Study on the Expression of Marker Genes for Multipotent Cells in the Retinal Pigment Epithelium Cells during Retinal Regeneration in the Adult Newt

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Content

Abstract

The newt is a vertebrate endowed with an outstanding ability, even in adult stage, to regenerate a missing neural retina (NR) in the eye from the retinal pigment epithelium (RPE) cells. In this animal, upon a retinal injury RPE cells initially lose their epithelial structure and re-enter the cell-cycle. During this process, it has been inferred that RPE cells are reprogrammed into multipotent cells which eventually regenerate a new NR and renew the RPE itself. However the nature of this RPE-derived cell remains unclear because it preserves some molecules indicating original characteristics (e.g., melanin pigments and a visual cycle protein RPE65). Here, to address what cell state the adult newt RPE cells are reprogrammed into for retinal regeneration, first we identified *Small eye* Pax6 of this animal, which can be a marker of the cells that have a potency to generate both NR and RPE (e.g., retinal stem/progenitor cells and immature/uncommitted RPE cells), and examined its expression patterns in RPE-derived cells by immunohistochemistry. As a result, we found that RPE cells which just re-entered the cell-cycle do not exhibit Pax6-immunoreactivity which was obviously detected in both the optic vesicle/cup of embryos and the ciliary marginal zone (CMZ) of larval/adult eyes. Pax6-immunoreactivity appeared in a small number of RPE-derived cells just before those are sorted out into two rudiment layers (pro-NR and pro-RPE), and finally localized along the pro-NR layer. Next we investigated expression of other stem-cell markers in addition to *Pax6* in RPE-derived cells by single-cell quantitative PCR (qPCR). We found that the RPE-derived cells before cell division, which still have a cell polarity and express *RPE65*, newly express pluripotency factors (*c-Myc*, *Klf4* and *Sox2*; but not *Oct4*) and a *Microphthalmia* factor *Mitf* as well as *Pax6*, all of which are suggested to be expressed in early optic vesicles.

To confirm this result, we carried out immunohistochemistry against Sox2. However interestingly, Sox2-immunoreactivity, which became restricted in pro-NR region of the optic vesicle as its protrusion proceeds, appeared in RPE-derived cells in a similar manner to that of Pax6-immunoreactivity. Taken together, these results lead us to an implication that the adult newt RPE cells are reprogrammed into multipotent cells similar to those of the early optic vesicle while preserving certain original characteristics - in other words, the adult newt RPE cells dedifferentiate in nature - , and then those multipotent cells are specified into two cell populations from which the pro-NR and pro-RPE layers are formed with a correct polarity. Moreover, our findings give an insight into a close similarity of RPE-derived multipotent cells in the adult newt with those implicated in retinal disorders such as *proliferative vitreoretinopathy* (*PVR*) in humans, inspiring biomedical approaches to induce retinal self-regeneration through a treatment of the RPE-mediated retinal disorders.

Abbreviation

Ab.: Antibody

CMZ: Cilliary Marginal Zone

CNS: Central Nervous System

DAB: 3, 3´-diaminobenzidine

GCL: Ganglion Cell Layer

HCG: Human Chorionic Gonadotropin

IHC: Immuno Histochemistry

INL: Inner Nuclear Layer

IPL: Inner Plexiform Layer

M.W.: Molecular Weight

ONL: Outer Nuclear Layer

OPL: Outer Plexiform Layer

PBS: Phosphate Buffer Saline

P.O.: Post Operation

PFA: Para Formaldehyde

RPE: Retinal Pigment Epithelium

RPESCs: Retinal Pigment epithelium Stem Cells

RT: Room Temperature

shRNA: Short Hairpin Ribonucleic Acid

Chapter I: General Introduction

1.1 Regeneration

According to a terse dictionary definition from *Stedman's Medical Dictionary*, *regeneration* is the "reproduction or reconstitution of a lost or injured part" or "a form of asexual reproduction." In general, the restoration of lost organ or body parts to the original previous functional state is known as regeneration (Carlson BM, 2007). Regeneration is one of the most fascinating phenomena as well as the oldest field in biology having a profound historical background longer than genetics and embryology (Okada TS, 1996). However it is also one of the most complex arenas of biological research. In ancient Greek mythology, the god Prometheus stole fire from Mount Olympus for the human species he created. As eternal punishment, Zeus, the supreme god had him chained to a rock where an eagle would peck out his liver during the day, only to have it regenerate each night. The regenerating ability made Prometheus alive.

In the 20th century, researchers have discovered in their experiments that the liver actually does have the prodigious regenerative capacity described in the legend of Prometheus of the Greek mythology (Hoehme S et al., 2010; Michalopoulos GK, 2007). Virtually all species from protozoa to humans possess the regeneration capacity but their degree of regenerative ability varies greatly. Planarian, starfish and some worms can regenerate most of their body, whereas many other species are able to regenerate only parts of specific tissues. The regeneration ability depend on body size in nature; i.e. the bigger the body size, the lower the regeneration ability; conversely the smaller the body size the higher the regeneration ability. In recent years, there has been considerable interest in building missing or damaged tissues and organs in humans through the application of bioengineering principles or through the use of stem cell technology pave the way of regenerative medicine (Stocum DL, 2006; Carlson

BM, 2007). Therefore combinatorial effort and knowledge of basic science researchers and medical doctors nowadays make us to be optimistic to make the miraculous concept of regeneration into reality by developing therapies to restore lost, damaged, or aging cells and tissues in the human body.

1.2 Newt as a model animal for regeneration study

To understand the cellular mechanism and molecular network of regeneration, it is very important to select some model animals those possess this capacity. Urodeles are assumed to possess something special for that they can back into developmental pathways if necessary in any stage of their life (Richardson MK and Chuong CM, 2009). Newt, an urodele amphibian, which has a tremendous ability to regenerate a wide range of body parts: limbs (Yokoyama H, 2008) lower jaw (Kurosaka H et al., 2008), portions of the heart (Singh BN et al., 2010, Brockes JP and Kumar A, 2002), ocular tissues including retina (Mitsuda S et al., 2005; Mizuno N et al., 1999) tail and central nervous system tissues, including spinal cord (Margotta V et al., 2002) and even part of the brain (Okamoto M et al., 2007, Berg DA et al., 2010). That is why newt is known as the champion in the racing track of regeneration. Unlike other regeneration competent animal like chick, fish and frogs, newt has an obvious advantage to study regeneration as they keep this ability even in adult stage. Additionally newt's retinal neuronal cells and RPE cells are comparatively larger in size which provides us with additional benefit to observe their cellular morphological change during regeneration. Nonetheless the increasing information on newt transcriptome has revealed that adult newt is closely comparable with other higher vertebrate and mammal, which is the greatest interest to regenerative medicine (Mihaylova Y and Aboobaker AA, 2013). Moreover they are a good model organism as female can retain live sperm in her cloacafor up to five months, allowing her to be inseminated on suitable time and place. Later they have fertilisation induced through hormonal stimulation. Another advantage of this species is their slow development,

so all the key stages of ontogenesis can be observed, from the oocyteto swimming tailbud embryos or larvae. Recently it has been possible to control their metamorphosis in the laboratory which can be an obvious means to study their regeneration in a control environment (Chiba C et al., 2012). Those advantages let them to be studied as a laboratory animal and for genetic manipulation experiment in the recent days (Casco-Robles MM et al., 2011).

1.3 Why Retina?

Although newt is capable of regenerating most of their body parts, my interest is on the newt's retinal regeneration. Retina is a part of central nervous system, being indispensable of seeing processes for its unique function. Most of the adult vertebrate including human are not able to regenerate their retina after getting any injury or cell damage, however some lower vertebrate like teleost fish (Sherpa T et al., 2008; Mensinger AF & Powers MK, 1999), birds and urodele amphibian (Hitchcock P et al., 2004) can regenerate their retina. The cells sources are varied for regenerating retina depending on both the species and the developmental stages (Del-Rio Tsonis K and Tsonis PA, 2003; Haynes T and Del Rio-Tsonis K 2004; Fischer AJ & Bongini R, 2010). Briefly, retinal repair/regeneration depends on three distinct cell sources; the first one being the RPE cells, the second one neural precursor in the cilliary marginal zone (CMZ) and the last one Müller cells. In this study, I have focused on the retinal regeneration through RPE transdifferentiation.

To understand retinal regeneration it is essential to know the retinal development (**Figure 1)** and its cellular organization. The retina is highly structured tissue, which is located in the inner most layer of the eye and like other regions of the vertebrate CNS (brain and spinal cord) derived from the neural tube. In early embryogenesis, the retina originates from neuroectodermal evagination of the diencephalons, called the optic vesicle. Subsequent

invagination processes of the surface ectoderm causes lens induction and resulting in the formation of the optic cup, which initially consist of the two cellular layers. The outer layer remains a single layer of cells that accumulate melanin and form RPE, and the inner layer of the optic cup becomes a multilayered neural retinal tissue (Fuhrmann S, 2010; Jean D, Ewan K, Gruss P, 1998; Kwan KM, 2012). In all vertebrates, the neural retina contains at least five types of retinal neurons (photo receptor cells, horizontal cells, bipolar cells, amacrine cells and ganglion cells) and one type of glia (Müller cells). The cell bodies of photoreceptor reside in the outer nuclear layer (ONL) and their axons in the outer plexiform layer (OPL). Photoreceptors have two segment- the inner segment and outer segment, the later one extended into the microvilli of the RPE. The inner nuclear layer (INL) contains the nuclei of bipolar cells, the horizontal cells, the Müller cells and the majority of amacrine cells. Vertical communication between the bipolar cells and the ganglion cells takes place in the next layer, the inner plexiform layer (IPL). The ganglion cell layer (GCL) contains the cell bodies of ganglion cells as well as displaced amacrine cells. The dentdrites of the ganglion cells connects to the IPL where as their axons extends into the opposite direction to form nerve fiber that eventually make the optic nerve (Dowling JE, 1987; Masland RH, 2012). The neural retina receives, integrates and transmits visual information to the brain through the optic nerve. In newt, after surgical removal of retina, it regenerates the entire retina from a single monolayer of RPE cells. Our lab has developed an efficient tracking system for newt's RPE cells by RPE65 protein which is a marker of mature RPE cells. As retina composed of different kinds of neurons and during regeneration all neurons are derived from a single source, i.e. the RPE; it is obviously matter of interest to study how a complete tissue is formed from RPE cells (**Figure 2**).

1.4 Retinal Pigment Epithelium (RPE)

RPE and retina is derived from the same origin. During development, the distal part of the optic vesicle becomes prospective RPE (pro-RPE) while the proximal part becomes neural retina (pro-NR). When the surface ectoderm evginates optic vesicle gets optic cup structure forming two layer pro-NR i.e inner layer and pro-RPE i.e. outer layer (**Figure 1)**. The optic vesicle stage cells are bipotential in nature, i.e. the pro-RPE cells are competent to form retina (Araki M, Okada TS, 1977; Horsford DJ et al., 2005; Westenskow PD, 2010) conversely the pro-NR cells are competent to develop into the RPE (Coulombre JL and Coulombre AJ, 1965; Stroeva OG, Mitashov VI, 1983). Depending on the intrinsic and extrinsic factor RPE cells are specified and differentiated into mature state. RPE is a single layer of cuboidal-columnar cells between the retina and the choroid in the posterior portion of the eye and extends over the ciliary body and the posterior iris. Functionally, this layer is related to both the choroid and the retina. It resembles a simple cuboidal epithelium of the choroid but it has no free surface under the retina, instead attached to Bruch's membrane of the choroid tissue. Bruch's membrane is formed by a network of collagen and elastic fibers sandwiched between the basement membranes of the choriocapillaris and the retinal pigment epithelium (Skeie JM, 2010; Zayas-Santiago A et al., 2011). The apical surface of these cells have microvilli and cylindrical cytoplasmic sheaths that enclose the ends of the retinal photoreceptors to nourish the outer portions of the rods and cones and phagocytose pieces of their outer segments which are continuously shed off (**Figure 3**).

The Bruch's membrane and well developed tight junctions between RPE cells provide a blood-retinal barrier that prevents materials from passing into or out the retina unless actively transported. The pigmented RPE cells along with choroid pigment, adsorbs scattering light. There is no firm attachment between the RPE and the underlying photoreceptor layer, so the retina can detach following head trauma, causing slow death in photoreceptors unless

surgically reattached. Retina remains closely apposed to and functionally dependent upon the RPE throughout life. Besides these RPE cells are also involved in the recycling of visual cycle intermediates (Young RW, 1976; Zinn KM and Marmor MF 1979). Thus RPE always plays role as an indispensable partner of neural retina and is an inevitable part of the visionary system.

1.5 Retinal regeneration in newt

Newts can regenerate their entire retina through transdifferentiation of the retinal pigment epithelium (RPE) cells after the neural retina is completely removed by surgical means even though in the adult stage (Chiba C et al., 2006; Del Rio-Tsonis KD and Tsonis PA, 2003; Grigoryan EN, 2001; Hitchcock P et al., 2004; Mitashov VI, 1996 Raymond PA, and Hitchcock PF, 2000; Reh TA, and Fischer AJ, 2001; Tsonis PA and Del-Rio Tsonis K, 2004). Upon retinectomy, RPE cells are detached from each other as well as from the basement membrane (Bruch's membrane), while re-entering the cell-cycle, forming cell aggregates [Stage E-1: this event occurs typically between day-5 and day-10 post-operation (po); almost all of the RPE-derived cells at this stage have entered the S-phase of the cell-cycle, but do not proceed into the M-phase; notably after retinectomy the volume of the vitreous cavity decreases and Bruch's membrane, which lies along the RPE, becomes folded, because blood vessels in the choroid dilate, allowing more blood to flow in as an inflammatory-like response]. The RPE-derived cells generate two rudimentary layers (pro-NR and pro-RPE) for prospective NR and RPE [Stage E-2: day-14 po; both cell-layers at this stage have partially lost melanin pigments; cell division becomes obvious from this stage; note that the volume of the vitreous cavity and the shape of Bruch's membrane almost recover between Stage E-1 and E-2]. Cells in the pro-RPE layer exit the cell-cycle and re-initiate pigmentation, while those in the pro-NR layer continue proliferation [Stage E-3; day-19 po; immunoreactivity (IR) to RPE65 (a marker for terminally differentiated RPE cells) in both layers drops sharply

from this stage]. Thereafter, the RPE layer becomes matured (from about day-28 po, RPE65-IRre-appears and microvilli start to extend), while a new NR is formed (neuronal cell differentiation and network construction have almost been completed between day-45 and day-65 po). Retinal regeneration in adult newt has been diagramaticaly shown in **Figure 2** (modified from Chiba C et al., 2006).

1.6 Retinal regeneration in other animal model

Retinal regeneration ability after injury is limited among the organisms. Other than urodeles amphibian, fish and bird have been reported to be able to regenerate retina (Del-Rio Tsonis K and Tsonis PA, 2003). However the cell sources and degree of regeneration ability varies among animal species and their ages. Müller glia cells of adult fish harbor retinal stem cell properties that after injury can proliferate and differentiate to generate almost all retinal neuron after injury (Lenkowski JR, Raymond PA, 2013; Goldman D, 2014). Adult mammal (mouse and rat) have come into focus for their retinal regeneration capacity too although in a limited range. The source of regenerating neuron of mammals are Müller glia as well that can regenerate a limited number of retinal neuron (amacrine cell) after retinal injury however it needs to be supplemented with extrinsic factor (Ooto S et al., 2004; Karla MO, 2008). In frog system (*Xenopus Laevis* and *Rana Catesbieanna* tadpole) RPE mediated retinal regeneration has been well documented where retina is removed leaving retinal vascular membrane (RVM). RPE cells located on the back of the eye dissociate and migrate to the RVM. These migrated RPE cells then proliferate and differentiate to the neural retina while remaining RPE on the back of the eye differentiate to repair the RPE layer (Chiba C, 2014; Lee DC, 2013; Reh TA, Nagy T, 1987). Another candidate source for retinal regeneration in adult amphibian and fish are cells that reside in cilliary marzinal zone (CMZ). CMZ contains some progenitor as well as retinal stem cell other than neuron that can be differentiated into retinal neuron even in the adult age. Those CMZ cells also can take part in the retinal regeneration after

injury (Locker M 2009; Miyake A and Araki M 2014). Embryonic chick is another widely and systematically studied animal model for RPE mediated retinal regeneration. Retinal regeneration in embryonic chick is restricted at optic cup or embryonic eye stage when RPE is immature. After retinal removal in the embryonic chick, RPE cells transdifferentiate to retinal neuronal cells. RPE cells dedifferentiate and lose their pigment. These cells proliferate to make neuroepithelium which later differentiate to all neuronal cells consisting different layer of the retina. However, the polarity is reverse of the newly formed retina, that is, ganglion cells which is normally placed closest to the lens is in opposite direction (back to the eye or far away from the lens) in regenerated retina. Second thing is that the transdifferentiated RPE cells cannot replenish the RPE layer itself although they successfully generate a new retina (Coulombre JL and Coulombre AJ, 1965; Luz-Madrigal A, 2014). Moreover RPE transdifferentiation and retinal regeneration can be induced and manipulated by administering FGF1 and FGF2 (Park CM and Hollenberg MJ, 1989; Park CM and Hollenberg MJ, 1991). Thus the retinal regeneration in embryonic chick is incomplete and nonfunctional although it is RPE mediated. On the other hand, after retinectomy in newt, RPE cells lose their epithelial characteristics and undergo a numbers of anonymous genetic and molecular remodeling following dedifferentiation into retinal progenitor cells as well as redifferentiating to new RPE cells (Sakami S et al., 2005; Chiba C et al., 2006; Chiba C and Mitashov VI 2007). Thus the regeneration here is a complete and fully functional process in newt model. Nevertheless, in case of chick models they are only capable of regenerating retina from RPE in embryonic stage instead of adult stage. On the contrary newt retinal regeneration takes place from mature RPE in the adult stage. That is why newt promises an incomparable platform as a model animal to understand cellular and molecular mechanism of retinal regeneration in adult vertebrate including mammals and the RPE mediated retinopathy

in human like proliferative vitreoretinopathy and retinoblastoma, (Chiba C 2014; Sadaka A and Giuliari GP 2012).

1.7 Previous studies

It is obvious that retinal regeneration in newt is a fascinating study of modern experimental biology however a little progresses have been done to understand such an interesting phenomena in nature. Based on the accumulated evidences and surrounding repots Dr. Chiba Chikafumi and Dr. Victor Ivanovich Mitashov proposed a hypothetical pathway for newt retinal regeneration (Chiba C, 2007). In that hypothesis (**Figure 4**), it was proposed that RPE cells after 10 days (stage E1) of retinectomy (surgical removal of retina) repress their marker gene RPE65 and concurrently reprogram to acquire retinal stem cell properties by expressing new regulatory genes *Pax6* and *Msi-1*(Chiba C et al., 2006; Kaneko J, and Chiba C, 2009) at the same time preserving original characteristics like RPE65 protein expression and pigmentation (arrow 1 in Fig 3). Then cells proliferate towards the formation of retinal progenitor cells (arrow 2 in Fig 3) and renew the RPE layer (arrow 3 in Fig 3) by 14 days of retinectomy (stage E2). After 14 days retinal progenitor cells express *Pax6, Msi-1 and Chx10-1* while still keeping a minute RPE65 protein and pigment granules. When the retinal progenitor cells become 1-2 cells thick (stage E3), they express Notch-1 by 19 days po (arrow 4 in Fig 3). The E3 stage's cells hardly contain pigment granules but still have RPE65 protein. At stage I-1, when the regenerating retina reaches 3-4 cells thickness, all cells lose RPE65 protein (arrow 5 in Fig 3). Thereafter retinal cell differentiation and synapse formation follows to complete regenerating retina (arrow 6 in Fig 3). Based on the proposed hypothesis by Chiba C and Mitashov VI 2007; a question raised if RPE derived cells after 10 days po really acquire a multipotent state that can differentiate into neural retina and RPE itself. First, in this hypothesis they have addressed the 10 days po RPE-derived cells as stem like cells supported by Msi-1 expression data which was up regulated in 10 days po RPE-

derived cells while down regulated in the pro-RPE and continue up regulated expression in pro-NR, however Msi-1 is was expressed in the intact RPE cells as well. Second, the *Pax6* expression in 10 days RPE-derived cells was taken by PCR data where cDNA was prepared from the RPE-choroid tissue. Although Msi-1 and Pax6 is the marker of retinal stem/progenitor cells, it is necessitated to examine the multipotency nature of the RPE-derived cells in detail in the cellular level. This is because Msi-1 is expressed in the intact RPE cells and the Pax6 data was obtained from the cDNA derived from RPE-choroid tissue that is not from the RPE-derived cells solely.

1.8 Purpose of the study

To address the question whether RPE-derived cells after 10 days po acquire multipotent state that resemble to the cells (like early optic vesicle state cells during eye morphogenesis) which give rise to both neural retina and RPE cells, I decided to work on some molecules that are widely accepted as retinal stem/progenitor marker in the field of retina/lens regeneration as well as eye development. In this case *Pax6* is the prime candidate because *Pax6* is known to express in the cells that differentiate into both retinal neuron and RPE (Collinson JM 2000; Canto-Soler MV, Ruben A 2006; Xu S et al., 2007). Moreover *Pax6* is known as a master control gene for RPE transdifferentiation for retinal regeneration in the classical embryonic chick model (Azuma N et al., 2005). Additionally I have made an effort to screen out other candidate molecules and their expression in RPE derived cells. Multipotency factors like *Nanog*, *Oct4*, Sox2, *Klf4*, *c-Myc* and other relevant genes that might be involved in the early process of retinal regeneration were searched from our newt *de novo* assembly transcriptome database (IMORI, http://antler.is.utsunomiya-u.ac.jp/imori/). Therefore in this study I have categorized my study in the following manner-

1. To identify the *Pax6* in newt

2. To screen good antibody for newt *Pax6*
3. To evaluate expression pattern of *Pax6* in newt developing eye
4. To identify *Pax6* expression in RPE derived cells and in regenerating retina
5. To evaluate relation between *Pax6* expression and Cell-cycle entry in RPE derived cells.
6. To search and identify expression of other multipotency factors and relevant factors associated with the early process of retinal regeneration in adult newt in RPE derived cells.
7. To define what cell state does acquire the RPE-derived cells after 10 days.

Chapter II: Identification of Pax6 in newt

2.1 Introduction

Pax6 is a developmental gene found in all vertebrates and invertebrate which is a DNA binding transcription factor. In the early development, *Pax6* plays very important role in eye morphogenesis in every seeing animal (Gehring WJ, 1996; Gehrin WJ, Ikeo K, 1999; Ashery-Padan R, Gruss P, 2001; Gehring WJ, 2002; Nilsson DE, 2004). Generally, *Pax6* exists in multiple isoforms in different animals each having regulatory effect on different kind of genes in different condition (Carriere C et al., 1995; Jaworski C, et al 1997; Pinson J, 2006; Bandah D et al., 2007). Thus *Pax6* function is context dependent. *Pax6* has two DNA binding domain known as paired domain and homeo domain (Walther C and Gruss P, 1991). The isoforms which contain both paired and homeo domains are known as canonical isoforms. On the other hand there might be some paired less *Pax6* isoforms (containing no paired domain but homeo domain only) are known as noncanonical isoforms (Lakowski J, et al, 2007). Newt has four canonical *Pax6* isoforms namely LL, LS, SL and SS. Although Pax6 mRNA sequences have been reported, their functional validation is yet to be examined in this animal, i.e. whether Pax6 function is conserved in newt as well (Mizuno M et al., 1997). That is why it is necessary to observe Pax6 function in this animal. Currently gene knock down through *shRNA* means is a popular tool in modern biotechnology. Here in this study I applied *shRNA*-transgenic method for knocking down Pax6 gene in newt to see how this affects eye development in this animal. I also screened good antibody for newt that can efficiently indentify canonical Pax6. Canonical Pax6 is believed to be an essential transcription factor for the bipotential cells of optic vesicle that generate neural retinal progenitor cells as well as RPE progenitor cells during development (Lakowski J et al., 2007).

2.2 Methodology

2.2.1 Western blotting

Conventional immunoblot analysis were performed with the following antibodies; the primary antibody: rabbit anti-Pax6, dilution (1:500; PRB-278P, Convance, Richmond, CA); rabbit anti-Pax6, dilution (1:500; PA-801, Affinity Bioreagents); chicken anti-Pax6, dilution (1:1500, Operon Biotechnologies, K.K.); mouse monoclonal anti-Pax6, dilution (1:250; AD-2.38, sc-32766, Santa Cruz Biotechnology); and the secondary antibodies: biotinylated anti-rabbit goat IgG, dilution(1:500; Vector); biotinylated anti-chicken goat IgG (1;500; Vector) and biotinylated anti-mouse goat IgG (1:500; Vector)

Protein isolation

Five newts were anesthetized with FA-100 (4-Allyl-methoxyphenol, Tanabe, Osaka, japan) for two hours then they beheaded and their head was rinsed in 70% ethanol. The eye balls were enucleated and collected into PBS×1 (ice cold). Then the anterior half of the eye was cut out and the posterior half (eye-cup) was placed on the nitro cellulose filter membrane (0.42 µm pore size, Millipore). Ten retinas were collected from eye-cups into eppendorf tube (1.5 mL) containing PBS×1 (ice cold). Then PBS×1 was removed as much as possible from eppendorf tube and lysis buffer (ice cold) [25 mM Tris, 150 mM NaCl, 1 mM EDTA · 2 Na, 1% Igepal CA-630, 1% proteinase inhibitor cocktail (P-8340, Sigma), 1% Sodium deoxycholate, 0.1% SDS, pH 7.5] was added 7µL per retina. The retina tissues were frozen with liquid nitrogen then sonicated for five minutes with sonicator (Iuchi, Vs-70U) filled with ice cold water. After sonication sample were spinning down. This freezing, sonication and spinning down process were done two times then finally centrifuged at 12000 rpm at 4° C for 10 minutes. Supernatants were collected into different tube (1.5 mL) and equal volume of 2× sample buffer [100 mM Tris-HCl (pH 6.8), 4% SDS, 20% Glycerol, 0.01% (w/v)

Bromophenol blue (Wako, Japan), 10% β- Mercaptoetanol (Sigma)] was added and mixed quickly. The samples were boiled in hot water for 5 minutes then allowed to be cool and was stored at -20° C for western blot analysis. Similar technique was applied for protein extraction from larvae. Additional care was taken during protein collection because a foamy layer was formed on the top after centrifugation. So protein was collected from the middle where a clear solution was found.

