Plasticity for axolotl lens regeneration is associated with age-related changes in gene expression

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Abstract

Mexican axolotls lose potential for lens regeneration 2 weeks after hatching. We used microarrays to identify differently expressed genes before and after this critical time, using RNA isolated from iris. Over 3700 genes were identified as differentially expressed in response to lentectomy between young (7 days post-hatching) and old (3 months post-hatching) axolotl larvae. Strikingly, many of the genes were only expressed in the early or late iris. Genes that were highly expressed in young iris significantly enriched electron transport chain, transcription, metabolism, and cell cycle gene ontologies, all of which are associated with lens regeneration. In contrast, genes associated with cellular differentiation and tissue maturation were uniquely expressed in old iris. Many of these expression differences strongly suggest that young and old iris samples were collected before and after the spleen became developmentally competent to produce and secrete cells with humoral and innate immunity functions. Our study establishes the axolotl as a powerful model to investigate age-related cellular differentiation and immune system ontogeny within the context of tissue regeneration.

Keywords

Axolotl, lens, microarray, newt, regeneration

Introduction

Regenerative ability varies across organs, developmental stages, and species. However, one generality that has been noted for highly and lowly regenerative vertebrates is that regenerative ability tends to decrease with age (Sousounis et al. 2014). Fetal and larval forms tend to possess an ability to regenerate tissue in a scar-free manner while adults, and especially mammals, show minimal potential for regeneration. These patterns suggest that regenerative ability is associated with age-related changes in cells that form tissues and organs, as well as maturation of systems that broadly regulate development and physiology (Seifert & Voss 2013). Exceptions include lens regeneration in adult newts (Eguchi et al. 2011) and fin regeneration in zebrafish (Itou et al. 2012).

Some amphibians are capable of regenerating their lens through a process called transdifferentiation. During embryonic development of salamanders, the lens is formed by invagination of the surface ectoderm, which later differentiates into cornea (Wolff 1895; Freeman 1963; Suetsugu-Maki et al. 2012). In contrast, progenitor cells that regenerate lens after lentectomy derive from the iris, which has a neural origin (Fuhrmann 2010; Graw 2010). Thus, transdifferentiation refers to a special type of regeneration where progenitor cells from a different tissue are the source of the regenerate.

The adult red-spotted newt (Notophthalmus viridescens) has long served as the primary salamander model for studies of transdifferentiation and lens regeneration. Soon after lentectomy, pigment epithelial cells (PECs) of the dorsal and ventral iris dedifferentiate; however, only PECs from the dorsal iris contribute progenitors for lens regeneration (Sato 1940). For many years, the axolotl (Ambystoma mexicanum) was thought to lack the newt’s lens regenerative potential; however, it was recently shown that axolotls can in fact regenerate lens from dorsal and ventral iris PECs during early larval development (Suetsugu-Maki et al. 2012). But, after approximately 28 days of post-hatching development, axolotl larvae lose the ability to regenerate lens. Thus, the axolotl provides an important new model to
identify age-related changes in gene expression that correlate with regenerative ability. In this study, we used microarray analysis to identify gene expression differences between irises collected from 7-day post-hatching larvae (referred to as young) and 3-month-old larvae (referred to as old). We collected tissues post-lentectomy to sample regeneration-associated transcripts from young iris and transcripts associated with a non-regenerative response in old iris. The genes that were expressed differentially between young and old axolotl larvae reveal age-related differences in transcription, metabolism, cell proliferation, differentiation, and immune response. We report further insights by comparing genes identified between young and old axolotl iris to genes that were identified recently from dorsal and ventral irises of newts (Sousounis et al. 2013).

