A Transcriptome for the Study of Early Processes of Retinal Regeneration in the Adult Newt, Cynops pyrrhogaster

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Abstract

Retinal regeneration in the adult newt is a useful system to uncover essential mechanisms underlying the regeneration of body parts of this animal as well as to find clues to treat retinal disorders such as proliferative vitreoretinopathy. Here, to facilitate the study of early processes of retinal regeneration, we provide 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 viridescens) thus far. On the other hand, de novo assembly transcripts of this transcriptome were functionally annotated. Coding sequence prediction in combination with functional annotation revealed that 76,968 in silico transcripts encode protein/peptides recorded in public databases so far, whereas 17,316 might be unique. qPCR and Sanger sequencing demonstrated that this transcriptome contains much information pertaining to genes that are regulated in association with cell reprogramming, cell-cycle re-entry/proliferation, and tissue patterning in an early phase of retinal regeneration. This data also provides important insight for further investigations addressing cellular mechanisms and molecular networks underlying retinal regeneration as well as differences between retinal regeneration and disorders. This transcriptome can be applied to ensuing comprehensive gene screening steps, providing candidate genes, regardless of whether annotated or unique, to uncover essential mechanisms underlying early processes of retinal regeneration.

Introduction

The newt has long been recognized as a master of regeneration from which the principles for the regeneration of body parts following traumatic injury can be learnt. This animal has an outstanding ability, when metamorphosed or sexually matured, or even as an aged adult, to regenerate missing body parts – including a part of a limb, the jaw, the tail (the spinal cord), the brain, the heart, the eye (the lens and the retina) – from remaining tissues at the site of injury. This takes place through dedifferentiation–redifferentiation/-transdifferentiation (or reprogramming) of terminally differentiated cells as well as recruitment of endogenous stem/progenitor cells, their proliferation and patterning, and physiological integration of regenerates into the body system [1,2]. Although ample endeavors have been made to understand these surprising phenomena for over a century, the underlying cellular mechanisms and molecular networks are still largely uncertain primarily because of our technical limitations. However, studies in this field are now moving forward with increasing speed by incorporating highly efficient technologies to analyze gene functions [3,4] and transcriptomes [5,6]. For studies of the newt whose genomic size is too large to be sequenced (estimated ~20 Gbp [7]), construction of an in silico transcriptome by RNAseq/de novo assembly is certainly cost effective, and the resulting data sets are highly informative for bench screening candidate genes.

Regeneration of the adult newt retina is a good system to study the early processes of regeneration, particularly the mechanisms underlying reprogramming, cell-cycle re-entry/proliferation and patterning, because of its simplicity. The cell source for retinal regeneration in the posterior eye is the retinal pigment epithelium (RPE) cells only. The RPE, which is a highly specialized monolayer lining the back of the neural retina, expresses specific molecular markers such as RPE65, allowing the RPE cells and RPE-derived cells to be tracked in an early phase of retinal regeneration [8]. In addition, this system has an obvious medical target, namely proliferative vitreoretinopathy (PVR) in which RPE.
cells proliferate and transform in response to retinal injury, leading to the loss of vision [9].

Among all newt species, the Japanese fire bellied newt (Cynops pyrrhogaster) is the best choice for the study of retinal regeneration since: 1) a surgical procedure to induce retinal regeneration has been established; 2) morphological stages of retinal regeneration have been defined in detail; 3) both in vivo and in vitro functional gene assay systems are being developed [4,8,10,11]. However, to facilitate the study of retinal regeneration in this species, one considerable obstacle still remains: limited information on genes. Thus, in the current study, to overcome this problem we carried out mRNA-seq/de novo assembly in this species, providing an in silico reference transcriptome specialized for the study of early processes of retinal regeneration.

Materials and Methods

Ethics statement

This study using the Japanese fire bellied newt C. pyrrhogaster was permitted by the University of Tsukuba Animal Use and Care Committee (AUCC). Surgical removal of the neural retina (retinectomy) and sacrifice were carried out under anesthesia [anesthetic: FA100 (4-allyl-2-methoxyphenol); DS Pharma Animal Health, Osaka, Japan] to minimize suffering [8]. No other in vivo experiments were done.

