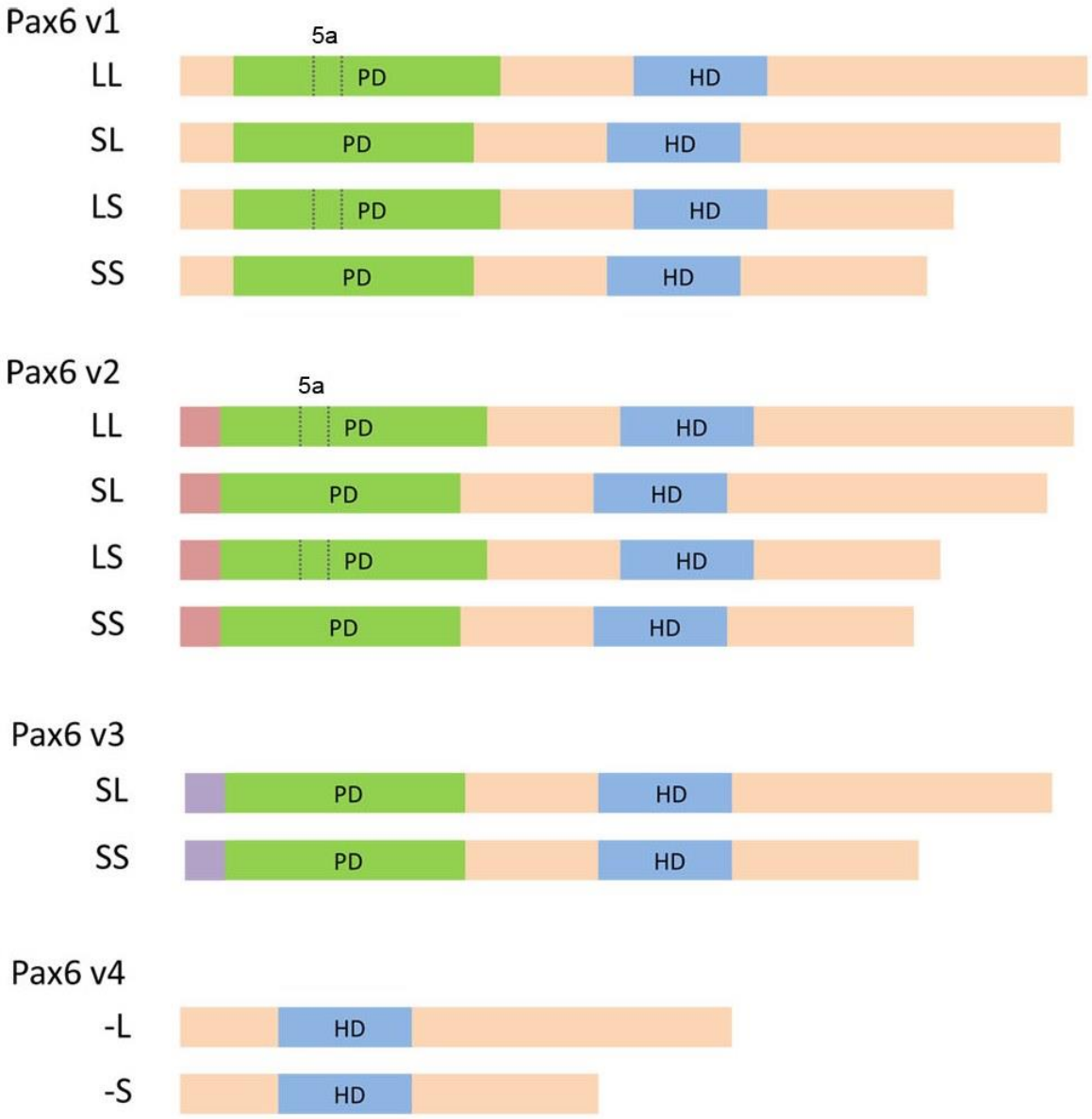

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Figure 8. Pax6 protein isoforms were deduced from these splice variants.

Pax6 v1: LL (49.9 kD), SL (48.4 kD), LS (47.3 kD), SS (45.8 kD); Pax6 v2: LL (48.3 kD), SL (46.8 kD), LS (45.7 kD), SS (44.2 kD); Pax6 v3: SL (47.1 kD), SS (44.5 kD); Pax6 v4: -L (31.5 kD), -S (28.9 kD). PD:paired-domein. HD: homeo-domain. The unique amino acid sequences of Pax6 v1, v2 and v3 exist before the PD. Pax6 v4 lacks the PD, and is classified as a paired-less type.

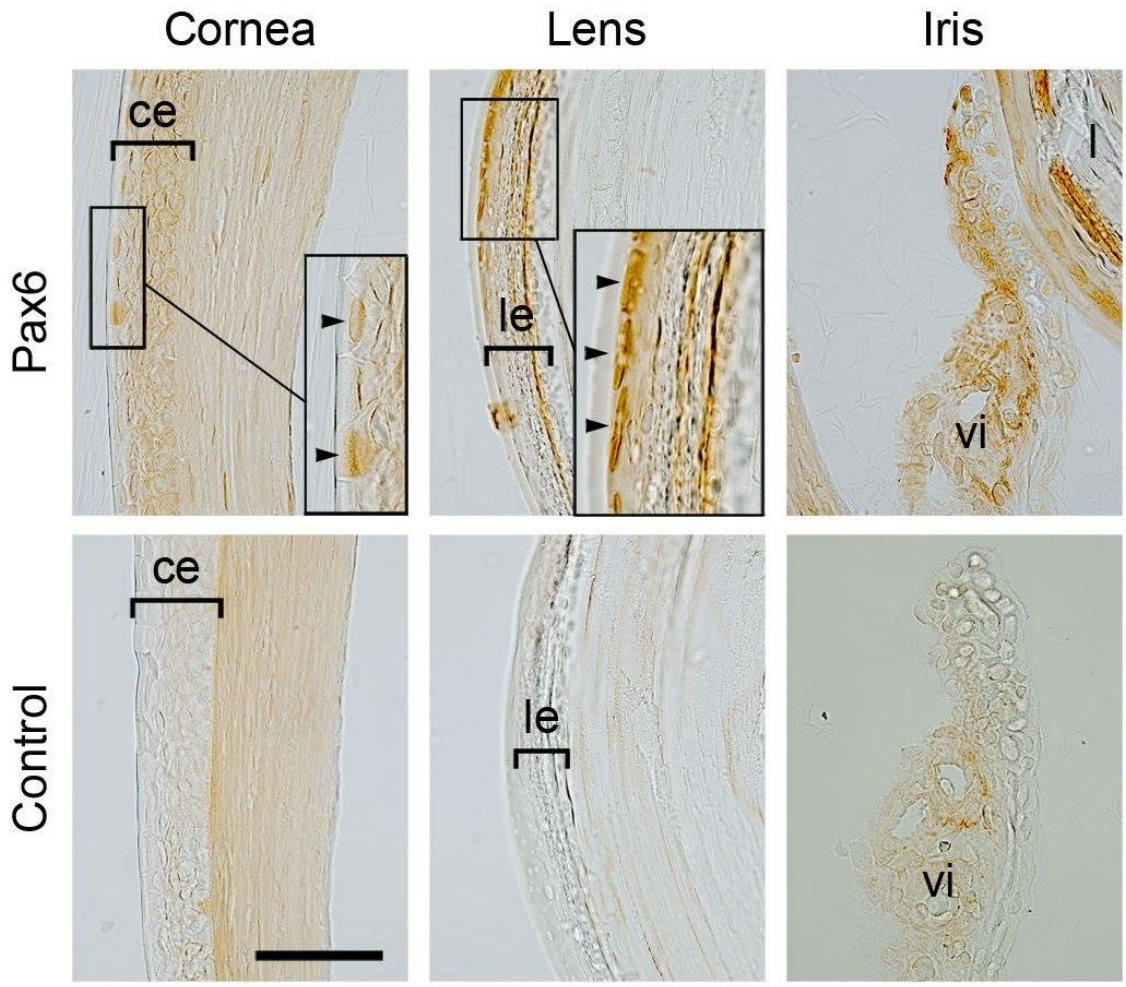

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Figure 9. Expression patterns of Pax6 variants in the intact eyes of the adult newt. Immunohistochemistry was carried out to localize Pax6 in the cornea, lens and iris.