Results

Gene expression during axolotl lens regeneration

Young and old axolotl larvae were lentectomized and 6 hours later whole iris rings were isolated for RNA extraction and Affymetrix microarray analysis. A total of 3751 probe sets (i.e. genes) were identified as statistically, differentially expressed between the young and old iris samples, and, of these, 1572 registered a > 2-fold difference in expression (Table S1). Approximately half of the differentially expressed genes were more expressed in young iris samples (N = 1809) and thus the remainder were more expressed in old iris (N = 1942) (Fig. 1A). Strikingly, many of the upregulated genes were highly differentially expressed between samples. For example, krt8, krt19, sfpce, itlin1, and col28a1 were 1324 to 41 times more abundant in young iris than old. Moreover, igll1, hbg1, hba2, css, mrc1, and slc6a13 were 533 to 34 times more abundant in old iris (Fig. 1B). Examination of expression estimates for all of the genes listed above, and 168 additional genes, suggests that they were only expressed in one of the iris samples. Affymetrix probe sets for these genes registered low, mean expression values for one of the samples, values that did not eclipse an empirically determined threshold for defining absence of expression (see Materials and Methods). Thus, these results show fundamental differences in transcription between young and old iris, with > 100 genes expressed in one sample but not the other. In addition to the genes listed above, we note that additional keratins (krt15, krt18) and collagens (col5a1, col12a1, and col29a1), and a biomarker of cell proliferation (shcbp1), were only expressed in regeneration competent young iris.

Gene Ontology (GO) enrichment analysis

Statistically significant genes (q < 0.05) that minimally exhibited a 2-fold difference between the iris samples were selected for Gene Ontology (GO) enrichment analysis. The genes identified from young iris samples enriched GO terms associated with regulation of gene expression, electron transport chain, cell cycle, DNA repair, oxidation—reduction process, and metabolic process (q < 0.05, Fig. 2A, Tables 1, 2 and S2). The genes that significantly enriched these terms are predicted to regulate transcription (ccnh, cdk7, gfa2a2, tatf5, tat9, tat13, and tat15), splicing (lsm1, lsm3, lsm5, lsm6, lsm7, phf5a, srnp1a, srnpd2, and srnpd3), ATP production (ndufa1−7, ndufa12, ndufb2, ndufb4−8, ndufc2, ndufs5, ndufs6, ndufv2, and ndufv3), intracellular protein levels (psma3, psma4, psma7, psmb2, psmb7, psmd12, and psmd8), DNA replication (chaf1a, gina1, pole2, dbf4, rpa2, and ryns), DNA repair (nsmce1, ral51, rad51ap1, trip13, and rpa2), and chromosome segregation (cdc27, cdc8, and kij20a). Overall, these expression results suggest that young iris was metabolically more active and proliferative than old iris, as would be expected if the former were initiating a larval dedifferentiation response (Reyer 1982). We note that these expression differences were quantitative and not absolute as the genes listed above were also expressed in old iris, at significantly lower levels, however.

A different set of GO terms were identified for old iris samples – immune response, defense response, cell communication, signal transduction, negative regulation of gene expression, and cell differentiation (q < 0.05, Fig. 2B, Tables 3, 4 and S3). Many of the genes that enriched these terms were only expressed in old iris, including factors associated with innate immunity (cd74, csth, csts, cfd, ctsg, igj, ighm, igll1, igsf1, f3lb, pros1, ccl19, tgbh2, mrc1, enp2, and ighm), and cellular growth and differentiation (hex5, fgf13, edar, vwc2, adfp, cntnap2). Many additional genes associated with cellular differentiation were expressed more highly in old iris than young, including cdh2, dner, gpm6a, ndrg2, ndrg4, numb, pirin, wsp1, notch, bmp2, bmp7, rb1, atf1, atf5, af6, jag1, fgfbp3, fgfr1, kit, cnmd1, smad7, igfbp3, igfbp6, hgf, tgbh1, tgbh2, cift, procn, igfals, and lhx2. Overall, the identified genes clearly indicate that a post-lentectomy immunological response was induced in old iris, a response that was not observed in young iris. In addition, in comparison to young iris, the results suggest that old iris was relatively more differentiated and presented less potential for cell proliferation. Indeed, negative regulators of DNA synthesis (enosf1) and cell cycle progression (mll5, kiss1r) were expressed more highly in old iris.