The field study did not involve endangered or protected species. The newts were originally captured by a provider (Mr. Kazuo Ohuchi, Misato, Saitama, 341-0037 Japan; http://homepage3.nifty.com/monmo51-kaeru/index.html) using a net from canals along the rice paddies located within ~25 km in diameter around a Miyayama area (35.130013, 140.013842) in Kamogawa city, Chiba prefecture, Japan [4]. No specific permissions were required for the location of capture.

Newt strain

The newts, which have been captured since 2008 (~300/year) (see Ethics statement), have been stocked/cultured in both the laboratory (Univ. of Tsukuba) and a field ‘Imori-no-Sato’ (Kazukawa/Kamitakai, Toride city, Ibaraki prefecture, Japan; http://imori-net.org/) [4]. This population belongs to Kanto group in Northern lineage [12] and is called ‘Toride-Imori’. In this study, sexually mature adult Toride-Imori (total body length: male, 9 cm; female, 11–12 cm) which had been reared in the laboratory were used.

Housing condition

In the laboratory, the animals had been reared in containers/aquarium tanks (~10 cm x base area of 30 cm² square; the water depth was 5–15 cm; a stone/a piece of kitchen sponge was placed therein, serving as land) at ~18°C under a natural light condition; they had been fed with frozen mosquito larvae (Akamushi; Kyorin Co., Ltd., Japan) every day and the containers/aquarium tanks had been kept clean [4].

Anesthesia

For retinectomy, the animals were anesthetized as follows: 0.3 ml of the anesthetic FA100 (4-allyl-2-methoxyphenol) was poured in 300 ml tap water (~22°C) in a bottle (of the bottom: ~7 cm; height: ~5 cm) with a lid; the bottle was sealed immediately and shaken several times so that the solution is mixed well; the newts (up to 5) were placed in this solution (i.e., 0.1% FA100) and the bottle was sealed again immediately; the bottle containing the animals was placed in the dark at room temperature (~22°C) for 2 h, allowing dark adaptation of the retina which makes the adherence between the neural retina and the RPE weaker. After this treatment, they were rinsed in distilled water (DW) and wiped with a paper towel. Under this condition, they did not show the pupillary reflex during surgery, and not awake for at least 4 h.

For sacrifice, intact animals and those of day-14 or later after retinectomy whose wound had closed were anesthetized as done for retinectomy. However, in the case of animals between 4 h and day-14 after retinectomy, an alternative anesthetic method was applied to avoid damage of RPE cells due to invasion of the hypotonic anesthetic solution into the eye chamber: the animals were injected with 100 ml of 20% FA100 (dissolved in PBS) into their abdominal cavity through a fine needle (27Gx3/4″, NN-2718S, Terumo, Tokyo, Japan) connected to a syringe (1 ml, SS-01T, Terumo); they fell asleep in 30 min. For animals within 4 h after retinectomy, additional anesthetic treatment was not done.

Retinectomy

To induce retinal regeneration, the neural retina was removed, together with the lens, from the left eye (the eyeball size: 2 mm in diameter) of a living animal as follows [8]; see Figure 1A): the mouth cavity of an anesthetized animal was stuffed with a roll of the absorbent cotton so that the eyeball is pushed out from the eyelid; the animal was held on the silicon bottom of an operating chamber so that the left eye faces up, using a U-shaped pin which was mounted on the neck of the animal and stuck onto the silicon bottom of the chamber; the animal on the chamber was placed under a binocular, and the dorsal half of the left eye was cut open along the position slightly below the boundary between the cornea and sclera using a blade and fine scissors; both the neural retina and the lens were carefully removed by a fine injection needle (27Gx3/4″, NN-2718S, Terumo) and forceps, while gently infusing a sterile saline solution (in mM: NaCl, 115; KCl, 3.7; CaCl₂, 3; MgCl₂, 1; D-glucose, 18; HEPES, 5; pH 7.5 adjusted with 0.5N NaOH) into the vitreous chamber through the same injection needle which was connected to a syringe (1 ml, SS-01T, Terumo) via a filter cassette (0.20 μm pore size, Cellulose, DISMIC-29CS, ADVANTEC, Japan); at this time the retinal margin containing the ora serrata (the tissue harboring the Retinal stem/progenitor cells) which remained along the base of the ciliary epithelium was also removed by forceps. After operation, the eye flap consisting of the iris and cornea was carefully placed back to its original position. The operated animals were placed on a paper towel (lightly wet with DW) in a plastic container (~5 cm x depth of the bottom: 19 cm x height: 4 cm) and allowed to recover, and then reared in an incubator (~22°C; the day-night cycle was 12 h:12 h) until use (up to 14 days in this study). In the mean time, the containers were kept clean and the animals were not fed to control the speed of regeneration. The stage of retinal regeneration and corresponding day post-operation (po) were determined according to previous criteria [8]; see Figure 1B).