The lower panel in each figure is a negative control with no primary antibody. In the cornea and lens, the region enclosed by a small box was enlarged in an inset panel and the nuclei with immunoreactivity are indicated by arrowheads. The abbreviations in the figures are as follows. (ce: corneal epithelium; le: lens epithelium; vi: ventral iris) Scale = 100 μm

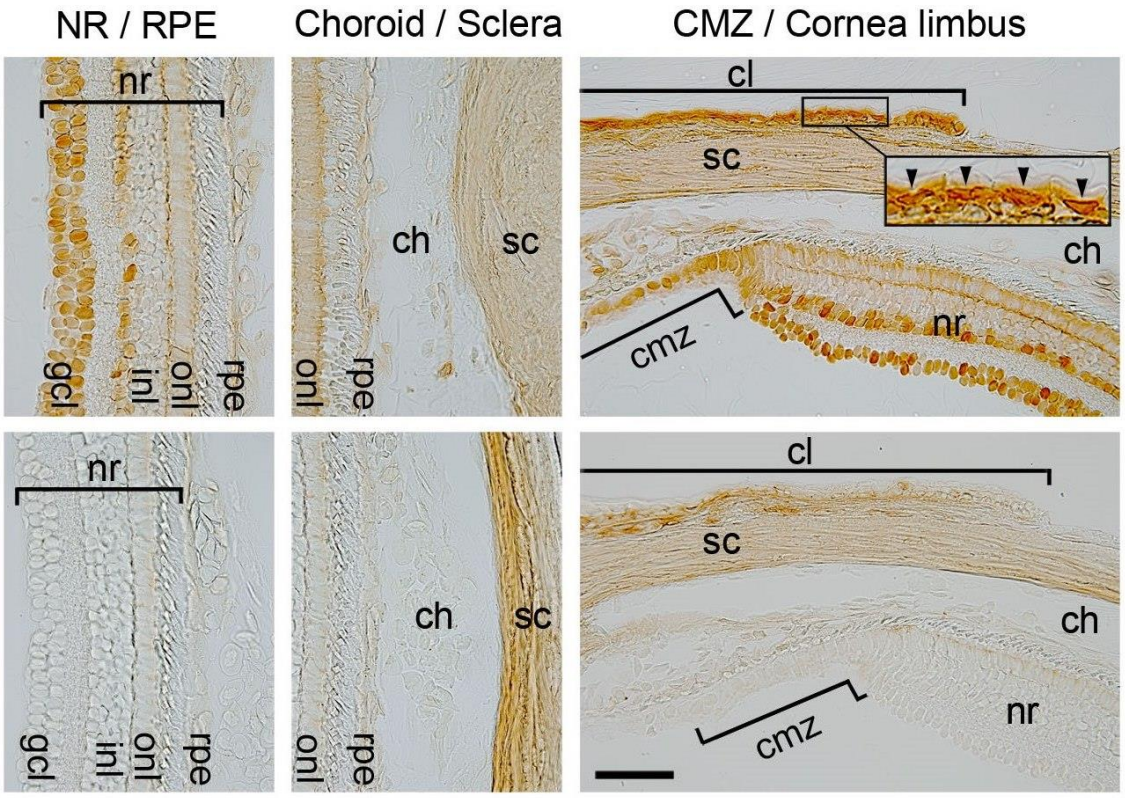

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Figure 10. Expression patterns of Pax6 variants in the intact eyes of the adult newt. Immunohistochemistry was carried out to localize Pax6 in the NR and RPE, choroid and sclera and CMZ and cornea limbus.

The lower panel in each figure is a negative control with no primary antibody. In CMZ and cornea limbus, the region enclosed by a small box was enlarged in an inset panel and the nuclei with immunoreactivity are indicated by arrowheads. The abbreviations in the figures are as follows. (nr: neural retina; rpe: retinal pigment epithelium; onl: outer nuclear layer; inl: inner nuclear layer; gcl: ganglion cell layer; ch: choroid; sc: sclera; cl: cornea limbus; cmz: ciliary marginal zone) Scale = 100 μm

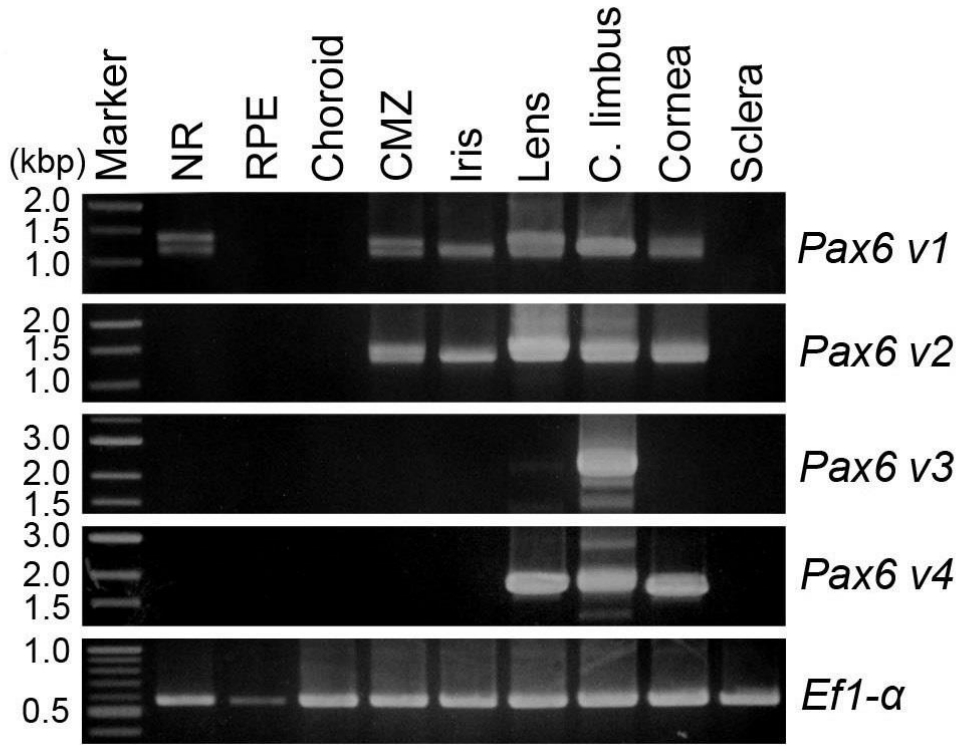

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Figure 11. PCR analysis with class-specific primer sets was carried out to determine the class of *Pax6* expressed in each part of the eye.

The transcript variants identified here are listed in Table 1. The eye tissues, except for the RPE, choroid and sclera, expressed different classes of *Pax6* variants in different combinations.