Validation with qPCR

Several genes were selected for independent validation of microarray expression estimates using quantitative polymerase chain reaction (qPCR). Using biological replicates, qPCR yielded highly similar estimates to those obtained by microarray (Fig. 3). Two of three genes (eya2 and mpo, but
not lect1) that were estimated as highly differentially expressed in young iris were validated, as were all six genes that were deemed as only expressed in old iris (slc6a13, slc6a20, cd74, ctsg, hbd, and hbg1). The qPCR estimates for lect1 did not reveal a significant difference between young and old iris, as was suggested by the microarray analysis. Overall, qPCR validated all but one of the microarray estimates (Fig. 3).

Comparison of gene expression patterns between axolotl and newt lens regeneration
Recently, RNA sequencing was used to identify genes expressed differently between regeneration competent dorsal and regeneration incompetent ventral iris during newt lens regeneration (Sousounis et al. 2013). We compared genes from Sousounis et al. (2013) that exhibited a > 2-fold

Figure 1. Microarray gene expression during axolotl lens regeneration. (A) Volcano plot of all the probe sets of the microarrays. Probe sets are color-coded based on the significance and the fold change between the samples. (B) Highly upregulated genes in young and old iris samples. N/A, not applicable.
Figure 2. Selected enriched GO terms in axolotl samples. (A) Selected enriched GO terms in young iris samples ($q < 0.05$). (B) Selected enriched GO terms in old iris samples ($q < 0.05$). Bars indicate the number of genes found with the corresponding GO term.

Table 1. Genes related to gene expression that were found to be significantly upregulated in the young axolotl iris.

| Function            | CCNC | CDK7 | E2F4 | MTERF | POLR2K | TAF13 | TAF9 |
|---------------------|------|------|------|-------|--------|-------|------|
| Transcription       |      |      |      |       |        |       |      |
| RNA processing      |      |      |      |       |        |       |      |
| EIF4E               |      |      |      |       |        |       |      |
| IARS2               |      |      |      |       |        |       |      |
| Protein processing  |      |      |      |       |        |       |      |

difference between dorsal and ventral iris at 4 or 8 days post-lentectomy (DPL) to genes identified as significant in our study. We found greater overlap of significant genes and enriched GO terms between regeneration competent newt dorsal iris and young axolotl iris than regeneration incompetent newt ventral iris and old axolotl iris (Fig. 4). In particular, GO terms for transcription, cell cycle, and metabolic process were identified in common between newt dorsal iris and young axolotl iris, while innate immune responses were identified in common between newt dorsal iris and old axolotl iris. The 96 genes that were expressed more highly in young axolotl and newt dorsal iris than old axolotl and newt ventral
Table 2. Genes related to electron transport chain, cell cycle, and DNA repair that were found to be significantly upregulated in young axolotl iris.

| Function        | Gene          | Gene          | Gene          | Gene          | Gene          | Gene          |
|-----------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Electron transport chain | ATP5D         | ATP5L         | ETFA          | NDUFA4        | NDUF3        | UQCR10        |
|                 | ATP5E         | COX6A1        | NDUFA1        | NDUF4         | NDUF5        | UQCR11        |
|                 | ATP5F         | COX6C         | NDUFA5        | NDUF5        | NDUF6        | UQCR2        |
|                 | ATP5J         | COX7B         | NDUFA6        | NDUF7        | NDUF8        | UQCR3        |
|                 | ATP5J2        | COX7C         | NDUFA7        | NDUF2        | NDUF3        | UQCR5        |
|                 | ATP5J3        |               |               |               |               |               |
| Cell cycle      | CCNH          | DYNLL1        | KRT18         | NUF2         | PSMA3        | PSMD14        |
|                 | CDC26         | E2F4          | LIN9          | NUF8         | PSMA4        | SKA2          |
|                 | CDC27         | E2F8          | MCTS1         | ORC6         | PSMA7        | SNA1          |
|                 | CDC28         | GINS1         | MRPL41        | PDCD2L       | PSMB2        | TOP2A         |
|                 | CDK7          | GORASP2       | NDUFA2        | PFDN1        | PSMB7        | RFC2          |
|                 | CHAF1A        | KIF20A        | NOLC1         | POLE2        | PSMD12       | RPA2          |
|                 | CHAF1B        | KIF23         |               |               |               |               |
| DNA repair      | ACTL6A        | EYA2          | INO80C        | POLG2        | PTTG1        | RBX1          |
|                 | CCNH          | FBXO6         | NEIL3         | POLR2K       | RAD17        | RFC2          |
|                 | CDK7          | GTF2H5        | NEMC1         | PRMT6        | RAD51        | RPA2          |
|                 | CHAF1A        | HMGA2         | POLE2         | PSMD14       | RAD51AP1     |               |