Collection of eyeballs

To collect eyeballs, the anesthetized animals (dried on a paper towel) were decapitated. The head was fixed on the silicon bottom of the operating chamber with a marking pin, and placed under the binocular. The eyeballs were carefully enucleated with fine scissors and forceps. In the case of retinectomized animals, especially before day-14 po, this operation was made with minute attention because their wounded eyes were very fragile.
Tissue samples for analyses

For de novo assembly transcriptome, the workflow is illustrated in Figure 1B. To obtain the transcriptome involved in early processes of retinal regeneration, especially for the study of reprogramming, cell-cycle re-entry/proliferation and patterning in full, retinectomized eyeballs (time po: 30 min, 2 h, 12 h, 24 h, 5 days, 10 days, 14 days) and the retina-less eye-cups (RLECs) of normal eyeballs were used. These retinectomized eyeballs should have contained RPE cells which just received somewhat onset-signals for regeneration [13,11] as well as those which have undergone mitosis (stage E-1) and segregation into two layers, i.e., rudiments for a new neural retina and RPE (stage E-2) [8]; see Figure 1B for the morphological stages of retinal regeneration. RLECs were used as a source of intact RPE cells (stage E-0). This sample was prepared as described previously [8] with some modifications: a normal eyeball was placed, the cornea side up.

Figure 1. Workflow from retinectomy to de novo assembly. A. Retinectomy. B. Sample collection, mRNA-seq and de novo assembly. Stage E-0: The RPE immediately after retinectomy. Stage E-1: Almost all RPE cells that have lost their epithelial characteristics and formed aggregates have entered the S-phase of the cell-cycle. Stage E-2: Partially depigmented cells are segregated into two rudiment layers (pro-NR and pro-RPE), which give rise to a new neural retina and the RPE layer itself. Under the current experimental conditions, regenerating retinas at Stage E-1 and E-2 are obtained at day-10 and -14 po [8]. doi:10.1371/journal.pone.0109831.g001
onto a filter membrane (MF™ Membrane Filter, 0.45 µm HA, HAWP01300, Merck Millipore, Darmstadt, Germany) in a 35 mm plastic dish (Falcon 353001, Becton Dickinson, NJ 07417-1886), and then cut along the equator by manipulating a blade and scissors; RNase-free PBS chilled on ice was gently poured onto the eyeball; after the dish was filled up with this saline, the anterior half of the eyeball containing the iris and lens was removed to make the eye-cup; the neural retina was carefully separated from the RPE with a fine pin and forceps, and then removed from the rest of the eye-cup, i.e., the RLEC, by cutting the optic nerve with fine scissors. This operation was completed within 5 min.