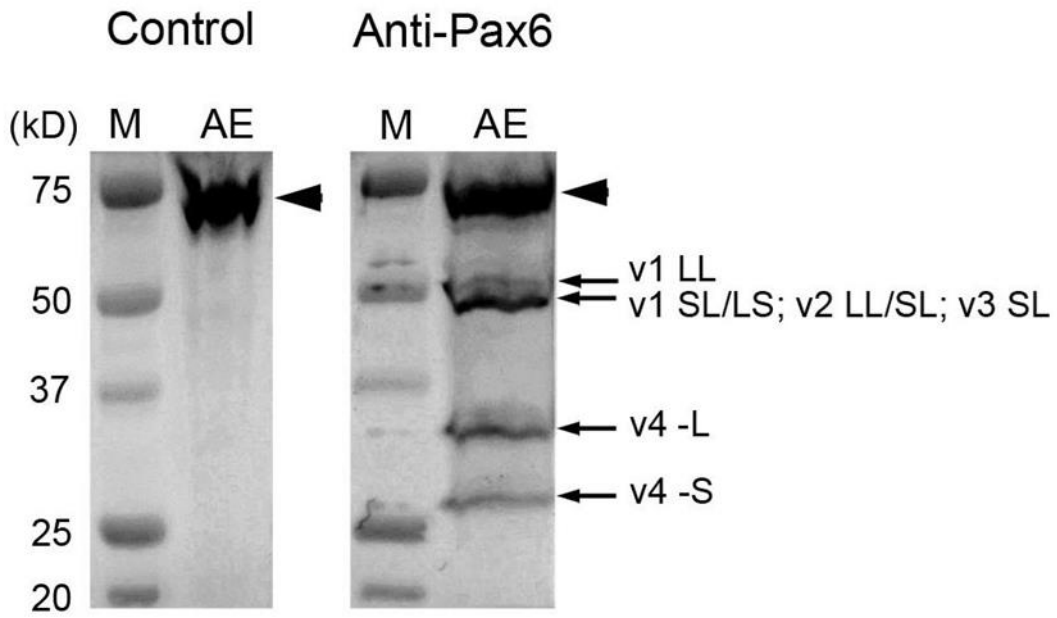

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Figure 12. Western blotting was carried out with anterior halves of the eyeballs to confirm the expression of Pax6 variants as proteins.

Two protein bands around 50 kD and the bands at ~31.6 kD and ~26.4 kD, corresponding to longer forms of Pax6 v1, v2 and v3 (canonical type) and -L and -S forms of Pax6 v4 (paired-less type), were specifically labelled by Pax6 antibody (arrows). Arrowheads: non-specific band which appeared even when the primary antibody was omitted (Control). *M*: marker; *AE*: anterior eye.

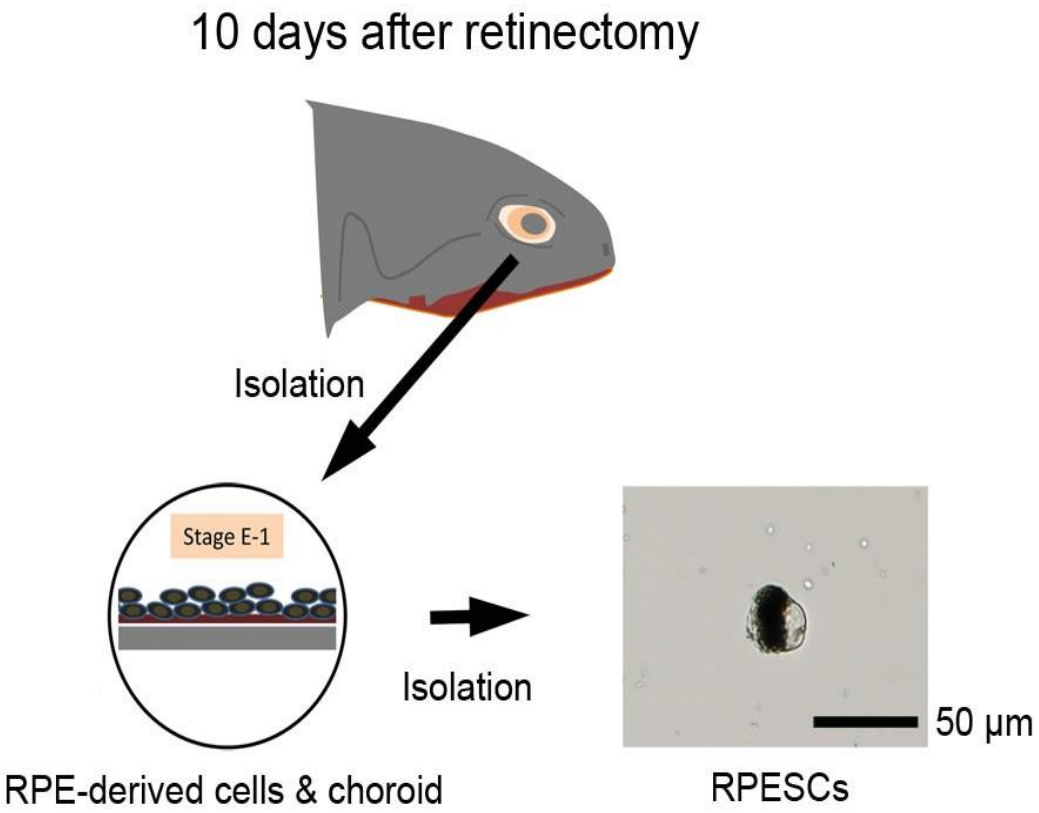

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Figure 13. The image of the sample collect.

To avoid the contamination of the basement membrane and colloid tissue that became fragile in a regeneration process, solitary RPESCs were identified by their morphological characteristics

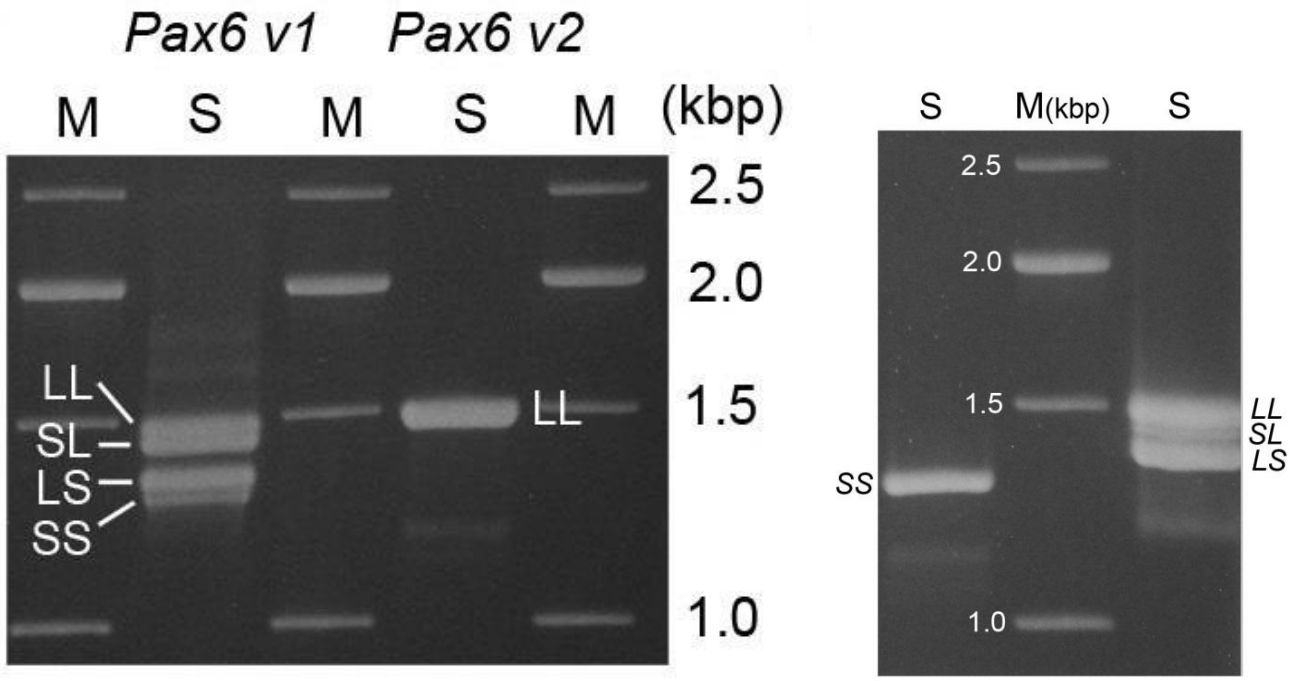

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Figure 14. *Pax6 v1* and *v2* express in RPESCs upon retinectomy.