Blotting

Proteins were separated on a 10% gradient gel (Ready Gel J and Mini-Protean, Bio-Rad) by SDS-PAGE and transferred to a PVDF membrane (Immun-Blot PVDF membrane for protein blotting, Bio-Rad) using Bio-Rad mini trans-blot cell (153BR25841). The blots were rinsed in TBST [100 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20] for 10 minutes and incubated in a blocking solution (2% Normal goat serum and 2% skim milk in TBST) containing Avidin D (1:50; Avidin/Biotin Blocking kit, Vector) for 1 hour. After rinse with TBST, the membrane was incubated with primary antibody, diluted in blocking solution containing Biotin (1:50; Avidin/Biotin Blocking kit, Vector) overnight at 4° C. The membrane was washed three times (15 minutes each) in TBST and incubated with the secondary antibody diluted in blocking solution for 90 minutes at room temperature. After incubation the membranes were washed again three times (15 minutes each time with TBST and then incubated with a mixture of Avidin/Biotin complex solution (Vectastain ABC Elite kit, Vector) for 30 minutes and reaction was visualized by DAB substrate kit (Vector). The negative control data was obtained by omitting the primary antibody (only blocking solution and Avidin D, 1:50).

2.2.2 Immunohistochemistry (IHC)

Normal eyeballs were enucleated from cold shocked newts and fixed in Zamboni's fixative solution for 5 hours at 4°C. The eyeballs were then washed at 15 minutes (twice), 30 minutes once, 1 hour and 2 hours interval once each time with the ice chilled PBS×1. After washing, eyeballs were immersed in 30% sucrose (Wako, Japan) in cold PBS×1 solution and kept in the refrigerator at 4°C for overnight. In the following day, the eyeballs were submerged in OCT (Sakura tissuetek, USA, Inc.) solution and made frozen inside the coldtom (Sakura, CM-41) at -35°C, and then frozen eyeballs were stored at -80°C for cryosection. The cryosection was made transversely at about 20 µm thickness. In case of embryos/larvae of different developmental stages, the fixation time was applied for 8 hours in Zamboni's solution and special care was taken to prevent any damage during washing and freezing the sample. In this study developmental stages of newts were determined according to Okada YK and Ichikawa M, 1947.

Conventional immunoperoxidase labeling was performed with the following antibodies. For the primary antibody: mouse monoclonal anti-Pax6, (1:100; AD-2.38, Santacruz biotech); Cy-3 conjugated anti-GFAP (1:2000, C9205, Sigma; Susaki K and Chiba C, 2007); mouse monoclonal anti-RPE65 (1:500; MAB5428, Millipore, Darmstadt, Germany).

For the secondary antibody: biotinylated anti-mouse goat IgG (1:500; Vector), tetramethylrhodamine-conjugated anti-mouse goat IgG (1:200; T2762; Life Technologies, MD 20850, USA). DAPI was used for cell nuclear staining (1:50,000; D1306; Life Technology). For double staining with RPE65 and DAPI: section was first stained with RPE65 antibody then counter stained with DAPI.

Peroxidase staining

Sections were rinsed in PBS×1, 0.5% Triton X-100 (Wako, Japan) in PBS, and PBS for 15 minutes each then incubated with 3.3% H_2O_2 in methanol for 10 minutes followed by a washing for 5 and 10 minutes with PBS×1 and incubated for 2 hours in a blocking solution (3% normal goat serum and in 0.3% Triton X-100 diluted in PBS) containing Avidin D (1:50; Avidin /Biotin Blocking Kit, Vector) at RT. The samples were washed for 5 and 15 minutes respectively with PBS×1 then incubated with primary antibody diluted in blocking solution containing biotin (1:50; Avidin/Biotin Blocking Kit, Vector) for ~15 hours (over night) at 4°C. For negative control, the sample was incubated with blocking solution only with biotin (1:50; Avidin/Biotin Blocking Kit, Vector), i.e. no primary antibody. The following day, samples were washed thoroughly in PBS×1, 0.5% Triton X-100 (Wako, Japan) in PBS×1, and PBS×1 for 15 minutes each then incubated in secondary antibody diluted with blocking solution for 4 hours at RT. After a complete wash (in PBS×1, 0.5% Triton X-100 (Wako, Japan) in PBS×1, and PBS×1 for 15 minutes each then incubated), the samples were incubated in a mixture of Avidin- Biotin complex (Vectastain ABC Elite Kit, Vector) for 90 minutes. The ABC (2% each in PBS×1) complex solution was prepared 30 minutes before use. The sections were washed with PBS×1 for 15 minutes (3 times) and the reaction was visualized with DAB substrate Kit (Vector). The DAB solution was prepared in MilliQ water (200 µL MilliQ water, 4 µL Buffer, 8 µL DAB stock solution, 4 µL H_2O_2). Sections were then washed thoroughly [in PBS, 0.5% Triton X-100 (Wako, Japan) in PBS, and PBS for 15 minutes each then incubated] and fixed in 4% PFA in PBS×1 for 15 minutes at RT. This step was performed for depigmentation of RPE cells layer. Rinse in PBS for 5 and 10 minutes respectively and incubated with 15% H_2O_2 /1.5% Sodium Azide (Wako, Japan) in PBS×1 for 4 hours at RT. At last samples were washed with D.W. for 15 minutes twice and mount on the slide glass with 90% glycerol in PBS×1 solution.

2.2.3 Preparation of *Pax6 shRNA*-transgenic construct

Based on the mRNA sequence of *Pax6* (DDBJ/GenBank Accession#: D88741) reported by Mizuno, M et al., 1997, I designed two *shRNA* in the region as every isoform of Pax6 can be knockdown (**Figure 5**).For negative control, I designed another *shRNA* from newt *β-crystalline* promoter (DDBJ/GenBank Accession#: AB113881).

The following three primers containing two restriction site-*MunI* (aattg) *and HpaI* (gtt) were dissolved in TE buffer to make a concentration as 250 pMol/µL.

Pax6-shRNA 1 (Forward):

attgAACATGCAGAACAGTCATAGCGGgaagcttgCCGCTATGACTGTTCTGCATGTTttttt
gtt

Pax6-shRNA 1 (Reverse):

aacaaaaaAACATGCAGAACAGTCATAGCGGcaagcttcCCGCTATGACTGTTCTGCATGT
Tc

Pax6-shRNA 2 (Forward):

aattgAACAGTCATAGCGGAGTCAACCAgaagcttgTGGTTGACTCCGCTATGACTGTTttt
ttgtt

Pax6-shRNA 2 (Reverse):

aacaaaaaAACAGTCATAGCGGAGTCAACCAcaagcttcTGGTTGACTCCGCTATGACTGT
Tc

Pax6-shRNA negative control (Forward):

attgAACGACGCCTGTCAGGAACTTCTAgaagcttgTAGAAGTTCCTGACAGGCGTCGTT
Tttttgtt

Pax6-shRNA negative control (Reverse):

aacaaaaaAAACGACGCCTGTCAGGAACTTCTAcaagcttcTAGAAGTTCCTGACAGGCGT CGTTc

For each set of *shRNA* 40 µL of forward and reverse primers were mixed and incubated for 5 minutes at 70°C. Then 20 µL annealing buffer (pre-heated at 70°C for 5 minutes) was added to the oligo mixture to make the volume 100 µL in a PCR tube and incubated for 5 minutes at 70°C followed by 1 hour incubation at room temperature resulted 100 µM of adapter molecule (about 1 µg/ µL). The resulting adaptors were ligated using the ligation kit (Nippon Gene) into the pACGGs-mCherry-pA (Clontech EGFP-N1 vector modified version and a kind gift from Dr. Elly Tanaka, Molecular Cell Biology and Genetics, Max Planc Institute, Leipzig, Germany) cassette between *MunI* and *HpaI* restriction site (**Figure 6**). For ligation reaction, 2 µL MilliQ water, 0.5 µL vector DNA (1 µg/ µL) which was cut open with *MunI* and *HpaI* and 2 µL insert DNA (adaptor) and 5 µL ligation solution (2X) were mixed and incubated for 5 minute at 16 °C. After ligation, these three constructs were transformed into the JM109 cells (Takara) and cultured with standard technique. The pCAGGs-mCherry-shRNA-pA plasmid was purified using maxi kits (Qiagen, Valencia, CA), recovered in nuclease free water and stored at -80°C.

2.2.4 Collection and preparation of fertilized newt eggs for microinjection

The female newts were injected with 50 µL Human Chorionic Gonadotropin (HCG; equivalent to 30 U) into the intra peritoneal space every other day. HCG helps to mature the egg. After three consecutive injections, newts start to lay the fertilized egg (Casco-Robles MM et al., 2010). Plastic sheets were put on the water into the newt tank as an imitation of plant leaves where they usually laid eggs. In the early morning of the following day eggs (stick on the plastic sheets because of jelly) were collected in a plastic box containing ice-

cold water (low temperature slows down egg differentiation). The jellied eggs were carefully isolated from the plastic sheets and kept them into a plastic cup containing cold 0.5X MHS placed into the ice.

After isolation, eggs were sterilized by washing with 70% ethanol followed by washing with 0.5X MHS three to five times. 0.5X MHS was exchanged with the dejelly solution [2% (w/v) thioglycolate in 0.5×MHS (MHS stock solution was prepared with70 g per liter NaCl, 1 g per liter KCl, 2 g per liter CaCl2 and 4 g per liter MgCl2·6H2O. It was stored at room temperature (22–24 °C) and used within 6 months] to remove the jelly capsule on egg. Eggs were transferred with sterile wide-bore pipette immediately after dejelling into the fresh 0.5X MHS to a separate plastic cup. To remove the jelly debris from the eggs it was carefully washed with 0.5X MHS several times. The one cell stage eggs with no damage were then placed into the well of Terasaki plate (one egg per well) filled with the microinjection medium. After transferring eggs into terasaki plate, it can be stored at 4°C for 1-2 hour/s.

Micro injection solution was prepared as follows*: I-SceI* buffer (10X) 1 µL, Phenol red (0.1%) 1 µL, *I-SceI* plasmid (1 µg/µL) 2 µL, I-SceI (5 U/ µL) 1 µL and Milli Q water 5 µL to make the total volume 10 µL. Then the solution was incubated at 37°C for 40 minutes for the activation of I-SceI meganuclease enzyme (**Table 1**).

During the incubation of microinjection solution, injection needle was prepared as described (Casco-Robles MM et al., 2010). One drop of microinjection solution was placed on the parafilm sheet and sucked 2 µL solution into the injection needle through its tip. Terasaki plate filled with eggs was placed under stereomicroscope and 4 nL solution was injected per egg. After injection eggs were kept at 14°C.

2.2.5 Rearing transgenic embryo and larvae

After two days of injection, eggs were carefully transferred from terasaki plate to agarose coated petridish containing 0.5X MHS that had been sterilized using syringe filter (0.2 µm pore size). Embryos were kept at 14°C until they reached at stage 35. The rearing solution was exchanged every other day. mCherry fluorescence was generally first observed in blastula stage and become stronger in neurola stage. During rearing, eggs with no expression or some damages, dying eggs were discarded. At stage 22 (tell-bud), the rearing solution was changed to 0.1X MHS from 0.5X MHS. Solution was exchanged every other day and embryos were regularly checked if there was dying or abnormal one. In case of dying or abnormalities, embryo was immediately removed from the dish and changed the solution to avoid microbial growth. When embryos grew to stage 35, they were transferred to a multi-well plate (one embryo per well) and reared them at room temperature.

2.3 Results

2.3.1 Detection of Pax6 isoforms by immunoblot

Pax6 protein bands were detected from newt's retinal proteins sample by immunoblot. So far four canonical isoforms of Pax6 were reported in embryonic newt retina (Mizuno M et al., 1997) and those were LL, SL, LS and SS (**Figure 7**). The epitop of rabbit anti-Pax6 (PRB) are present on the c-terminal region of LL and SL isoforms. This antibody bound two canonical Pax6 isoforms (M.W. 49 kD and 47 kD) namely LL and SL. Moreover some low molecular weight protein bands were also detected (**Figure 8**).

LS/SS, chicken anti-Pax6 (Operon Biotechnologies, K.K.), also a C-terminal binding antibody, whose epitope is on the C-terminal region of LS and SS isoforms of newts Pax6. LS/SS antibody detected another two canonical isoforms of Pax6 (M.W.~50 kD and 45 kD)

namely LS and SS in newt's normal retina. Interestingly, LS/SS also bound some low molecular weight proteins bands (**Figure 8**). Recently many researchers have reported paired-less/short fragment of Pax6 in different animal model, however with ambiguous function (Ninkovic J et al., 2010; Kim J and Lauderdale JD 2008; Kim J and Lauderdale JD 2006; Short S and Holland LZ 2008; Bandah D et al., 2007). Another C-terminal binding antibody, PA1, whose epitope also iss on the C-terminal region but close to the homeodomain (**Figure 7**). Theoretically, this antibody was expected to bind all four newts Pax6 canonical isoforms, however it could bind only LS and LL isoforms (**Figure 8**). On the other hand AD2.38, a mouse monoclonal anti-Pax6 (Santa Cruz Biotech) antibody, whose epitope is on the N-terminal region i.e. in paired box containing region (**Figure 7**). It bound at least three canonical isoforms LL, SL, SS of newt's Pax6 protein extracted from normal retina (**Figure 8**). The most important feature of this antibody is that, it did not bind any low molecular weight Pax6 related protein/pairedless/noncanonical Pax6. The results were compared with negative control data (**Figure 8,** control).

2.3.2 Pax6 immunolocalization in newt normal retina with AD 2.38 by IHC

Mouse monoclonal anti-Pax6 Ab. (AD2.38, Santa Cruz Biotechnology) was successfully able to localize Pax6 expressing cells in newt retina of the normal eye-ball section. Most cells in the GCL were stained. However the staining pattern of all the cells was not similar. Almost all the amacrine cells, some horizontal cells were found to be stained in the INL (**Figure 9**)**.** The staining intensity of horizontal cells was weaker than that of other cells. To be surprised, it reacts with the Müller glial cell body which in zebrafish plays a crucial role in injury induced retinal regeneration (Thummel R et al., 2008). All the observations were compared with the control data (**Figure 9**, C). Besides this, the retinal progenitor/stem cells that reside in CMZ also positively stained for Pax6 however intensity is higher in progenitor cells than that of the stem cells.

2.3.3 *shRNA*-transgene construct knockdowns Pax6

As *Pax6* is a developmental regulatory gene, the knockdown effect is vivid in newt as has been observed in other animals (Hill RE et al., 1991; Grindley JC, 1995). I found mainly three phenotypes of that small eye in general, and in most severe cases eyeless and head less (**Figure 10**). I also observed that *shRNA-2* is more effective than *shRNA-1*. The eggs that were injected with *shRNA-2* resulted into all three phenotypes where as those injected with *shRNA-1* resulted into only small eye phenotype. Moreover *Pax6* knockdown affects the normal developmental process of newt in terms of their abnormal body formation that includes bending body, small size, atypical tail etc.

In the section of head of those three phenotypes I observed small eye with tiny lens and retina in small eye phenotype where as I could not find any eye field in the eye less phenotype comparing with the control group where I observed normal eye development as like as normal embryo (**Fig 11**). For this observation I set a parameter that is m-Cherry fluorescence expression intensity. I divided all the injected embryo into three groups based on the intensity of fluorescence: strong (Fluorescence>60); moderate (Fluorescence>40<60); and week (Fluorescence<40) **Table 2.**

To determine what extent *shRNA* was able to knockdown Pax6 protein expression in newt, I carried out western blot analysis for Pax6 protein extracted from eyeless and headless animal and compared with animal treated with control *shRNA*. I observed no protein band in eyeless and headless animal where as I detected two protein bands in control group i.e. SL, SS (**Fig 12**).

2.3.4 Pax6 expression in developing retina and RPE cells

During development, like other vertebrate newt also follows a similar cascade of events for eye morphogenesis. Neural ectoderm which is the primary cell source of both neural retina and retinal pigment epithelial (RPE) cells. All most all cells of early optic vesicle (Stage-23) expressed Pax6 evenly. In late optic vesicle (Stage-24) where proximal and distal regions are known as pro-neural retina and pro-RPE expressed Pax6 as an equivalent manner. Pax6 expression was also observed both in pro-neural and pro-RPE in eye cup (Stage-29) and in lens epithelium. This expression become prominent in the proliferating neural progenitor, differentiated ganglion cells, iris pigment epithelium cells compared to other retinal neuronal cells and expression obviously started to decrease in the RPE cells in embryonic eye (Stage-32). In late embryonic eye (Stage-37), Pax6 expression, for the first time disappear completely from the RPE layer while keeping expression in differentiated ganglion cells, amacrine cells, horizontall cells, Müller glia, lens epithelium, cornea as well as in ciliary marginal zone in embryonic eye i.e. Stage- 43 (**Figure 13 and 14**). *In situ* hybridization with newt eye tissue also has been reported to express *Pax6* in neural retina, lens, cornea epithelium as well as in developing human eye (Rio-Tsonis KD, 1995). RPE65, a marker protein for mature RPE cells was first immunolocalized in early embryonic eye (Satge-35) in the centre region and was localized along the RPE line at stage- 43 (**Figure 14**).

2.4 Discussions

2.4.1 AD2.38 (Mouse monoclonal anti-Pax6 Ab.) is compatible for newt Pax6

Four canonical Pax6 isoforms, namely LL, LS, SL and SS, have been reported in the embryonic newt eye by Mizuno M et al., (1997). In the present study, I used a chicken polyclonal anti-Pax6 antibody to newts LS/SS isoforms as well as I bought two commercially available antibodies PRB and PA1 as part of searching good probe. Then I checked their

compatibility to newts Pax6 by immunoblot analysis. Newts Pax6 was detected successfully by two Abs., PRB and LS/SS however, PA1 was found less compatible for newts Pax6 in immunoblot analysis. PRB bound two canonical isoforms, LL and SL of molecular weight 49 kD and 47 kD respectively. On the other hand LS/SS was able to bind also two another canonical isoforms, LS and SS of molecular weight ~50kD and 45kD respectively. Moreover, both of these two C-terminal binding antibodies (PRB and LS/SS) also bound some low molecular weight protein. On the other hand in immunoblot analysis, AD2.38 efficiently bound at least three newts canonical Pax6 isoforms. In addition, AD2.38 did not react with any low molecular weight proteins which revealed its high specificity for canonical Pax6 isoforms only.

Consequently, I used AD2.38 for the detection of Pax6 expression pattern focusing on the RPE, cilliary marginal zone (CMZ) and normal retina with IHC. This N-terminal binding antibody looked very efficient to localize Pax6 in the CMZ cells and retinal neural cells (ganglion cells, amacrine cells, bipolar cells and some horizontal cells also). Conversely, RPE cells were not detected as Pax6 positive.

Retinal stem/progenitor cells that reside in the CMZ of some adult vertebrates (Tropepe V et al., 2000; Kubo F et al., 2003; Moshiri A et al., 2005) showed canonical Pax6 expression (**Figure 9**). So far, AD2.38 was found as more reliable tool for the detection of canonical pax6 in the regenerating retina, especially for RPE-derived stem-like cells. Taken together all the observations, I was encouraged to use AD2.38 as a marker antibody for the identification of Pax6 in the developing eye as well as early to regenerating retina by IHC.

2.4.2 *shRNA*-transgenic method is an effective means for knocking down *Pax6* gene functions

Pax6 shRNA which I designed based on the mRNA sequence reported by Mizuno M et al., 1997 showed effectively knockdown Pax6 in newt. That is why I observed small eye newt and in severe case eye less and abnormal-head animal. This result reveals *shRNA*-transgenic means can be a useful technique to manipulate genes functions as like *Pax6* in this animal model as mammal (Peng S et al., 2006). I also observed abnormal development in the anterior posterior axis in animal as reported in other animal model (Li Y et al., 2000). I performed western blott analysis with the protein of control *shRNA* injected animal and *shRNA-2* injected animal (eyeless and head less). Western blot data also suggest that eye formation as well as brain and normal development is sharply affected by the degree of *Pax6* knockdown with the *shRNA*. In this study I found that *shRNA-2* is more effective than *shRNA-1*. In case of *Pax6 shRNA-1,* 33.3% and 75% animal having small eye showed moderate and strong fluorescence respectively. On the other hand *shRNA-2* showed more severe effect: 33% and 50% having strong fluorescence were headless and eyeless respectively (**Table 2**). The eye/head section revealed that Pax6 knockdown directly affect eye morphogenesis in this animal as well as brain development (**Fig 11**) which suggest that the reported *Pax6* mRNA sequence (Mizuno, M. et al, 1997) is evolved from the same locus and it is functionally conserved in newt like other vertebrate including mammals (Matsuo T et al., 1993).

2.4.3 Pax6 expression is conserved in newt

During early vertebrate development, cells of the ectoderm invaginate to give rise to the neural tube. The cells of the neural ectoderm (the cells that surround the neural tube) then grow outward on either side, producing the optic vesicles (**Figure 1**). Contact and molecular signaling between the optic vesicle and the surface ectoderm induces the formation of the

lens from the surface ectoderm (**Figure 1**). In addition, signals from the surface ectoderm including fibroblast growth factor 2 (FGF2) induce the inner layer of the optic cup to become neural retina (Pittack C et al., 1997) and several transcription factors including *Pax6* and *Chx10* are essential for neural specification (Belecky-Adams T et al., 1997). On the other hand, the outer layer of the optic cup is under the influence of factors from the underlying mesenchyme such as activin which in turn could activate transcription factors like *Microphthalmia* factor (*Mitf*) that are able to specify the RPE fate (Fuhrmann S et al., 2000). I examined the the expression pattern and regulatory role of developmentally important transcription factor *Pax6* in the processes of retina and RPE formation and their maturation.

Linage tracing experiment in *Xenpus laevis* has been reported that retina, RPE and lens arise as a consequence of cell-autonomous function of *Pax6* (Chow RL et al., 1999).In newt embryonic/larval development, expression patterns of Pax6 were highly conserved, as anticipated. Pax6-immunoreaction (Pax6-IR) was observed in (i) the neuroepithelial cells of the early optic vesicle (St. 23), (ii) the pro-NR region in the optic vesicle (late stage; St. 24)/cup (St. 27), (iii) retinal progenitor cells in immature retinas (St. 30); (vi) the CMZ, (v) the pro-RPE region in the optic vesicle (late stage; St. 24)/cup (St. 27), (vi) immature RPE cells which had melanin pigments but not RPE65-imminoreactivity (St. 30), as well as (vii) some types of NR cells (ganglion, amacrine, horizontal and Müller glia cells). A similar expression pattern was observed in other vertebrate also (Hitchcock PF et al., 1996). From this observation it is obvious that a relation between RPE maturation and Pax6 expressison was conserved in newt as was reported in other vertebrate that *Pax6* together with *Mitf* is essentially required to activate the downstream genes for melanogenesis in RPE (Bäumer N et al., 2003; Raviv S et al., 2014). Therefore Pax6 is required for the lineage specification until immature RPE but disappear in mature one or *Pax6* down regulation occurs in RPE cells

for their maturation but continuous expression is necessary for the retinal neuronal cells differentiation and specification.

2.5 Data analysis

In the transgenic analysis, bright-light and fluorescence images of embryos/larvae were acquired using a digital camera (C-5060; Olympus, Japan) attached onto a fluorescence stereomicroscope (Leica M165 FC) with a filter set for mCherry (Exciter: XF1044, 575DF25; Emitter: XF3402, 645OM75; Opto Science, Japan). Embryos/larvae with visible red fluorescence throughout the body were selected, and then subdivided into 'weak', 'moderate' and 'strong' on the basis of their fluorescence intensity; fluorescence intensity was estimated as the average luminance by analyzing the fluorescence images of embryos/larvae which were acquired with a 40x objective lens, using a function of Photoshop Extended CS5 graphics software (Adobe); embryos/larvae with an average luminance value of 40-60 were categorized as 'moderate', and those with higher and lower values were categorized as 'strong' and 'weak' respectively. For the immunohistochemical analysis, bright light and fluorescence images of tissue sections were acquired using a CCD camera system (DP73; cellSens Standard 1.6; Olympus) attached to a fluorescence microscope (BX50; Olympus).