Table 3. Genes related to cell differentiation that were found to be significantly upregulated in the old axolotl iris.

| Gene          | Gene          | Gene          | Gene          | Gene          | Gene          | Gene          | Gene          | Gene          | Gene          |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| A2M           | CBLN1         | CTGF          | EXT2          | IRF1          | KRT8          | NUMB          | SLC7A11       | TRAPPCC9      |               |
| ARGHAP2       | CCL19         | CTSV          | FHL1          | IRF8          | MGMT          | PIR           | STEAP4        | UHRF2         |               |
| B2M           | CDH2          | DNER          | GNA12         | JUN           | MSI1          | PPDFF         | TDRKH         | ZFP36L1       |               |
| BMP2          | CHRD1L        | EDAR          | GPM6A         | KIT           | NDRG2         | PSAP          | TGBF1         | ZFFM2         |               |
| BNIP3         | CREBL2        | EPAS1         | HERC4         | KMT2E         | NDRG4         | SEMA4A        | TGBF2         | ZSCAN2        |               |
| CAMK4         | CREM          | ERAP1         | HES5          | KRT19         | NOTCH1        | SKIL          | TMEM176B      |               |               |

Table 4. Genes related to immunity which were found to be significantly upregulated in old axolotl iris.

| Gene          | Gene          | Gene          | Gene          | Gene          | Gene          | Gene          | Gene          | Gene          | Gene          |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| ADCY2         | CCL19         | CTSG          | ENPP2         | IGJ           | KIT           | PCBP2         | PROS1         | TGFβ2         |               |
| ADCY3         | CD59          | CTSH          | ERAP1         | IGLL1         | MR1           | PLD2          | SFTPD         | TLR2          |               |
| APOA4         | CD74          | CTSS          | FTH1          | IRF1          | NFL3          | POLR2L        | SPPL2B        | TRIM11        |               |
| B2M           | CHIT1         | CXCL10        | HLA-E         | IRF8          | NOTCH1        | PRF1          | TGBF1         | TRIM35        |               |
| CAMK4         | CLU           | ECM1          | HSP90AA1      | JUN           |               |               |               |               |               |

iris provide important new candidates for functional studies (Table 5). Also, 20 genes that were commonly upregulated in regeneration incompetent irises in both species implicate these as candidate inhibitors of regeneration. We discuss several genes identified from this bioinformatics analysis below.

**Discussion**

Regenerative ability varies greatly among vertebrates but is generally much higher during early life stages (Seifert & Voss 2013; Sousounis et al. 2014). In this study the early transcriptional response of iris to lentectomy was compared between young and old axolotl larvae that differed in regeneration competence. Using only three replicate Affymetrix GeneChips per treatment, > 3700 differentially expressed genes were identified statistically and many of these genes exhibited 10–100-fold expression differences between treatments. The many highly differentially expressed genes identified in our study are probably explained by the presence and absence of different cell types between young and old iris tissue and age-related changes in cellular differentiation. We discuss both of these explanations below, and then discuss new gene expression insights that were gained by comparing our results with those obtained recently from newts.

**Ontogeny of immunity correlates with loss of regenerative ability in the Mexican axolotl**

In axolotls, the spleen is the organ where erythrocytes, lymphocytes, and thrombocytes are produced and released into the blood (Charlemagne 1972). Although the axolotl spleen