RPE-derived cells were isolated together with the choroid tissues from eyeballs at 10 days after retinectomy (Stage E-1) and the expression of each class of *Pax6* transcript was examined by PCR with class-specific primer sets. Left: All four transcript variants of *Pax6 v1* and *v2* were amplified. Right: For Pax6 *v2*, changing to PCR conditions allowed the detection of three other variants. *S*: sample; *M*: marker.

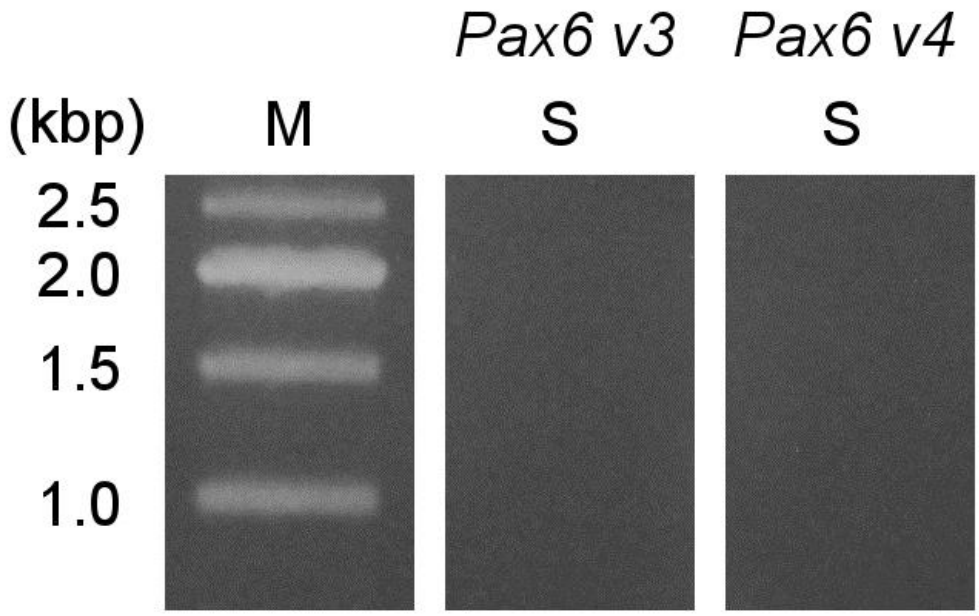

Figure 15. *Pax6 v3* and *v4* do not express in RPESCs upon retinectomy.

RPE-derived cells were isolated together with the choroid tissues from eyeballs at 10 days after retinectomy (Stage E-1) and the expression of each class of *Pax6* transcript was examined by PCR with class-specific primer sets. No transcripts were detected for *Pax6 v3* and *v4*. S: sample; M: marker.

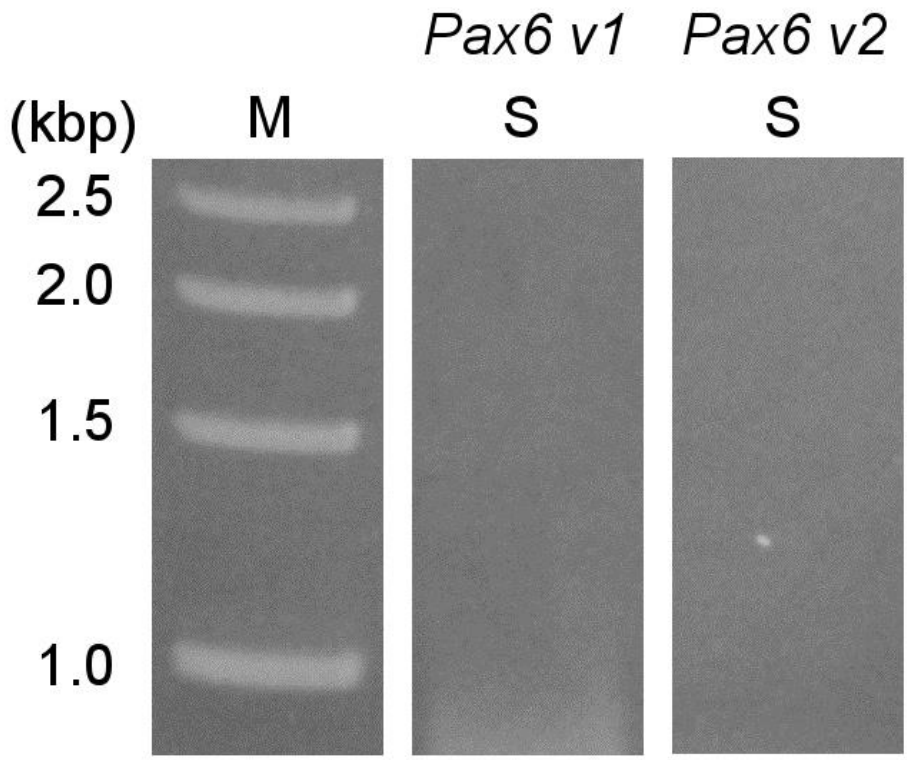

Figure 16. No product bands were detected in the choroid tissues after RPE-derived cells had been removed.

The choroid tissues after RPE-derived cells had been removed were examined by PCR with class-specific primer sets. No transcripts were detected for *Pax6 v1* and *v2*. S: sample; M: marker.

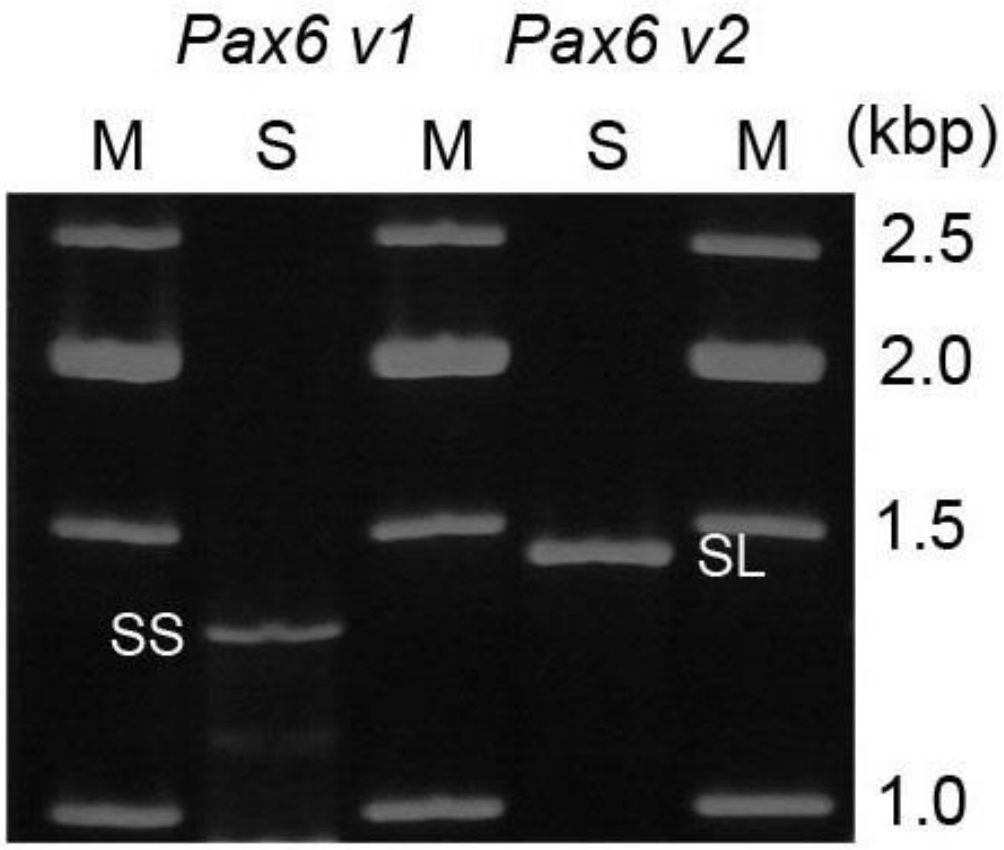

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Figure 17. Single-cell PCR was carried out with 100 solitary RPESCs.

