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Gene expression signatures in the newt irises during lens regeneration

Evgeny Makarev[a], Mindy K. Call[a], Matthew W. Grogg[a], Donald L. Atkinson[b], Brett Milash[b], Shannon J. Odelberg[b], Panagiotis A. Tsonis[a,*]

[a] *Department of Biology and Center for Tissue Regeneration and Engineering, University of Dayton, Dayton, OH 45469-2320, USA*
[b] *University of Utah Health Science Center, Salt Lake City, UT 84132, USA*

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Abstract **Lens regeneration in adult newts is possible by transdifferentiation of the pigment epithelial cells (PECs) of the dorsal iris. The same cells in the ventral iris are not capable of such a process. To understand this difference in regenerative competency, we examined gene expression of 373 genes in the intact dorsal and ventral irises as well as in irises during the process of lens regeneration. We found similar signatures of gene expression in dorsal and ventral with several cases of even higher levels in the ventral iris. Such transcriptional activity in the regeneration-incompetent ventral iris was unexpected and calls for a revision of our views about mechanisms of lens regeneration induction.**

Keywords: Newt; Regeneration; Lens; Transcription

1. Introduction

The pigment epithelial cells (PECs) possess a remarkable plasticity and are able to transdifferentiate to retinal or lens cells [1,2]. This ability is most impressive in adult newts where after lens removal, PECs from the dorsal iris dedifferentiate and then redifferentiate to a different cell type to regenerate the removed lens [3]. Interestingly, PECs from the ventral iris do not participate in lens regeneration. Consequently, it has been thought that induction events, such as re-entry of the cell cycle and transcriptional activation, must take place exclusively in the dorsal iris [4–6]. Such a process would ensure that only one lens can be regenerated. However, it has been shown that ventral PECs do re-enter the cell cycle, although their rate of re-entry appears to be lower than dorsal PECs. An unresolved issue is whether there are factors expressed specifically in the dorsal iris that regulate lens regeneration. If such factors exist, their function could be studied in the ventral iris in an attempt to induce it to transdifferentiate to a lens, thus possibly opening the way for lens induction in other animals as well. In a previous study we showed that *six-3* over-expression as well as BMP pathway inhibition were able to elicit a lens from the ventral iris [6]. But surprisingly these factors were not dorsal-specific. They are expressed in the intact ventral iris and in the ventral iris after lens removal. The same was true for pax-6 expression. Both *six-3* and *pax-6* are considered to act as eye master genes and it is surprising that they would be active in a developmentally inactive tissue. We reasoned, therefore, that in order for regeneration to occur from the dorsal iris the expression of these genes had to be elevated specifically in the dorsal iris. To determine on a more global level whether differential gene expression is important for lens regeneration from the dorsal iris, we decided to study gene expression using a custom newt microarray containing 373 genes, known to be expressed in regenerating tissues. This set of cDNAs should be able to provide us with concrete information about patterns of gene expression in the newt iris and their correlation to the mechanisms of lens transdifferentiation.

2. Materials and methods

2.1. Microarray analysis

Microarray slides containing quadruplicate spots of 521 cDNA fragments representing 373 regeneration-enriched newt genes were prepared at the Huntsman Cancer Institute Microarray Core Facility at the University of Utah. These cDNAs were isolated from regeneration blastema. Tissues were collected from the intact dorsal and ventral iris as well as from irises at 8 days postlentectomy and were flash frozen in liquid nitrogen. RNA was extracted from these tissues, amplified, and purified using a RiboAmp RNA amplification procedure from Arcturus Engineering. Preparation of the probes, hybridization, and data collection was performed as previously described [7]. Intensity data from low-quality spots were removed and the values of the remaining spots for each cDNA fragment were averaged and log2 ratios were calculated between the lentectomized and control samples. We observed no significant spatial variation on any of the microarrays and the median intensity value for each array was nearly identical, so no further normalization was performed. Spots with a signal less than 20% greater than the background signal were eliminated from further analysis. The data were analyzed using Spotfire DecisionSite for Functional Genomics (Spotfire Inc.).