For quantitative PCR (qPCR), both the right (intact) and left (retinectomized) eyeballs of animals at day-10 and -14 po were used; that is, the right eyeballs were used for day-0 po sample containing intact RPE (stage E-0), and the left ones were for samples of day-10 po (stage E-1) or day-14 po (stage E-2). Tissue samples for RNA isolation were prepared as follows. After the animal was decapitated under anesthesia, the right and left eyeballs were collected in different dishes (filled with RNase-free PBS) on ice. The right eyeball was immediately dissected into the RLEC, and the RPE sheet together with the choroid tissues was isolated by separating these from the sclera using a fine pin and forceps. On the other hand, the left eyeball of day-10 or -14 po, which was put on the filter membrane and soaked in chilled RNase-free PBS as done for the right eyeball, was carefully opened from the wound at the time of retinectomy using a fine pin and scissors; after the anterior part of the eyeball containing the iris and the ciliary marginal zone was carefully removed, RPE -derived cells in the posterior eye were collected together with the choroid tissues as done for the right eyeball. After blood cells in the choroid were removed as much as possible by shaking them in the dish, each day samples were transferred into different tubes (containing RNase-free PBS) on ice with a pipette (3.5 ml Transferpipette, Sarsted, D-51588 Numbrecht, Germany). For one round of RNA purification, this process was repeated for at least 5 animals, collecting 5 each-day samples (good samples only) at once.

Library construction and sequencing for de novo assembly transcriptome

A total of 14 RLECs and 97 retinectomized eyeballs (30 min, 14; 2 h, 13; 12 h, 13; 24 h, 13; 5 days, 13; 10 days, 15; 14 days, 14) were harvested, under a conventional nuclease free condition, one by one in a 50 ml tube containing liquid nitrogen, and stored in –80°C until use (see the workflow in Figure 1B). Total RNA was purified from them all by the Isogen protocol (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions and evaluated with an Agilent 2100 Bioanalyzer (Agilent Technology, Santa Clara, CA 95051). Using a qualified RNA sample (384.3 ng/µl; RIN: 8.7), a normalized cDNA library (insert size: 300–400 bp) was constructed with a TrueSeq RNA Sample Prep Kit (Illumina, San Diego, CA 92122) followed by Duplex Specific Nuclease normalization (Illumina) according to the manufacturer’s instructions. Subsequently, paired end sequencing (101 bp read x2) was carried out by Illumina HiSeq2000 and a set of raw read data [403,817,536 reads; 40,786 Mbases; % of high quality base (Q≥30): 92.74; Mean quality score: 35.92] was...
produced after base calling and Chastity filtering (CASAVA ver.1.8.1., Illumina). These raw reads were filtered to remove reads with adaptors and low-quality sequences (reads with unknown sequences ‘N’) as follows: first, the software cutadapt [14] was used to trim adapters, and then the trimmed reads and reads containing ‘N’ are discarded using in-house scripts. Finally, 330,837,190 clean reads (101 bp each) were yielded for the following de novo assembly.

**De novo assembly**

To obtain contigs and in silico transcripts (IS-transcripts), the clean reads, obtained after filtering the raw reads, were assembled using de novo assemblers. There is no consensus in terms of the best de novo assemblers. So, three widely used algorithms were applied: Trinity [15] (version 2012-10-05; http://trinityrnaseq.sourceforge.net/), Trans-ABySS [16] [for Trans-Abyss, version 1.4.4 (http://www.bcgsc.ca/platform/bioinfo/software/transabyss); for ABySS, version 1.3.5 (http://www.bcgsc.ca/platform/bioinfo/software/abyss)] and Velvet-Oases [18] [for Velvet [19], version 1.2.01 (http://www.ebi.ac.uk/~zerbino/velvet/); for Oases, version 0.2.02 (http://www.ebi.ac.uk/~zerbino/oases/)]. Trinity was run with ‘–min_kmer_cov 2’. By applying the ‘–min_kmer_cov 2’ parameter (the default is 1), only k-mers that occur more than once are assembled. This parameter is used to reduce memory requirements and runtimes. Generally, this will eliminate super rare transcripts and sequencing errors, but will not usually affect assembly quality. ABySS was run with k-mer values form 51 to 95 in steps of 2, and then all 23 assemblies from AbySS were merged into one assembly using Trans-ABySS. Velvet was run with k-mer values from 45 to 95 in steps of 5, and

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**Figure 3. Comparisons with other newt transcriptomes.** Black circles: IS-transcripts of this study. Blue circles: cDNAs and EST-contigs reported in *C. pyrrhogaster*. Red circles: cDNAs and EST-contigs reported in *P. waltl*. Green circles: cDNAs, EST-contigs and IS-transcripts reported in *N. viridescens*. The values in each circle (written in corresponding color) mean the number and ratio of IS-transcripts, cDNAs or EST-contigs.