Chapter III: Pax6 expression in RPE-derived cells and in regenerating newt retina

3.1 Introduction

It is believed that the adult newt RPE cells dedifferentiate by losing their original characteristics as iris pigment epithelium (IPE) cells do. During lens regeneration, IPE cells dedifferentiate by losing their original pigment epithelial characteristics. They lose pigmentation, re-enter the cell cycle, proliferate to regenerate the new lens. The dedifferentiated proliferating IPE derived cells express *Pax6* (Del-Rio Tsonis K and Tsonis PA, 2003). On the other hand, RPE-derived cells preserve pigmentation and RPE65 protein expression until Stage E-3 (Chiba C et al., 2006). Nonetheless, I have assumed that adult newt RPE cells are reprogrammed into multipotent cells which give rise to both a new NR and RPE (Chiba C & Mitashov VI, 2007). In fact, almost all of the RPE-derived cells at Stage E-1, which seem homogeneous with regards to their pigmentation and RPE65-immunoreactivity (RPE65-IR), uniformly express a neural stem-cell marker Musashi-1 in their cytoplasm (Kaneko J & Chiba C, 2009). Therefore, these RPE-derived cells were called 'stem-like cells' (Chiba C & Mitashov VI, 2007). However, it remains elusive what state the stem-like cells are in. In this study, I address the nature of adult newt RPE-derived stem-like cells by means of immunohistochemistry for the identification Pax6 because Pax6 is widely accepted as retina/RPE stem cells marker in wide range of animals, thus obtaining valuable insight into similarities and differences between retinal disorders and regeneration.

3.2 Methodology

Surgical operations

Sexually mature adult *C. pyrrhogaster* newts (total body length: male, ~9 cm; female, 11-12 cm) were originally caught from a restricted area (~25 km in diameter) of Kamogawa city (Chiba prefecture, Japan) and were reared in the laboratory (Casco-Robles MM et al; 2011). Surgical removal of the NR (retinectomy) was performed as described previously (Chiba C et al., 2006). In brief, after the adult newts were anesthetized in 0.1% FA100 (4-allyl-2-methoxyphenol; DS Pharma Animal Health, Osaka, Japan) solution in the dark at room temperature (RT, ~22°C) for 2 h, the dorsal half of the left eye was cut open along the position slightly below the boundary between the cornea and sclera, and the NR was carefully removed together with the lens. At this time, the retinal margin containing the *ora serrata* (the tissue harboring the retinal stem/progenitor cells) was also removed. After operation, the eye flap consisting of the iris and cornea was carefully placed back in its original position. The operated animals were allowed to recover in a moist container [<5 newts/container (19 cm x 14 cm x 4 cm)], and then reared in an incubator (~22°C; 12 h : 12 h day : night cycle) until use. The stage of retinal regeneration and corresponding day po were determined according to previous criteria (Chiba C et al., 2006).

Collection of eyeballs and preparation of tissue samples

Newts were sacrificed under anesthesia to minimize suffering. To collect eyeballs, the animals were decapitated, then placed under a binocular and the eyeballs were carefully enucleated with fine scissors and forceps (Susaki K & Chiba C, 2007).

Immunohistochemistry

Immunolabelling of tissue sections was performed as described previously in **chapter II**, with some modifications. For retinectomized eyeball sections, an antigen retrieval step was added before the process of immunolabelling: after the 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 dissecting microscope, eyeball sections 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°C for 10 min, and then rinsed in PBS twice for 5 min each time. During incubation in the sodium citrate buffer, the corneal and scleral tissues became coiled and dissolved into the buffer solution. This treatment obviously decreased the background staining of tissues (possibly due to some blood-related content in the choroid), while sustaining immunoreactivity, raising the signal to noise ratio.

Pax6 was localized with a previously developed immunoperoxidase labeling procedure (**Chapter II**) with the ABC/DAB (Vectastain ABC Elite kit, DAB substrate kit, Vector Laboratories) system and RPE65 (mouse monoclonal anti-RPE65 antibody ; 1:500; MAB5428; Millipore) and PCNA (Human autoantibody to PCNA 1:500; gift from Dr. T. Saito; Chiba et al., 2006) were localized with an immunofluorescence procedure(Chiba C et al., 2006). In the case of double labeling with Pax6 (monoclonal anti-Pax6 antibody ; AD2.38, sc-32766; Santa Cruz Biotechnology) and RPE65 antibodies (both are mouse monoclonal antibodies), immunoperoxidase labeling of Pax6 without the DAB reaction was followed by immunefluorescence labeling of RPE65, and finally Pax6-immunoreactivity was visualized with DAB (substrate kit, Vector Laboratories). The secondary antibody used in this experiments: biotinylated goat anti-mouse IgG antibody for Pax6 (1:500; Vector Laboratories), tetramethylrhodamine-conjugated goat anti-mouse IgG antibody for RPE65 (1:200; T2762; Life Technologies), Alexa Fluor 488-conjugated goat anti-human IgG for

PCNA (1:1000; A-11013; Life Technologies). After labeling, cell nuclei were counterstained with DAPI (1:50,000 in PBS×1; D1306; Life Technologies) for one hour in room temperature.

Western blot

Conventional western blot was performed as was done in **Chapter II**. Protein was extracted from RPE-choroid tissues from 20 regenerating eyes and 20 normal eyes.

3.3 Results

In adult retinal regeneration, if RPE-derived stem-like cells are comparable to cells in the optic vesicles/cups or to immature RPE cells, cells of the CMZ (retinal stem/progenitor cells harbor) should express Pax6. The expression was not observed at all in day-0 and day-5 po RPE-derived cells. However, dedifferentiation process was started after 5 days po. I observed depigmentation, cell dissociation i.e disruption of epithelia (**Figure 15**). Unexpectedly, Pax6-IR was first detected only in a small population of RPE-derived cells at Stage E-1 (day-10 po). At 10 days after retinectomy, the dissociated RPE cells aggregated and among them some cells expressed Pax6 (**Figure 16**). This became obvious in the few ensuing days in which the RPE-derived cells were segregated into two rudimentary layers (pro-NR and pro-RPE). At Stage E-2 (day-14 po), Pax6-immunoreactivity was observed along the pro-NR layer almost uniformly but very sparsely in cells located along the pro-RPE. Pax6-immunoreactivity in the pro-RPE layer became undetectable by Stage E-3 (day-19 po) (**Figure 17**). On the other hand, thickness of the Pax6-immunoreactive (Pax6-IR+) pro-NR layer or regenerating NR increased. As cell differentiation proceeded, the number of Pax6-IR+ retinal progenitor cells decreased while Pax6-IR+ NR cells appeared in the following order: ganglion cells (St. I-1) → amacrine cells (St. I-2) → horizontal cells (St. I-3) → Müller glia cells (St. I-3 to L-1) (**Figure 18**). Although Pax6 was not detected in all the RPE-derived

cells at 10 days po, western blot detected a thin, weak but distinguished protein band corresponding to newt LL Pax6 isoform in protein extracted from 10 days po RPE-choroid tissues (**Figure 19**). Moreover, I observed only about 12% RPE-derived cells expressed Pax6 while almost all (about 98%) the RPE-derived cells at 10 days po expressed PCNA indicated re-enter the cell cycle (**Figure 20**).

3.4 Discussion

The RPE-derived cells at Stage E-1 most likely were not comparable to the cells that I had predicted based on the Pax6 expression pattern. In the IHC experiment for Pax6 expression, I observed a small population of the RPE-derived cells in the aggregate positively stained but the signal was so faint that I decided to reconfirm the presence of Pax6 protein in RPE-derived cells applying western blot technique. From this observation it is obvious that Pax6 is just started to express in the RPE derived cells. Moreover, all the RPE derived cells (~98%) express PCNA (proliferative cell nuclear antigen), which is an established marker for cell cycle entry in newt (Chiba C et al., 2006). PCNA may persist into the proliferating cells as well. On the other hand only about 12% of the RPE derived cells showed Pax6-IR +. As speculated from this observation RPE-derived cells enter the cell-cycle independently where there is no relation between cell-cycle entry and Pax6 expression in these cells.

In addition, it appeared as if two cell populations appeared abruptly in the RPE-derived cells between Stages E-1 and E-2, forming the pro-NR layer (Pax6-IR+) and the pro-RPE layer (Pax6-IR-) by Stage E-2. The Pax6 expression pattern that I observed in the developing retina in **Chapter II** was similar to that in the regenerating retina. However in case of development, the RPE cells also exhibit Pax6 expression until they reach to maturation. On the contrary in case of regeneration, once the RPE derived cells get partitioned as pro-RPE and pro-NR, Pax6-IR was observed only in the pro-NR but not in the pro-RPE. This indicates RPE derived

cells in pro-RPE do not necessarily return to a stage like immature RPE of development. Thus, together with the previous findings (Chiba C et al., 2006, Chiba C & Mitashov VI, 2007) and from this study as I observed that after Stage E-3, the process of retinal regeneration seemed like a recapitulation of embryonic retinal development.

Chapter IV: Expression of marker genes involved in the early phase of retinal regeneration

4.1 Introduction

To facilitate the study of retinal regeneration in newt, one considerable obstacle still remains: limited information of genes. Recently, our laboratory has provided a *de novo* assembly transcriptome and inferred proteome of the Japanese fire bellied newt (*Cynops pyrrhogaster*), which was obtained from eyeball samples of day 0-14 after surgical removal of the lens and neural retina. This transcriptome (237,120 *in silico* transcripts) contains most information of cDNAs/ESTs which has been reported in newts (*C. pyrrhogaster*, *Pleurodeles waltl* and *Notophthalmus viridescence*) so far (Abdullayeva I et al., 2013; Looso M et al., 2013). On the other hand, *de novo* assembly transcriptomes reported lately for *N. viridescence* only covered 16-31% of this transcriptome, suggesting that most constituents of this transcriptome are specific to the regenerating eye tissues of *C. pyrrhogaster*. A total of 87,102 *in silico* transcripts of this transcriptome were functionally annotated. Coding sequence prediction in combination with functional annotation revealed that 76,968 *in silico* transcripts encode proteins/peptides recorded in public databases so far, whereas 17,316 might be unique. In this study, I attempted to search the presence and expression level of genes that are regulated in association with cell reprogramming, cell-cycle re-entry/proliferation, and tissue patterning in an early phase of retinal regeneration through qPCR analysis.

4.2 Methodology

Retinectomized (left eye) animals were decapitated under anesthesia at day-10 and day-14 po, then eyeballs were collected into RNase-free PBS. Left eyes were used for day-10 and -14 samples, and right eyes were treated as day-0. Eyeballs were cut open, the anterior part containing the iris and the ciliary marginal zone was removed carefully, and RPE cells (day-

0) or RPE-derived cells (day-10 and -14) were collected together with the choroid tissues (blood cells in the choroid were removed as much as possible by gently pippeting up and down them in RNase-free PBS). Each day samples were immediately used to purify total RNA (5 samples/tube; NucleoSpin® RNA kit; Macherey-Nagel GmbH & Co. KG, Düren, Germany). First strand cDNAs were synthesized (SuperScript™ II Reverse Transcriptase; Life Technologies, Carlsbad, CA) with 30 ng of total RNA. The resulted cDNA was diluted 100x, and then used as a template for qPCR (**Figure 21**). qPCR was performed by a LightCycler® Nano system (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions of Fast Start Essential DNA Green Master (Roche) or Fast Start Essential DNA Probes Master (Roche), with 45 cycles. Target *IS*-transcripts genes: *Ef1α*, *RPE65*, *CRALBP/RLBP1*, *ZO1*, *Otx2*, *Musashi1a/c*, *Cyclin D1*, *CDK4*, *Histone H3*, *c-Myc*, *Klf4*, *Sox2*, *N-Cadherin*, *α-SMA*, *Vimentin*, *Pax6*, *Chx10/Vsx2*, *FGFR1*, *FGFR3*, *Mitf*, *Wnt2b*, and their PCR primers and probes [selected from the Roche Universal Probe Library (https://www.roche-applied-science.com)] are listed in **Table 3**. The DDBJ/GenBank accession number of the cDNA corresponding to each *IS*-transcript is also mentioned in **Table 3**.

For each gene, qPCR, which was always run simultaneously with day-0, -10 and -14 samples, was repeated using more than three sets of independently collected samples. The relative expression level of each transcript was calculated as follows: the amount of transcript was first compensated for *Ef1α* (the mean from several rounds of qPCR) in the same sample, and then normalized against that of the day at which the average value from all samples was highest. Data was presented as a bar graph showing the mean ± SEM. Statistical differences were evaluated by Mann-Whitney's U test or Sheffe's test following the Friedman test.

4.3 Result

The current transcriptomes contained much information of genes which have not been identified in *C. pyrrhogaster* but whose transcription has been hypothesized to be regulated in early processes of retinal regeneration. Here, for qPCR, I selected 20 genes (**Table 3**): for unidentified genes *CRALBP/RLBP1*, *ZO1*, *Cyclin D1*, *CDK4*, *Histone H3*, *c-Myc*, *Klf4*, *N-Cadherin*, *α-SMA*, *Vimentin*, *Chx10/Vsx2*, *Mitf*, *FGFR1*, *FGFR3*. qPCR results (**Figure 22**) revealed that the expression levels of many genes characterizing RPE cells (*RPE65*, *CRALBP/RLBP1*, *ZO1*, *Otx2*, *Musashi1a/c*) gradually decreased upon retinectomy, while the expression of genes for the cell-cycle (*Cyclin D1*, *CDK4*, *Histone H3*) and growth signaling (*FGFR1*, *FGFR3*) was up-regulated to reach a maximum level by day-10 po when almost all RPE cells had entered the cell cycle (Stage E1; **Figure 20**). Intriguingly, pluripotency factors except for *Oct4* (i.e., *c-Myc*, *Klf4*, *Sox2*) were expressed between day-0 and day-10 po; *Oct4* was not found in the current transcriptomes. Up-regulation of these three factors and lack of *Oct4* expression were also reported in early lens and limb regeneration of *N. viridescence* (Maki N et al., 2009). *Mitf*, a marker gene for immature or uncommitted RPE cells (Bharti K et al., 2012), was also expressed by day-10 po but then its expression level decreased. These expression patterns of genes might reflect the reprogramming of RPE cells into a multipotent cell state. On the other hand, marker genes for retinal stem/progenitor cells (*Pax6*, *Chx10/Vsx2*) were expressed, later than *Mitf*, at day-14 po when two progenitor layers for the prospective neural retina and RPE just appear (Stage-E2; see **Figure 17**). Interestingly, the expression of *Wnt2b* was coincidental. Wnt/β-catenin signaling has been suggested to promote differentiation of the RPE while protecting cell-fate switching of the uncommitted RPE into the neural retina in embryonic eye development (Yoshikawa T et al., 2012; Bharti K et al., 2012).

4.4 Discussion

It is obvious that, the provided *in silico* transcriptomes covering information of genes which are expressed in eyeballs containing Stage E-0 to E-2 regenerating retinas will certainly facilitate the investigations of adult newt retinal regeneration study. Gene expression patterns revealed by qPCR demonstrate the usefulness of the transcriptomes for the study of early processes of retinal regeneration. This resource can be applied to the next comprehensive gene screening steps to uncover essential mechanisms underlying reprogramming, cell-cycle re-entry/proliferation, and patterning of the RPE derived cells in adult newt retinal regeneration. Thus by a comparative study on the genes expression profile between newt RPE derived cells and human iPS cells derived RPE cells might provide us with some useful information toward the recovery of RPE mediated retinopathy. For example in *PVR* (proliferative vitreoretinopathy), after retinal injury RPE cells migrate to the site of injury and form an epiretinal membrane leading to the loss of vision in humans. In this case, RPE cells transform into mesenchymal cells such as myofibroblasts, probably by passing through a stem-cell state (Chiba C, 2014). In the newt, expression of mesenchymal markers (*N-Cadherin*, *Vimentin*) seemed to be up-regulated by day-10 po and then decreased while the relative expression level of a marker of myofibroblasts, *α-SMA* (smooth muscle actin), obviously decreased (**Figure 22**). Taken together the qPCR results and Pax6-IR data, in contrast to humans, in the newt RPE-derived cells (possibly in a stem-cell state) properly and evidently generate retinal stem/progenitor cells. Moreover, the newt transcriptomes could be a good tool to identify or screen candidate genes involved in early processes of retinal regeneration.

Chapter V: Identification of multipotency factors in RPE-derived cells

5.1 Introduction

In *in silico* transcriptome data base I found some marker genes that have role in the early process of retinal regeneration and observed expression changes during E0 to E2 stages. Then I decided to carry out qPCR experiment again for isolated RPE (intact) and RPE-derived cells (Satge E1, 10 days po) because in the previous chapter (**Chapter IV**) I performed qPCR based on the *in silico* transcript sequences with the cDNA made from RPE-choroid tissues which might contain other cells (Chiba C et al., 2006) especially blood cells (**Figure 23**). Isolated cells qPCR is a more reliable tool for accurate determination of genes expression study. That is why for better resolution of gene expression result in the RPE-derived cells I attempted to pick up the solitary isolated RPE/RPE-derived cells to make cDNA for authentic target gene expression profile in them. Nowadays single cell qPCR is even more powerful technique for particular gene expression assay in a given time or condition (Ståhlberg A and Bengtsson M, 2010; Sanchez-Freire V et al 2012). In this chapter I have focused on the genes *c-Myc, Klf4 and Sox2* (pluripotent genes) as well as *Mitf* and *Pax6* in the isolated and single cell level expression. In addition I have performed IHC (for Sox2) for protein level expression in the developmental, adult normal and regenerating tissues.

5.2 Methodology

Cell isolation and cDNA synthesis

For single-cell qPCR, both the right (intact) and left (retinectomized) eyeballs of animals at day-10 po were used; that is, the right eyeballs were used for day-0 po sample containing intact RPE (stage E-0), and the left eyeballs were for day-10 po (stage E-1) sample. For one round of the experiment, three animals were sacrificed under anesthesia, and the right and left

eyeballs were collected in different plastic dishes (Falcon 35-3001; Becton Dickinson) filled with RNase-free PBS on ice. The right eyeball (day-0) was dissected into the eye-cup, the NR was removed, and then the RPE sheet together with the choroid tissues was isolated by separating these from the sclera using a fine pin and forceps (Susai K and Chiba C 2007); left-hand pathway in **Figure 24**. On the other hand, the left eyeball (day-10) was carefully opened from the wound at the time of retinectomy using a fine pin and scissors, the anterior part of the eyeball containing the iris and ciliary marginal zone was carefully removed, and then RPE-derived cells in the posterior eye were collected together with the choroid tissues as was done for the right eyeball; right-hand pathway in **Figure 24**. After blood cells in the choroid were removed as much as possible by shaking carefully and slowly them in the dish, three each-day samples were transferred into different 15-ml tubes containing 1 ml of elastase (1 mg/ml; Porcine pancreas; Ref:11027891001; Roche Diagnostics) in EDTA solution (in mM: 115 NaCl, 3.7 KCl, 10 EGTA, 18 D-glucose, 10 HEPES, 0.001% phenol red, pH 7.5 adjusted with 0.3N NaOH) with a 3.5 ml transfer pipette (Sarsted, D-51588 Nümbrecht, Germany), and incubated for 90 min at 28°C. The tissue samples were rinsed with a chilled newt 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) several times, and then dissociated by gentle trituration with the transfer pipette. The cell suspension was transferred into a 1% agarose-coated 35 mm plastic dish (Falcon 35-3001) placed on ice box. This trituration and cell collection steps were repeated 5-6 times.

cDNA synthesis and pre-amplification were carried out, under conventional nuclease-free conditions, using TaKaRa CellAmp Whole Transcriptome Amplification Kits (Real Time) Ver.2 (code#: 3730; Takara, Japan) according to the manufacturer's instructions. In brief, solitary RPE cells or RPE-derived cells, which were identified by their morphological characteristics (**Figure 24 and 25**), were picked up from the dish by a microtip (703Y, Ina

Optika, Japan) attached to a micropipette (set at 0.5 µl; Pipetman P20; Gilson) under a dissecting microscope. For 100-cell qPCR analysis, 100 cells were picked up and transferred into a PCR tube (No T-02F, Ina Optica) containing 50 µl RNase-free PBS, and settled on the bottom of the tube by centrifugation for 1 min at 3,000 g. After centrifugation, PBS in the tube was discarded leaving behind ~0.5 µl, a 4.5 µl reaction mixture (containing cell lysis buffer, recombinant RNase inhibitor, RT dT primer 2, dNTP mixture, RNase free dH_2O) was added. For one-cell qPCR analysis, one cell (0.5 µl) was directly transferred into the tube containing the 4.5 µl reaction mixture. In both samples, cDNA synthesis was followed and then resulting cDNAs were amplified non-selectively by PCR (95°C, 1 min → 50°C, 1 min →72°C, 3 min: 1 cycle; 95°C, 30 sec → 67°C, 1 min → 72°C, 3 min: 20 cycles; 72°C, 1 min). The amplified cDNA samples were stored at -20°C until use.

Quantitative PCR (qPCR)

The amplified cDNA samples were diluted 10x, and then used as a template for qPCR. qPCR was performed by a LightCycler® Nano system (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions of FastStart Essential DNA Green Master (Roche) or FastStart Essential DNA Probes Master (Roche), with 45 cycles. For each gene examined in this study, the DDBJ/GenBank accession number, primers, probes [selected from the Roche Universal Probe Library (https://www.roche-applied-science.com)] and expected size were given in **Table 4** The specificity of PCR was confirmed by standard gel-electrophoresis and DNA sequencing.

Western blott

Western blot was performed for the validation of Sox2 antibody as was described in **chapter II**.

Antibodies for primary antibody: rabbit anti-Sox2 antibody (1:660 for IHC and 1:1000 for western blot; ab97959; Abcam) and for secondary antibody: biotinylated goat anti-rabbit IgG antibody (1:500; Vector Laboratories); for negative control, mouse or rabbit antibodies (IgG) which have no specific reactivity to newt tissues (e.g. anti-dsRed antibody; 632543 or 632496; Clontech, CA 94043, USA) were applied, instead of the primary antibody, at the same concentration of primary IgG.

Immunohistochemistry

Histochemistry was done as was described in **chapter II** (for normal eye section and larvae) and **chapter III** (for regenerating eye tissue).

5.3 Results

I investigated whether the RPE-derived cells express other stem-cell markers. To perform this, a single-cell qPCR technique was adopted, since in this animal the availability of antibodies is quite limited, and empirically *in situ* hybridization seems to have insufficient sensitivity (or signal to noise ratio) to detect low concentrations of mRNAs whose transcription just started. Candidate genes were identified using a newt *de novo* assembly transcriptome database (IMORI, http://antler.is.utsunomiya-u.ac.jp/imori/) **Chapter IV**.

I first carried out qPCR analysis using 100 harvested cells (**Figure 26**). Pluripotency factors (*c-Myc*, *Klf4* and *Sox2*), *Mitf* and *Pax6* were always detected in day-10 samples but never in day-0 samples ($n \geq 4$), although *RPE65* was detected in both day samples (n=4); the relative expression levels of *RPE65* were not significantly different between samples. I next tried qPCR with one-cell samples (**Figure 27 and Table 5**). On day-0, as observed from 100-cell samples, none of the genes examined were detected but in a total of 17 cells, *RPE65* was detected (*RPE65+* cells). On the other hand, on day-10, from a total of 19 *RPE65+* cells, 13

cells (68%) showed the expression of target genes (**Figure 27**), although the combination of genes detected varied individually (day-10 in **Table 5**).

Such a fluctuation in the detection of each target gene in day-10 samples may be explained by the probability of occurrence of a reverse transcription (RT) reaction under a condition of low concentrations of mRNA (the substrate of the RT polymerase), which should be true for the current one-cell samples; that is, the detection rate should depend on the concentration of mRNA under this condition. Thus, all data was cumulated (n=19), revealing that the detection rates of target genes (**Figure 27**) showed a similar pattern to that (*c-Myc>Klf4>Sox2*; *Mitf>Pax6*) of their relative expression levels obtained in 100-cell samples (**Figure 26**).These observations suggest that mRNAs of all target genes may be present in a single day-10 cell at different concentrations. It is noteworthy that one particular cell (d-10 Single #18 in **Table 5**) that expressed all target genes. Taken together, these findings prove that RPE cells are reprogrammed into a unique multipotent state.

To visualize the expression of these multipotency factors, immunohistochemistry was attempted once again. I searched for antibodies for c-Myc, Klf4, Sox2 and Mitf of this species, and finally found a good antibody for Sox2. Antibody was validated by western blot analysis and it could bind successfully a protein band around 37 KD corresponding to newt Sox2 (**Figure 28**). In addition both developing and adult eyes, the patterns of Sox2-IR were consistent with those reported in other vertebrates(Lin YP et al., 2009; Ma W et al., 2009; Matsushima D et al., 2011): Sox2-IR was observed in (i) the neuroepithelium cells of the early optic vesicle (St. 23), (ii) the pro-NR region in the optic vesicle (late stage; St. 24)/cup (St. 27), (iii) retinal progenitor cells in immature retina (St. 29, 30, 32) in **Figure 29**; (vi) the CMZ, as well as (v) some types of NR cells (amacrine and Müller glia cells) in **Figure 30** and in **Table 6**.

Unexpectedly, but interestingly, in the adult retinal regeneration, the patterns of Sox2-IR expression until Stage E-3 were almost the same as those for Pax6: Sox2-IR was not detected in day-0 (Stage E0) po tissue (**Figure 31)**. Signal was detectable in a small number of RPE-derived cells at day-10 po (**Figure 32**), and was obviously observed along the pro-NR layer at day-14 po (**Figure 33**).