RPE-derived cells, which still preserve their original morphological characteristics 10 days after retinectomy, i.e., RPESCs, were isolated from RPE-choroid tissues. Consistent with the results in figure 11, the only classes of *Pax6* detected in RPESCs were *Pax6 v1* and *v2*. However, under this condition, only one transcript variant was amplified for each class (*SS* for *Pax6 v1* and *SL* for *Pax6 v2*). *S*: sample; *M*: marker.

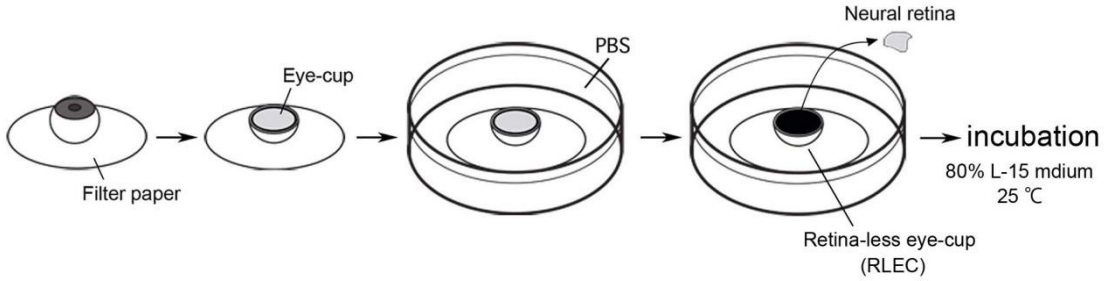

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Figure 18. The procedure to make RLEC.

The NR was removed from the posterior half (eye-cup) of the eyeball in PBS and the retina-less eye-cup (RLEC) was incubated in 80 % L-15 medium at 25 ℃

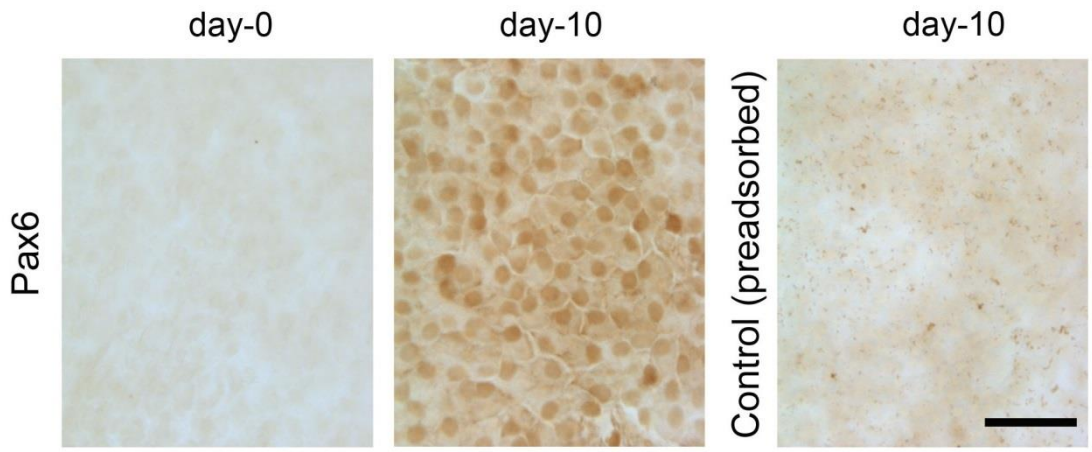

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Figure 19. Immunostaining of RPE cells in the RLECs was carried out with Pax6 antibody.
Pax6-immunoreactivity was not detected on day-0 but was observed in the nuclei of RPE cells on day-10. The right-hand panel is a negative control for day-10 with a preadsorbed primary antibody. Scale = 200 μm.

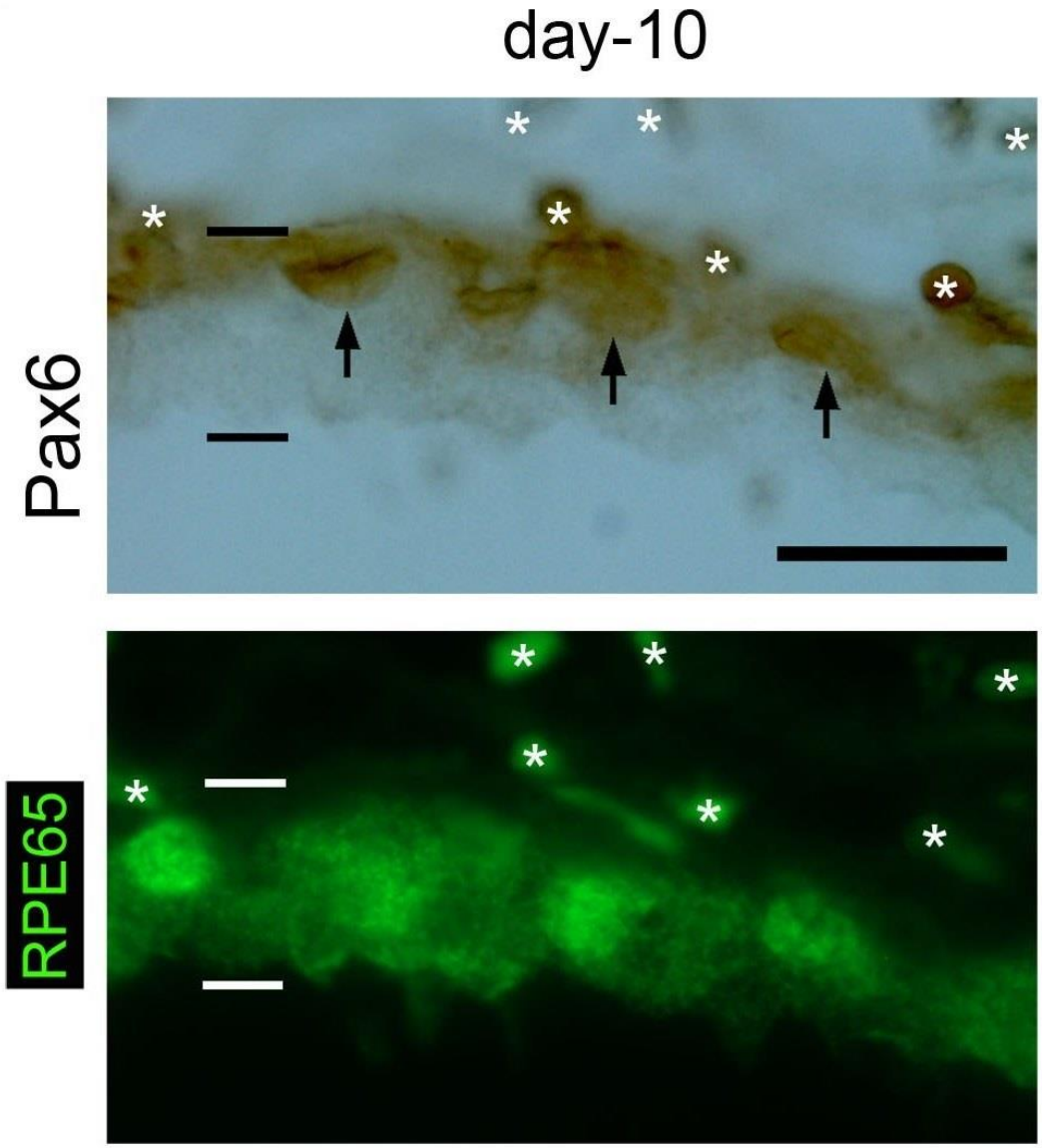

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Figure 20. Pax6-immunoreactivity in RPE cells was confirmed by double staining of a section of a day-10 RLEC with RPE65 antibody.