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then the contigs produced by Velvet at each k-mer value were further assembled into transcripts using Oases. Finally, the best result (k-mer = 75) was selected as output. Although the transcript data sets assembled at different k-mer values were generally merged using Oases-M, we just treated Velvet-Oases as a single k-mer assembler without Oases-M.

In this study, IS-transcripts deduced by Trinity were further analyzed by annotation as well as coding sequence (CDS) and protein/peptide prediction.

Annotation
Functional annotation of IS-transcripts was carried out by aligning them first to protein databases such as NCBI NR (release-20130408), Swiss-Prot (release-2013_03), KEGG (release 63.0) and COG (release-20090331) by blastx program (E-value threshold: e-5), and then to NCBI NT by blastn program (E-value threshold: e-5). Gene Ontology (GO) annotation was carried out by the Blast2GO program (v2.50) with NR annotation, and the data was classified by WEGO software [20]. Metabolic pathway analysis was carried out with the help of the KEGG database.

Coding sequence (CDS) and protein/peptide prediction
Both the nucleotide sequence (5’-3’) and protein/peptide CDS of IS-transcripts were predicted using proteins with highest ranks in the functional annotation by blastx program with NR, Swiss-Prot, KEGG and COG (see above). IS-transcripts that could not be aligned to any databases were scanned by ESTScan (v3.0.2) to predict CDSs [21]. This program compensates for the frame shift errors.

Quantitative PCR (qPCR)
Each day samples (5 samples/tube), which were prepared and harvested under a conventional nuclease free condition (see above), were immediately used to purify total RNA (NucleoSpin RNA kit; Macherey-Nagel GmbH & Co. KG, Duren, Germany). First strand cDNAs were synthesized (SuperScript™ II Reverse Transcriptase; Life Technologies, Carlsbad, CA) with 30 ng total RNA, diluted 100x, 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. ID numbers of target IS-transcripts (Ef1a, RPE65, CRALBP/RLBP1, ZO1, Otx2, Musashi1A/c, Cyclin D1, CDK4, Histone H3, c-Myc, Klf4, Sox2, N-Cadherin, z-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 S1. The

Figure 4. Functional annotation. A. Summary of annotation. B. E-value-, similarity (% identity)- and species-distribution in NR annotation. Species-distribution indicates that many of the C. pyrrhogaster IS-transcripts are close to genes of amniotes as well as those of amphibians such as Xenopus (Silurana) and Xenopus laevis, rather than fishes (e.g., Danio rerio, 2.9%; Oryzias latipes, 1.9%; these are included in ‘other’). Interestingly, within amphibians, the newt seems to adhere to X. (Silurana) rather than to X. laevis.
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Table 1. GO classification.

| Biological process                                                                 | Count  |
|-----------------------------------------------------------------------------------|--------|
| cellular process                                                                  | 29,570 |
| single-organism process                                                           | 25,093 |
| metabolic process                                                                 | 21,542 |
| biological regulation                                                             | 19,602 |
| regulation of biological process                                                  | 18,451 |
| response to stimulus                                                              | 14,838 |
| multicellular organismal process                                                   | 13,061 |
| developmental process                                                             | 11,328 |
| signaling                                                                         | 11,013 |
| cellular component organization or biogenesis                                     | 10,934 |
| localization                                                                      | 10,576 |
| positive regulation of biological process                                         | 8,574  |
| establishment of localization                                                     | 8,345  |
| negative regulation of biological process                                         | 7,416  |
| immune system process                                                             | 3,363  |
| locomotion                                                                        | 3,320  |
| reproduction                                                                      | 2,907  |
| reproductive process                                                              | 2,768  |
| biological adhesion                                                               | 2,223  |
| multi-organism process                                                            | 2,222  |
| growth                                                                            | 1,992  |
| rhythmic process                                                                  | 442    |
| cell killing                                                                       | 97     |