In the tables (see Section 3), we have averaged all signals for a given gene (if multiple cDNAs for a given gene were present on the microarray) that appear to have produced a reliable signal. We did not include in these averages results that did not appear to produce reliable signals. We have included the following comparisons: regenerating dorsal vs. normal dorsal (RD:ND); regenerating ventral vs. normal ventral (RV:NV); normal dorsal vs. normal ventral (ND:NV); regenerating ventral vs. regenerating dorsal (RV:RD). We have included all genes that exhibited a differential expression pattern where the average ratio was $|x| \geqslant 0.7$ (log2 scale) in at least one of the four comparisons (RD:ND, RV:NV, ND:NV, and RV:RD).

2.2. QPCR design

For our experiments iCycler iQ Real-Time Detection System and SYBR Green Supermix (#170-8882) from Bio-Rad were used. Also for cDNA synthesis iScript cDNA Synthesis Kit (Bio-Rad#170-8882) was used and RNA for cDNA synthesis meets the following requirements: Purity – A260/A280 = 2; Integrity: It should be two clear bands of rRNA on denaturing agarose gel electrophoresis; gDNA

*Corresponding author. Fax: +1 937 2292021.
E-mail address: panagiotis.tsonis@notes.udayton.edu (P.A. Tsonis).

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Table 1
Primers used for QPCR validation

	Probes		Primers	Tm (IDT)	Product size
1	Jun B	F	TACAGAAAGGAGCCATCCACACTC	58.1	194
		R	CCCACCTCGCAAACAGAACAAA	58.0	
2	Glucosamine, GN65	F	GTATCAAACCCAACCAGACCAAGA	56.7	159
		R	ATCGCCAAGTCCCATTCCA	56.4	
3	Actin-related protein 2/3 complex subunit 1B	F	CGAAAGCAACCGCATTGTGACCT	60.3	185
		R	AGCAGATGGAAATAACCCTGGAAC	56.8	
4	MMP3/10a	F	CAATCCATCCATCTGTGACCCAAC	57.6	246
		R	TCAAATCCCTGAAGAGCCCA	55.8	
5	MMP9	F	AGGCTGCGAACAGTAGATGAG	56.4	171
		R	GACCAACAATCACCATAGACGGCA	59.0	
6	DD223d unknown No homology	F	ATGGGTGGATAGGGCAAGGA	57.9	165
		R	ACCGAGTGTGAAGCGAGCAAA	59.1	
7	Small EDRK-rich factor 1A (spliced)	F	TTCAACGAAAGCAGAGGCAAAGGG	59.8	198
		R	GCAACAGACGGACGAATACCAAGA	58.8	
8	Tubulin – homolog of Mus alpha 6	F	TGCACACAGAGCAACAGACCAGAA	60.5	171
		R	TGGAGAAGGTATGGAGGAAGGAGA	58.3	
9	Epithelial-cadherin	F	ATGAAGAAGGTGGTGGAGGAGAAG	58.0	139
		R	GCTGGGCGAGGACGGTATTG	60.4	
10	Cardial α2 actin	F	CACCATCTCCAGAGTCCAG	54.5	130
		R	TCGTGAGAAGATGACACAGAT	53.2	
11	Profilin 2	F	TACGGCTGAAGAGGTCG	53.8	180
		R	AACGGCAATGCTGACTG	53.3	
12	Tubulin α2	F	GTGCTGGACCGAATGC	54.0	140
		R	GACTTCTTGCCATAATCTACAG	50.8	
13	Vimentin	F	CATCAACCTGGAGTTCAAG	50.6	213
		R	GTCCACCTGCCTGCG	56.8	
14	Galectin 9	F	GCCTCAGCGATGGGAC	56.1	134
		R	CGAAAAGCGTGGATTGA	50.2	
15	Stromal cell-derived factor 2	F	AACAATCAGGAAGTCAGCG	52.7	153
		R	TTCCCCTGTAATGGTCAAG	51.7	
16	Cathepsin L	F	GGCTTATGGACCAGGCT	54.5	130
		R	GTGTCATTGGCAGAGTTGT	53.0	

contamination: It should be difference in at least 12 cycles between "minus" and "plus" controls during QPCR. We compared gene expression between 4 cDNA libraries: intact dorsal (ND), intact ventral (NV), 8th day of regeneration dorsal (RD), 8th day of regeneration ventral (RV). Each experimental point was done in triplicate. The 16 genes that we selected to validate are listed in Table 1. Since the purpose of this experiment is to validate the microarray patterns, these genes were selected randomly. For normalization an internal control (house-keeping gene rpL27) was used. Data analysis was done with the Pfaffl [8] and Ramakers et al. [9] methods.