5.4 Discussion

Here I examined three pluripotency factors *c-Myc*, *Klf4* and *Sox2* (Takahashi K et al., 2007), a *Microphthalmia* factor *Mitf* (Nguyen M & Arnheiter H, 2000) as well as *Pax6*. Recent *in vitro* studies in mammals have suggested that a set of pluripotency factors is able to induce cell-type switching of RPE cells into multipotent cells (Hu Q et al., 2010). However, I must note that the expression of Oct4 or Nanog, the pluripotency factors that generates iPS cells in mammals (Takahashi K et al., 2007; Hu Q et al., 2010), is not detected in early processes of retinal regeneration (Nakamura K et al., 2014). *Mitf* is known, in other vertebrates, to be expressed in (i) the neuroepithelium cells of the early optic vesicle, (ii) cells of the pro-RPE region in the optic vesicle (late stage)/cup, or (iii) immature/uncommitted RPE cells in embryonic/larval eyes (**Table 6**). In those cells, it has been suggested that loss of function of this factor is enough to induce their fate-switching into the NR (Nguyen M & Arnheiter H, 2000). I isolated RPE cells (day-0 sample) and RPE-derived cells (day-10 sample) from the intact and retinectomized eyeballs respectively, which were collected from animals at day-10 po (**Figure 24**), and selected solitary cells manually under a dissecting microscope that served for cDNA synthesis followed by qPCR. For day-0 sample, RPE cells were identifiable by their characteristic morphology (there is a polarity with a pigmented apical part and a non-pigmented basal part containing the nucleus). However, in the day-10 sample, visual cell identification was not easy because of the possible contamination of pigmented cells of different origins. Therefore, only those cells resembling the normal RPE cells were selected,

i.e., a fraction of the RPE-derived cells whose morphological change was slightly delayed (**Figure 25**). In this study it was evident that the RPE cells newly express *c-Myc, Klf4, Sox2, Mitf* and *Pax6* while retaining the expression of RPE65, as observed by both mRNA and protein levels, being converted into a unique state of multipotent cells termed RPESCs (**Figure 34**). Pluripotency factors c-Myc, Klf4 and Sox2 might work to reprogram or initialize the RPE cells as demonstrated in *in vitro* studies in mammals (Hu Q et al., 2010), although in this system Oct4 or Nanog is unlikely to participate, as observed in chick RPE reprogramming into the NR (Luz-Madrigal A et al., 2014) as well as in other regeneration systems of body parts in adult newts (Maki N et al., 2009). As day 10 po RPE derived cells did not show Pax6-IR uniformly, I supposed Sox2-IR might be observed in all the RPE derived cells. However the result was similar to that of Pax6-IR. Some population of the RPE derived cells at day10 po showed Sox2-IR while other did not. Sox2-IR was found apparently in the pro-NR but not in the pro-RPE at day14 po. Thus together with the qPCR data and Sox2-IR data suggest that Sox2 increase in the pro-NR while decrease in the pro-RPE.

5.5 Data analysis

In single-cell qPCR analysis, 100-cell qPCR for each gene, which was always run simultaneously with day-0 and -10 po samples, was repeated using more than four sets of independently collected samples. The expression level of each gene (i.e., the amount of transcripts estimated from a Cq value) was compensated for *Ef1α* in the same sample. For one-cell qPCR, samples in which *RPE65* was detected served for further analysis of gene expression. Data was presented as a bar graph showing the mean ± SEM (**Figure 26 & 27**). Statistical differences were evaluated by Mann-Whitney's U test or Sheffe's test following the Friedman test.

Chapter VI: General discussion

Based on the accumulated data in this study, I illustrate a summary of the current results and a deduced scenario in **Figure 34**. In the adult newt, the RPE is a sole cell source for regeneration of missing NR in the posterior eye. The adult newt RPE is a highly specialized monolayer as in other vertebrates (Chiba C et al., 2006). I can exclude the possibility that the intact RPE contains 'retinal stem/progenitor cells resembling RPE cells (or RPE65-IR+) which have been committed to regenerate the NR only, because the RPE is not filled up by other tissues than the RPE itself (Chiba C et al., 2006). Moreover, the current single-cell qPCR revealed that the intact RPE cells do not express retinal stem/progenitor markers.

Upon retinectomy, RPE cells are detached from each other as well as from the basement membrane, losing their epithelial characteristics to become cell aggregates, while entering the S-phase of the cell-cycle. These RPE-derived cells are segregated into two rudimentary layers (the pro-NR and pro-RPE layers) with correct polarity, eventually regenerating a new NR while renewing the RPE itself. Previous study demonstrated that almost all of the RPE-derived cells which entered the S-phase of the cell-cycle at day-10 po (Stage E-1) uniformly express a neural stem-cell marker Musashi-1 in their cytoplasm, and that the expression of Musashi-1 is sustained along the pro-NR layer at day-14 po (Stage E-2) but down-regulated along the pro-RPE layer (Kaneko J & Chiba C 2009). These observations led us to imply that the RPE cells are reprogrammed into multipotent cells by Stage E-1, and specified into two cell populations (**Figure 35)** possibly depending on their surrounding microenvironment (or niche) between Stage E-1 and E-2.

In this study I revealed that the RPE cells newly express *c-Myc*, *Klf4*, *Sox2*, *Mitf* and *Pax6* while retaining the expression of RPE65, as observed by both mRNA and protein levels, being converted into a unique state of multipotent cells (termed RPESCs here for

convenience; in **Figure 34**). Pluripotency factors c-Myc, Klf4 and Sox2 may play some role to reprogram or initialize the RPE cells as demonstrated in *in vitro* studies in mammals (Hu Q et al., 2010), although in this system Oct4 or Nanog is unlikely to participate, as observed in chick RPE reprogramming into the NR (Luz-Madrigal A et al., 2014) as well as in other regeneration systems of body parts in adult newts (Maki N et al., 2009). My review of the gene expression patterns during eye morphogenesis (**Table 6**) suggests that the cells which express c-Myc, Klf4 and Sox2 as well as Pax6 and Mitf are only those of the early optic vesicle. Hence, it seems that the adult newt RPESCs correspond to the neuroepithelial cells of the early optic vesicle which generates both the NR and the RPE. In other words, the adult newt RPE cells seem to dedifferentiate in nature. Previous study suggests that the presence of RPE65 and melanin pigments in the RPE-derived cells for a long period (~20 days after retinectomy) may be due to their slow degradation or excretion (Chiba C et al., 2006).

However, this is not so simple because both Pax6- and Sox2-antibodies, which clearly labelled the optic vesicle cells, did not label the RPESCs uniformly. In the tissue of Stage E-1 (day-10 po), though single-cell qPCR detected *Pax6* and *Sox2* mRNAs even in RPE-derived cells which had not reached a standard progression in which cell shape resembles a normal RPE cell (**Figure 24**), the antibodies labelled only well advanced cells just forming the pro-NR layer. A possible explanation is that either the amount of proteins in the RPESCs is very small or some kind of modification occurs on both proteins, preventing access of the antibodies to their epitopes. The latter explanation can be excluded because I detected Pax6 protein in a western blot (**Figure19**). If the former explanation is true, at least with regards to Pax6 and Sox2, then adult newt RPESCs may not need the concentrations equivalent to those in optic vesicle cells to behave as multipotent cells.

Establishing a primary pattern of tissue organization at an early stage of regeneration is essential for reconstruction of different tissue types with the correct polarity from a single cell

source. Pax6/Sox2-IR+ cells appeared in RPESCs from Stage E-1 to E-2, forming the pro-NR layer (**Figure 34**). On the other hand, the cells with no immunoreactivity formed the pro-RPE layer. Since mitotic figures became obvious in RPE-derived cells between Stages E-1 and E-2, these cell populations seem to become sorted according to the correct polarity (inside: cells forming pro-NR; outside: cells forming pro-RPE) while their proliferation became activated during this period.

However, this process did not seem to be perfect: a small number of Pax6/Sox2-IR+ cells were distributed irregularly in the pro-RPE layer (termed 'displaced pro-NR cells' here). Interestingly, since the displaced pro-NR cells (arrow head in **Figure 17**) participated in renewal of the RPE, it appears that the RPESCs directed towards the regeneration of the NR still had the potency to re-differentiate into the original cell type. Pax6/Sox2-IR in displaced pro-NR cells disappeared in between Stages E-2 and E-3, but along the pro-NR layer Pax6/Sox2-IR seemed to be sustained until Stage I-1 from which neuronal cell differentiation starts, suggesting that an opposite regulation of Pax6/Sox2 expression occurs on the renewing RPE and regenerating NR.

Considering their following morphological and physiological changes (Chiba C et al., 2006; Chiba C and Mitashov VI, 2007), the pro-NR and pro-RPE layers seem like to be comparable to the pro-NR and pro-RPE regions in the optic vesicle (late stage)/cup. However, unlike the pro-RPE region (Pax6-IR+/Sox2-IR-) in the optic vesicle/cup, the pro-RPE layer (Sox2-IR-) was seldom labelled with Pax6-antibody (**Figure 17**). In retinal development, Pax6-IR seemed to be regulated in association with cell differentiation: as for the RPE, Pax6-IR observed in immature cells disappeared as they matured while expressing RPE65 (**Figure 13&14**). In retinal regeneration, RPE65-IR reappeared along the renewing RPE between Stages I-3 and L-1 at day-28 po (Chiba C et al., 2006) . Thus, compared to the developmental process, disappearance of Pax6-IR in the renewing RPE seems to occur far earlier than

maturation of the RPE, implying different regulatory mechanisms for Pax6 between development and regeneration. As discussed for RPESCs, the process of RPE renewal might not require Pax6 at the concentrations equivalent to those for development of the RPE.

Taken together, to our knowledge, this study provides the first set of evidence for the natural reprogramming of somatic cells for the regeneration of body parts in the newt at the single cell level. Furthermore, these results revealed that adult newt retinal regeneration contains many important issues that need further investigation, such as reprogramming, multipotency, cell specification, niche, tissue patterning, as well as EMT and proliferation. However, to apply this knowledge to medicine, the extent to which this system is unique to this animal must be evaluated. In humans, upon retinal injury, RPE cells lose their epithelial characteristics and acquire the ability to migrate and proliferate, transforming into mesenchymal cells such as myofibroblasts (Chiba C, 2014; Salero E et al., 2012). In *PVR*, the RPE-derived myofibroblasts are a major component of the epiretinal membrane which covers the wound of the NR but eventually contracts together with the NR, leading to the loss of vision. Thus, the initial response of human RPE cells to retinal injury resembles that of adult newt RPE cells. However, this phenomenon is categorized as EMT because the destination of RPE cells is mesenchymal cells but not the NR (Chiba C, 2014). Recent studies in stem-cell biology have implied that human RPE cells pass through a multipotent state during EMT. *In vitro* studies suggest that RPE-derived proliferative cells in humans are capable of expressing C-MYC and KLF4 as well as PAX6 and MITF (but not OCT4 or NANOG), and behave like stem cells termed as RPESCs (Salero E et al., 2012). Interestingly, in human RPESCs, SOX2 expression seems to be negligible (Salero E et al., 2012), being different from adult newt RPESCs. Since Sox2 is considered to be a decisive factor of neural competence in the retina (Ma W et al., 2009; Matsushima D et al., 2011), this factor, and possibly its co-factors also, might be one of the keys to directing RPESCs toward retinal regeneration. This study

revealed that human and newt RPESCs have a close similarity in their intrinsic properties. In near future, comparative/integrated studies of these regeneration-competent and -incompetent models at the molecular level, for example by a comprehensive single-cell transcriptome analysis, would likely provide essential insight to induce retinal regeneration in human RPESCs as well as to understand the molecular mechanism of adult newt retinal regeneration.

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Table 1: Micro injection solution for Pax6 knocking down

Reagents	Volume (µL)
I-SceI buffer (10X)	1
Phenol red (0.1%)	1
I-SceI plasmid (1 µg/µL)	2
I-SceI (5 U/ µL)	1
Milli Q water	5
Total volume	10 µL

Control shRNA

	Fluorescence intensity		
	Weak (n=6)	Moderate (n=8)	Strong (n=8)
Undetected	4 (100%)	6 (100%)	7 (100%)
Small Eye	0	0	0
Eyeless	0	0	0
Headless	0	0	0
Abnormal	2	2	1

Pax6 shRNA-1

	Fluorescence intensity		
	Weak (n=15)	Moderate (n=11)	Strong (n=6)
Undetected	12 (100%)	6 (66.7%)	1 (25%)
Small Eye	0	3 (33.3%)	3 (75%)
Eyeless	0	0	0
Headless	0	0	0
Abnormal	3	2	2

Pax6 shRNA-2

	Fluorescence intensity		
	Weak (n=16)	Moderate (n=11)	Strong (n=7)
Undetected	9 (90%)	5 (62.5%)	0
Small Eye	1 (10%)	3 (37.5%)	1 (16.7%)
Eyeless	0	0	3 (50%)
Headless	0	0	2 (33.3%)
Abnormal	6	3	1

Table 2 Effects of *shRNAs* on eye morphogenesis in the newt

Transgenesis was carried out and larvae showing mCherry fluorescence in the whole body were selected (see Methods). In the control *shRNA*, which was designed from the newt crystallin promoter (DDBJ/GenBank Accession #: AB113881), abnormalities were not recognized in larvae regardless of the fluorescence intensity of mCherry (an indicator for the relative expression level of *shRNA*), except for a curved tail fin, which was also observed in another control group with an empty construct. Therefore, the animals showing such an abnormality (numbers shown in the last row of each table) were omitted from data analysis. In *Pax6 shRNA-1*, a small eye phenotype was observed in 33.3% and 75% of animals which showed moderate and strong fluorescence, respectively. On the other hand, effects of *Pax6 shRNA-2* seemed to be much severe: 33% and 50% of animals showing strong fluorescence exhibited headless and eyeless phenotypes, respectively. An aspect that is correlated to the severity of the cranial part should be noted, namely that the total length along the body axis tended to decrease. Such abnormalities were consistent with those reported in other vertebrate.

Table 3: List of target genes, their accession number, qPCR primers, probe number and corresponding product size

Gene Name	Forward Primer	Reverse Primer	Product Size	Roche UPL Probe	Accession#
Efl α-1	cgtgacatgaggcagactgt	Tagaggccttctgggctgat	100bp	#25	AB00558
RPE65	tgctgctggaaaggatttga	Gctttctctgcatttctcttcac	95bp	#48	AB095018
CRALBP/RLBP1	agacagaggagtcccgtgag	Acgggctcgaatgaacctaag	149bp		AB904159
ZO1	accgaaagcagatgtcctacc	Aagggactggtttaggctcg	143bp		AB904168
Otx2	accaccaactgacaggagca	Agactggaagcaccgtagcc	118bp		AB052937
Musashi1a/c	aggtaatggctcgtttcttgc	Aaagttctgccattgaccctg	113bp		AB353845 AB353846
Cyclin D1	ggtggaaccgctgaagaa	Gccacgaacatgcaggtag	60bp		AB904160
CDK4	ggaccagttgggcaagatt	Gcataaaggcacatcgtggt	92bp	#82	AB904157
Histone H3.3	ggtgtcaagaagcctcacagata	Ctccctcaccaagcgctgaa	120bp		AB904163
c-Myc	ctcagaagaagaacaggatgacg	Atccaggcttcctgccagtag	101bp		AB904156
Klf4	acgcacacaggagagaagc	Ttccggtagtgacgggtaag	92bp	#48	AB904164
Sox2	acctgagggacatgatcagc	Gtttatggaggagggcacgg	119bp		AB074258
N-Cadherin type1	tggacgaaagaccaatccat	Tgaagtccccaatatcaccag	81bp		AB897892
α-SMA	tcccccagaacgcaagta	Gtagacagcgaagccaggat	60bp		AB904155
Vimentin	agctggagcagctgaagg	Ttctcgttggtgagctggt	103bp		AB904167
Pax6	tctgggcaggtattacgagac	Cgatcttgctcaccacctc	95bp		D88741
Chx10/Vsx2	gaggcgtcacagaaccatct	Taatgcgcatcgttgaagg	72bp		AB904158
FGFR1	ccccttgcagagacaggtaa	Ccagcataacgccagagc	68bp	#25	AB904161
FGFR3	gactgcctgtgaagtggatg	Tcgctttggtgagtgtagacc	64bp	#110	AB904162
Mitf	cgtagcaagatccgtgatgtc	Cagcagttcatccgagcat	78bp	#25	AB904165 AB904166
Wnt2b	ccgggcacttatgaacttgc	Ttagcgagcaagatccgctg	107bp		AB205148

Table 4: Single cell qPCR primers and probes of target genes with their accession number

Name of Genes	Accession#	Forward Primer	Reverse Primer	Product Size	Probe#
Ef1α	AB00558	cgtgacatgaggcagactgt	tagaggccttctgggctgat	100	
RPE65	AB095018	tgctgctggaaaggatttga	gctttctctgcatttctcttcac	95	48
Mitf	AB904165	cgtagcaagatccgtgatgtc	cagcagttcatccgagcat	78	25
Pax6	D88741	tctgggcaggtattacgagac	cgatcttgctcaccacctc	95	58
Sox2	AB074258	gtttatggaggagggcacgg	acctgagggacatgatcagc	119	
c-Myc	AB904156	ctcagaagaagaacaggatgacg	atccaggcttcctgccagtag	101	
Klf4	AB904164	ggtcaagggctggttagtcc	tgaggaggacacttgatggc	101	

day-0

Cell No.	RPE65	c-Myc	Klf4	Sox2	Mitf	Pax6
D0 Single #1	31.352	—	—	—	—	—
D0 Single #2	31.104	—	—	—	—	—
D0 Single #3	31.077	—	—	—	—	—
D0 Single #4	40.078	—	—	—	—	—
D0 Single #5	41.263	—	—	—	—	—
D0 Single #6	34.752	—	—	—	—	—
D0 Single #7	25.441	—	—	—	—	—
D0 Single #11	27.172	—	—	—	—	—
D0 Single #12	26.601	—	—	—	—	—
D0 Single #13	21.479	—	—	—	—	—
D0 Single #15	25.578	—	—	—	—	—
D0 Single #16	29.321	—	—	—	—	—
D0 Single #18	23.241	—	—	—	—	—
D0 Single #19	24.724	—	—	—	—	—
D0 Single #20	25.411	—	—	—	—	—
D0 Single #21	26.527	—	—	—	—	—
D0 Single #22	24.585	—	—	—	—	—
Detection ratio (%)	**100**	**0**	**0**	**0**	**0**	**0**

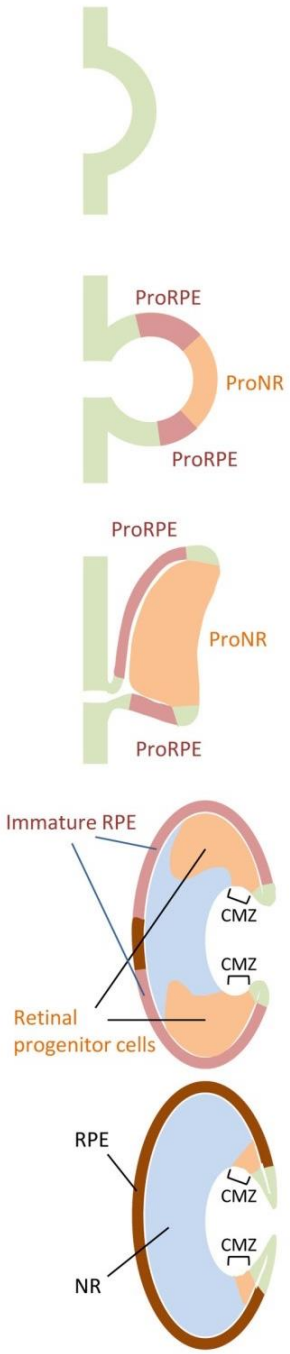
day-10

Cell No.	RPE65	c-Myc	Klf4	Sox2	Mitf	Pax6
D10 Single #1	30.987	—	—	—	—	35.304
D10 Single #2	31.141	—	—	—	—	—
D10 Single #3	31.393	—	—	—	—	—
D10 Single #4	30.694	36.053	—	—	37.964	—
D10 Single #5	31.337	—	38.659	—	—	—
D10 Single #6	31.217	—	—	—	—	—
D10 Single #7	31.201	—	—	—	—	—
D10 Single #8	31.229	40.188	—	39.852	36.735	—
D10 Single #9	40.606	—	—	—	39.434	—
D10 Single #10	33.865	37.991	—	26.767	36.497	36.888
D10 Single #11	38	35.139	—	28.079	41.306	—
D10 Single #18	37.464	34.862	37.966	40.047	37.136	36.635
D10 Single #19	38.793	—	—	—	—	—
D10 Single #20	38.626	35.52	—	—	—	—
D10 Single #22	26.168	—	36.463	—	37.496	37.476
D10 Single #23	27.322	37.129	36.204	—	—	36.793
D10 Single #24	27.829	—	—	—	—	—
D10 Single #25	28.551	—	36.915	—	—	—
D10 Single #26	27.715	—	—	—	—	38.727
Detection ratio (%)	**100**	**36.8**	**26.3**	**21.1**	**36.8**	**31.6**

Table 5: Raw data tables mentioning Cq values for each gene of day-0 po and day-10 po single RPE cell.

In both day-0 and day-10 single RPE cell, *RPE65* was used as a marker for RPE and RPE-derived cell as well as the efficiency determinant of cDNA synthesis and amplification by the pre-amplification step. Although day-0 RPE cell expressed *RPE65*, did not express other genes. On the other hand, *RPE65* positive day-10 cell randomly express other genes but one cell expressed all the target genes in the same time (red box). Horizontal bars indicate no detect ion.

Table 6: Inferred expression patterns of Pax6, Mitf, c-Myc, Klf4, Sox2 and RPE65 during retinal development.



		Pax6	Mitf	c-Myc	Klf4	Sox2	RPE65
Optic vesicle (Early)		✓	[1,2,3,4] ✓	[5] ✓	[4] ✓	✓	ND
Optic vesicle (Late)	ProNR	✓	[1,2,3,4] ND	[5] ✓	[4] ✓	✓	ND
	ProRPE	✓	[1,2,3,4] ✓	[5] ✓	[4] ✓	ND	ND
Optic cup	ProNR	✓	[1,2,3,4] ND	[4,5,6] ✓	[4] ✓	✓	ND
	ProRPE	✓	[1,2,3,4] ✓	[4] ✓	[4] ✓	ND	ND
Immature retina	Retinal progenitor cells	✓	[1,2,3,4] ND	[5] ND	[4] ✓	✓	ND
	Immature RPE cells	✓	[1,2,3,4] ✓	[4] ✓	[4] ✓	ND	ND
	CMZ	✓	[1,2,3,4] ND	[4,5,6] ✓	[4] ✓	✓	ND
Mature retina	NR	✓ (G, A, H, M)	[1,2,3,4] ND	[5,6] ND	[7,8] ✓ (G)	✓ (A, M)	ND
	RPE	ND	ND	ND	ND	ND	✓
	CMZ	✓	[1,2,3,4] ND	[4,5,6] ✓	[4] ✓	✓	ND

√ : expression (determined in this study); ND: no detection (determined in this study); √ : expression (inferred from other studies; #: reference are given at page 67); ND : no detection (inferred from other studies; #: reference are given at page 67); NR: neural retina; RPE: retinal pigment epithelium; CMZ: ciliary marginal zone; G: ganglion cells; A: amacrine cells; H: horizontal cells; M: Müller glial cells

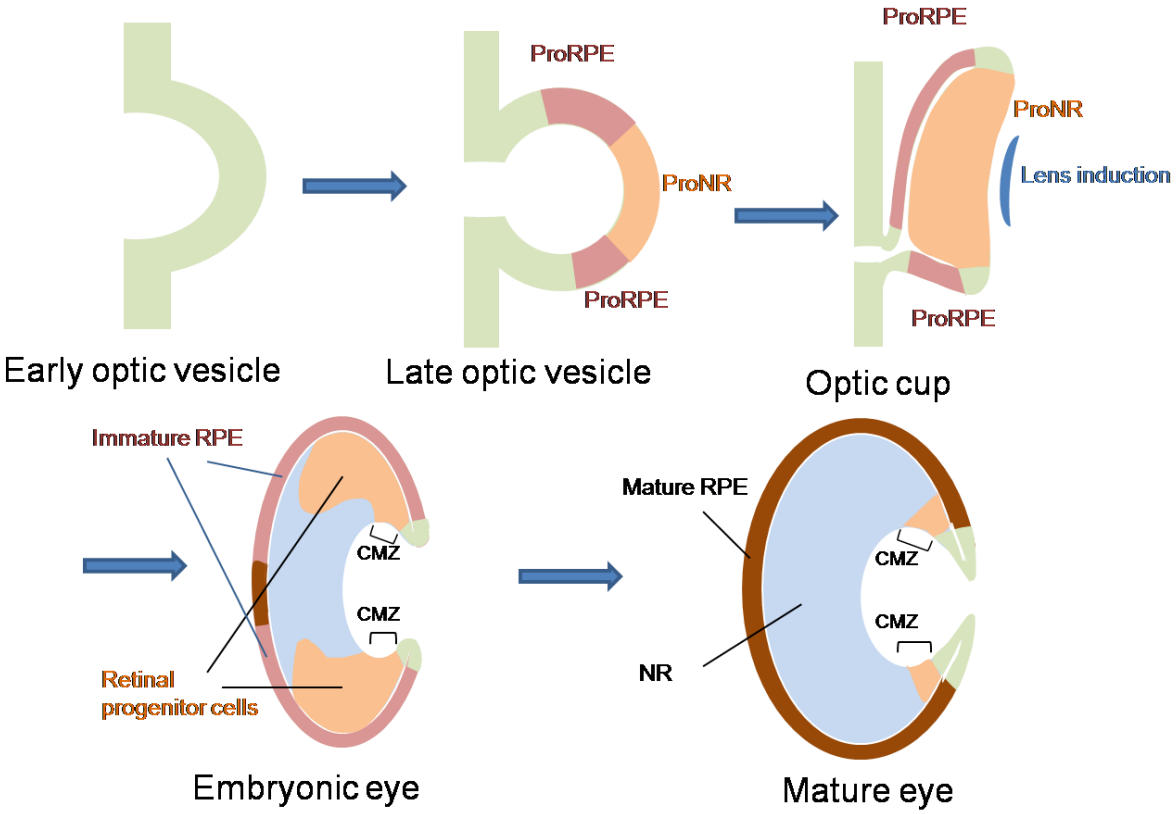


Figure 1: Retina and RPE development.