| Cellular component                                                               | Count  |
|----------------------------------------------------------------------------------|--------|
| cell                                                                             | 29,452 |
| cell part                                                                        | 29,452 |
| organelle                                                                        | 21,715 |
| membrane                                                                         | 15,252 |
| organelle part                                                                   | 13,910 |
| membrane part                                                                    | 9,970  |
| macromolecular complex                                                           | 8,397  |
| membrane-enclosed lumen                                                           | 6,813  |
| extracellular region                                                              | 2,349  |
| cell junction                                                                     | 1,989  |
| synapse                                                                          | 1,713  |
| extracellular region part                                                         | 1,520  |
| synapse part                                                                     | 1,205  |
| extracellular matrix                                                             | 894    |
| extracellular matrix part                                                         | 336    |
| nucleoid                                                                         | 72     |

| Molecular function                                                               | Count  |
|----------------------------------------------------------------------------------|--------|
| binding                                                                          | 26,423 |
| catalytic activity                                                               | 13,617 |
| transporter activity                                                              | 2,690  |
| molecular transducer activity                                                     | 2,323  |
Table 1. Cont.

| Molecular function                  | Count  |
|-------------------------------------|--------|
| enzyme regulator activity           | 2,160  |
| receptor activity                   | 2,005  |
| nucleic acid binding transcription factor activity | 1,913  |
| protein binding transcription factor activity | 1,264  |
| structural molecule activity        | 1,085  |
| electron carrier activity           | 223    |
| channel regulator activity          | 207    |
| antioxidant activity                | 90     |
| chemorepellent activity             | 66     |
| receptor regulator activity         | 58     |
| translation regulator activity      | 50     |
| chemoattractant activity            | 30     |
| metallochaperone activity           | 10     |
| morphogen activity                  | 3      |
| nutrient reservoir activity         | 3      |
| protein tag                         | 1      |

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Figure 5. CDS prediction. Blastx predicted CDSs in 71,511 IS-transcripts (for the length distribution, see the graph ‘Blast’; for protein/peptide sequences, see Table S5) while ESTscan predicted CDSs in 22,773 IS-transcripts (for the length distribution, see the graph ‘ESTScan’; for protein/peptide sequences, see Table S6).
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DDBJ/GenBank accession number of the cDNA corresponding to each IS-transcript is also mentioned in Table S1. Most cDNAs were cloned and their sequences, determined by the Sanger method, were deposited in DDBJ.

For each gene, qPCR, which was always run simultaneously with day-0, -10 and -14 po 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. The data were presented as the mean ± SEM. Statistical differences were evaluated by Sheffe’s test following the Friedman test.
Three transcriptomes deduced by different de novo assemblers

There is no consensus in terms of the best de novo assemblers. So, we tested three widely used algorithms, Trinity [13], Trans-ABySS [16] and Velvet-Oases [18]. Each algorithm produced a large number of IS-transcripts: Trinity, 237,120 (N50 (the length of an IS-transcript whose order is 50% of all IS-transcripts): 2,737 nt), Trans-ABySS, 635,930 (N50: 1,800 nt); Velvet-Oases, 99,566 (N50: 4,001 nt) [Figure 2]; note that these sets of IS-transcripts (transcriptomes) can be downloaded from our repository site ‘IMORI’ (http://anlcr.is.utsunomiya-u.ac.jp/imori/). These three transcriptomes were compared with each other by blast (NCBI; v2.2.27+*x64-linux; E-value threshold: e-5), with other transcriptomes (cDNAs, ESTs, and IS-transcripts) in three species (C. pyrrhogaster, Pleurodeles waltl, Notophthalmus viridescens), which were collected from databases in DDBJ/NCBI and from sequence repository sites for N. viridescens: Red Spotted Newt Resource Page (RSNR P http://sandberg.cmb.ki.se/redspottednewt/ [5] and Newt-Omics: http://newt-omics.mpil-bn.mpg.de/ [6] (Figure 3). ESTs were assembled into contigs by the CAP3 program [22], before comparison. This analysis revealed that the current transcriptome in early regenerating eyes (day 0–14 po) contains >78% information of cDNAs/ESTs (i.e., mRNA) reported in these three species so far, except for 59% of ESTs in P. waltl; notably, in C. pyrrhogaster ~82% of cDNAs and ~95% of EST-contigs were matched (Figure 3). In addition, the current transcriptome covered ~44% and ~67% of the IS-transcripts for N. viridescens (RSNR P and Newt-Omics, respectively). On the other hand, these N. viridescens data covered only ~31% and ~16% of the current transcriptome, respectively. Thus, the current transcriptome comprehensively covers transcript information in C. pyrrhogaster.