Nuclei of RPE cells whose cytoplasm was stained with RPE65 antibody were labelled with Pax6 antibody (arrows). Horizontal bars: thickness of the RPE layer. Asterisks: small nuclei in the choroid, which have nonspecific staining as well as autofluorescence under this condition. Scale = 40 μm.

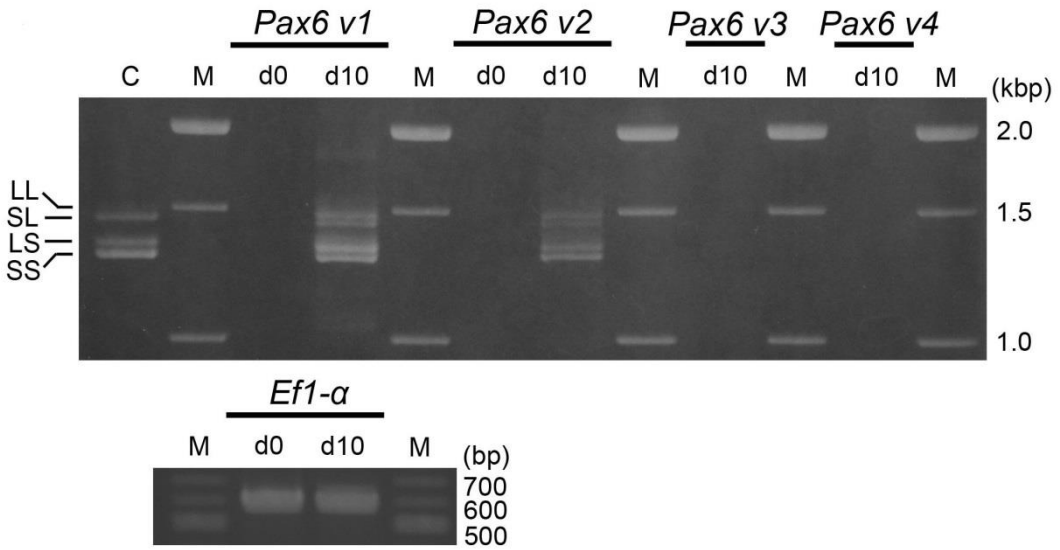

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Figure 21. PCR analysis was carried out with RPE-choroid tissues collected from the RLECs.
PCR analysis was carried out with RPE-choroid tissues collected from the RLECs immediately after the NR was removed (day-0; *d0*) and with those after 10 days of incubation (day-10; *d10*). All four transcripts of *Pax6 v1* and *v2* were newly expressed after 10 days of incubation, but for *Pax6 v3* and *v4*, no transcripts were detected, even in *d10* samples. *C*: positive control with a cDNA sample prepared from the cornea. *M*: marker.

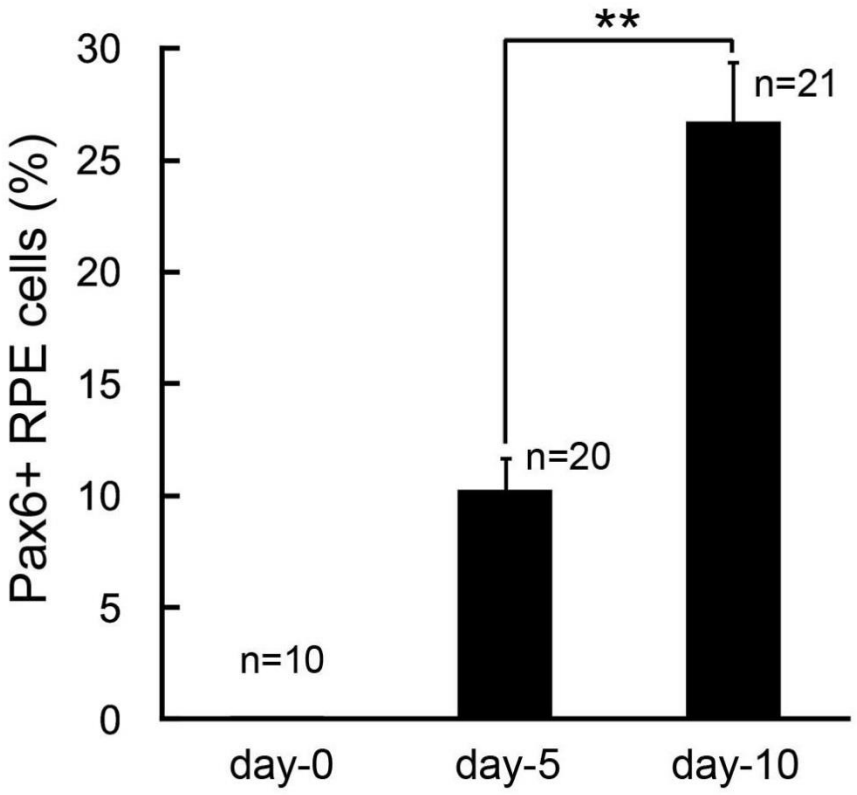

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Figure 22. Pax6-immunoreactive (Pax6+) RPE cells in the RLEC gradually increased in number upon retinectomy.

Pax6+ nuclei in the RPE appeared within 5 days and significantly increased between 5 and 10 days (Mann-Whitney *U* test, **: $p < 0.00001$). The Pax6+ nuclei were distributed uniformly. The number of nuclei was counted in the area farther than 50 μm from the peripheral margin of the RPE. Note that mitotic figures were hardly observed in RPE cells in this period.