Neural epithelium of neural tube gives rise to early optic vesicle which later differentiate into pro neural retina (pro-NR) and pro retinal pigment epithelium (pro-RPE). With the surface ectodermal envagination optic vesicle gets optic cup shape forming two distinct layer- outer layer (pro-NR) and inner layer (pro-RPE). Both RPE and retina maturation starts at the center however some progenitor cells still remain at the peripheral retina (CMZ).

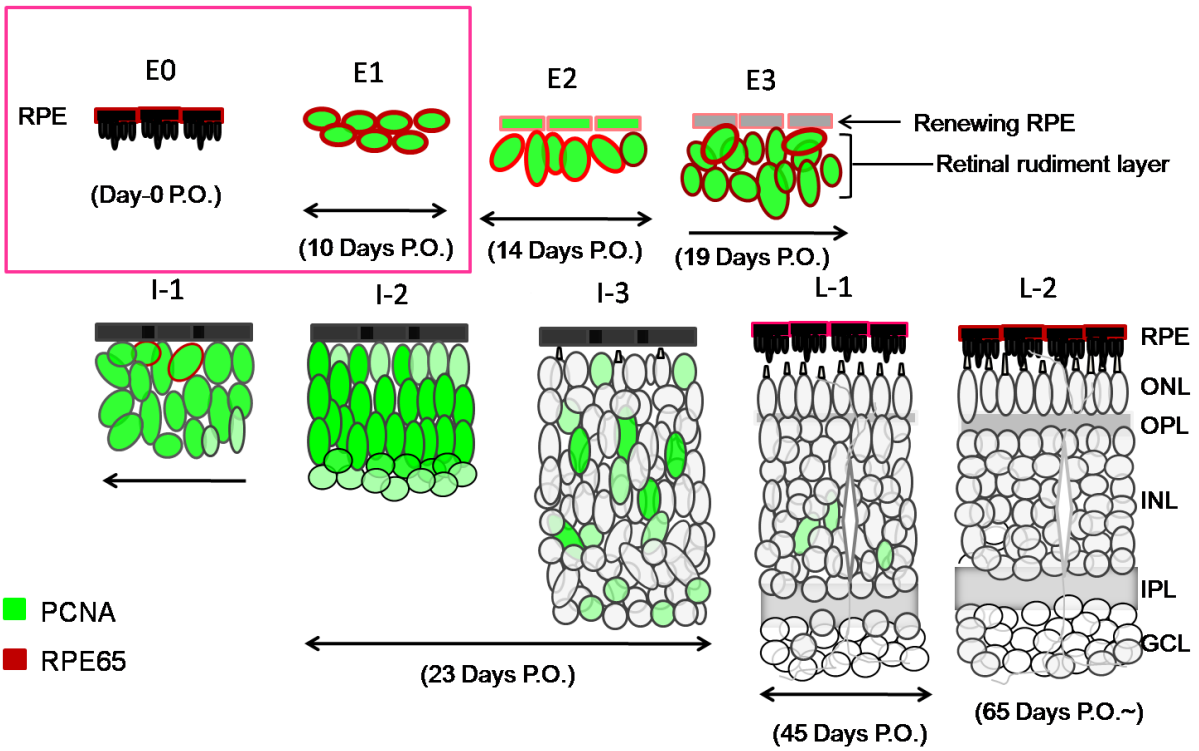


Figure 2: Stage of retinal regeneration (modified from Chiba C et al 2006).

Retinal regeneration process in newt is divided into three major stages. Early stage (E), intermediate stage (I) and later stage (L); these three stages are further subdivided as early stage-E0 (day0), E1 (day10), E2 (day14) and E3 (day19); intermediate stage- I1, I2 and I3 (19 days to 23 days, these stages are defined based on thickness of retinal rudiment cell layer), later stage- L1 and L2 (45 days 65 days when regenerated retina is appeared completely).

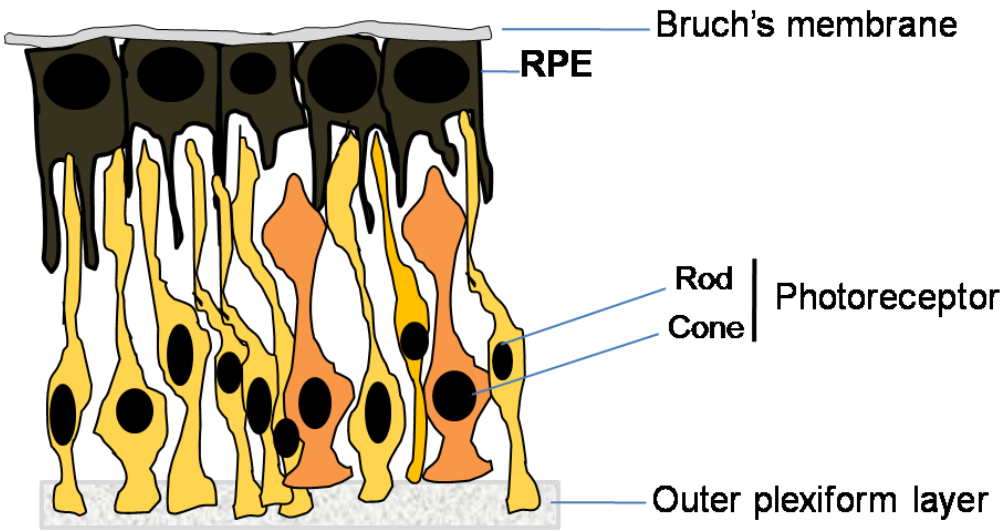


Figure 3: Organization of Bruch's membrane, RPE cells and photoreceptor cells

The RPE cells have two poles, the basal pole and the apical pole. The basal pole of RPE cells are attached to the Bruch's membrane and the apical pole containing microvilli are extended into the photoreceptor outer segment. Bruch's membrane and the tight junction between RPE cells provide a blood-retinal barrier.

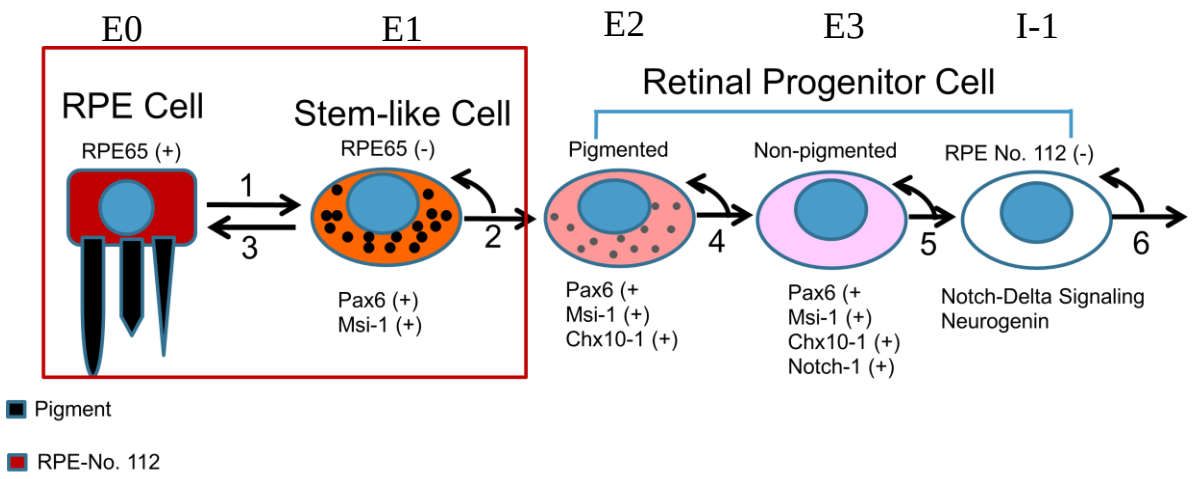
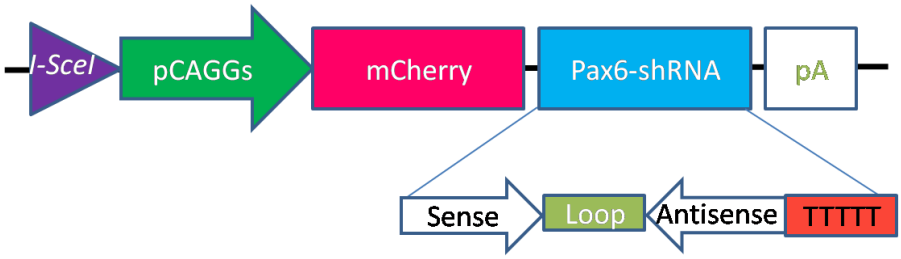


Figure 4: Working hypothesis modified from Chiba C and Mitashov VI, 2007.

In this hypothesis, they proposed that RPE cells after retinectomy (surgical removal of retina) switch to stem like cells while they express retinal stem cell marker Pax6 and Mushashi-1 decrease their marker RPE65 and proliferate. These stem like cells can derive both retinal progenitor and RPE itself. The retinal progenitor cells following expression of other neural retinal progenitor markers- Chox-10 and Notch-1 continuing proliferation toward the complete regenerated retina. Black and red color reflects pigment granule and RPE65 protein respectively.

Figure 5: Target sites of *shRNAs.*

5′ parts of cDNAs encoding isoforms (LL, LS, SL, SS) of *Cynops pyrrhogaster Pax6* (DDBJ/GenBank Accession #: D88741). Boxes show the sequences based on which the *shRNA*-1 and *sRNA*-2 were designed.



shRNA1: 5' aattgAACATGCAGAACAGTCATAGCGGgaagcttgCCGCTATGACTGTTCTGCATGTTttttgtt 3'

shRNA2: 5' aattgAACAGTCATAGCGGAGTCAACCAgaagcttgTGGTTGACTCCGCTATGACTGTTttttgtt 3'

Control shRNA: 5'aattgAACGACGCCTGTCAGGAACTTCTAgaagcttgTAGAAGTTCCTGACAGGCGTCGTTTTTTTTgtt 3'

Figure 6: *shRNA* transgene construct.

shRNA-1 and *shRNA-2* were made against *Pax6* mRNA transcript as to knockdown all four canonical isoform. Control *shRNA* was designed against newt *β-crystalline* promoter. Every *shRNA* was then inserted into pACGGs-mCherry-pA (Clontech EGFP-N1 vector modified version and a kind gift from Dr. Elly Tanaka, Molecular Cell Biology and Genetics, Max Planc Institute, Leipzig, Germany) cassette between *MunI* and *HpaI* restriction site.

Figure 7: Epitope sites of antibodies on newt's four canonical Pax6 isoforms.

LL, SL, LS and SS (Mizuno M et al, 1997) containing paired domain (Green box) and homeo domain (orange box), Blue, pink, red and green colored sequence indicating epitope region of AD2.38, PA1-801, Pax6 (LS/SS) and PRB-278P antibodies respectively.

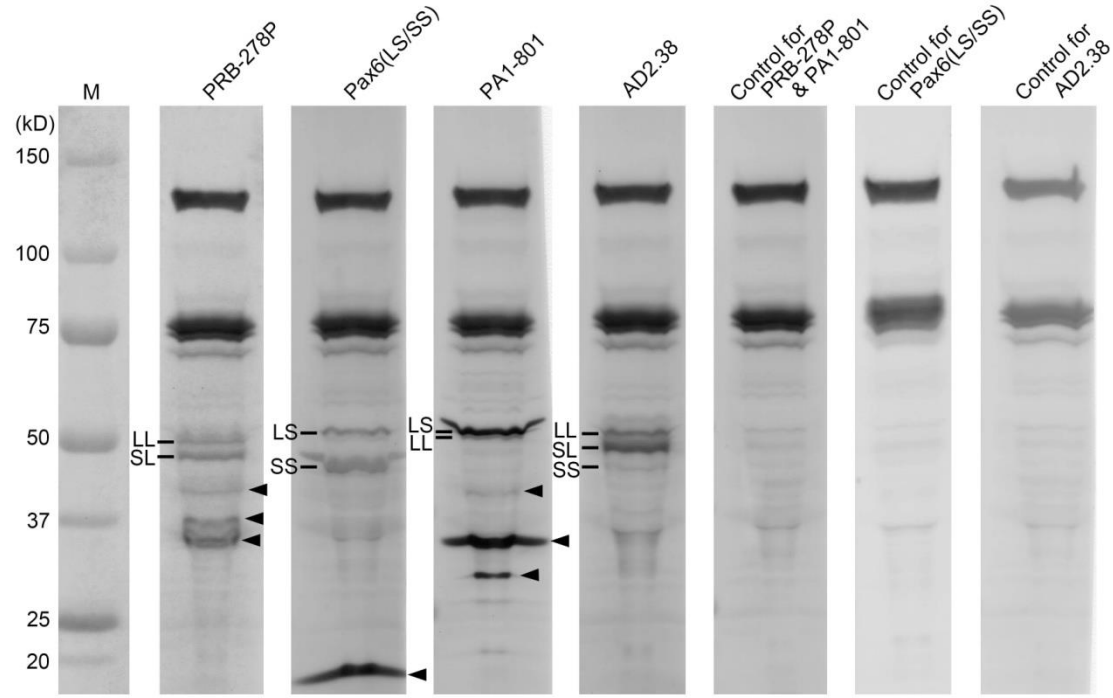


Figure 8: Western blot of the protein extracted from normal neural retina for Pax6 against PRB-278P, Pax6 (LS/SS), PA1-801 and AD2.38 anti Pax6 antibodies.

PRB-278P could label LL and SL isoform however some low molecular weight protein band was also detected. Pax6 (LS/SS) which was designed to label LS and SS could efficiently detect these two isoforms but this one also bound with low molecular weight protein. PA1-801 which should label all four isoforms theoretically but practically detect LS and LL isoform as well as some low molecular weight protein band. AD2.38 label successfully at least three canonical isoform- LL, SL and SS but it did not react with low molecular weight protein band. Results were compared with negative control where primary antibody was not used instead secondary antibody was used only.

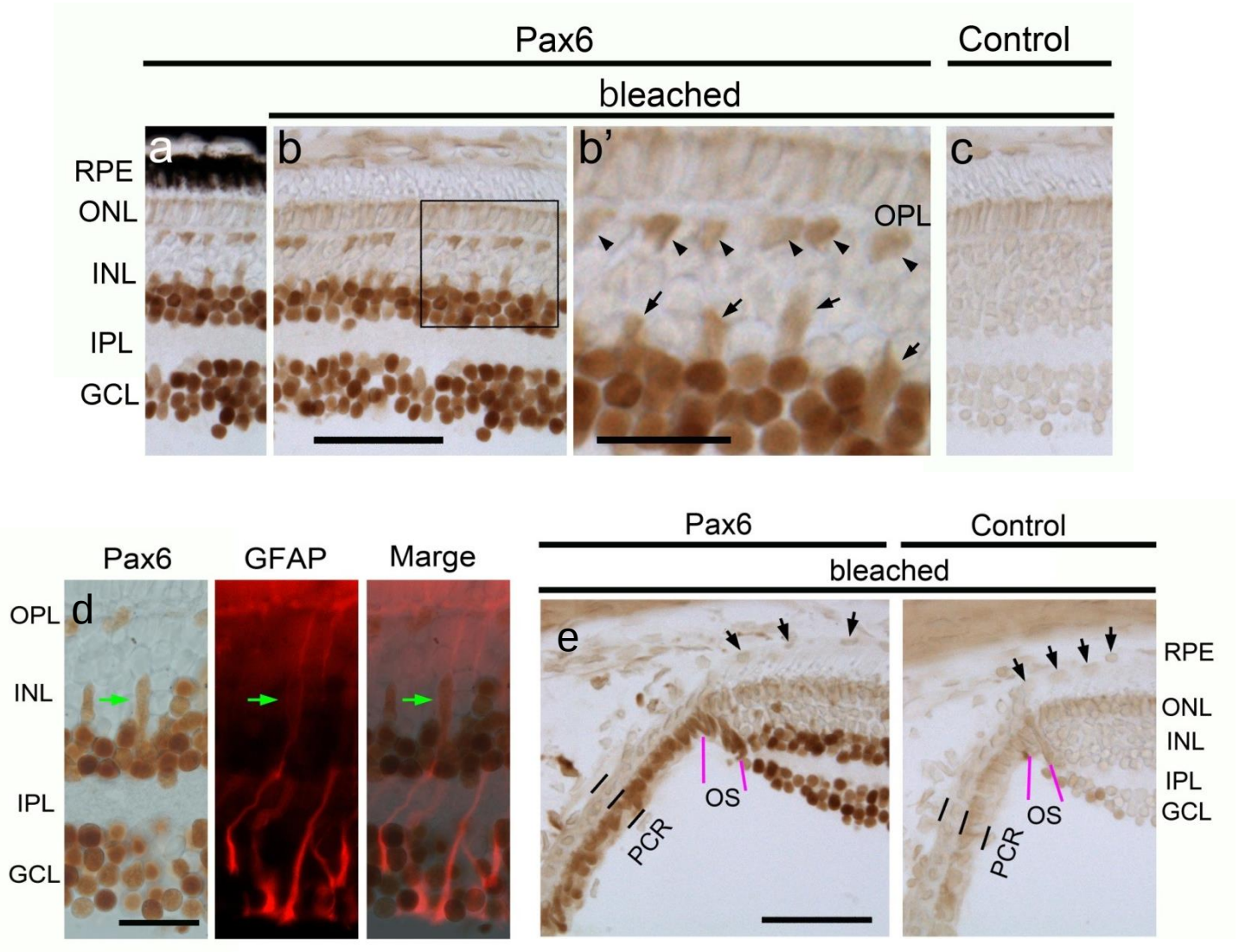


Figure 9: Immunolocalization of Pax6 in central retina and CMZ.

AD2.38 labelled almost all of the cell bodies (nuclei) of amacrine and ganglion cells, which are located in the inner one third of the inner nuclear layer (*INL*) and in the ganglion cell layer (*GCL*). Labelling intensity was not uniform. This antibody also labelled cell bodies (nuclei) of Müller glia cells (arrows) and horizontal cells (arrowheads) (**b**). a: before bleaching; band b: after bleaching. b': enlargement of the box in b_2. Pax6-immunoreactivity

in Müller glia cells (green arrow) were confirmed by double labelling with a GFAP antibody (red) which visualizes the apical and basal processes of Müller glia cell (Susaki K and Chiba C, 2007) **d.e,** Immunohistochemistry of the peripheral retina (after bleaching). The ciliary marginal zone (CMZ), which is comprised of *ora serrata* (*OS*) and *pars ciliaris retinae* (*PCR*: the partially pigmented inner layer of the ciliary epithelium), containing the retinal stem/progenitor cells, was labelled with AD2.38. Arrows indicate nuclei of RPE cells with no labelling. *ONL*: outer nuclear layer; *OPL*: outer plexiform layer; *IPL*: inner plexiform layer. Scale bars: 100 μm for **a, b** and **c**; 40 μm for **b'** and **d**.

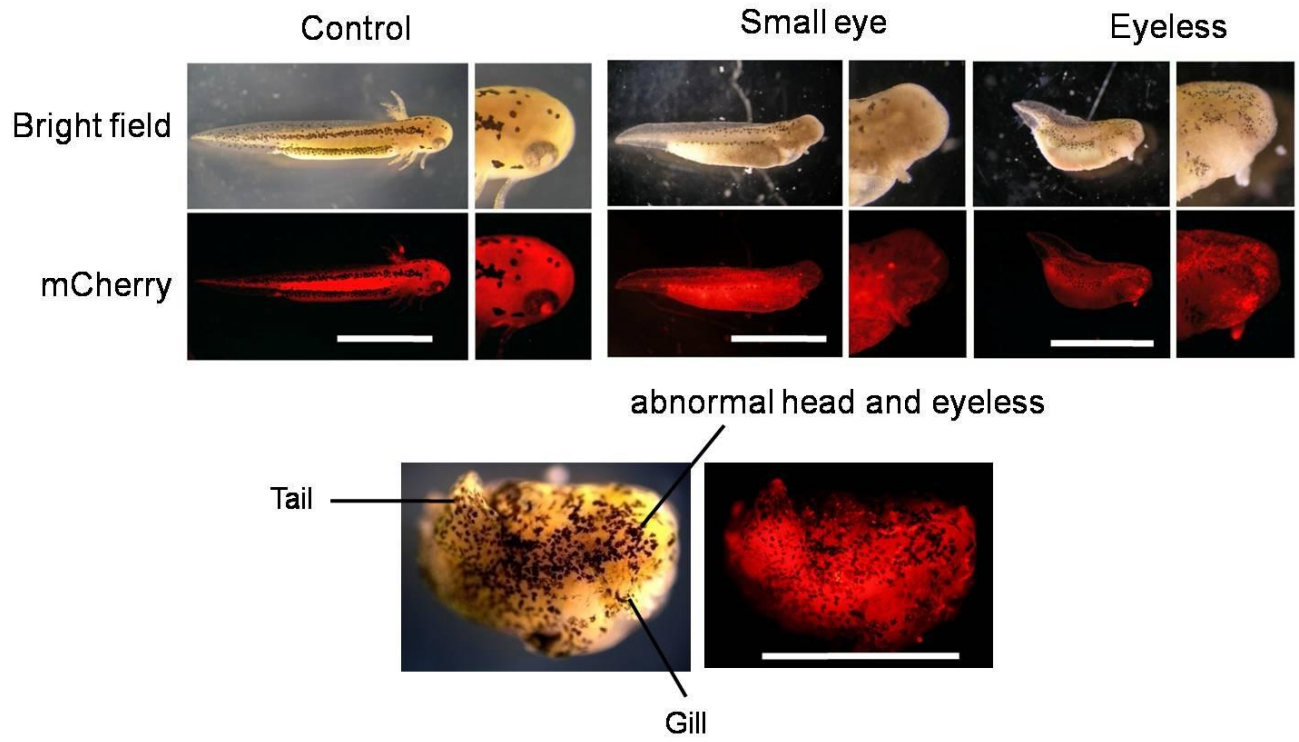


Figure 10: Functional identification of *Pax6* by *shRNA*-transgenesis morphologically.

Knock-down of *Pax6* was carried out with two *Pax6 shRNAs* which were designed to degrade all four transcript variants (LL, LS, SL, SS) of *Pax6*. Each was inserted into the pACGGs-mCherry-pA (Clontech EGFP-N1 vector modified version and a kind gift from Dr. Elly Tanaka, Molecular Cell Biology and Genetics, Max Planc Institute, Leipzig, Germany) transgene construct which allows the expression of both a reporter mCherry and *shRNA* in the whole body of the animal. Control *shRNA* was designed from a region in the newt crystallin promoter. *Pax6 shRNAs* affected eye morphogenesis as well as body growth along the anterior-posterior axis

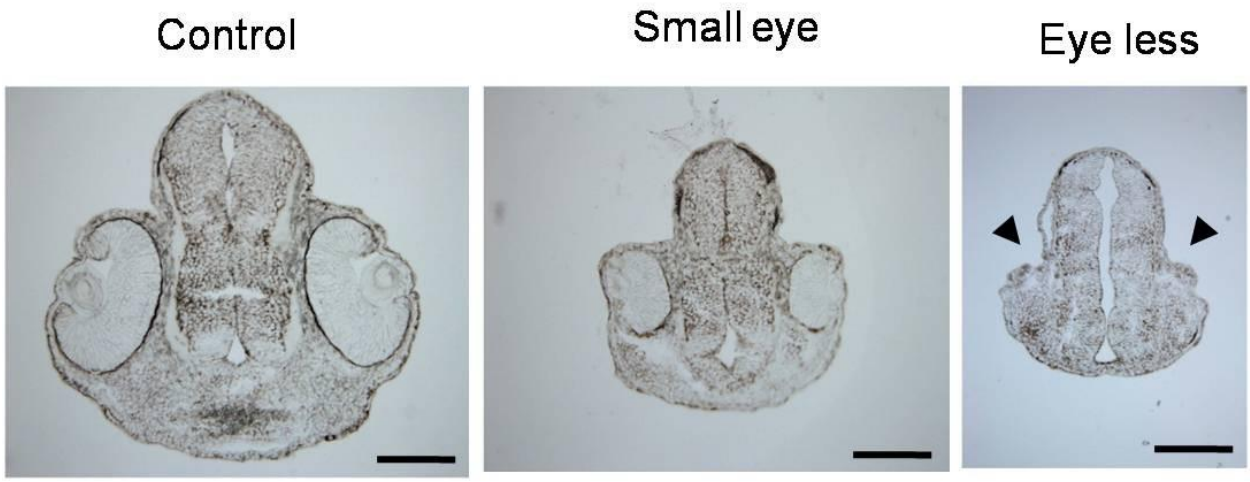


Figure 11: Tissue section showing Pax6 knockdown effect on eye morphogenesis

Control *shRNA* virtually exerts no effect on eye development however *shRNA*-1 and *shRNA*-2 both results small eye where eye field is very small with tiny lens. Moreover *shRNA*-2 severely affects eye development resulting no eye at all (eyeless, arrow head). Scale bar: 100µm

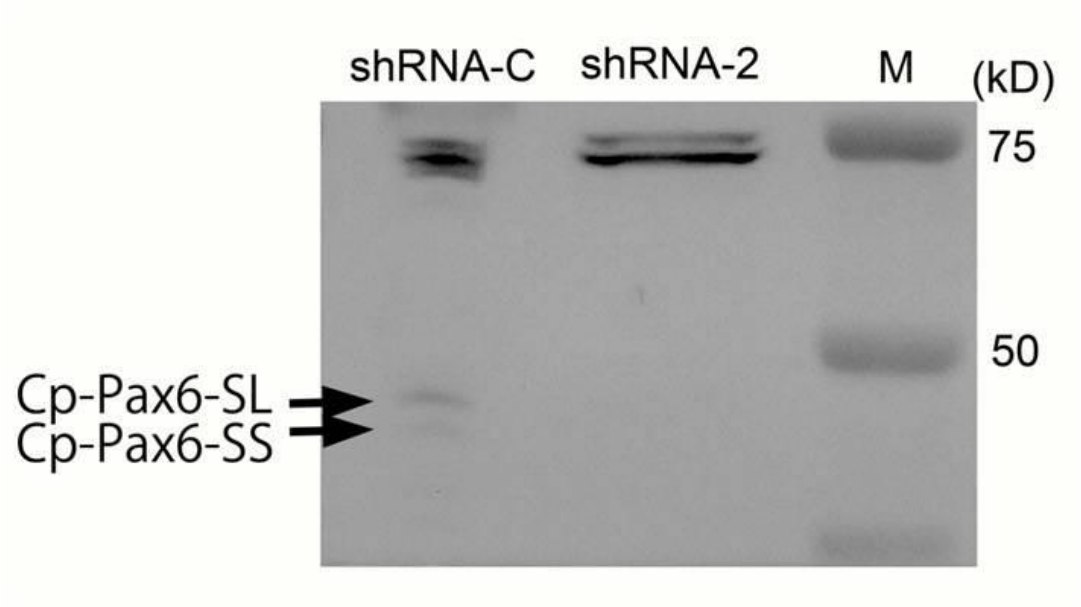


Figure 12: *shRNA* knockdowns Pax6 protein

Western blot with protein extracted from larvae treated with control *shRNA* and *shRNA-2* (eyeless and headless animal) revealed that *shRNA-2* were able to knock down Pax6 efficiently because no Pax6 protein band was detected in eyeless and headless animal's protein while two bands corresponding to newt SL, SS Pax6 were detected in protein of animal treated with control *shRNA*.