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begins to differentiate during the later stages of embryonic development, maturation is not completed until larvae reach 2–3 months of age. Immunoglobulin M (IgM) synthesizing lymphocytes are first observed approximately 35 days post-hatching in the spleen, and then 56–70 days after hatching in serum (Fellah et al. 1989). This explains why genes encoding heavy and light chain components of IgM (ighm, igj, igll1, igsf1) and hemoglobin gamma A (hbg1) were highly expressed in the old iris samples but not in young iris samples. The axolotls that provided the older iris tissue were approximately 84 days post-hatching and thus had circulating lymphocytes and erythrocytes. In support of this explanation, genes associated with immune cells and system responses were uniquely expressed in old iris, including genes associated with macrophages (mrc1, ctsh, ctsg), basophils/mast cells (hdc), and B-cells (multiple immunoglobulins, lrrc8d), and processes ranging from coagulation (f13b, pros1), lymphocyte homing and migration (ccl19, enpp2, wasf3), complement (cfd), and antigen presentation and processing (cd74, cits). These gene expression results are consistent with the above timeline for axolotl spleen development (Fellah et al. 1989) and hemoglobin switching (Page et al. 2010), and clearly show that some humoral and immunological gene expression responses to injury change with aging. These differences between the early and old iris are absolute and robust; if no B-cells are present in a tissue sample, no B-cell-associated transcripts will be measured. In future studies, it will be important to more broadly sample the larval period, as such a design could better resolve age-related changes in gene expression that are quantitative in nature. Such a design would be informative for understanding how injury and non-injury responses change with aging, perhaps comparing in parallel regeneration competent and incompetent tissues. Our results suggest that such a study could be readily performed using axolotls, and such a study would probably provide important new insights about the maturation of
tissues and physiological systems within the context of tissue regeneration.

**Cellular differentiation also correlates with loss of regenerative ability**

Cells differentiate and tissues mature as an organism ages. In general, cells become more differentiated and less stem-like with aging and may show lower potential for dedifferentiation, cell cycle re-entry, and patterning (Sousounis et al. 2014). During the aging process of vertebrates with low potential for regeneration (e.g., mammals), cells may differentiate toward fates that are more appropriate for tissue repair and less permissive for regeneration. Independent of immune system function, our results support the idea that axolotl iris differentiates with aging to a point that it is no longer capable of dedifferentiation. Indeed, we identified a
number of regulators/biomarkers of cellular differentiation that were more highly expressed in old iris, including *notch*, *bmp2*, *bmp7*, and *rb1*. Interestingly, Grogg et al. (2005) were able to induce lens regeneration by inhibiting bone morphogenetic protein 4 (BMP4) and BMP7 expression in regeneration incompetent ventral iris PECs of the newt, but similar treatments did not induce lens regeneration in axolotl (Grogg et al. 2005). This suggests that age-related changes in regenerative ability may involve transcriptional changes across multiple signaling pathways; and such changes may specify non-regenerative cellular phenotypes. Two lines of evidence support this idea. (1) We observed genes expressed in young iris that promote cell proliferation and genes expressed in old iris that function to restrict DNA synthesis and cell proliferation. For example, *enosf1* and *ctnp2* were highly expressed in old iris. *enosf1* encodes an anti-sense transcript that downregulates thymidylate synthase, an enzyme that functions in thymine biosynthesis, while *ctnp2* catalyzes the rate-limiting step in cytosine synthesis. These patterns suggest that lentectomy causes an imbalance of nucleotide precursors in old iris, a molecular pathology that is not optimal for supporting cell proliferation. (2) Johnson (2013) recently showed that the expression of genes for cell proliferation and collagen synthesis declined with aging in axolotl brain; both of these patterns were observed in our study. Genes that are permissive for lens regeneration are expressed highly early in the larval period but are gradually or suddenly downregulated during development. We note that the loss of lens regenerative plasticity in the Mexican axolotl occurs after the first 28 days of post-hatching development, which associates with not only the initiation of immune system function but also gonadal differentiation (Gilbert 1936). Disentangling the effects of local and peripheral factors on regenerative capacity can be tested by grafting young iris cells into regeneration incompetent older eyes, or by moderating the immune response of older axolotls, as was done recently in a study of macrophage function during axolotl limb regeneration (Godwin et al. 2013).