3. Results

3.1. Regulation in dorsal or ventral iris

These comparisons were undertaken in order to see differences between the intact irises and irises during lens regeneration. In other words, to examine differences in gene expression in dorsal or ventral irises, due to initiation of the regeneration process.

3.1.1. Comparison between intact normal dorsal iris (ND) with dorsal iris during regeneration (RD). Several genes that are responsible for tissue remodeling [10], such as *MMP9, galectin, cathepsin, collagenase, TIMP1* seem to be up-regulated during dedifferentiation. This is to be expected, since the process of dedifferentiation is marked by loss of pigmentation and remodeling. Other genes that seem to be up-regulated are several cytoskeletal protein coding genes and *H+ transporting ATPase* (Table 2). Very few genes (mainly those with no known homology) seem to be down-regulated during dedifferentiation (Table 3).

3.1.2. Comparison between intact normal ventral iris (NV) with ventral iris during regeneration (RV). Interestingly, during the regeneration process, genes that were found to be up-regulated in the dorsal iris were up-regulated in the ventral iris as well, even though the ventral iris does not contribute to regeneration (Table 2).

Table 2
Differentially regulated genes along the dorsal-ventral irises

Gene name/homology	RD:ND	RV:NV	ND:NV	RV:RD
No homology	2.191792079	1.960721314	−0.562404657	0.87964592
MMP9	1.923385139	1.817683645	0.047084251	0.372243203
Galectin 9	1.71490151	1.53721827	−0.067776697	0.54341548
Interferon regulatory factor I	1.651564559	1.733963983	−0.353343165	0.492271273
Alpha 6 tubulin	1.562679276	1.057754098	0.273350158	0.433858952
No homology	1.532512331	1.609600262	−0.122136189	0.592606709
Cathepsin L	1.355308533	1.061093452	0.078541035	0.284224289
No homology	1.202847378	0.659383629	−0.366737067	0.851760652
No homology	1.143917295	0.861587837	0.109822359	0.122632849
No homology	1.121170787	1.247927814	−0.044346187	0.590618464
Immune responsive protein 1	1.071896328	1.011308878	−0.181549422	0.461738048
Notophthalmus collagenase	1.051188823	1.89155884	0.348338623	0.771725988
Variable lymphocyte receptor-like gene	1.030488005	1.08710777	−0.058943765	0.539614276
No homology	1.012759776	1.279508446	0.269605432	0.936472627
Ferritin, heavy poly peptide 1	1.003957285	0.737356601	−0.026277219	0.029549368
No homology	0.995830619	1.665737417	0.39783504	0.629604895
No homology	0.957327579	0.71595014	−0.150120588	−0.252898881
Stromal cell-derived factor 2-like 1	0.939628073	0.995304883	0.300033962	0.529686404
Guanylate nucleotide binding protein 4	0.931329266	0.621388933	−0.023979163	0.311224023
Chloride channel, nucleotide sensitive 1A	0.924293056	0.923079567	0.194495527	0.388262391
Fibulin 2-like	0.919413455	0.844231686	−0.278215132	0.081287866
ATPase, H+ transporting, lysosomal subunit C	0.879010059	0.90228957	0.195570658	0.457332666
Heterogeneous nuclear ribonucleoprotein M	0.877881952	1.28836317	0.726537303	0.95044215
TIMP1	0.870079701	0.630342976	−0.262315589	0.330327295
Myosin alkali light 1av	0.865387859	1.154470223	0.257250823	0.316279634
Profilin 2	0.816549876	0.115953498	−0.37314796	−0.619858232
No homology	0.800798377	0.545831494	−0.137608799	0.239403937
No homology	0.78082676	0.92880451	n.i.s.	0.490733899
Cold-inducible RNA binding protein	0.763822662	0.521449971	0.614936176	0.113921291
No homology	0.753151452	0.458873499	−0.156463562	0.158091757
No homology	0.738826266	0.441089922	0.328856367	0.04084412
Complement component C1q alpha	0.717487556	0.641492573	−0.353938445	0.524274582
No homology	0.700008141	0.775154589	0.056304667	0.2384327
DnaJ (Hsp40) homolog, subfamily B, member 6	0.697791599	1.006592194	0.211569931	0.96371162
No homology	0.672231019	1.082291441	−0.102798506	0.455060128
Eukaryotic translation elongation factor 1 beta 2	0.648320051	0.563452824	0.297154248	0.854822912
Baculoviral IAP-repeat containing protein 2	0.636873105	0.865064872	0.001148684	0.689535548
Peptidoglycan recognition protein (MGC108330)	0.628512689	0.800828097	−0.117144625	0.255973372
Proteaosome subunit c3	0.600187196	0.752581655	0.279316544	0.551659886
ATPase, H+ transporting, lysosomal, V1 subunit H	0.592192437	0.712815726	0.117596349	0.515885679
Ribosomal protein S27 (metallopanstimulin)	0.546530648	0.71809839	0.447298697	0.507360789
Jun b	0.532309318	0.873907848	0.155217038	0.51591953
Elafin-like #1	0.51178992	1.312530046	−0.194203378	0.674307611
No homology DD223d	0.495849702	1.109973725	0.173445474	1.241963232
No homology	0.371960932	0.89926198	−0.288685503	0.370437547
No homology	0.359600734	0.639129506	0.713875768	0.838533325
small EDRK-rich factor 1A (telomeric)	0.333039523	0.730058863	0.509058129	0.808423229
Ribosomal protein 37a	0.303486829	0.626880839	0.441573623	0.773118847
MMP3/10a	0.293609402	0.791912237	−0.320245044	0.324199607
Hypoxia induced gene 1 (MGC81854)	0.22962918	0.646389747	0.32344754	1.073069123
Type 1 cytoskeletal 12 keratin (LOC398464)	0.214832825	1.550596186	n.i.s.	1.334083584
No homology	0.10690408	0.372469666	0.304385429	0.885329548
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2	0.04104392	0.770203615	0.724251123	1.015193939
Annexin A1-1	−0.018194988	0.078180618	0.429699386	0.848230467
Actin-related	0.630902393	0.23357597	−0.244516502	−0.40175653
Glucosamine, GN65	0.334316825	−0.21143992	−0.124565511	−0.45911250
Cadherin	−0.296380454	−0.20507679	−0.319848861	0.161319802
Tubulin α2	0.226281857	−018471720	0.004111931	−0.24288805
Vimentin	0.232857232	0.119469382	0.001670135	0.072639419