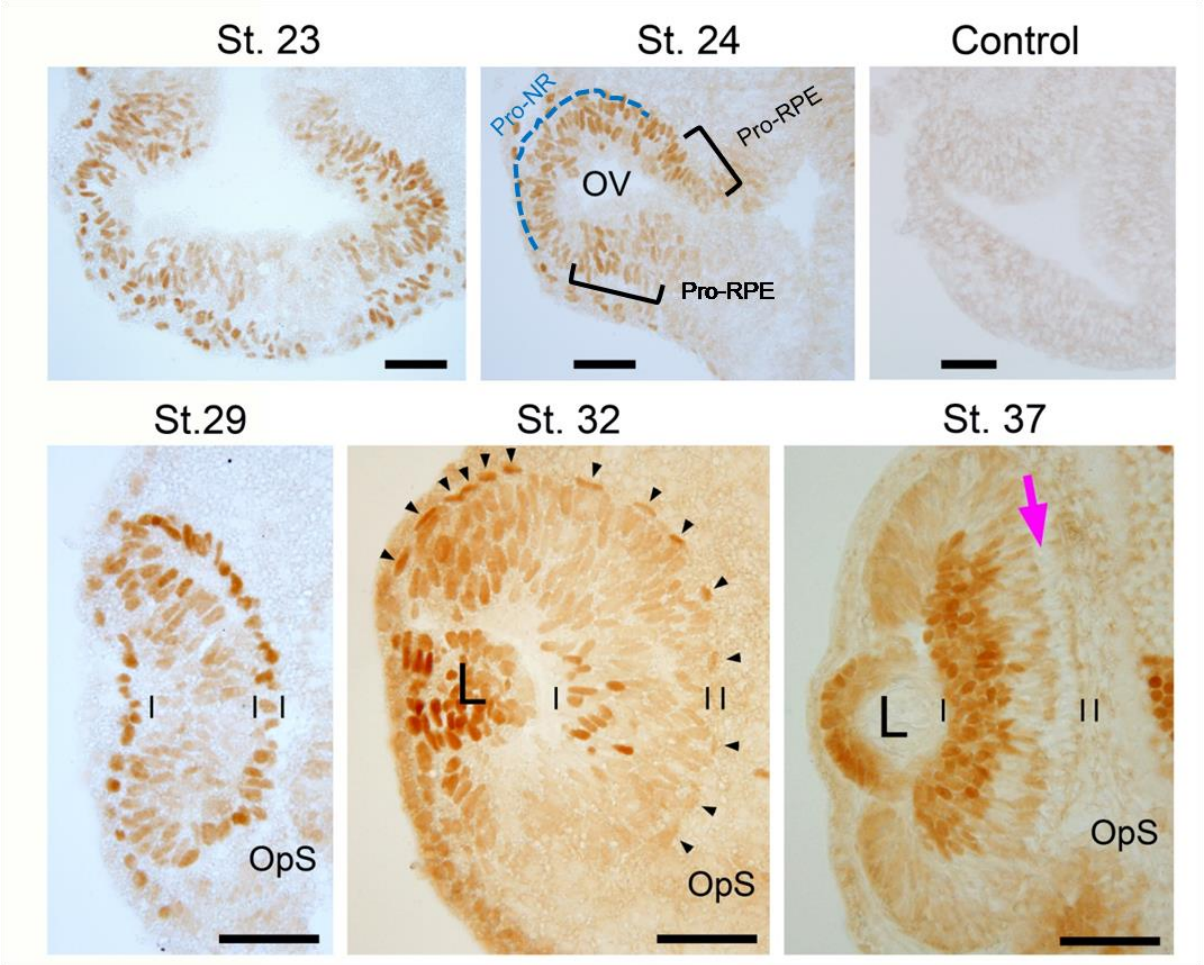


Figure 13: Pax6 expression pattern and its regulation during retina and RPE development. Pax6 expression was localized thoroughly in the cell's nucleus of early optic vesicle (neural epithelium, St.23) which continues to the pro-RPE and pro-NR (St24 and 29). With the development of retina and RPE expression become stronger in the differentiated ganglion cells, proliferating retinal progenitor cells (marginal zone) and peripheral iris pigment cells than in the cells of central retina while Pax6 expression starts decreasing in the RPE cells specially in the central area (St.32) and completely disappears (St.37). OV: Optic Vesicle, L: Lens; Cells between double vertical bar are RPE and the region between a single vertical bar and double vertical bar is neural retina, OpS: Optic Stalk, Scale bar: 100 µm

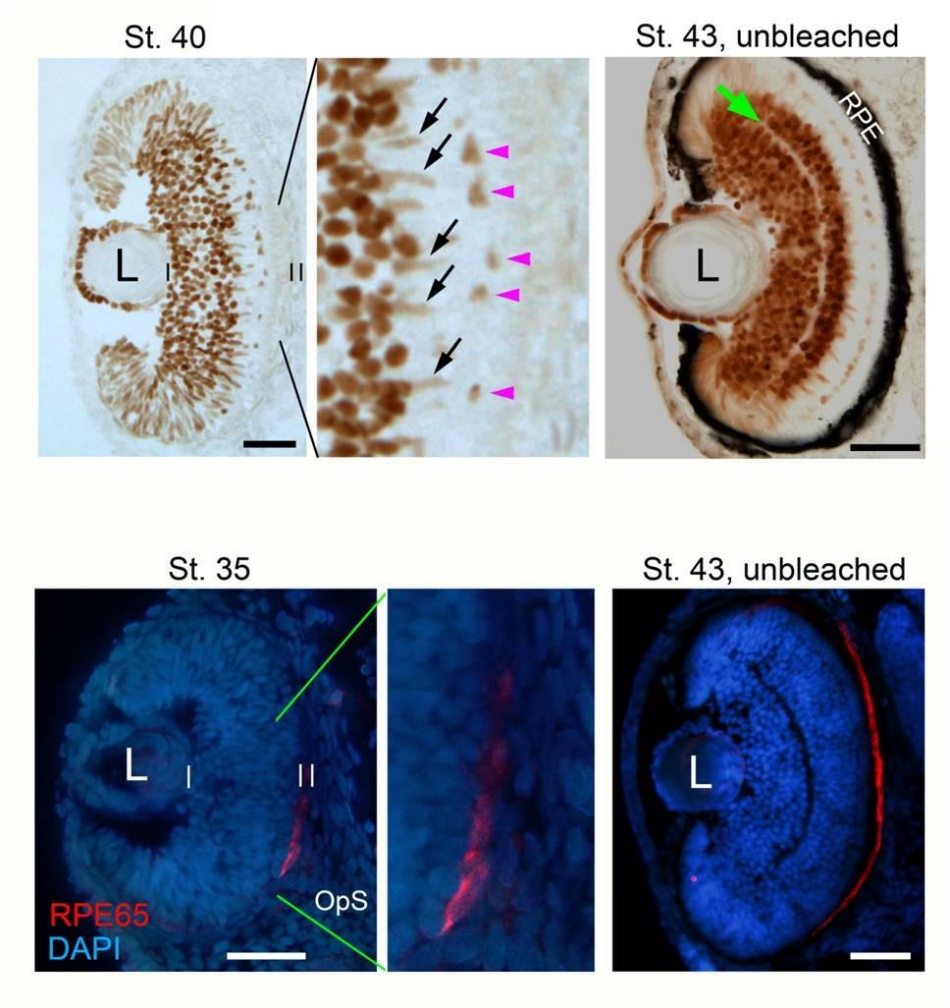


Figure 14: Pax6 and RPE65 expression in the embryonic eye.

On the NR side, Pax6 expression increased in certain types of cells as they differentiated in the following order: ganglion/amacrine cells (St. 30) → horizontal cells (St. 37) → Müller glia cells (St. 40). It should be noted that Müller glia cells in this animal expressed Pax6 even in the mature NR (indicated with the black arrow) as well as horizontal cells (indicated with the pink arrow head). RPE65, a maturation marker of RPE cells, initiated expression from the corresponding area at St. 32 (red) and extended along the RPE layer (St. 43).

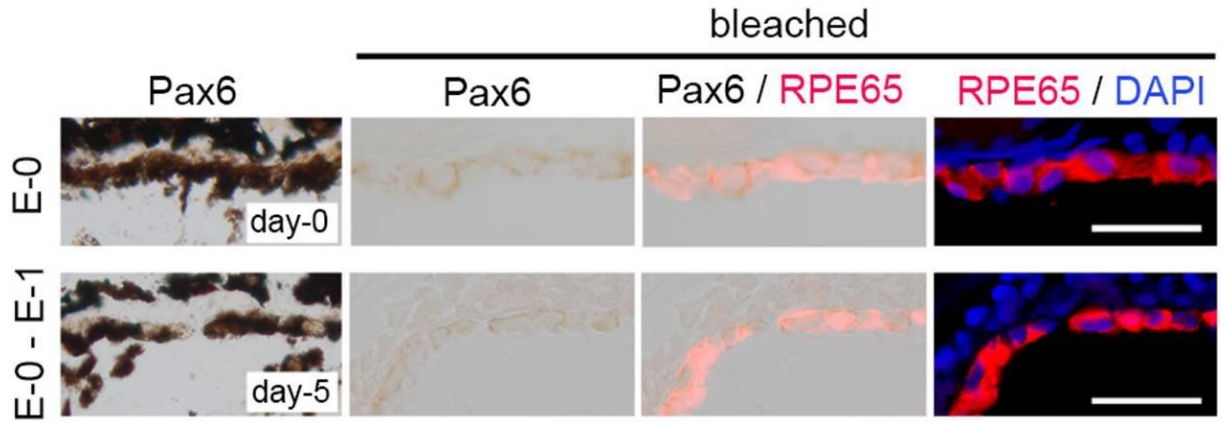


Figure 15: Pax6 signal was not detected in day-0 and day-5 po RPE cells.

Tissues were double stained with RPE65 antibody (red fluorescent) to specify RPE cells followed by nuclear stained with DAPI (blue fluorescent). However it is noteworthy that RPE cells started to lose epithelial (disrupt cell to cell adhesion) decreasing pigment granule in day-5 comparing with day-0 section. Scale bar: 40µm

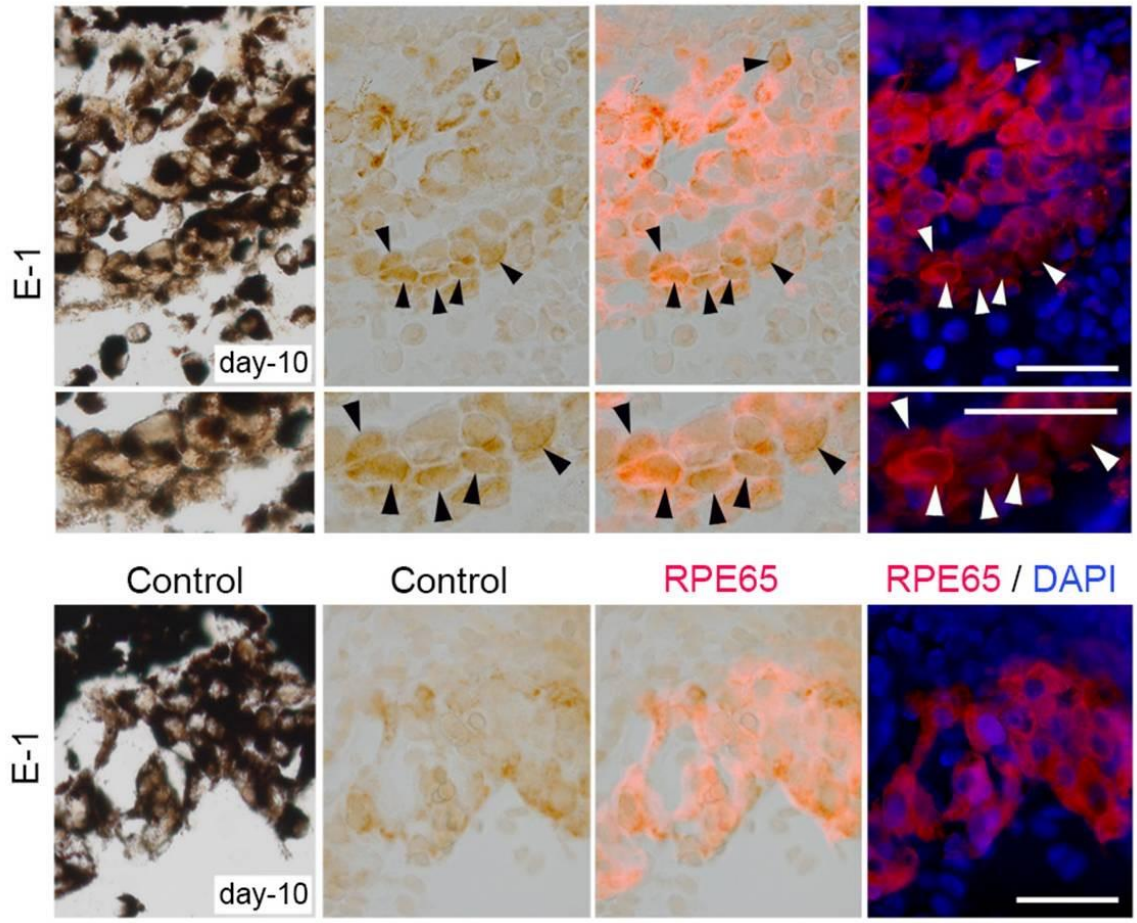


Figure 16: Pax6 expression was first detected in some d-10 (E1 stage) RPE-derived cells. Tissues were bleached (pigment removal) following staining with RPE65 (red) and DAPI (blue) for a purpose of the clear visualization of Pax6 signal in the nucleus of RPE-derived cells. Positive signal was observed in the nucleus of some RPE-derived cells. RPE-derived cells form aggregate in this stage after completely losing epithelial character. Scale bar: 40µm

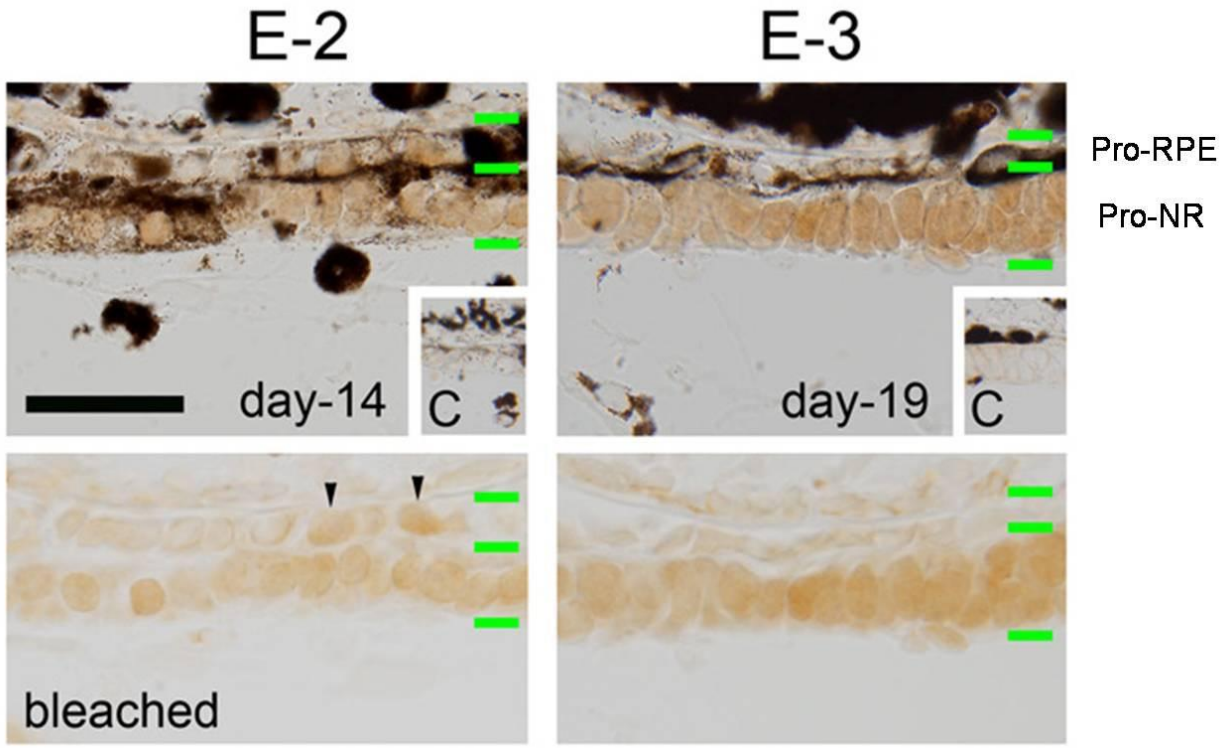


Fig 17: Pax6 expression patter in 14 and 19 days po retinectomized eye.

Stage E-2 to E-3, when the two rudimentary layers formed (St. E-2) at around day-14, Pax6-IR became localized along the inner layer (pro-NR layer) almost uniformly, but only in a small number of nuclei (arrowheads) along the outer layer (pro-RPE layer). Pax6-IR in the pro-RPE layer became undetectable by day-19 when the constituent cells mostly exit the cell-cycle (St. E-3). On the other hand, cells in the pro-NR layer (or the regenerating NR), which continue to proliferate (Chiba C et al., 2006), continued Pax6-IR. Scale bar: 40 µm

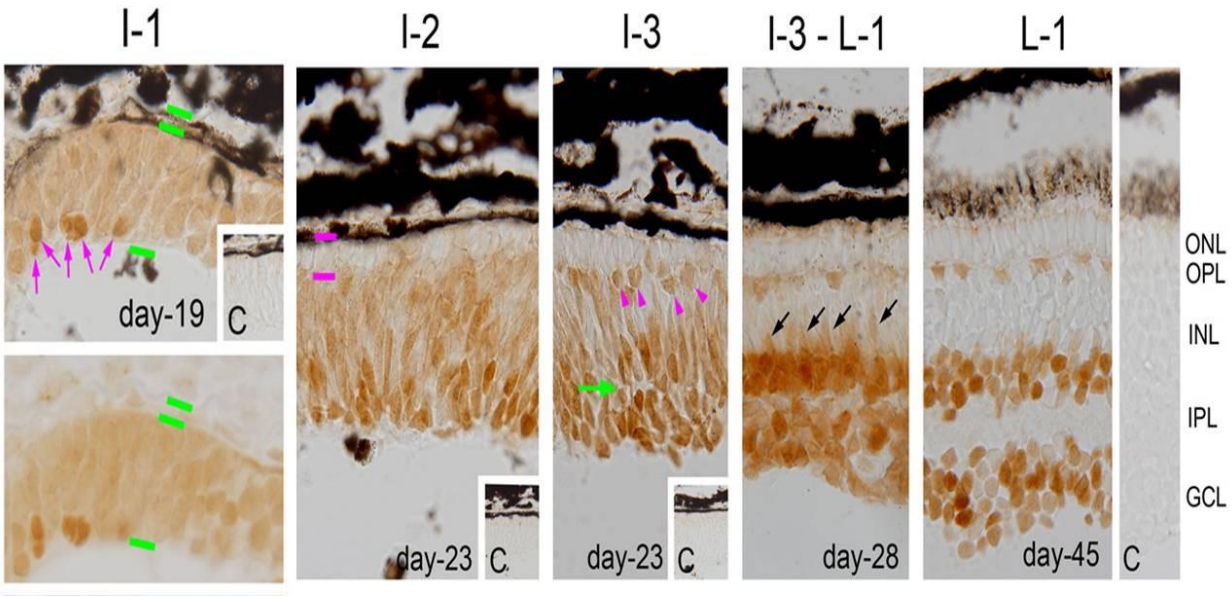


Figure 18: Pax6 expression pattern in the intermediate and later phase of regenerating retina. Expression was detected in all the proliferating cells while strong signal observed in some differentiating cell (arrow) however there was no signal in the RPE (green double bar) layer (I-1, lower panel: after bleach). Photoreceptor cells (double pink bar) and ganglion cells appeared first (I-2) in regenerating retina, the first one did not express while the later one expressed as was observed in development. As regeneration was advancing inner plexiform layer (IPL) appeared (green arrow) along with horizontal cells (arrow head) which were Pax6 positive (I-3) there after Müller cells also Pax6 positive (black arrow) and outer plexiform layer (OPL) seemed like normal neural retina (L-1). C: negative control

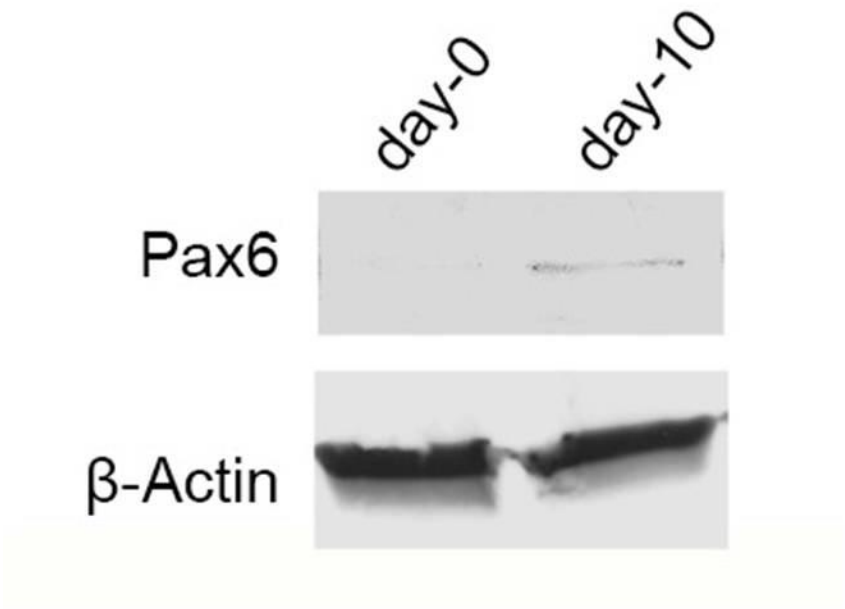


Figure 19: Pax6 protein was detected by western blot in d-10 RPE-choroid tissues. Protein extracted from d-0 (E0 stage) and d-10 (E1 stage) RPE-choroid tissues to perform western blot for the identification of Pax6 presence. A weak and thin but distinguished band correspond to newt LL-Pax6 was detected in protein of d-10 (E1 stage) RPE-choroid tissues. β-Actin was used as internal control.