Characterization of the current transcriptome

Functional annotation. To further characterize the current transcriptome (237,120 IS-transcripts), physiological functions of each IS-transcript were predicted by annotation, which carried out by blast with NR, NT, Swiss-Prot, KEGG, COG and GO databases. Finally, a total of 87,102 IS-transcripts (range: 201–19,064 nt; N50: 1,202 nt) were annotated (Figure 4A; for details, see Table S2). For example, in NR annotation, 82,482 IS-transcripts (94.7% of all annotated transcripts) were enriched; 67.7% of them had an E-value of <e-5, and 41.8% had a similarity of >60%, indicating that many of the NR-annotated IS-transcripts contain the sequence information of genes highly homologous to those found in NR database (Figure 4B); species distribution seemed to reflect the position of this animal in phylogeny (Figure 4B); 39,895 of the NR-annotated IS-transcripts were also dealt GO terms (Figure 4A). As shown in GO classification (Table 1) as well as in COG- and KEGG-classification (Table S3 and S4), these IS-transcripts were assigned to various functional categories and pathways. On the other hand, the remaining 150,018 IS-transcripts (range: 201–11,726 nt; N50: 290 nt) were not annotated.

Inferred proteome. To validate the current Trinity-deduced transcriptome, we first selected 20 genes (Table S1) with cDNA samples prepared from RPE-choroid tissues or RPE-derived cells-choroid tissues which were harvested from intact (day-0) or retinectomized (day-10 or day-14) tissue samples. For example, in NR annotation, 82,482 IS-transcripts (94.7% of all annotated transcripts) were enriched; 67.7% of them had an E-value of <e-5, and 41.8% had a similarity of >60%, indicating that many of the NR-annotated IS-transcripts contain the sequence information of genes highly homologous to those found in NR database (Figure 4B); species distribution seemed to reflect the position of this animal in phylogeny (Figure 4B); 39,895 of the NR-annotated IS-transcripts were also dealt GO terms (Figure 4A). As shown in GO classification (Table 1) as well as in COG- and KEGG-classification (Table S3 and S4), these IS-transcripts were assigned to various functional categories and pathways. On the other hand, the remaining 150,018 IS-transcripts (range: 201–11,726 nt; N50: 290 nt) were not annotated.
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 E-1). 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. viridescens [23]. A microphthalmia factor Mitf, a marker gene for immature or uncommitted RPE cells [24], was also expressed by day-10 po but then its expression level decreased. On the other hand, marker genes for retinal stem/progenitor cells (Pax6, Chx10/Vsx2) were expressed at day-14 po when two rudiment layers (pro-NR and pro-RPE) for the prospective neural retina and RPE just appear (Stage E-2). 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 [11,24].

In PVR leading to the loss of vision after retinal injury in humans, RPE cells transform into mesenchymal cells such as myofibroblasts, probably by passing through a stem-cell state [9]. 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 6B).

Consequently, the current transcriptomes could be a good tool to identify or screen candidate genes which might be involved in early processes of retinal regeneration.

Implications for reprogramming of adult newt RPE cells into a multipotent state

In the adult newt, the RPE is a sole cell source for retinal regeneration in the posterior eye [8]. Upon retinectomy, RPE cells are detached from each other and leave the basement membrane, forming cell aggregates, while entering the S-phase of cell-cycle (Stage E-1). This event typically occurs between day-5 and day-10 po. The RPE-derived cells at Stage E-1 form the pro-NR and pro-RPE layers by day-14 po (Stage E-2).