Genes in red (satisfying $|x| > 0.7$; see Section 2) and in blue (not in that category) were selected for QPCR analysis. (For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)
n.i.s.: non-interpretable signal.

3.2. Regulation along the dorsal-ventral axis

These comparisons were undertaken in order to see differences between the regeneration-competent dorsal iris and the regeneration-incompetent ventral iris both before and after lentectomy.

3.2.1. Comparison between ND and NV. Overall we can see that levels of gene expression along ND and NV vary little with some genes possibly expressed at slightly higher levels in the dorsal iris (i.e. *heterogeneous nuclear ribonucleoprotein M*

Table 3
Genes showing mostly down-regulation

Gene name/homology	RD:ND	RV:NV	ND:NV	RV:RD
No homology	−1.775609109	−0.290882313	−0.096283064	n.i.s.
No homology	−1.259259341	n.i.s.	n.i.s.	n.i.s.
No homology	−0.965052409	n.i.s.	n.i.s.	n.i.s.
No homology	−0.711734613	−0.601414161	−0.650479871	n.i.s.
No homology	−0.687275516	−0.766315475	−0.644919129	n.i.s.
Osteonectin (secreted protein, acidic, cysteine-rich)	−0.435398869	−0.77103128	−0.020573987	−0.037651281
Ly-6/urokinase-type plasminogen activator receptor (uPAR) superfamily	−0.157042582	−0.826111357	n.i.s.	−0.230658717
Actin, alpha 2, smooth muscle, aorta	−0.104519399	−0.912041524	−0.463272337	−0.351769044
Solute carrier family 25, adenine nucleotide transporter	0.067273669	−0.704719724	−0.141566425	−0.132409871
No homology	n.i.s.	−0.92004439	n.i.s.	n.i.s.
No homology	n.i.s.	n.i.s.	−0.74627459	n.i.s.

Genes in red (satisfying $|x| > 0.7$; see Section 2) were selected for QPCR analysis. (For interpretation of the references to colour in this table legend, the reader is referred to the web version of this article.)
n.i.s.: non-interpretable signal.

and *ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2*).