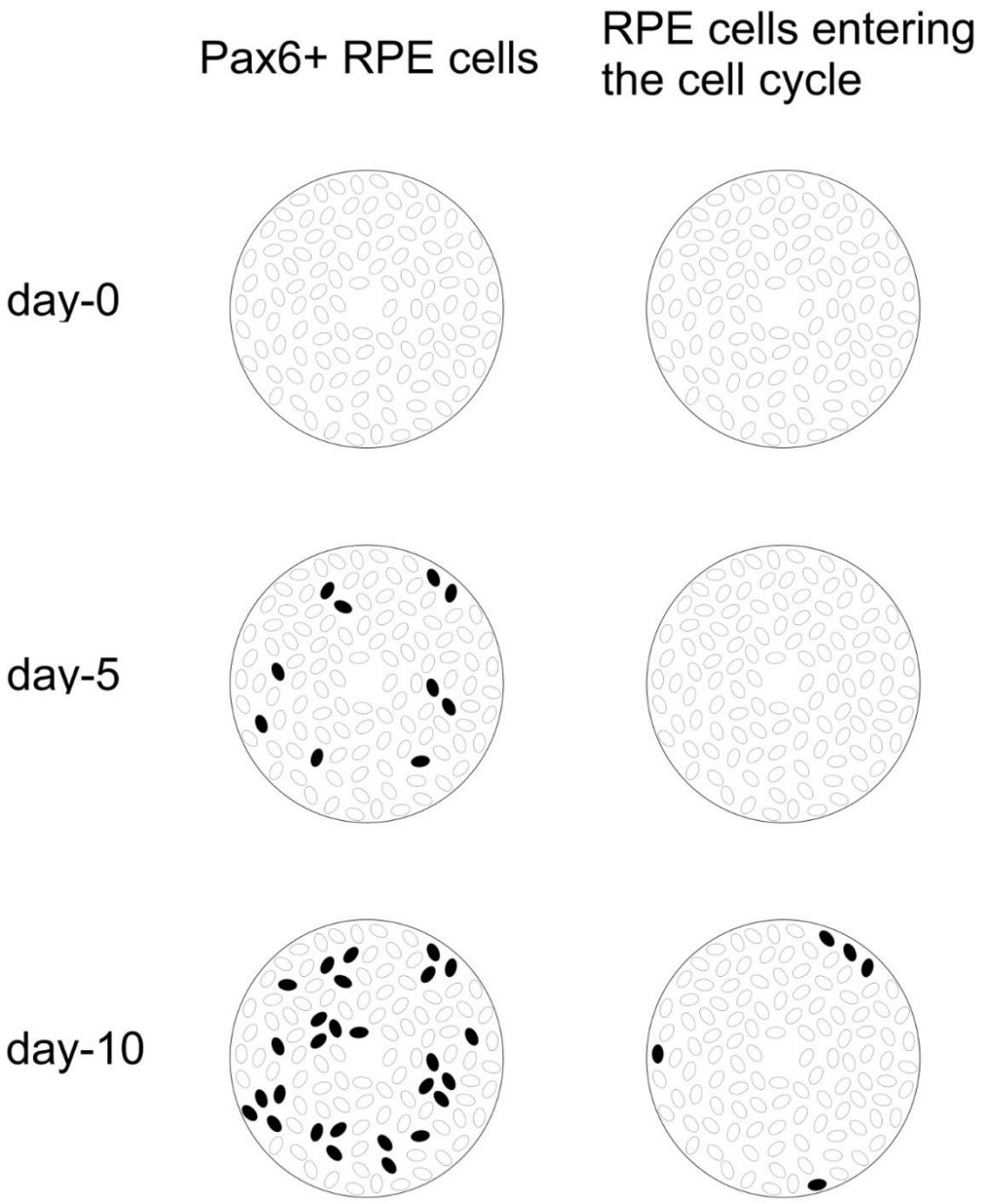

Figure 23. Spatial distribution pattern of Pax6+ cells and cells which enter the cell cycle on the same RPE sheet.

The nuclei of the RPE cells which are Pax6+ or enter the cell cycle are shown with black ovals in day-0,5,10. The range that confirmed staining at the RPE sheet is shown with large circle.

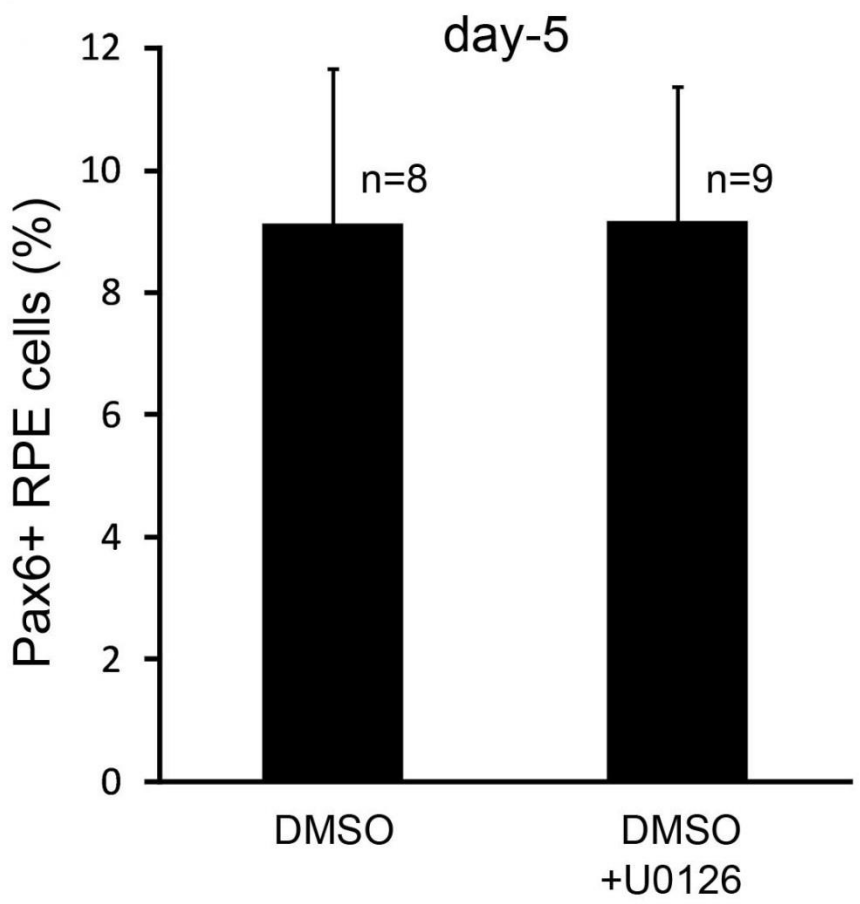

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Figure 24. Relationship of Pax6 expression and MEK1/2-ERK1/2 signaling cascade.

A MEK1/2-specific inhibitor, U0126 (5 μM), did not affect Pax6 expression in RPE cells after 5 days of incubation compared to the solvent (0.25 % DMSO) only.

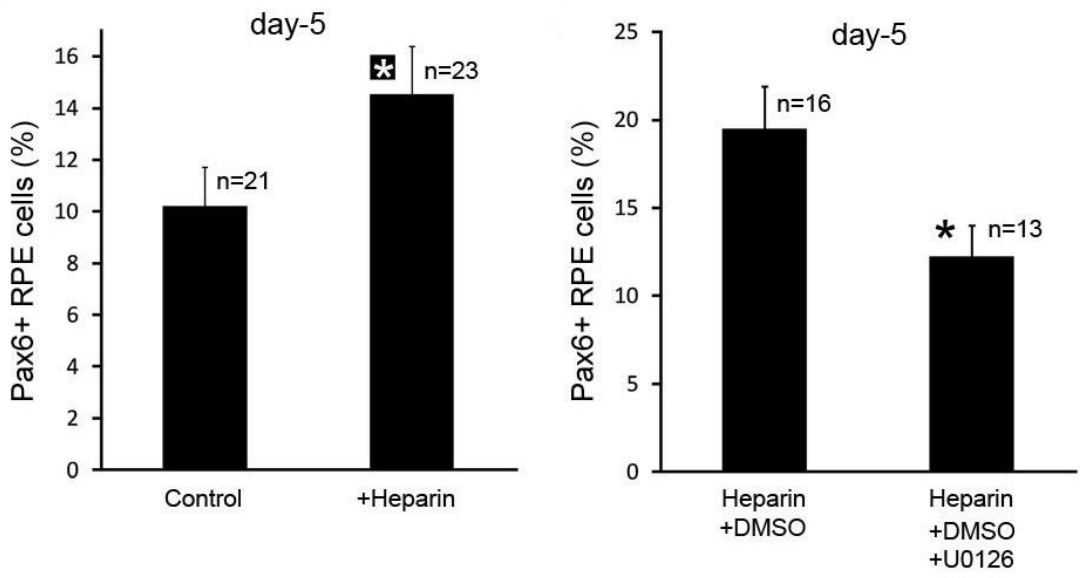

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Figure 25. Relationship of Pax6 expression and heparin.

Left: The ratio of Pax6+ RPE cells in the day-5 RLEC tended to increase in the presence of heparin (7.5 μg/ml). White asterisk: Jonckheere-Terpstra test, p=0.0421. Right: The effect of heparin on Pax6 expression in RPE cells was significantly inhibited by U0126. Asterisk: Mann-Whitney's U-test, p=0.0485.