In the previous studies [25,26], we demonstrated that an RNA-binding protein Musashi-1, whose expression is restricted in the nucleus of the intact RPE cells, changes its location into the cytoplasm upon retinectomy, although the amount of the transcripts tends to be decreased as suggested in the current qPCR. This pattern of Musashi-1 expression was observed in almost all of the RPE-derived cells at Stage E-1 uniformly. As the regeneration proceeds to Stage E-2, Musashi-1 expression was down-regulated along the pro-RPE layer, while sustained along the pro-NR layer [26]. Since the cytoplasmic expression of Musashi-1 is a character of neural stem/progenitor cells [27], these observations led us to an implication that the RPE cells are reprogrammed into a multipotent state of cells by Stage E-1, and specified into 2 cell populations forming the pro-NR and pro-RPE layers between Stage E-1 and E-2. However, the nature of the RPE-derived cells remains unclear.

The current qPCR revealed that gene expression suggesting multipotent properties of cells is up-regulated upon retinectomy, while gene expression for original RPE characters is down-regulated, giving us an insight for the cell reprogramming. Interestingly, certain pluripotency factors (c-Myc, Klf4, Sox2) as well as Mitf were first detected in day-10 samples (Stage E-1). On the other hand, Pax6 was detected only in day-14 samples. In the previous study, we detected Pax6 expression at day-10 po, being earlier than Chx10-1/Vsx1 (a retinal progenitor marker) at day-14 po [29]. This inconsistency may be due to the difference in the template for PCR: in the previous study, we used a PCR-amplified library of cDNAs as the template, and therefore the detection sensitivity should have been higher. Taken together, our results suggest that the intact adult newt RPE cells are not comparable to either immature/uncommitted state of RPE cells or retinal stem/progenitor cells, as well as a possibility of reprogramming of RPE cells into such multipotent cells upon retinectomy. On the next stage of this study, we must carry out immunohistochemistry and single-cell qPCR to address the expression of these factors in the RPE-derived cells at Stage E-1 (our study on this subject was published [29] while the current article was under review).

The current qPCR with day-14 samples detected a factor (Chx10/Vsx2) suggesting the presence of retinal progenitor cells and that (Wnt2b) inferred to be involved in the RPE differentiation. Sox2, which is also a marker of retinal stem/progenitor cells [30], increased in its expression level between day-10 and day-14, whereas c-Myc, Klf4 and Mitf were declined. These expression patterns seem to support our previous implication that the RPE-derived cells are specified into two cell populations from which the pro-NR and pro-RPE layers are formed, leading a possibility that the cells in the pro-NR and pro-RPE layers might be correspond to the retinal progenitor cells and RPE cells which just started their differentiation, respectively. In addition, the expression of α-SMA, which is observed in the choroid tissues (data not shown), was obviously declined to a minimal level at day-14, revealing a contrast to human PVR in which the RPE-derived multipotent cells transform into myofibroblasts. Thus, in the adult newt system, the RPE-derived cells (possibly in a multipotent state) directly generate retinal progenitor cells for regeneration of a missing neural retina.

In the current study, we did not carry out further analyses because the purpose of the study was to establish a good transcriptome database. However, our study would move onto the next stage at histological and functional levels.

Conclusions

To facilitate investigations of adult newt retinal regeneration, we provided in silico transcriptomes covering information of genes which are expressed in eyeballs containing Stage E-0 to E-2 regenerating retinas. Gene expression patterns revealed by qPCR demonstrate the usefulness of the transcriptomes for the study of early processes of retinal regeneration. This tool can be applied to the next comprehensive gene screening steps to uncover essential mechanisms underlying reprogramming, cell-cycle re-entry/proliferation, and patterning in adult newt retinal regeneration.

Supporting Information

Table S1 Primers and probes for PCR targets. (XLSX)
Table S2 Summary of annotation. (XLSX)
Table S3 COG classification. (XLSX)
Table S4 KEGG classification. (XLSX)
Table S5 Proteins/peptides predicted by blast. (XLSX)
Table S6 Proteins/peptides predicted by ESTScan. (XLSX)
Table S7 Unknown proteins/peptides predicted by ESTScan.

[XLSX]

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Author Contributions

Conceived and designed the experiments: KN FT CC. Performed the experiments: KN MRI MT HW WI AK. Analyzed the data: KN RMCR FT CC. Contributed reagents/materials/analysis tools: FT CC. Wrote the paper: KN FT CC.