3.2.2. Comparison between RD and RV. A quite surprising picture emerges by comparing the ventral iris (RV) and dorsal iris (RD) during regeneration. Several of these genes appear to be expressed at slightly higher levels in the ventral iris, even though RV does not contribute to lens regeneration. Clearly, *MMP9, galectin, cathepsin*, and *collagenase* and are among several genes that are up-regulated in RV (Table 2).

3.3. Validation by real-time QPCR

We selected 16 genes based on the microarray data for verification of expression by QPCR. QPCR is an independent technique that is commonly used to verify microarray data. We show that all the selected genes were expressed in a similar manner when examined by QPCR. Microarray data indicated that 12 of these 16 genes exhibited differential expression between regenerating and intact tissues at the level of $|x| \geqslant 0.7$ (log2 scale) as described in the methods (marked red in Tables 2 and 3). These were: *MMP9, alpha6 tubulin, galectin, cathepsin L, stromal cell-derived factor 2, profilin 2, junb, DD223d* (no homology), *small EDRK-rich factor*, *alpha2 actin, MMP3/10a* and *actin related*. All of them, except small EDRK, are in good agreement with the microarray data and clearly verify the up-regulation in the ventral iris as well (Fig. 1). We also selected four other genes, which do not fall into this category and do not show such variation in regulation by microarray analysis. These genes were: *cadherin, tubulin alpha2, vimentin* and *glucosamine* (marked blue in Table 2). As expected, the QPCR data agree in general with the microarray values (the reader should note that in some cases the values are too small for any difference to be significant). Overall the QPCR data confirmed the main conclusion of the microarray data, *i.e.*, that similar activity exists in the dorsal and in the ventral iris.

4. Discussion

In this paper we have examined the expression of 373 newt genes in the iris of the adult newt before and after lentectomy. The dorsal iris PECs are responsible for lens regeneration via transdifferentiation, while, interestingly, the ventral counterparts never participate in the process of regeneration [4,6]. The reason for this difference in PEC response has not yet been elucidated. Understanding the molecular mechanisms underlying this difference in response to lentectomy would provide insights as to why regeneration is not possible from the ventral iris as well as from irises of other animals, including mammals. Given the fact that the ventral iris cells do undergo some regeneration-related activities, such as cell cycle re-entry, it is important to examine genetic activity in these cells. In a previous study we have shown that regulatory genes, such as *six-3, BMPs* and *pax-6*, which are involved in induction of lens regeneration, are expressed in the ventral iris as well, indicating that the presence of such regulatory genes alone might not be a sufficient for regeneration. Rather, up-regulation over normal levels might be the key for induction of lens regeneration. These surprising results prompted us to question how widespread this kind of gene regulation might be in the ventral iris. To answer this question, we employed microarray analysis using an available array of 373 newt genes that were known to be enriched for genes expressed in the regeneration blastema [7]. Even though the limited number of cDNAs in the microarray might provide some bias, we did find that several genes are in fact up-regulated in both dorsal and ventral iris during regeneration. What is more striking is that several of these genes are involved in tissue remodeling, such as *MMP9, TIMP, collagenase* and *cathepsin*. These factors are known to be regulated in response to injury or amputation of the limb in order to re-organize the extracellular matrix and lead to dedifferentiation. Our data clearly show that the ventral iris undergoes similar events, even though it does not contribute to regeneration. These observations provide a new paradigm in the field of lens regeneration in which the ventral iris is not a passive tissue, as it was thought to be, but undergoes similar events as the regeneration-competent dorsal iris at least as it is judged from the gene expression signatures that we observed. This might suggest different mechanisms for lens regeneration. In one, both dorsal and ventral iris initiate the same events in response to lens removal, but then there is an inhibitor in the ventral iris that prevents the completion of the regenerative process. Alternatively, completion of regeneration might be prevented by the lack of one or more factors required for the final stages of the regenerative process or that up-regulation of gene expression in the ventral iris may be a mechanism of negative regulation in the dorsal iris. Finally, another possibility is that up-regulation over normal gene expression levels is required for the formation of a new lens.