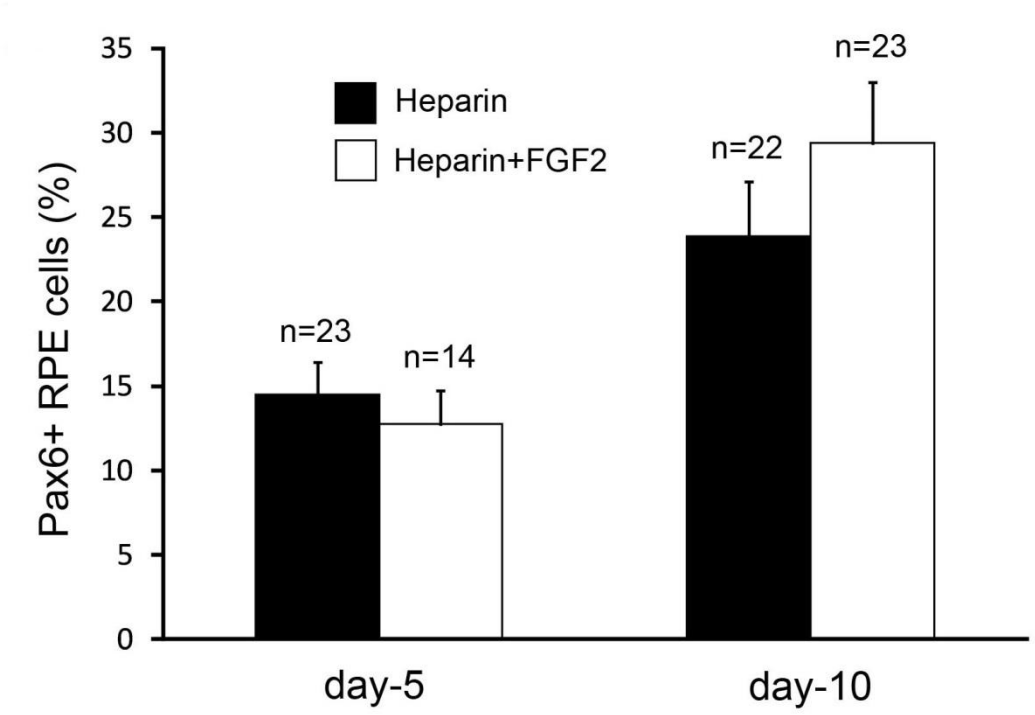

Figure 26. Relationship of Pax6 expression and FGF2.

Administration of FGF2 did not influence the effect of heparin on Pax6 expression in RPE cells after 10 days of incubation.

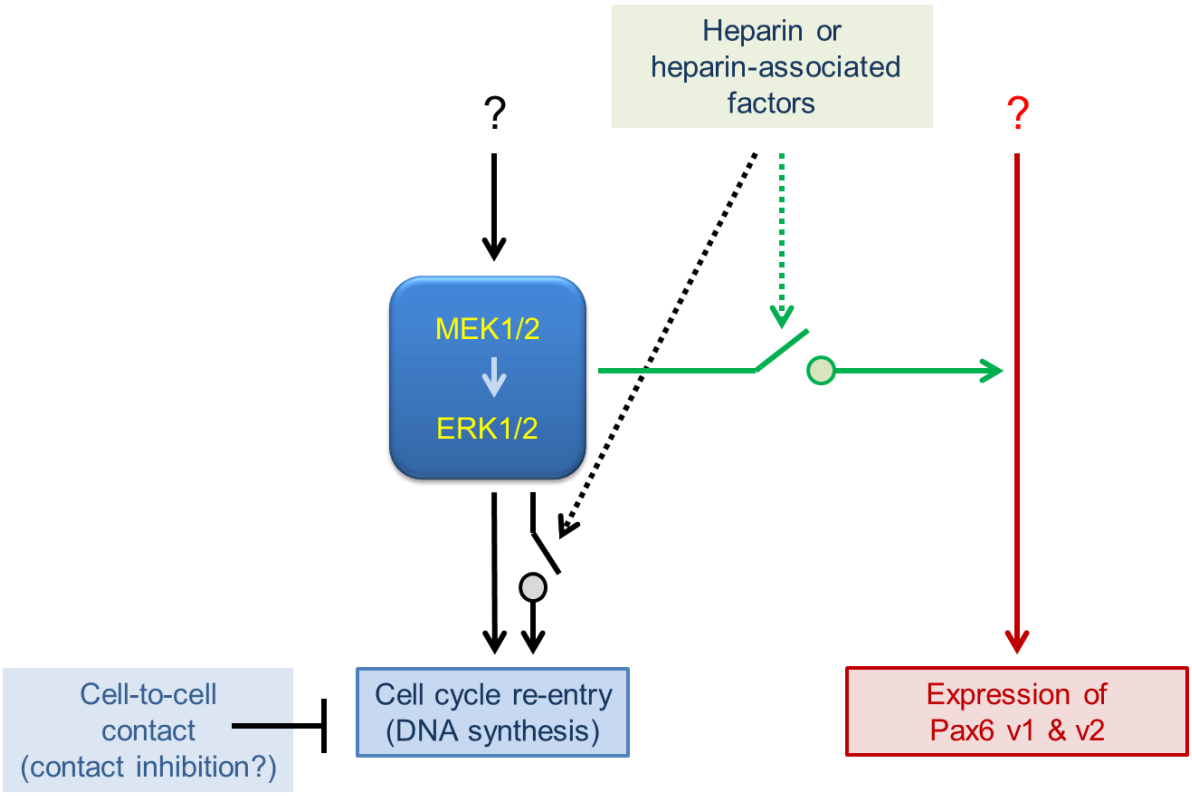

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Figure 27. Hypothetical pathways involved in cell cycle re-entry and Pax6 expression in RPE cells after retinectomy.

The pathways illustrated in red and green were predicted in this study. After retinectomy, expression of the two classes of Pax6 (Pax6 v1 and v2) in RPE cells is triggered through a pathway separate from the MEK1/2-ERK1/2 pathway, which is involved in cell cycle re-entry (Yoshikawa et al., 2012). The presence of heparin, or heparin-associated factors, seems to link two separate pathways (*turn on*), promoting Pax6 expression in a manner dependent upon MEK1/2 activity. Similar effects of heparin have been observed on cell cycle re-entry (Yoshikawa et al., 2012).

7. Tables

Table 1. *Pax6* transcripts expressed in the adult newt eye.

	NR	RPE	Choroid	CMZ	Iris	Lens	Cornea limbus	Cornea	Sclera
Pax6 v1	LL, LS SL, SS	-	-	LL, LS SL, SS	LS SS	LL, LS SL, SS	LL, LS SL, SS	LL, LS SL, SS	-
Pax6 v2	-	-	-	LL, LS SL, SS	LS SS	LL, LS SL, SS	LL, LS SL, SS	LL, LS SL, SS	-
Pax6 v3	-	-	-	-	-	SL, SS	SL, SS	SL, SS	-
Pax6 v4	-	-	-	-	-	-L, -S	-L, -S	-L, -S	-

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Table 2. *Pax6* class-specific primers and standard PCR conditions.

Class	Sense primer 5′-3′	Antisense primer 5′-3′	PCR conditions	Expected size (bp)
Pax6 v1	cgctggagcagttcaacatgcaga	gccttcaaattgtgcaaaggtcccttg	94 °C, 2 min	*LL* : 1,492
			98 °C, 10 sec 68 °C, 2 min } 50 cycles	*SL* : 1,450 *LS* : 1,341
			68 °C, 2 min	*SS* : 1,299
Pax6 v2	ggaacatgattccatgcagcacagttc	gccttcaaattgtgcaaaggtcccttg	94 °C, 2 min	*LL* : 1,496
			98 °C, 10 sec 63.3 °C, 30 sec 68 °C, 2 min } 50 cycles	*SL* : 1,454 *LS* : 1,345 *SS* : 1,303
			68 °C, 2 min	
Pax6 v3	cgagttcatggctcattagcagcacaa	gccttcaaattgtgcaaaggtcccttg	94 °C, 2 min	*SL* : 2,328
			98 °C, 10 sec 68 °C, 3 min } 50 cycles	*SS* : 2,177
			68 °C, 3 min	
Pax6 v4	atgcactttagctcacgaactcgcgaa	gccttcaaattgtgcaaaggtcccttg	94 °C, 2 min	*-L* : 2,001
			98 °C, 10 sec 68 °C, 3 min } 50 cycles	*-S* : 1,850
			68 °C, 3 min	

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