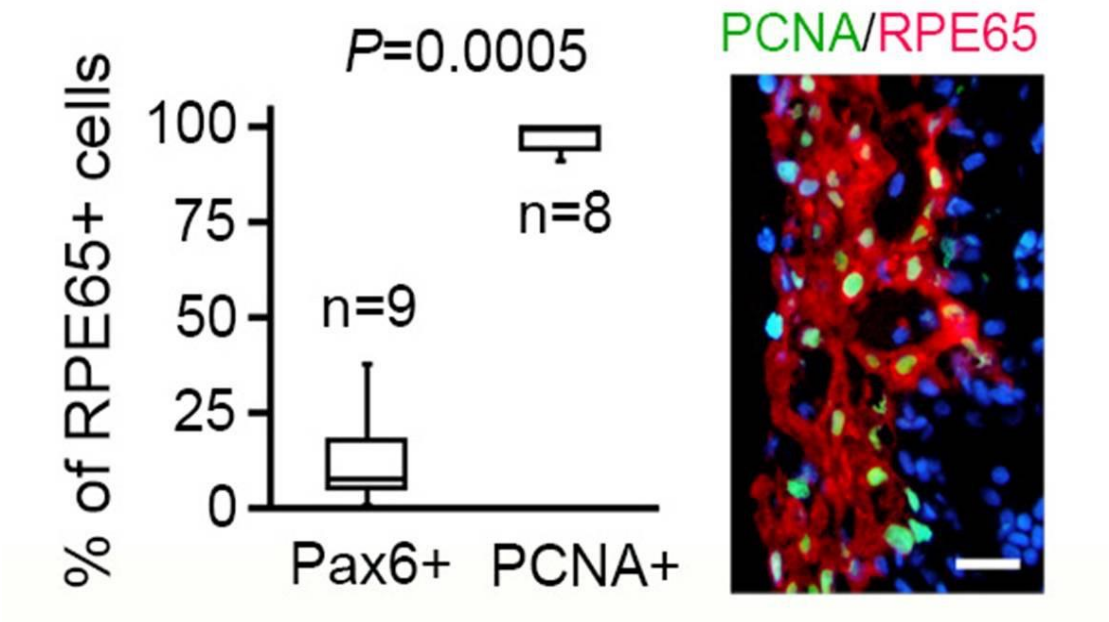


Figure 20: RPE cells that enter the cell cycle and express Pax6 in day-10 po tissues. Tissues were double stained with RPE65 (red fluorescent) and PCNA (green fluorescent)/Pax6 (DAB) followed by counter staining with DAPI (blue fluorescent) to visualize cell nucleus for counting the number of RPE cells enter into the cell cycle and express Pax6. For PCNA experiment 8 adjacent eye tissues section from 4 animals and for Pax6 experiment 9 adjacent eye tissues section from 4 animals were analyzed. The box and whisker plot shows about 98% RPE cells enter into the cell cycle while only about 12% RPE cells express Pax6. Data was presented as a box plot graph or as a bar graph showing the mean ± SEM. Statistical differences were evaluated by Mann-Whitney's U test or Sheffe's test following the Friedman test.

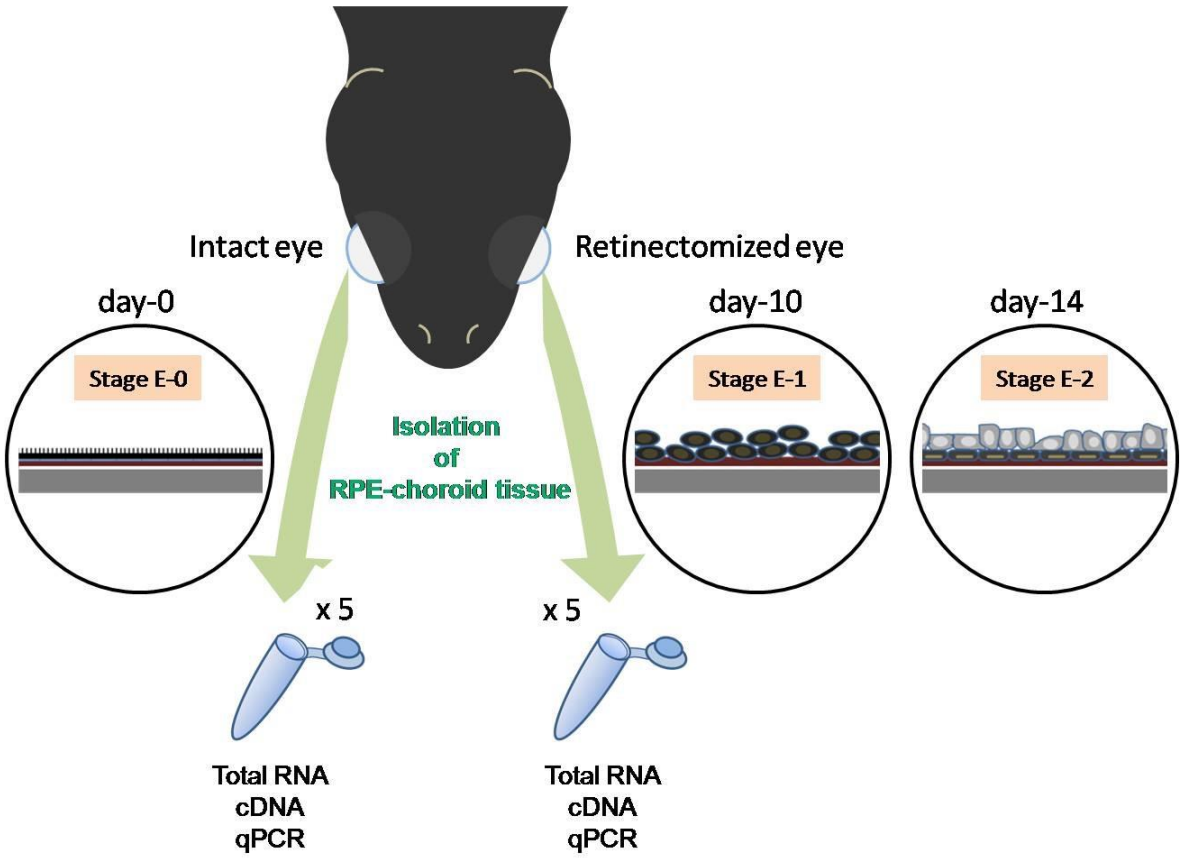


Figure 21: cDNA preparation from RPE-choroid tissues.

Intact eye and retinectomized eye were enucleated and anterior half was cut along with lens (intact eye) and iris pigment epithelium out (neural retina was removed from intact eye) then RPE-choroid tissues were collected using a fine micro niddle for total RNA isolation followed by cDNA syntehis for qPCR analysis.

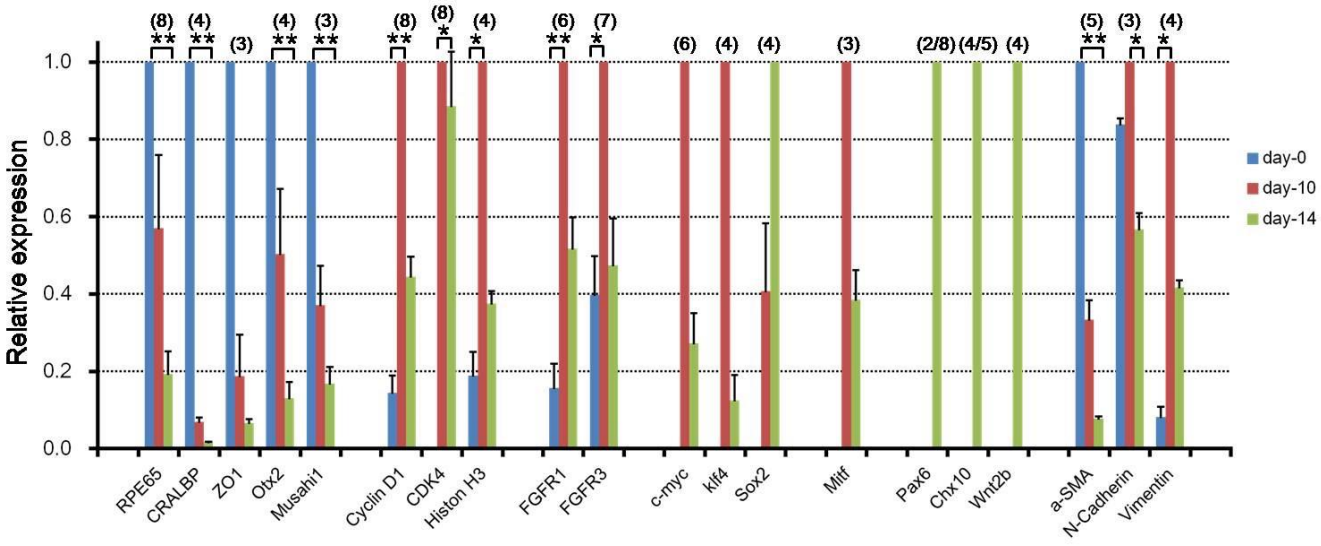


Figure 22: Identificataion genes predicted importantly involved in the early process of retinal regeneration by qPCR. 20 selected genes which have been suggested or inferred to be regulated in an early phase of retinal regeneration were found in *in silico* transcriptomes. Changes in their relative expression level until day-14 po were examined. Results represent means and SE. The number in parenthesis indicates the number of independent sample sets (see Methods) except for *Pax6* and *Chx10*, whose expression was detected in 2 of 8 sample sets and 4 of 5 sample sets, respectively at day-14 p.o only. Statistical significance based on Sheffe's test following the Friedman test (*: $p< 0.05$; **: $p< 0.01$), except for *Pax6* and *Chx10*.

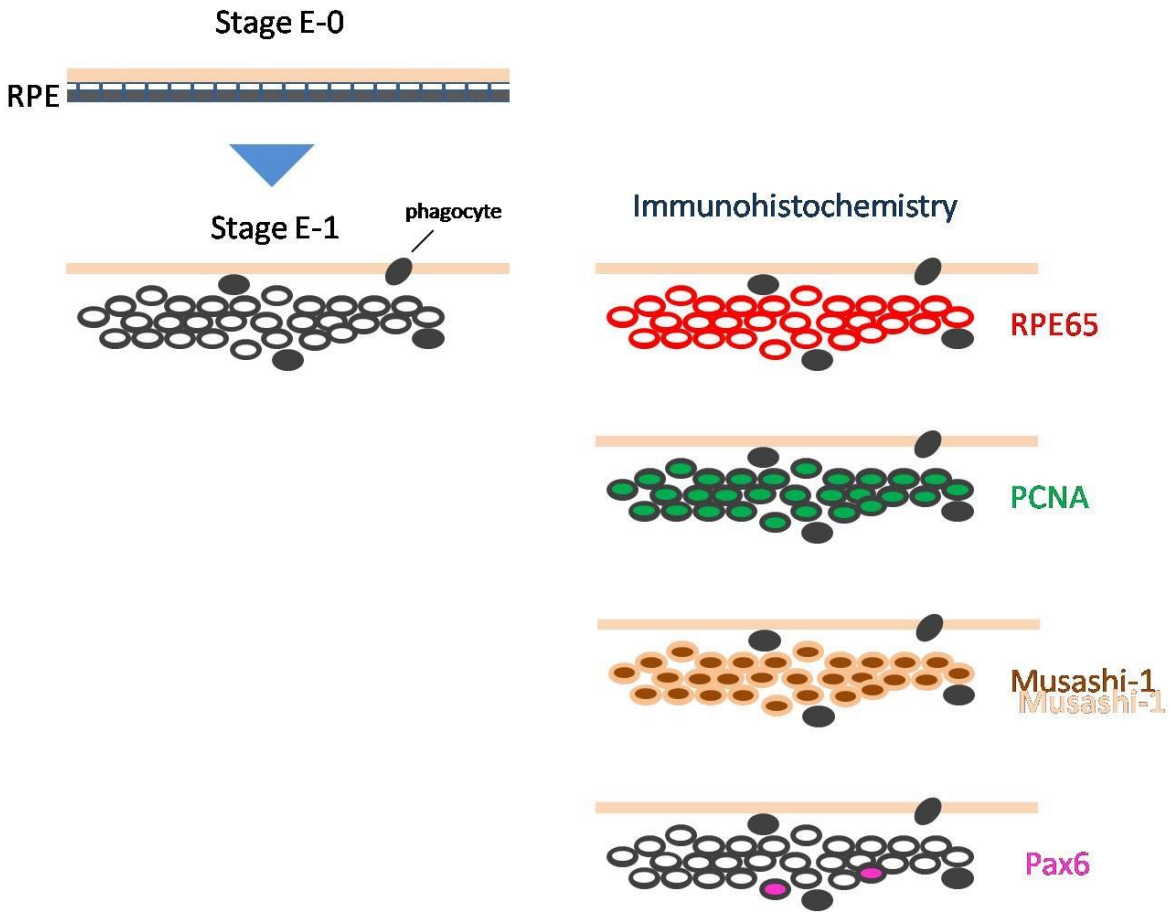


Figure 23: Aggregation of RPE-derived cells and their expression pattern of RPE65, PCNA, Musashi-1 (Kaneko J and Chiba C, 2009) and Pax6.

After 10 days of retinectomy (Stage E1), some pigmented (darker than RPE-derived cells) cells are found in the cells aggregates (Chiba et al., 2006) looks like RPE-derived cells but actually they are blood cells (phagocytic cell). Immunohistochemical studies reveal their corresponding expression pattern for each antibody.

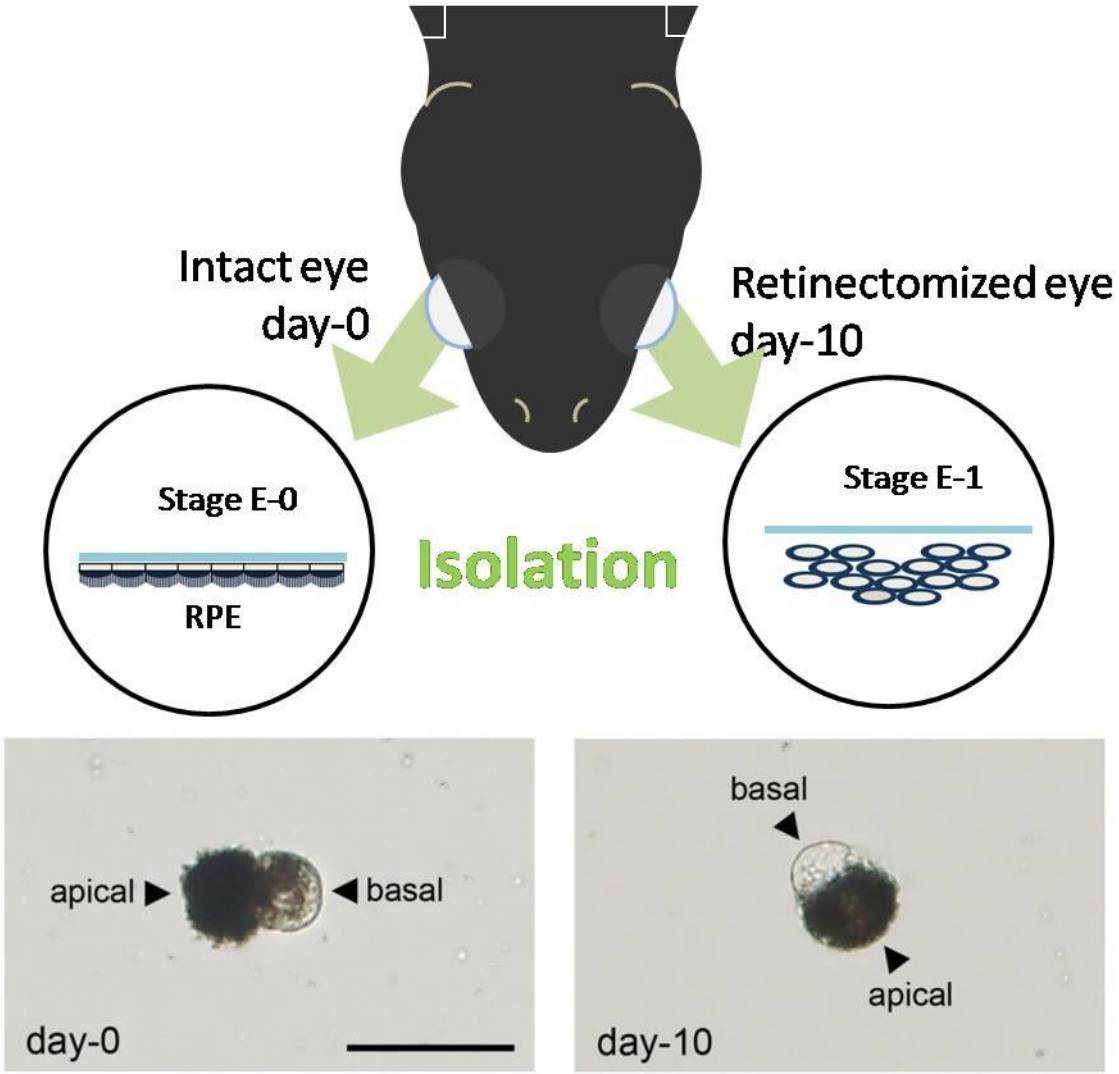


Figure 24: Single RPE cell isolation.

Single RPE cell was isolated for the analysis of multipotency factor from both the d-0 and d-10 eye. d-10 RPE cells having basal and apical pole and pigment almost closely like d-0 RPE was taken for the qPCR analysis because cells were sorted manually under microscope. That is why cell polarity and pigment were treated as marker for this manual cell sorting technique.

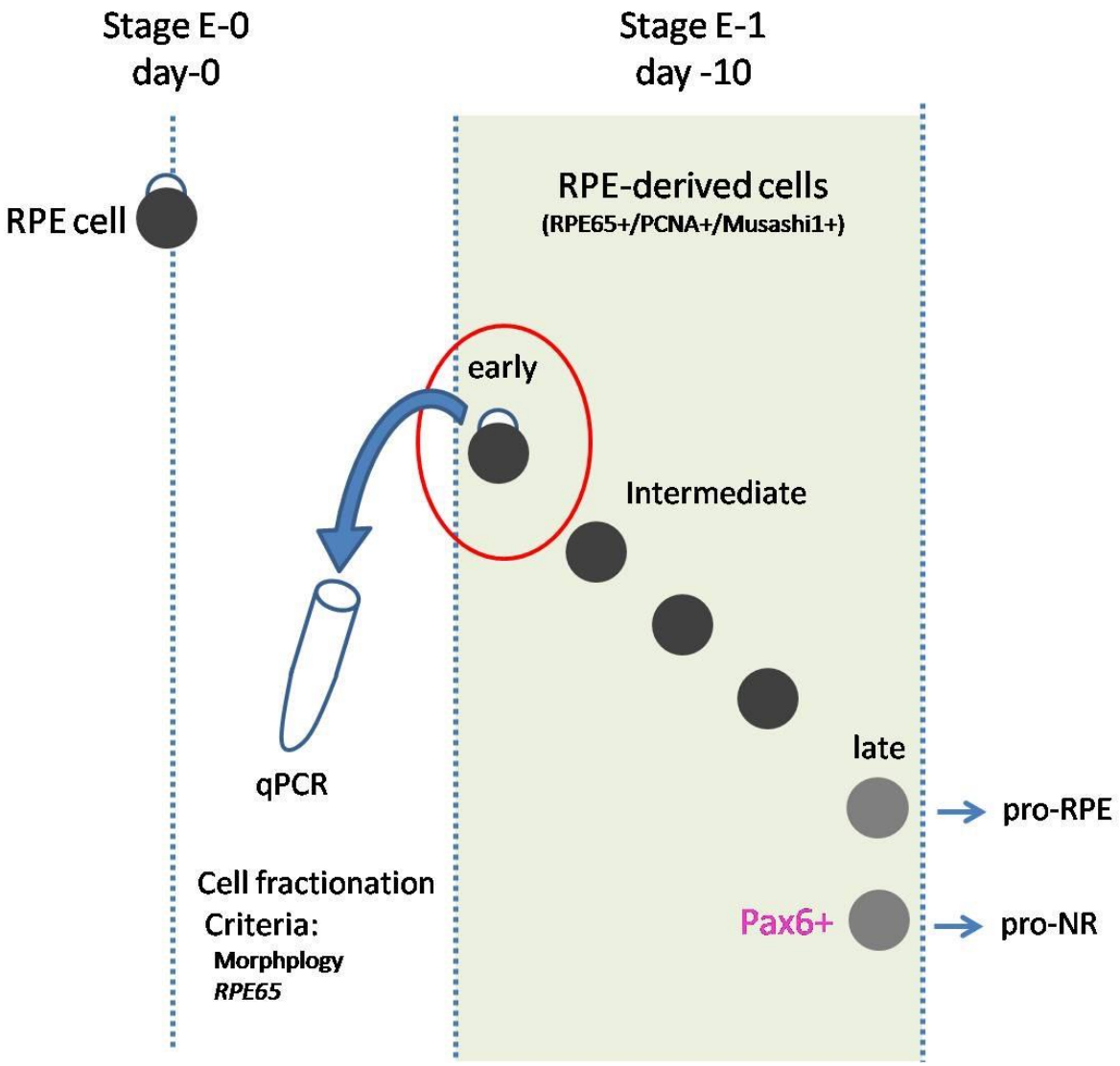


Figure 25: RPE-derived cells fractionation.

RPE-derived cells after 10 days po (Stage E1) seemingly exist in different pace of morphological change and so far reprogramming. The cells that were closely resembled to the intact RPE were taken for qPCR analysis.

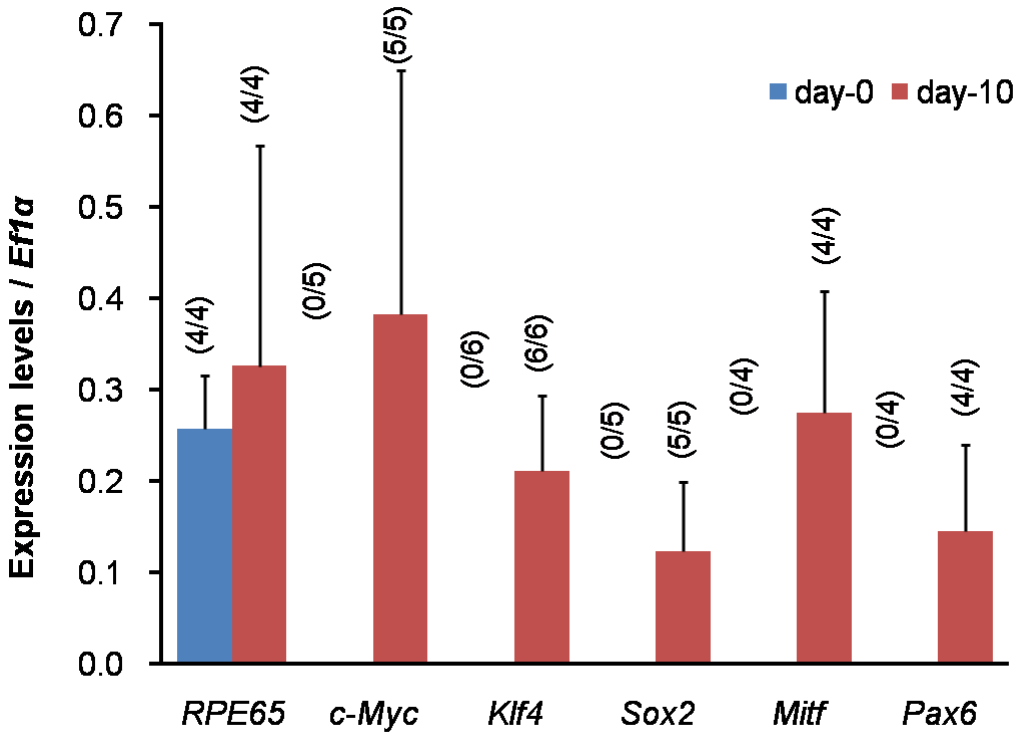


Figure 26: 100 isolated RPE-derived cells' qPCRfor target genes.

First, 100 cells at day-0 and day-10 were harvested into different tubes to prepare each-day cDNA sample, real time PCR was carried out using these samples for *RPE65*, *c-Myc*, *Klf4*, *Sox2*, *Mitf*, *Pax6* as well as *Ef1α*. A comparison of the expression levels of genes that compensated for *Ef1α* is shown on the graph. Green and red column represent day-0 (E0) and day-10 (E1) RPE cell's genes expression respectively.

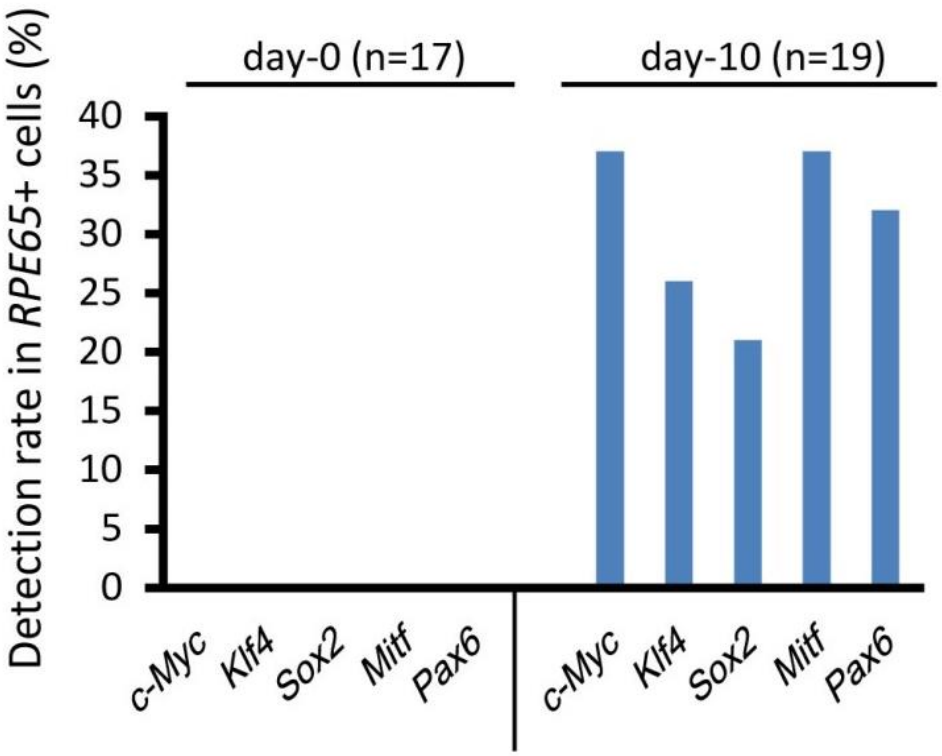


Figure 27: One solitary RPE-derived cell qPCR for target genes.