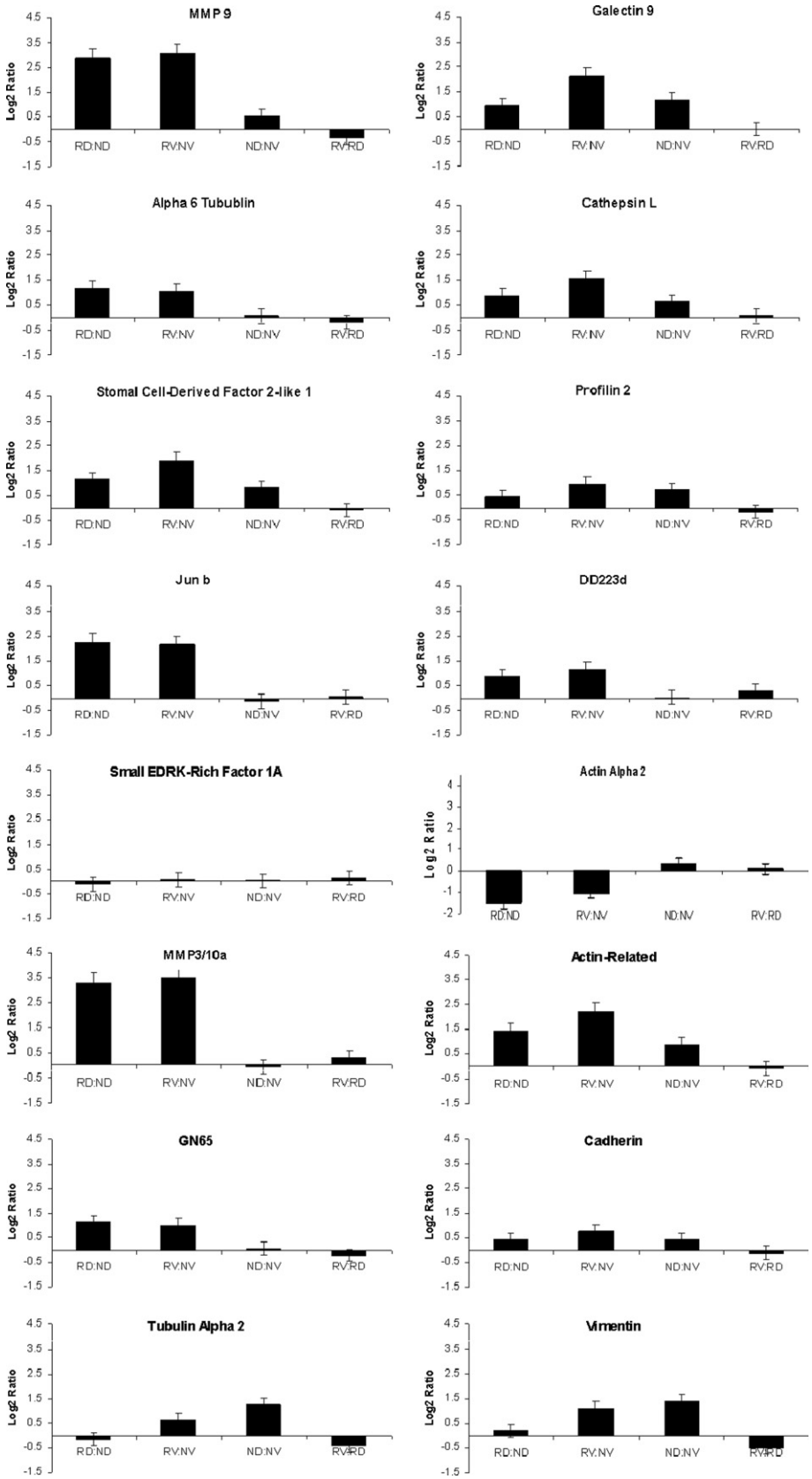

Fig. 1. Examination of 16 genes (marked red and blue in Tables 1 and 2; see Section 2 for criteria and text) by QPCR. The same comparisons as in the tables are presented. Note the general patterns of similar regulation in the dorsal and the ventral iris as well. Most of these genes were up-regulated during regeneration in both the dorsal and ventral iris. Only actin alpha 2 was down-regulated during regeneration.

We can now start designing experiments to determine which of these mechanisms might be controlling lens regeneration. For example, a comparative study with other animals incapable of lens regeneration, such as axolotl and mice, might provide useful information. Answers to these questions would help explain why higher animals are unable to regenerate their lenses and could lead to new methods for inducing lens regeneration. In a more general sense, given that the adult newt is capable of regenerating its retina through transdifferentiation of the retinal pigment epithelial cells, the results of our studies might also be applicable to retinal regeneration. If so, the study of the mechanisms of lens regeneration in newts could have an impact for the treatment of several diseases that lead to blindness.

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