**Identification of new candidate genes for lens regeneration**

Finally, we compared lists of genes that were compiled from two different lens regeneration models. We compared genes that were identified as differentially expressed between young and old axolotl iris to genes identified as differentially expressed between dorsal and ventral regions of the adult newt iris. The objective was to determine if gene expression was similar for regeneration competent and incompetent samples, even though they were derived from different species and experimental paradigms (the effect of aging versus patterning on regenerative ability). Somewhat surprisingly, given low power to detect homologous expression results between lowly replicated studies that derive expression estimates from different technologies, and given reports indicating axolotls and newts employ different mechanisms to accomplish the same regenerative outcome.

### Table 5. Genes found to be upregulated in both newt and axolotl lens regeneration in regeneration competent or incompetent iris.

| Function                        | Gene upregulated in regeneration competent iris |
|---------------------------------|-------------------------------------------------|
| Transcription                   | CIRH1A, ENY2, POLR1D, RBBP7, ZNF182, ZNF451     |
| RNA processing                  | CPFS3, MPHOSPH10, PDCD11, PNPT1, SNRPA1         |
| Translation                     | C12orf65, MRPL19, MRPL53, MRPS27, MRPS28, QRS1L |
| Protein processing              | CRELD2, PFDN4, TIMM13, TIMM8A, TIMM9, VPS13A, WDR77 |
| Electron transport chain        | P4HA1, PHPT1, ATPSD, UQCRQ, CMC1, COX16, ETFA, NDUFS5 |
| Metabolic process               | BCAT1, CYP51A1, CPSF3, MPHOSPH10, PDCD11, PNPT1, SNRPA1 |
| CYP26A1                         | DHR512, G2T2, MRPL19, MRPL53, MRPS27, MRPS28, QRS1L |
| Extracellular matrix            | COL12A1, DFT, HTRA1, MXRA5                     |
| Cell cycle                      | CDC27, DSCC1, KIF11, LIN9, NUF2, RCR1, RPS6KB1 |
| CHAF1A                          | CDC48, GINS1, KIF20A, NCPG2, PKB, RPS6KB1       |
| DBF4                            | HAUS1, KIF23, NDC80, PDCD2L, RNASEH2A, TOP2A   |
| Other                           | BAX, C19orf60, C7orf25, PRKIR, RP9, TMPO        |
| C11orf10                        | C6orf162, CASP3, NAMCE1, RHT1, SSNA1, TOR1AIP1 |
| C16orf68                        | C6orf203, LMNB1, NUP85, RNFT1, STOML2           |

| Gene upregulated in regeneration incompetent iris |
|--------------------------------------------------|
| ACTA2, CPAMD8, KDM4C, NR2F1, SLCTA3, STXB4, ZNF510 |
| CHRDL1, HSPA8, LAMC2, NRCAM, SLC6A13, TRIM11, ZNFX1 |
| CHST11, KCNMB2, LAMB2, RGMB, SLIT2, TCTC         |
Proposed model for lens regeneration in newts and axolotls. Lens regeneration competent tissues (young axolotl iris and newt dorsal iris) have potent cells that can be activated and carry out similar events leading to transdifferentiation. Newt ventral iris and old axolotl iris contain more differentiated cells that lack pluripotency and the ability to be activated post-lentectomy.

(e.g., Sandoval-Guzman et al. 2014), we identified common gene expression responses. In particular, genes associated with cholesterol metabolism (cryp51a, lss, fdps, sqle), retinoic acid synthesis (rdh13, cyp26a), and mitosis/regulation of cell proliferation (e.g., dsccl, pbk, lin9, romo1) were identified for regeneration competent axolotl and newt iris. Thus, genes identified from regeneration competent iris, and presumably dedifferentiating and proliferating PECs, probably comprise a conserved regulatory network underlying transdifferentiation (Fig. 5). Upregulation of cyp26a in regeneration competent iris is interesting because it acts to attenuate retinoic acid signaling, a metabolite required for lens regeneration in adult newt and Xenopus (Thomas & Henry 2014). Among genes that were expressed in common between regeneration incompetent axolotl and newt iris, we note repressive axon guidance molecules (slit2, rgbm), neurotransporters (slcl1a3, slcl6a13) indicating possible roles in cell to cell attraction or repulsion (Kim et al. 2014), and m2f1, a transcription factor that specifies neural cell fates and negatively regulates retinoic acid signaling (Neuman et al. 1995; Yamamizu et al. 2013). These genes further support the idea that regeneration incompetent iris is associated with higher expression of differentiation markers. Overall, our comparative analysis shows that regenerative ability of salamander iris is associated with cholesterol biosynthesis and retinoic acid synthesis and signaling.