Numbers in parenthesis are the ratio of detection (dominator: sample number). The same experiments were performed with one-cell sample as were done for 100 RPE cells. Cumulative detection rates of genes-*c-Myc, Klf4, Sox2, Mitf and Pax6* in *RPE65+* cells are shown in

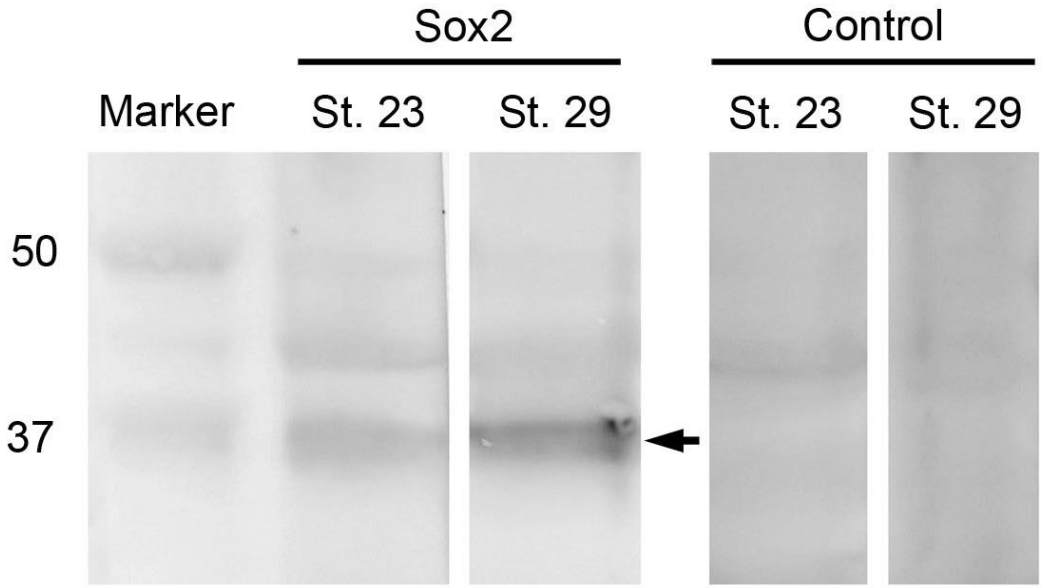


Figure 28: Western blot of the protein extracted from embryos (St. 23 and St. 29) for Sox2. Sox2 antibody (ab97959, Abcam) labelled protein bands corresponding to the newt Sox2 (~34 kD) in both stages. In control experiment anti dsRed antibody (anti-dsRed antibody; 632543 or 632496; Clontech) was used instead of primary antibody and no band was detected corresponding to Sox2.

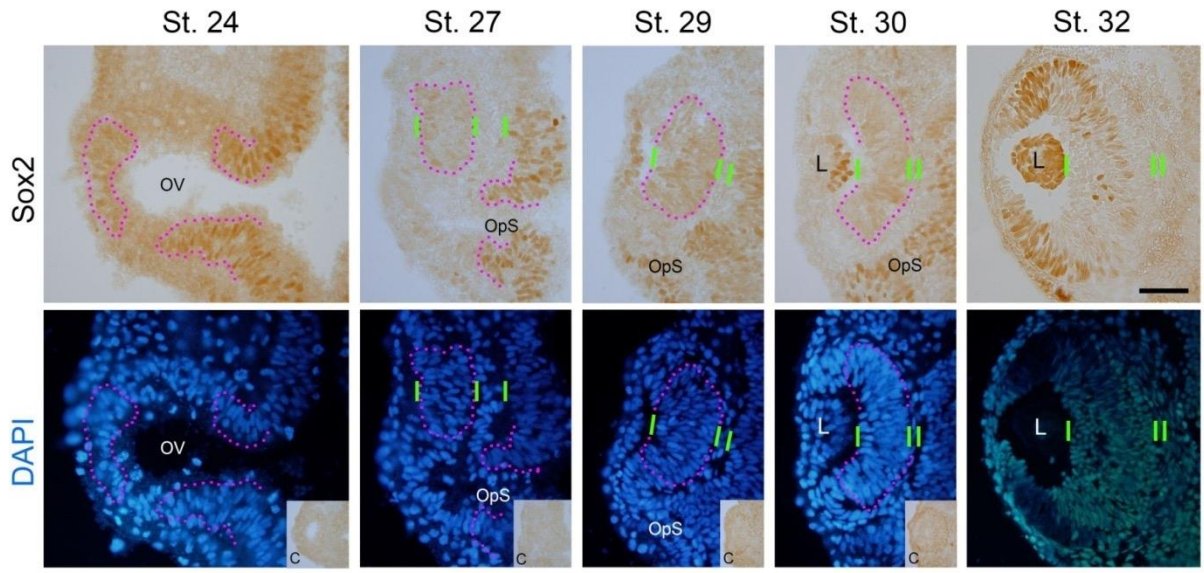


Figure 29: Immunohistochemistry of developing retinas (after bleaching) for Sox2 expression pattern.

Sox2-immunoreactivity, which was observed uniformly in nuclei of the early optic vesicle, was restricted in the pro-NR regions in the late optic vesicle (St. 24) to the optic cup (St. 29). The immunoreactivity observed in the retinal stem/progenitor cells in the central retina (St. 30) became restricted in the peripheral retina as neuronal differentiation started from the central retina while the retina grew in the periphery (St. 32). Inset panels labelled with 'c': negative control; DAPI: a nuclear marker; pink dotted lines: Sox2-immunoreactive area; short green lines: borders of NR and the RPE; *OV*: the optic vesicle; *L*: the lens; *OpS*: the optic stalk.

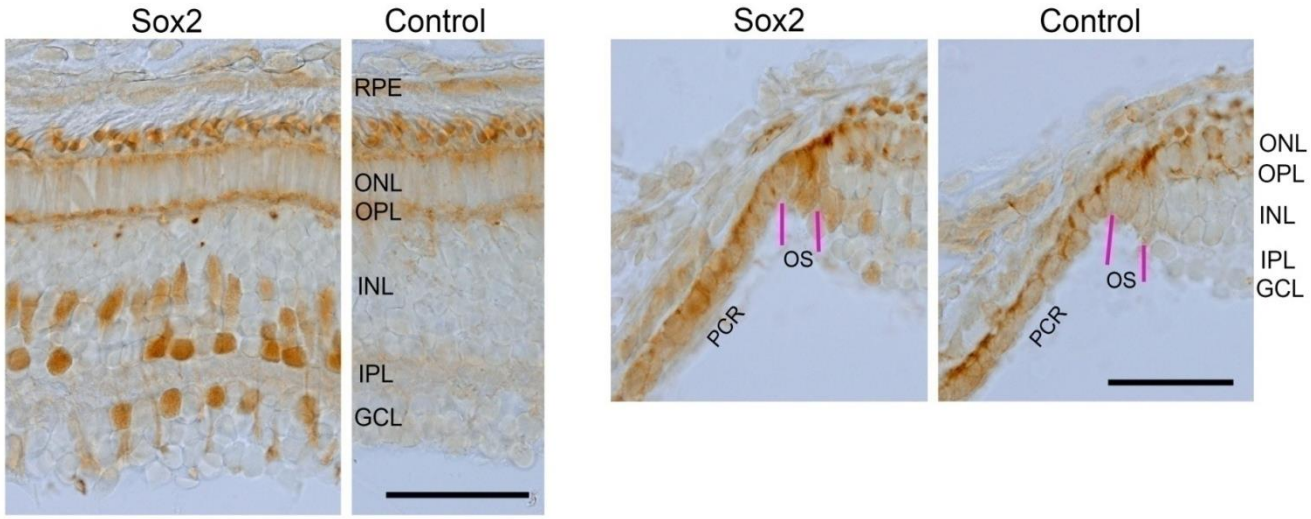


Figure 30: Immunohistochemistry of adult normal retina (after bleaching) for Sox2 expression pattern.

ab97959 labelled a small number of cell bodies (nuclei), possibly those of amacrine cells, which line both the outer and inner margins of the inner plexiform layer (*IPL*), as well as cell bodies and basal processes of Müller glia cells. In the ciliary marginal zone (CMZ), *ora serrata* (*OS*) was also labelled, but in *pars ciliaris retinae* (*PCR*) immunoreactivity was not clear. *ONL*: outer nuclear layer; *OPL*: outer plexiform layer; *IPN*: inner nuclear layer; *GCL*: ganglion cell layer. Scale bars: 100 μm.

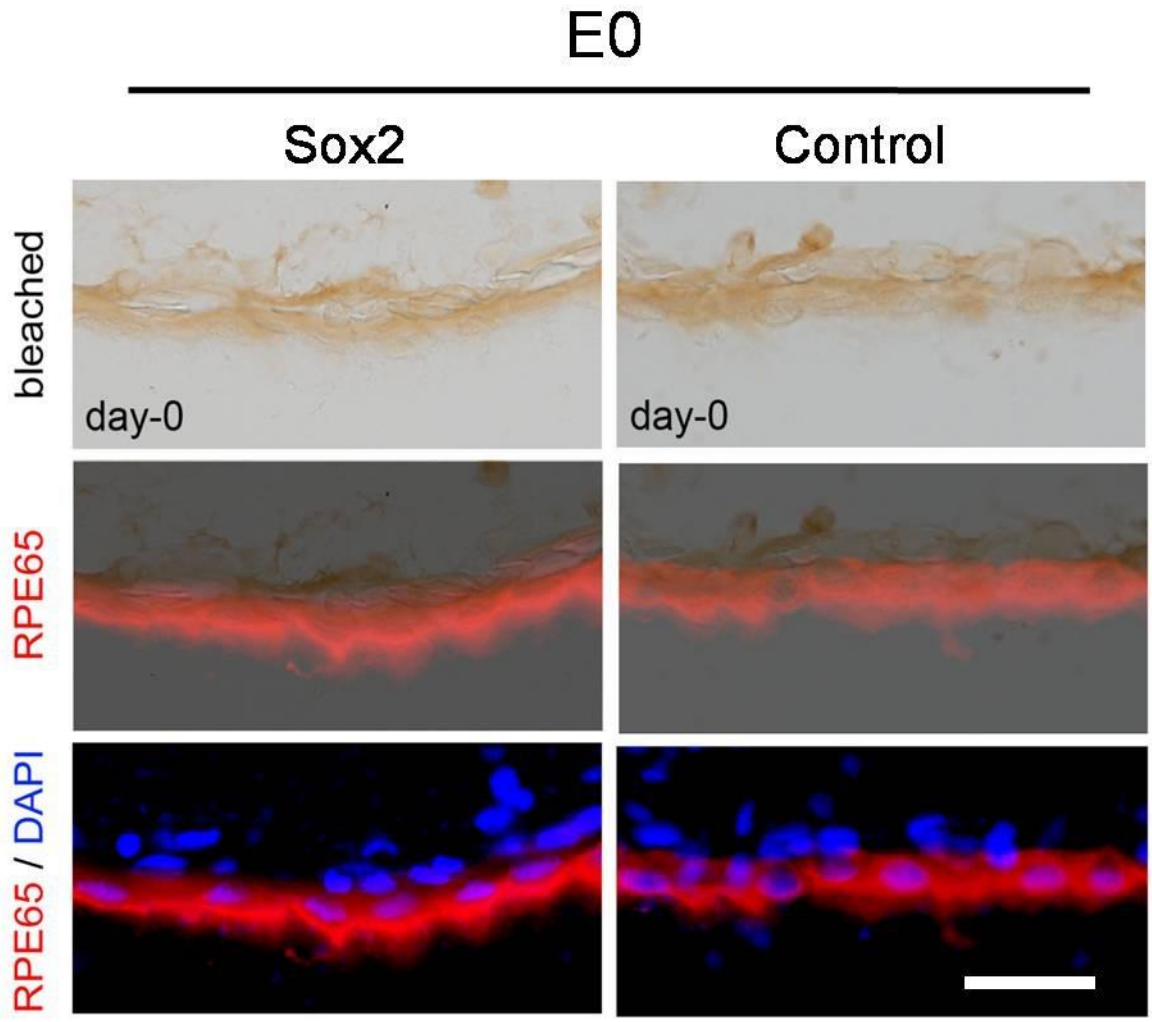


Figure 31: Sox2 immunoreactivity in d-0 po (E0 stage).

IR was not detected in tissues but RPE65 signal (red fluorescence) was detected on the RPE cells along the RPE line. Tissues were bleached (depigmentated) and counter stained with DAPI to visualize the cell nucleus. Scale bar: 50µm.

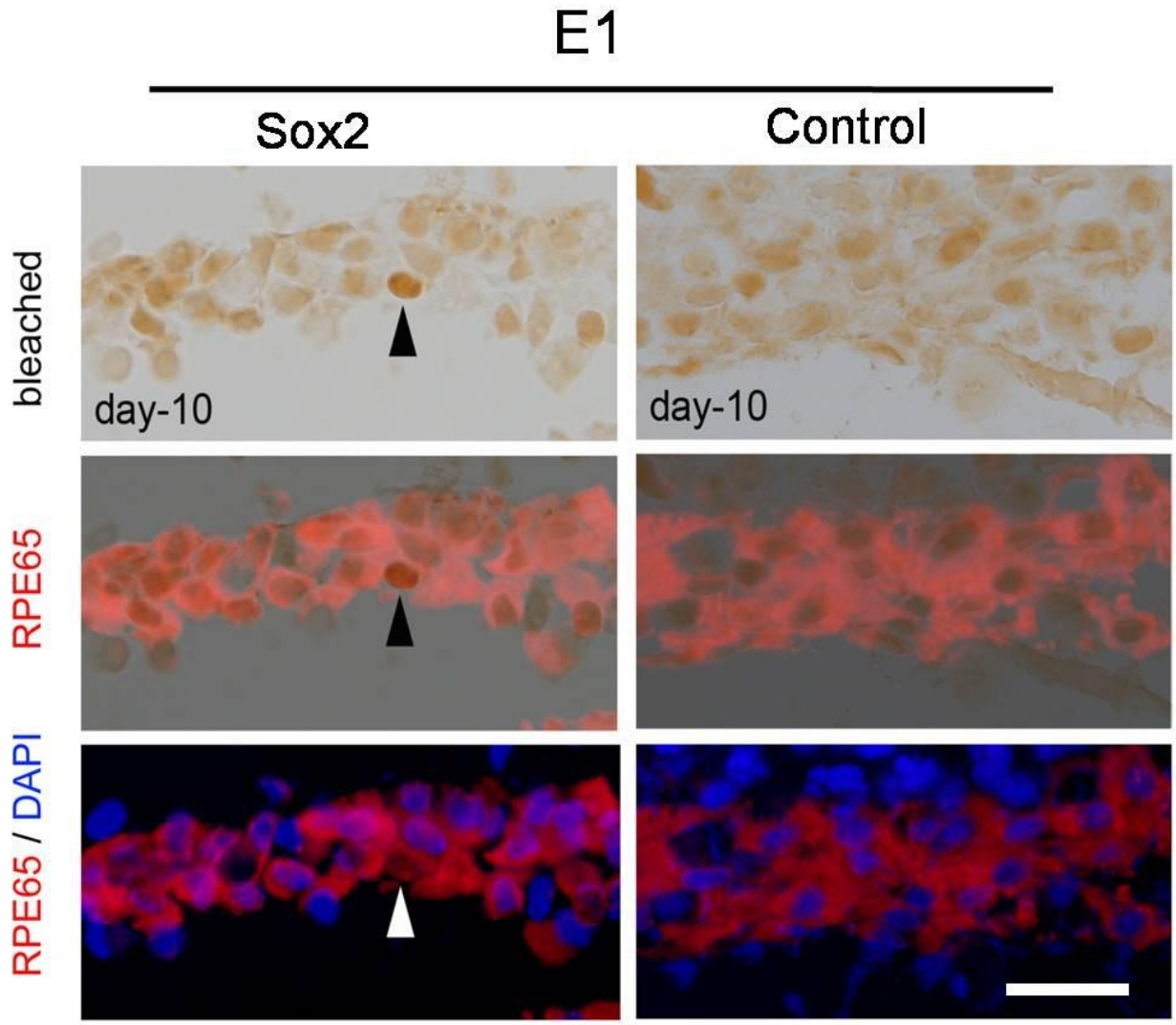


Figure 32: Sox2 was first detected in 10 days po (E1 stage) in some RPE derived cells. Tissues were bleached and stained with RPE65 antibody for the identification of RPE cells followed by counter staining with DAPI for the visualization of cell's nucleus (blue fluorescence). Negative control experiment was done using dsRed antibody instead of Sox2 antibody. Scale bar: 50μm.

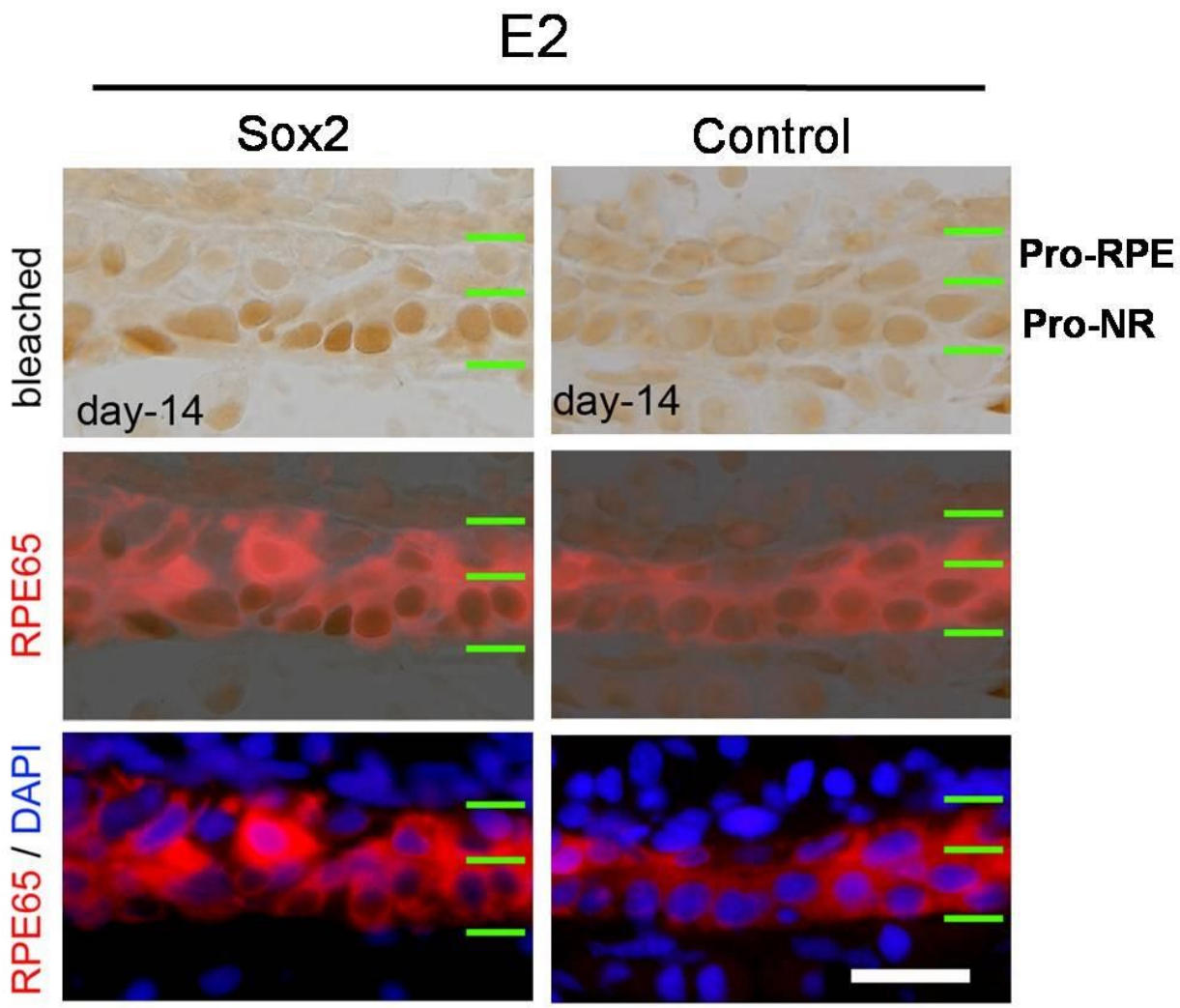


Figure 33: Sox2 expression pattern in 14 days po (E2 stage) tissues.

Immunoreactivity was detected only in the outer layer i.e. in the neural retinal rudiment layer (pro-NR) but not in the inner layer (pro-RPE).Tissues were depigmented and RPE65 immunolocalization was used to define RPE cells following nuclear staining with DAPI. Adjacent tissues were used for control experiment where dsRED antibody was used instead of Sox2 primary antibody. Scale bar: 50µm.

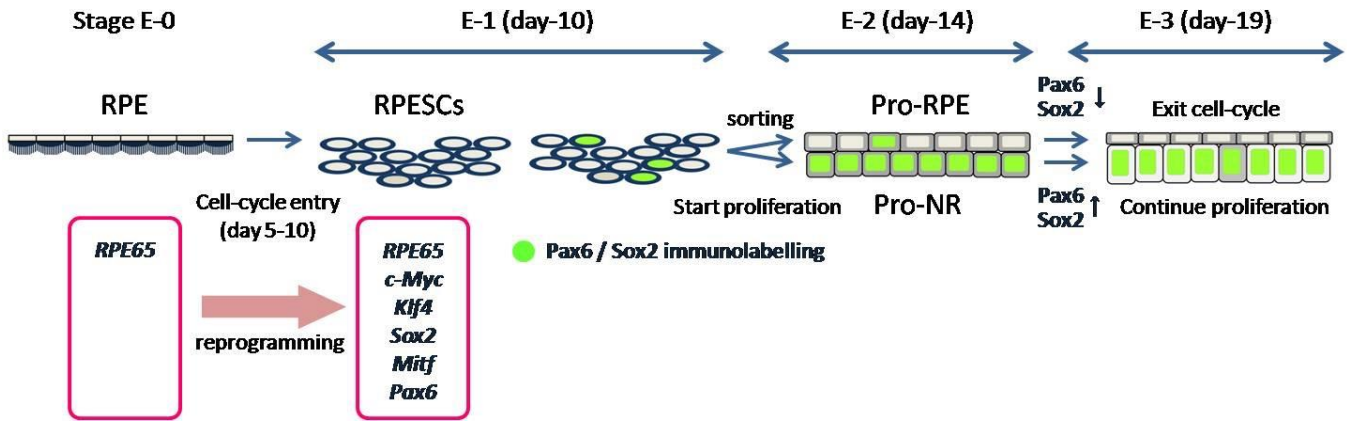


Figure 34: New scenario of retinal regeneration from RPE cells in adult newt.

After surgical removal of neural retina RPE cells lose their epithelial character forming cells aggregate and enter into the cell cycle between d-5 to d-10 p.o. These RPE-derived cells following expression of multipotency factor- *c-Myc*, *Klf4*, *Sox2* and retinal stem cell marker *Pax6* reprogrammed into RPE stem cells (RPESCs) while keeping expression their marker *RPE65* along with the *microphtalmia* factor *Mitf* which is a marker of immature RPE. Pax6 and Sox2 immunolocalization was detected in some population of RPEsCs simultaneously sorted into layer i.e. pro-RPR and pro-NR. Pax6 and Sox2 expression decrease in the rpo-RPE while increase in the pro-NR layer and cell proliferation continues in the pro-NR while pro-RPE layer exit the cell cycle.

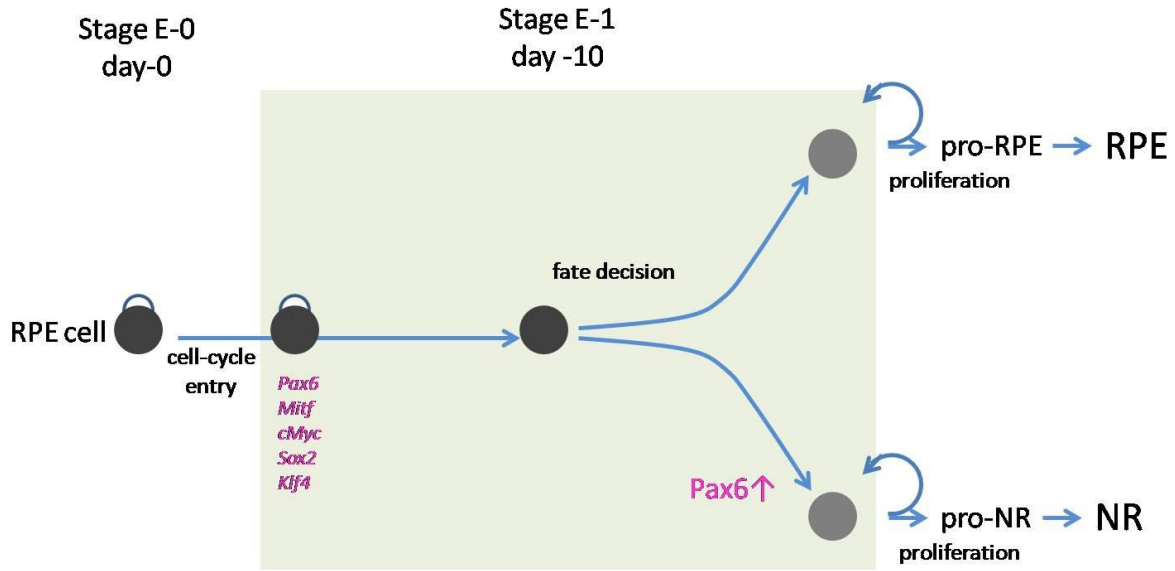


Figure 35: RPE-derived cell fate decision.

RPE-derived cells choose their fate into pro-RPE or pro-NR around stage E2. However it is still not clear that how this fate decision is made.