**Materials and Methods**

**Animals and operations**

*Ambystoma mexicanum* embryos and larvae were purchased from the Ambystoma Genetic Stock Center in Lexington, KY. The young iris samples were collected from individuals that were raised from embryos to 7 days post-hatching. For older animals, 3-month-old axolotl larvae...
(3–5 cm) were purchased. Axolotls were anesthetized in 0.1% (w/v) ethyl-3-aminobenzoate methanesulfonic acid (MS222; Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline. Using a sharp scalpel, an incision was introduced in the cornea. Lenses were removed with fine forceps ensuring that lens fibers and capsules were removed without damaging adjacent tissues. Six hours post-lentectomy, axolotls were anesthetized in MS222 and whole eye balls were removed in calcium- and magnesium-free Hanks’ solution where they were dissected according to the method of Bhavsar et al. (2011). Briefly, a fine scalpel was used to make a hole in the eye and scissors were used to separate the anterior and posterior eye parts. Iris pieces were separated from neural retina and cornea and placed in Eppendorf tubes with RNAlater solution (Life Technologies, Grand Island, NY, USA). Three microarray replicates were created for the young and old iris samples by pooling tissues from 11 7-day-old and four 3-month-old axolotl larvae, respectively. This same procedure was used to create a second, independent group of replicates for qPCR.

**RNA extraction, reverse transcriptase reaction and qPCR**

Methods that were used to isolate RNA, synthesize cDNA, and perform qPCR are detailed in Sousounis et al. (2013). qPCR conditions were optimized initially using PCR and gel electrophoresis. Primer sequences and qPCR settings are listed in Table S4. Gene expression estimates were calculated relative to the expression of a housekeeping gene (*eef1a1*).

**Microarrays**

The University of Kentucky Microarray Core Facility performed microarray analysis according to standard Affymetrix protocols. All RNA samples were quantified using an Agilent BioAnalyzer. RNA expression profiling was conducted using custom Amby_002 microarrays (Huggins et al. 2012). The six RNA samples were labeled and hybridized to independent microarray GeneChips and scanned. Background correction, normalization, and expression summaries were accomplished using the robust multi-array average (RMA) algorithm (Irizarry et al. 2003).

**Statistical analysis**

To identify significant genes between young and old iris samples (microarray and qPCR analyses), t-tests were performed assuming unequal variances and independent samples. Multiple testing used a false discovery rate cutoff of 0.05 (Benjamini & Hochberg 1995) and was performed by calculating *q*-values for individual probe sets. This was accomplished by dividing the number of probe sets expected to be false positives at or below the *P* value for a given probe set by the total number of probe sets detected at or below that *P* value. Genes with *q* < 0.05 were considered significant.

The method described by Buechel et al. (2011) was used to identify significant probe sets that were expressed in one sample but not the other. Briefly, a probe set was considered non-expressed if its expression estimate failed to exceed a threshold value that was identified in the saddle region of each array’s signal intensity histogram. Gorilla software was used to identify significantly enriched GO terms for significant genes that showed > 2.0-fold difference in expression between young and old iris samples (Eden et al. 2009). GO terms with *q* < 0.05 were considered significant.

**Comparative transcriptomics**

Newt genes that showed > 2-fold difference in expression between dorsal versus ventral iris (Sousounis et al. 2013) were compared to significant genes identified by contrasting young and old axolotl iris samples. Newt and axolotl genes with the same human gene annotation were assumed to be orthogonal and presumptive gene functions were deduced from the literature. Enriched GO terms were also compared between the species. Venn graphs were created using VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s website:

Table S1. Microarray gene expression data and statistical analysis.

Table S2. Gene Ontology enrichment analysis of young axolotl iris samples.

Table S3. Gene Ontology enrichment analysis of old axolotl iris samples.

Table S4. List of primers used for qPCR.